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Sustainable Bioprocesses for the Technological Enhancement of Sorghum Flour: Applications in Wheat-Sorghum Composite Bread

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“It always seems impossible until it’s done.”

Nelson Mandela

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Preface

This dissertation grew out of a deep interest in finding sustainable and innovative ways to feed a growing global population amidst environmental challenges. Sorghum, an adaptable and resilient crop, holds great promise for diversifying our food sources. However, incorporating it into everyday foods such as bread presents unique challenges, particularly in achieving the quality that consumers expect.

In this work, I explored how techniques like sprouting and lactic acid bacteria fermentation could help unlock sorghum's potential by enhancing its qualities for food applications. Through these studies, I investigated not only how sorghum's properties change at a molecular level but also how it could contribute to the sensory and functional aspects of bread. I also looked at ways to address bread staling, a key factor in maintaining food quality and reducing waste.

My hope is that this research offers valuable insights for integrating sorghum into Western diets, supporting the creation of nutritious, and high-quality food products. Ultimately, I wish for this work to contribute to sustainable food production, inspiring the use of resilient crops that can help nourish both people and the planet in a meaningful way.

Miriam

Summary

In response to the global need for sustainable food security amid rising populations and climate change, research into climate-resilient crops has gained urgency. Sorghum, valued for its nutritional properties and drought resilience, holds promise for Western diets. However, its limited technological performance—particularly in dough formation and bread quality—impedes its application in staple foods like bread. Bioprocessing methods, such as sprouting and fermentation, present a promising approach to enhance sorghum's structural and functional properties by modifying its starch, protein, and fiber content. These modifications may broaden sorghum's applicability in composite food products widely consumed worldwide.

This Ph.D. thesis investigates the molecular and techno-functional changes in sorghum flour induced by bioprocessing, with a particular focus on breadmaking applications. The first research phase examines the molecular modifications in sorghum flour through sprouting and lactic acid bacteria (LAB) fermentation, emphasizing the effects on protein and starch fractions and their contribution to flour functionality. These insights lay the groundwork for incorporating sorghum into composite formulations. The second phase explores the impact of LAB-fermented sorghum flour on the quality parameters of wheat-sorghum composite doughs and breads, highlighting how bioprocessing can be strategically utilized to optimize dough properties and improve bread sensory properties, crumb structure, and flavor. The third phase addresses bread staling dynamics—a critical quality factor influencing shelf life and consumer acceptance. This section evaluates wheat-sorghum breads formulated with untreated and LAB-fermented sorghum flour, analyzing the roles of fresh and freeze-dried fermented sorghum in modulating water mobility, starch retrogradation, and texture during storage. By elucidating sorghum’s transformations across molecular and techno-functional levels, this Ph.D. thesis offers a foundational perspective on the potential of bioprocessed sorghum for sustainable food production. Moreover, these findings highlight the role of bioprocessed sorghum in facilitating the integration of climate-resilient crops into Western diets.

General introduction

I. Food system future projections

The global food system is at a critical juncture. According to the FAO's World Future Projections, the global population is projected to reach 9.7 billion by 2050 (FAO, 2018). The population growth will be accompanied by a significant increase in food demand. The meta-analysis conducted by van Dijk et al. (2021) examined five representative scenarios, encompassing divergent but plausible socio-economic futures, and found that global food demand is expected to rise by 35% to 56% between 2010 and 2050. When factoring in the effects of climate change, this range shifts to an increase of 30% to 62%.

The escalating demand for food will exert considerable pressure on agricultural systems and environmental sustainability. Indeed, addressing the challenge of feeding a growing global population brings forth new obstacles, including ensuring food safety and security, promoting ecosystem sustainability, fostering economic growth, and advancing social equity. These objectives are closely aligned with the United Nations' 17 Sustainable Development Goals (United Nations, 2015) (Figure 1).

The projected population growth, coupled with current dietary patterns, is likely to intensify the negative impacts of food production on global environmental stability, including increased greenhouse gas emissions, pollution, biodiversity loss, and the depletion of water and land resources (Willett et al., 2019). In this context, the imperative to reimagine food systems with climate change mitigation strategies has never been more pressing (Herrero et al., 2020; Zurek et al., 2022). Moreover, food systems must adapt to the impacts of climate change, which are already affecting crop health and productivity due to changing temperatures, shifting precipitation patterns, and the increasing frequency of extreme weather events (Dhankher and Foyer, 2018; Zurek et al., 2022). The latest report from the Intergovernmental Panel on Climate

Change (IPCC, 2022) underscores the detrimental effects of climate change on food production and its contribution to malnutrition (Zurek et al., 2022).

Figure 1. United Nations –17 Sustainable Development Goals (taken from https://www.un.org/sustainabledevelopment). [1]

Moreover, the transformation of global food systems is urgently required not only to reduce greenhouse gas emissions and to adapt to climate changes effects, but also to ensure equitable food distribution worldwide, meeting the challenge of providing healthy diets for all (Zurek et al., 2022). Currently, it is estimated that over 820 million people suffer from inadequate food access, while many others consume poor-quality diets that lead to micronutrient deficiencies and contribute to a growing incidence of diet-related obesity and non-communicable diseases, such as coronary heart disease, stroke, and diabetes (Willett et al., 2019).

Considering these pressing challenges, there is a growing recognition of the need for innovative approaches to food production, with research playing a key role in addressing it.

[1] The content of this publication has not been approved by the United Nations and does not reflect the views of the United Nations or its officials or Member States

One such approach is the widespread adoption and valorization of climate-resilient crops, which offer a promising solution to mitigate the adverse effects of climate change on agricultural productivity, while simultaneously ensuring food security and environmental sustainability (Dhankher and Foyer, 2018; Chadalavada et al., 2021; Hossain et al., 2022). These crops, designed to withstand extreme weather conditions, could play a pivotal role in addressing the future demands of a rapidly growing population.

II. Climate resilient crops to address global food challenges

Pulses and grains have been recognized as playing a crucial role in addressing the growing global demand for food. However, current agricultural practices, characterized by intensive monoculture, excessive water use, and the heavy application of chemical inputs, are unsustainable in the long term. These methods contribute to soil degradation, biodiversity loss, and an increase in greenhouse gas emissions (Willett et al., 2019; Zurek et al., 2022).

To secure global food supplies in the face of climate change, one of the most promising strategies is the adoption of climate-resilient crops. Many underutilized plant species, such as quinoa, millet, sorghum, and teff, as well as pulses and fruits like zapote and chaya, have excellent nutritional profiles and traits that are beneficial for adapting food production systems to climate change. However, the increasing simplification of food systems has resulted in the loss of these species and varieties, reducing biodiversity and limiting access to nutrient-dense options from sustainable food systems (Willett et al., 2019). Several authors have highlighted the importance of climate-resilient crops in ensuring food security under changing environmental conditions (Dhankher and Foyer, 2018; Willett et al., 2019; Renzetti et al., 2023). The integration of resilient crops into agricultural systems, combined with sustainable farming practices, is critical for stabilizing food production and ensuring the agricultural sector's adaptation to a rapidly changing climate. In this context, the Food and Agriculture Organization of the United Nations declared 2023 as "The International Year of Millets," aiming to "unleash the potential of millets" as resilient crops that provide affordable and nutritious food options (FAO, 2022). Millets encompass a variety of grains including pearl, proso, foxtail, barnyard, little, kodo, browntop, finger and guinea millets, as well as fonio, teff and sorghum (or great millet) (FAO, 2022). Unleashing the potential of millets may not only contribute to sustainable development by

empowering smallholder farmers and combating hunger, but they also promote biodiversity and support the transformation of agri-food systems (FAO, 2022). Scaling up the cultivation of these crops is a key strategy in advancing the 2030 Agenda for Sustainable Development and achieving the Sustainable Development Goals (United Nations, 2015).

Climate-resilient crops like millets could offer solutions to the substitution of primary cereals, (such as wheat) in western countries, which suffer climate crises in term of crop health, productivity and grain quality (Dhankher and Foyer, 2018; Vogel et al., 2019; Rumler and Schönlechner, 2021; Rumler et al., 2022). Enhancing crop resilience to climate-induced stressors is essential for maintaining yields and mitigating the risk of crop failure, especially in regions highly vulnerable to climate variability. It could also reduce the reliance on wheat imports in regions like sub-Saharan Africa (Renzetti et al., 2023), where only a few countries (Ethiopia, Kenya, Sudan, Nigeria, South Africa, and Zimbabwe) have some capacity to produce wheat (Tadesse et al., 2019).

Consequently, promoting the cultivation of resilient crops could contribute to the diversification of food systems in these regions, reducing reliance on wheat. Simultaneously, these crops could represent a valuable source of novel ingredients in Western countries, aiding in the response to emerging challenges in global food systems.

III. *Sorghum bicolor* (L.) Moench: opportunity and challenge

Global Significance and Emerging Research Trends

Sorghum (*Sorghum bicolor* (L.) Moench) ranks as the fifth most important cereal globally, with an annual production of 62.1 million tons and a cultivation area covering 40.8 million hectares (FAOSTAT, 2021). It is predominantly grown in arid and semi-arid regions such as Africa and Asia, where it serves as a vital staple food and plays a significant role in food security (Belton and Taylor, 2004; Dicko et al., 2006; Ratnavathi CV, 2014; Hariprasanna and Rakshit, 2016). While sorghum flour is traditionally used in numerous African and Asian food products (flat breads and pancakes, dumplings and couscous, porridges, gruels, non-alcoholic fermented beverages, opaque and cloudy beers) (Belton and Taylor, 2004; Ratnavathi, 2014), sorghum is the basis for relatively few food products in the Western world, where products have positive sensory feedback

from consumers (Khoddami et al., 2023). Its primary application in Europe and America is as animal feed (Palavecino et al., 2020). Even though America leads in sorghum production and exports (primarily the United States, Mexico, and Argentina), only 3% of the sorghum produced globally is destined for human consumption. This is especially true in Europe and America, where the grain's potential as a food ingredient remains largely underutilized (Ratnavathi, 2014; Palavecino et al., 2020).

Over the last 20 years, there has been a general increase in publications on 'Sorghum Properties', with the most significant growth occurring between 2010 and 2022. During this period, the number of publications nearly tripled, reflecting growing interest in sorghum's role in sustainability, health, and climate resilience (Figure 2).

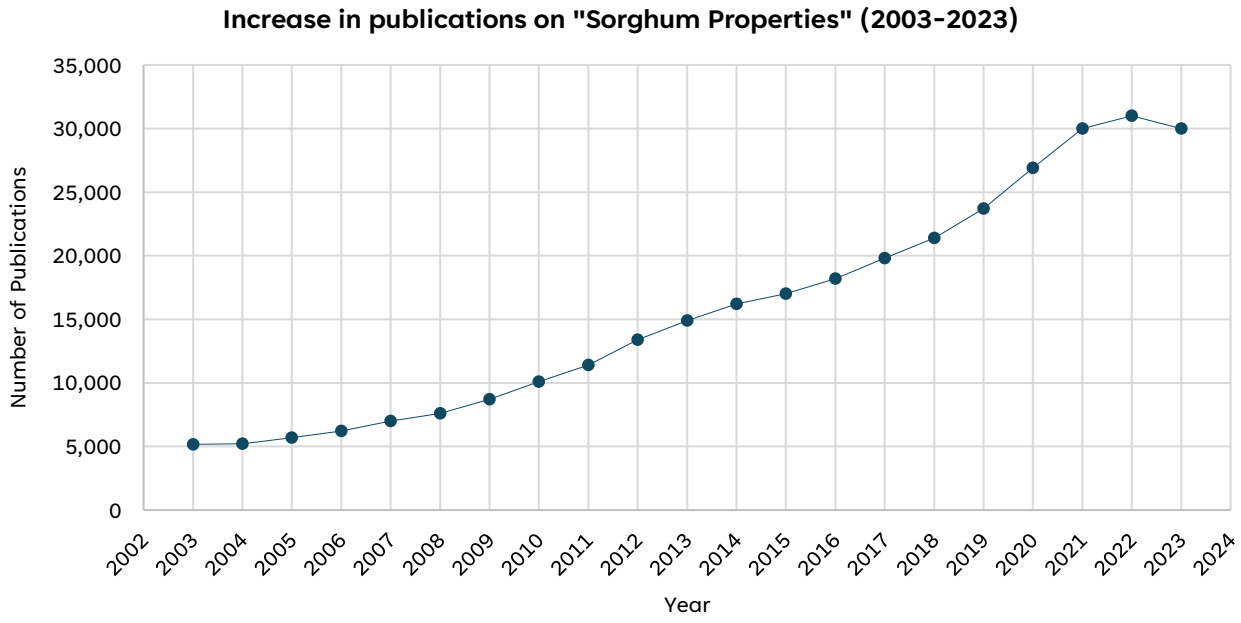

Figure 2. Increase in Publications on 'Sorghum Properties' (2003-2023). Source: Google Scholar.

The interest in exploiting its potential in western diets seemed to be the main factor, mainly due to its agronomic and nutritional properties. Indeed, sorghum is a drought-resistant crop that thrives in diverse environments, requires minimal cultivation input, and is well-suited to marginal lands and resistant to biotic and abiotic stresses (Tari et al., 2013; Abdel-Ghany et al., 2020; Renzetti et al., 2023). These characteristics make it an attractive candidate for broader applications in both food and non-food products.

Nutritionally, sorghum is a valuable source of both macronutrients, with its grain composed of carbohydrates (starch, dietary fibers, soluble sugars) (~80%), followed by proteins (~8-19%) and lipids (~1-4%). (Rhodes et al., 2017; Serna-Saldivar and Espinosa-Ramírez, 2019). Sorghum is also a source of micro-nutrients such as minerals (phosphorus, potassium, iron, and zinc), B-vitamins and fat soluble vitamins (A, D, E, K) (de Morais Cardoso et al., 2017), as well as phenolic compounds such as phenolic acids, flavonoids (especially 3- deoxyanthocyanins) and tannins, with many potential health benefits (Awika and Rooney, 2004; Dykes and Rooney, 2006, 2007; Duodu and Awika, 2019; Xiong et al., 2019). Furthermore, sorghum is gluten-free, presenting a promising alternative for individuals with celiac disease or gluten-related disorders (Ciacci et al., 2007; Pontieri et al., 2013).

Critical issues related to the consumption and use of sorghum

While the consumption of sorghum appears to have significant environmental and nutritional advantages, with potential health benefits, there are significant critical issues related to both the digestibility of its nutrients and the technological functionality of the flour. Poor nutrients digestibility appears to be related both to the presence of anti-nutritional factors and to the inherent structure of sorghum grain, which results in its difficulty in being attacked by digestive enzymes. In addition, the structure and conformation of polymers such as starch and protein determine sorghum technological functionality, negatively impacting the potential applications of this grain on a larger scale. Sorghum starch and protein structure, nutrient digestibility and the technological functionality of sorghum flour will be presented in the next paragraphs.

Starch and protein structure

To address the critical challenges associated with the consumption and application of sorghum in food formulations, it is essential to examine the structural characteristics of its major components, particularly starch and proteins. As the predominant constituents of sorghum flour, starch and proteins play a pivotal role in determining both the nutritional and technological functionalities of sorghum, which have potential implications for its food applications.

Sorghum starch is primarily located within the endosperm and is organized into granules composed of amylose (approximately 20-30%) and amylopectin, held together by hydrogen bonds (Serna-Saldivar and Espinosa-Ramírez, 2019). These granules exhibit distinct structural features,

including indentations, surface pores, and radial channels that extend to a central cavity at the hilum, which are critical for facilitating enzymatic reactions (Zhu, 2014). Sorghum starch granules possess a pseudocrystalline structure, characterized by alternating amorphous and semi-crystalline layers, with thicknesses ranging between 100 and 400 nm (Pflugfelder and Rooney, 1986; Pérez and Bertoft, 2010; Zhu, 2014). The semi-crystalline regions are composed predominantly of amylopectin, which is resistant to water penetration and enzymatic degradation, whereas the amorphous regions, rich in amylose, allow for water diffusion and facilitate enzymatic action (Pflugfelder and Rooney, 1986).

The protein composition of sorghum is dominated by prolamins known as kafirins, which are stored in protein bodies within the endosperm (Figure 3) (De Mesa-Stonestreet et al., 2010). Kafirins are classified into three major types based on their molecular weight and solubility: α-kafirins (66-84%), β-kafirins (8-13%), and γ-kafirins (9-21%) (De Mesa-Stonestreet et al., 2010). The α-kafirins, with a molecular weight of 23-25 kDa, are rich in nonpolar amino acids and form primarily intramolecular disulfide bonds. In contrast, β-kafirins (18 kDa) contain sulfur-rich amino acids and engage in both intra- and intermolecular disulfide bonding. γ-kafirins (20 kDa), characterized by a high content of proline, cysteine, and histidine, are highly crosslinked, contributing to their structural rigidity (De Mesa-Stonestreet et al., 2010). The β- and γ-kafirins, which are localized at the periphery of the protein bodies, form extensive disulfide crosslinks that impede the digestion of the more digestible α-kafirins located in the core (Duodu et al., 2003). Additionally, the high hydrophobicity of kafirins further reduces their susceptibility to enzymatic degradation (Duodu et al., 2003). Sorghum protein bodies are also embedded in a matrix of non-kafirin proteins, which serve to anchor them to the surrounding starch granules, thereby impacting both the digestibility and functionality of sorghum in food systems (Figure 3) (Hamaker and Bugusu, 2003). This intricate structural organization of sorghum starch and proteins presents a significant challenge for improving the bioaccessability of nutrients and optimizing sorghum's use in various food applications.

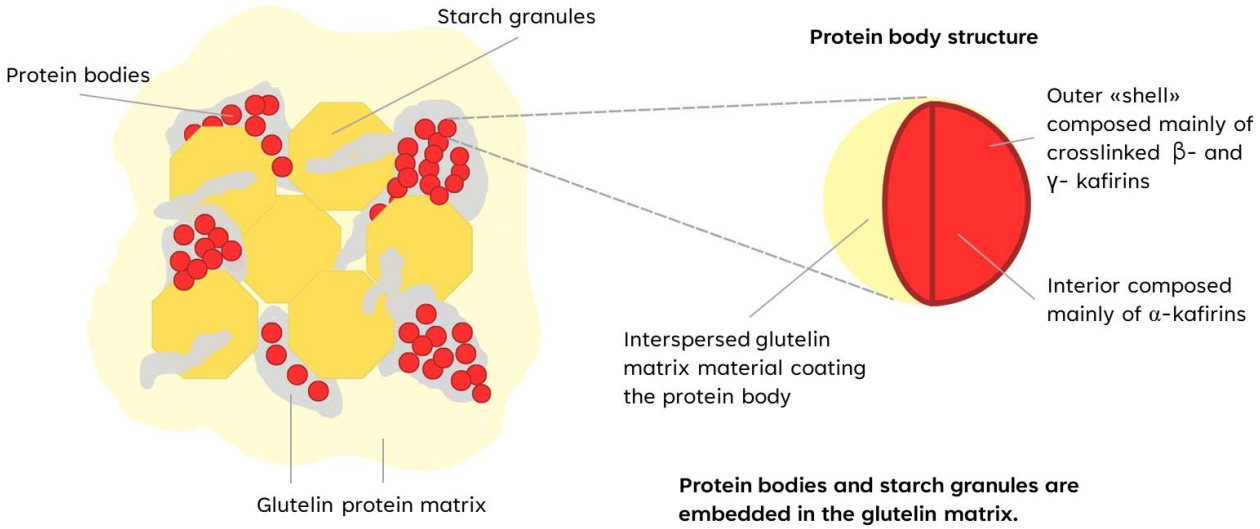

Figure 3. Sorghum protein bodies in relation to starch and the glutelin matrix and schematic representation of a protein body. Adapted from De Mesa-Stonestreet et al. (2010).

Digestibility of nutrients

Several factors have been identified as contributing to the digestibility of sorghum, including its physical grain structure, starch properties, protein composition, protein body structure, protein cross-linking, and the phenolic content and composition of the grain (Bean et al., 2011).

Sorghum starch digestion is generally regarded as the least efficient among grains (Zhu, 2014). Depending on the context, this low digestibility can be seen as either advantageous, such as in the production of low glycemic index foods, or problematic in regions where sorghum is a staple food and a primary energy source. Starch digestion is influenced by multiple factors, including granule size, crystalline structure, degree of crystallinity, surface properties, polymerization degree, interactions with non-starch components (such as proteins and tannin), and the amylose-to-amylopectin ratio (Mahasukhonthachat et al., 2010; Barros et al., 2012; Bean et al., 2019). Additionally, the interaction between starch and proteins, as well as the encapsulation of starch within protein matrices, limits enzyme access, contributing to the higher levels of resistant starch in sorghum compared to other grains (Benmoussa et al., 2006; Mahasukhonthachat et al., 2010; Zhu, 2014).

However, a significant challenge with sorghum is the poor digestibility of its proteins (Axtell et al., 1981; Duodu et al., 2003; Belton and Taylor, 2004). The digestibility of kafirins, the major storage proteins in sorghum, is heavily influenced by their structural arrangement and organization (Duodu et al., 2002, 2003). Their digestibility is further reduced by interactions with other components, either through chemical bonding or physical barriers (Duodu et al., 2003). Protein-protein cross-linking, particularly in γ- and β-kafirin, contributes to this reduction, as these proteins, located at the periphery of protein bodies, encapsulate α-kafirin, thus impeding digestion (Duodu et al., 2003). Additionally, exogenous factors such as interactions with non-protein components—polyphenols, starch, phytates, lipids, and cell wall components—further affect sorghum protein digestibility (Duodu et al., 2003).

Bioactive components, which are more abundant in sorghum than in other grains, have been studied for their positive health effects, such as maintaining health or preventing chronic disease (de Morais Cardoso et al., 2017; Amarakoon et al., 2021). However, like other cereals, sorghum contains several antinutritional factors that can significantly reduce its nutritional benefits. The limited absorption of certain nutrients in sorghum can have negative dietary implications, particularly in populations where it serves as a staple food (Bean et al., 2019; Duodu and Awika, 2019). Phytic acid, tannins, and protease inhibitors can reduce sorghum protein and starch digestibility. Phytic acid not only binds to starch and proteins, reducing their digestibility, but also chelates positively charged minerals such as zinc, iron, calcium, magnesium, potassium, and manganese, forming insoluble complexes that hinder mineral absorption (Afify et al., 2011; Bean et al., 2019). Tannins, while strong antioxidants, have a greater negative impact on nutrient bioavailability, particularly those with higher molecular weight or more complex structures (Mkandawire et al., 2013; Barros et al., 2014; de Morais Cardoso et al., 2017). Polymeric tannins, in particular, play a key role in the formation of resistant starch due to their strong interaction with amylose (Barros et al., 2014; de Morais Cardoso et al., 2017). Moreover, tannins can form complexes with proteins, further reducing their digestibility, especially in pigmented sorghum varieties (Afify et al., 2012; Duodu and Awika, 2019). Certain sorghum varieties also contain protease inhibitors (e.g., trypsin, chymotrypsin) and amylase inhibitors, as well as lectins, which further reduce protein and carbohydrate digestibility, as well as mineral bioavailability (de Morais Cardoso et al., 2017).

Sorghum technological functionality for food applications

When sorghum is incorporated into various products, several functional challenges arise. As for the digestibility, the technological functionality of sorghum's starch and proteins is not solely dictated by their intrinsic properties but also by interactions with other biopolymers, such as starch-protein interactions and those involving tannins and phenolic acids.

In terms of technological functionality, sorghum flour is characterized by relatively high gelatinization temperatures and a generally low degree of gelatinization (Taylor et al., 2006; Zhu, 2014; Serna-Saldivar and Espinosa-Ramírez, 2019). This behavior is largely attributed to the inherent characteristics of sorghum starch, including its amylopectin-to-amylose ratio and the chain length of amylopectin (Taylor et al., 2006). The amylose/amylopectin ratio plays a crucial role in determining key technological properties of sorghum starch, such as its rheological behavior, gelatinization profile, retrogradation tendency, gelling properties, and digestibility (Ratnavathi, 2018). These properties, however, are further modulated by interactions within the surrounding matrix, including proteins and tannins. As noted by Taylor et al. (2006), sorghum starch's gelatinization is hindered by its entrapment in a hydrophobic protein matrix, which is stabilized by cross-linking through disulfide bonds. Native sorghum starch granules also exhibit resistance to enzymatic degradation (Serna-Saldivar and Espinosa-Ramírez, 2019) and possess low β-amylase activity (Taylor et al., 2006).

Additionally, sorghum cell wall polysaccharides differ from those in wheat and barley, predominantly consisting of insoluble glucuronoarabinoxylans, which may introduce further technological implications (Taylor et al., 2006).

Unlike wheat proteins, sorghum proteins, particularly kafirins, lack the ability to form gas-holding, viscoelastic doughs, limiting their application in leavened products (Taylor and Dewar, 2001). Kafirins are more hydrophobic compared to wheat gluten, which makes them less capable of absorbing water and forming elastic networks (Duodu et al., 2003). This poor dough formation further restricts the technological functionality of sorghum in specific food applications (De Mesa-Stonestreet et al., 2010). Moreover, during thermal processing, outer β- and γ-kafirins undergo polymerization via disulfide bonds, encapsulating the α-kafirins. This process leads to the formation of web-like or sheet-like structures, significantly affecting the functionality of both

sorghum proteins and starch, and thus the technological and nutritional properties of sorghum-based products (Hamaker and Bugusu, 2003).

Furthermore, tannins in sorghum directly impact the sensory properties of sorghum-based products, often resulting in bitter and astringent flavors. While decortication can reduce the astringency by removing tannin-rich outer layers, this process can also reduce the concentration of beneficial secondary compounds such as phenolics (Khoddami et al., 2023).

Sorghum application in western style bread

Sorghum flour has long been a staple in the production of traditional flatbreads such as roti, injera, and kisra in various parts of Asia and Africa (Ari Akin et al., 2022). Despite its longstanding use in these regions, its application in Western-style leavened bread remains limited. This is largely due to the fact that sorghum lacks the viscoelastic properties inherent to wheat proteins, particularly gluten, which is essential for the formation of gas-holding dough. As consumer interest in climate-resilient crops grows, more research has been conducted to assess sorghum's potential in leavened bread production (Rumler and Schönlechner, 2021; Ari Akin et al., 2022; Rumler et al., 2022). In regions where wheat is not widely produced, or where imports are expensive, partial replacement of wheat flour with sorghum flour could offer both economic and environmental benefits. Furthermore, climate change has made it increasingly difficult to maintain consistent wheat yields in many Western countries, creating a need for alternative grains like sorghum (Rumler and Schönlechner, 2021).

One of the main challenges in utilizing sorghum for breadmaking lies in its protein composition. Due to its inability to form a gas-trapping network during fermentation, sorghum, like other gluten-free grains, produces doughs with poor structure, leading to lower-quality bread (Espinoza-Herrera et al., 2021). Sorghum prolamins, particularly kafirins, are rich in hydrophobic amino acids such as glutamine, alanine, leucine, and proline, which makes them difficult to hydrate. The presence of disulfide bonds further exacerbates this issue by contributing to dough stiffness and rigidity (Belton et al., 2006; Espinoza-Herrera et al., 2021). Additionally, the encapsulation of kafirins in protein bodies prevents effective interaction with other dough components during mixing, thereby limiting the formation of a cohesive network necessary for gas retention (Espinoza-Herrera et al., 2021).

When sorghum flour is blended with wheat flour, the dilution of gluten proteins typically leads to reduced dough quality. Studies have shown that this results in decreased water absorption, dough stability (Rumler and Schönlechner, 2021). The inclusion of 10-15% sorghum in wheat flour has been associated with lower extensibility of the final dough (Seleem and Omran, 2014; Dube et al., 2021). Farinograph data, although somewhat inconsistent due to variations in experimental conditions and flour types, generally support the observation that sorghum negatively impacts the rheological properties of wheat dough (Rumler and Schönlechner, 2021).

As the proportion of sorghum in bread formulations increases, a corresponding decline in bread quality is observed. Studies report an increase in firmness and decrease in specific volume, crumb porosity, and elasticity with increasing sorghum content (Elkhalifa and El-Tinay, 2002; Angioloni and Collar, 2012; Sibanda et al., 2015; Rumler and Schönlechner, 2021; Ari Akin et al., 2022). Sensory analyses have shown that sorghum flour can negatively affect the sensory properties of wheat bread, with the extent of the impact varying depending on the level of inclusion and the panelists' familiarity with sorghum's flavor. These differences in perception may vary significantly between African and Western countries (Keregero and Mtebe, 1994; Sibanda et al., 2015; Dube et al., 2021; Rumler and Schönlechner, 2021; Ari Akin et al., 2022; Khoddami et al., 2023).

In addition to impacting the quality of fresh bread, sorghum flour also influences the staling process, which leads to a gradual deterioration in texture and flavor over time (Fadda et al., 2014). However, research on the staling of wheat-sorghum composite bread is limited. Initial studies suggest that sorghum can accelerate the quality degradation compared to pure wheat bread (Hugo et al., 2000; Angioloni and Collar, 2012). However, these authors investigated only texture and sensory properties of bread over time, while a deeper understanding of sorghum’s role in staling would be essential for improving both shelf life and the overall quality of these products.

IV. Potential of bioprocessing: sprouting and fermentation

Bioprocesses such as sprouting and fermentation hold considerable potential to support the sustainable transition of agrifood systems by enhancing agricultural productivity and sustainability, promoting healthy and sustainable plant-based diets, and improving food waste management practices (Herrero et al., 2020; Jahn et al., 2023). By enhancing food security,

improving nutrition, promoting sustainable agriculture, and reducing environmental impact, they align with several Sustainable Development Goals (SDGs) set for the 2030 agenda, such as *life on land* (SDG n. 15), *responsible consumption and production* (SDG n. 12), *sustainable cities and communities* (SDG n. 11), *zero hunger* (SDG n. 2), and *good health and well-being* (SDG n. 3).

The trend of eating foods containing sprouted and fermented grains has increased in recent years (Olaerts and Courtin, 2018). Sprouting and fermentation are treatments well perceived by consumers demanding healthy and bio-processed foods (Lemmens et al., 2019; Diez-Ozaeta and Astiazaran, 2022). Potentially, sprouting and fermentation can improve the nutritional and technological functionality of grains and pulses (Nkhata et al., 2018). The use of sprouted and/or fermented grains and pulses in leavened bread formulations could be an effective strategy to increase the nutritional value of bread, which is a worldwide consumed food.

The use of fermentation and sprouting in processing sorghum flour has long been a traditional practice in African and Asian countries. Traditional African foods made from sorghum and millet, such as porridges, alcoholic beverages, gruel, pancakes, flatbreads, breakfast cereals, and infant foods, typically undergo lactic acid fermentation, malting, or both during their production (Belton and Taylor, 2004; Oluwafemi, 2020). However, some of these foods present also food safety risks, associated with the growth of opportunistic pathogens and/or their toxins production (Oluwafemi, 2020). Recent studies have also focused on the application of bioprocessing to sorghum flour to improve the nutritional and functional properties of sorghum, with the focus of harnessing its potential as a new ingredient for breadmaking, with focus related to sustainability and climate change issues, which may fit into future food systems projections (Cardone et al., 2021; Verni et al., 2024).

Sprouting and its impact on grains functionality

During sprouting, water uptake activates the seed's metabolism, mobilizing reserves for radicle growth (Nonogaki et al., 2010; Benincasa et al., 2019; Lemmens et al., 2019). This process induces significant biochemical changes in grains: starch is broken down by α-amylase, nitrogen fractions shift towards oligopeptides and free amino acids, triacylglycerols are hydrolyzed, and the ratio of saturated to unsaturated fatty acids increases. Anti-nutritional factors like phytate, trypsin inhibitors, and tannins decrease, while bioactive compounds such as phenolics, phytosterols,

folates, and GABA increase (Benincasa et al., 2019; Lemmens et al., 2019). As a result, starch and protein digestibility improves, and minerals and bioactive compounds become more accessible to digestive enzymes (Lemmens et al., 2019). These changes make sprouted grains nutrient-rich and high in antioxidants, qualifying them as 'functional foods' (Benincasa et al., 2019). Sorghum sprouting has been studied to modify and improve the nutritional, physicochemical and functional properties of sorghum (Rashwan et al., 2021). In sorghum, sprouting has been reported to increase both the availability and digestibility of starch and protein, protein solubility, bioavailability of minerals and bioactive compounds, and to reduce the anti-nutritional compounds (Afify et al., 2011, 2012; Lemmens et al., 2019; Saithalavi et al., 2021).

From a technological perspective, sprouting can modify the functionality of grains, affecting their use in baking and other food applications. While sprouting can improve the nutritional profile of grains, it can also lead to challenges in dough processing and baking performance. Excessive enzymatic activity, particularly from proteases and α-amylases, can degrade gluten and starch, resulting in a loss of dough structure, a sticky crumb, and large holes in baked goods (Liu et al., 2017; Marti et al., 2018). This is due, in part, to the hydrolysis of high molecular weight glutenin macropolymers, which are essential for the formation of a strong, elastic gluten network (Baranzelli et al., 2018). Similarly, the hydrolysis of starch and other polysaccharides can reduce dough viscosity and limit the gelation capacity of amylose, further compromising the quality of the final product (Doehlert and McMullen, 2003; Lemmens et al., 2019). Managing sprouting time revealed to be a good strategy to obtain high quality leavened products, by enhancing the hydrolysis of damaged starch into fermentable sugars, which increases carbon dioxide production and improves dough height (Marti et al., 2017). Additionally, sprouted grains have been shown to improve bread shelf life by slowing crumb firming and reducing staling, thanks to the action of amylases on starch retrogradation (Liu et al., 2017).

Even in sorghum, the technological properties of the starch fraction have been reported to worsen after sprouting (Li et al., 2017; Marchini et al., 2021b). Upon sprouting, the proteases and amylases of sorghum act reducing protein-starch interactions (Lemmens et al., 2019), but at the same time, amylases can breakdown starch granule structure, thus limiting the ability of the granules to gelatinize (Li et al., 2017; Lemmens et al., 2019). However, some studies have reported that post-sprouting drying conditions (Marchini et al., 2021b) and sprouting time and

temperature (Singh et al., 2017; Cardone et al., 2021) tailor the effects of sprouting, including the technological functionality. In particular, the time-temperature combinations of the sprouting and post-sprouting drying treatments influence the degree of grain modification through the modulation of the enzyme activity (Singh et al., 2017; Cardone et al., 2021; Marchini et al., 2021b), providing interesting insights into modulating sprouting conditions and improving the applicability of sprouted sorghum flour in food formulations.

Fermentation and its impact on grains functionality

Fermentation is another bioprocess that has been widely used to improve the nutritional and functional properties of grains. By lowering the pH and producing antimicrobial compounds, fermentation helps to inhibit the growth of harmful microorganisms, thus enhancing food safety and extending the shelf life of fermented products (Nkhata et al., 2018; Jahn et al., 2023). In addition to improving food safety, fermentation also reduces anti-nutritional factors in cereals such as phytic acid and oxalates, thereby increasing the bioavailability of essential minerals like calcium, iron, and zinc (Gobbetti et al., 2019). The presence of microbial residues and fermentation metabolites may also have positive effects on human health by interacting with the gut microbiome, although further research on human studies is needed to confirm these benefits (Jahn et al., 2023). Fermentation has been reported to influence the technological properties of food, by enhancing flavor and texture, and shelf life (Gobbetti et al., 2019), which can support shifts toward sustainable diets (Jahn et al., 2023).

In breadmaking, fermentation has been shown to significantly enhance the technological properties of dough and the quality of the final product (Garrido-Galand et al., 2021). Sourdough fermentation leads to several advantages on a technological level In terms of loaf volume; softness, resilience, and color; crust thickness, friability, crispiness, and color, flavor (taste and aroma of crumb and crust), and shelf-life (microbial spoilage as well as bread staling) (Katina et al., 2006a; Arendt et al., 2007; Gänzle et al., 2008; Pico et al., 2015; De Vuyst et al., 2017). Some studies have shown that sourdough starters enhance the activity of α-amylase and other enzymes, modifying starch and protein structure, which affects amylopectin retrogradation (Fadda et al., 2014). Additionally, microbial exopolysaccharides (EPS) help stabilize the dough and influence moisture retention and crust structure during storage (Fadda et al., 2014).

Sourdough fermentation has also been reported to lower the glycemic index of bread, reduce phytate content, and release bioactive compounds such as GABA, which has been associated with health benefits such as blood pressure regulation and improved cognitive function (Coda et al., 2010; Nakamura et al., 2013; D'Alessandro and De Pergola, 2014; Gobbetti et al., 2014, 2019).

Although the general benefits of fermentation are well-established, research on the fermentation of sorghum flour has primarily focused on its nutritional improvements. Most studies report enhanced starch and protein digestibility, a reduction in antinutritional factors, as well as increased availability of phenolics (Elkhalifa et al., 2004a; Svensson et al., 2010; Pranoto et al., 2013; Mohapatra et al., 2019; Adebo and Medina-Meza, 2020; Garzón et al., 2020; Ge et al., 2020; Olojede et al., 2020b; Rashwan et al., 2021; Ofosu et al., 2022).

Studies examining the impact of fermentation on the techno-functional properties of sorghum flour are summarized in (Table 1). However, these studies often rely on spontaneous fermentation, replicating traditional household practices (Hugo et al., 2003; Elkhalifa et al., 2004b, 2004a, 2005, 2006, 2017; Ge et al., 2020). Such processes involve the development of a complex and location-specific microbial ecosystem, making them difficult to reproduce consistently. To promote the inclusion of sorghum in Western diets and support the demand for sustainable food systems, it is necessary to transition from spontaneous to rationally designed fermentation processes. Of the limited studies on sorghum fermentation in breadmaking, only 8 have examined its impact on bread quality, while most focused on flour, sourdough, or dough characterization (Table 1). Additionally, most of the bread applications have been restricted to gluten-free formulations (Schober et al., 2007; Galle et al., 2012; Karrar et al., 2020; Olojede et al., 2020a, 2022). Research on the effects of sorghum fermentation with selected LAB strains in conventional bread, where sorghum partially replaces wheat flour, remains scarce (Hugo et al., 2003; Istianah et al., 2018; Karrar et al., 2020). Expanding this research could broaden sorghum's application in widely consumed products, appealing to a larger consumer base. In most cases, fermented sorghum has been incorporated as fresh sourdough, with few studies investigating the use of dried fermented sorghum flour (Table 1). Furthermore, only a limited number of studies have explored the effect of sorghum fermentation on bread staling, focusing primarily on texture characterization (Hugo et al., 2003; Schober et al., 2007).

Table 1. Studies on the effect of fermentation on the technological properties of sorghum flour, sourdough, dough and bread. S = sorghum; WS = wheat-sorghum; GF = gluten-free.

Reference	Inoculum	Target sample	Type of fermented sorghum	Sample characterization
Hugo et al. (2003)	Spontaneous fermentation	WS bread	Sourdough/ Dry fermented flour	pH and titratable acidity (TTA); flour color, moisture, protein, total starch and enzyme susceptible starch, pasting properties and optimum absorption; In vitro protein digestibility; Bread moisture content, volume and specific volume, crumb firmness (3 days of storage), sensory evaluation.
Elkhalifa et al. (2004a)	Spontaneous fermentation	S flour	Fermented flour	pH, TTA, starch digestibility, resistant starch and total starch, swelling power, iodine absorption capacity, water retention and starch solubility
Elkhalifa et al. (2004b)	Spontaneous fermentation	S flour	Fermented flour	Starch isolation, water retention, starch solubility, stability and clarity of starch pastes, freeze thaw stability
Correia et al. (2005)	Spontaneous fermentation/ *L. fermentum, L. bulgaricus, L. lactis, P. pentosaceus, P. cerevisiae*/ *L. bulgaricus* + *S. thermophilus*	S flour	Fermented flour	pH and TTA, reducing and total sugars, soluble proteins, total amino acids, insoluble proteins, starch content, High resolution ^{1}H NMR, Fourier Transform Infrared FT-IR
Elkhalifa et al. (2005)	Spontaneous fermentation	S flour	Fermented flour	Protein solubility, least gelation concentration, bulk density, WHC, OHC, emulsifying capacity and stability, foaming capacity
Elkhalifa et al. (2006)	Spontaneous fermentation	S flour	Fermented flour	Aggregation state and thiol content of proteins, total and damaged starch, pasting properties, light and electronic microscopy, titratable acidity and pH determination, SDS page
Schober et al. (2007)	*L. plantarum*	GF sourdough GF bread	Sourdough	Sourdough pH, TTA, and consistency, protein modification (SE-HPLC), starch damage, rheological analysis; Bread crumb grain, texture (7 days of storage), microscopic analysis (LSCM)
Galle et al. (2011)	*L. casei* FUA3185, *L. casei* FUA3186, *L. buchneri* JCM1115, *W. cibaria* 10M	S Sourdough GF dough	Sourdough	Sourdough extraction and characterization of EPS, pH, cell counts, RNA isolation and cDNA synthesis, rheology; Dough metabolite and sugar analysis; viscosity of cell suspensions
Galle et al. (2012)	*L. reuteri* Y2, *L. reuteri* VIP, *W. Cibaria* MG1	S Sourdough GF dough GF bread	Sourdough	Sourdough determination of cell counts, pH and metabolite formation, determination and analysis of oligosaccharide and EPS, Batter pH and TTA; Dough gaseous release and dough development (Rheofermentometer), rheology; Bread pH and TTA, texture
Pranoto et al. (2013)	Spontaneous fermentation/ *L. plantarum*	S flour	Fermented flour	pH, TTA, protein, in-vitro protein digestibility (IVPD), starch content and in-vitro starch digestibility (IVSD), pasting properties
Ogunsakin et al. (2015)	*P. pentosaceus* + *S. cerevisiae*	GF bread	Sourdough	Bread proximate analysis, determination of Ca, K, Mg, bread height, weight, sensory evaluation, visual shelf life (mold growth)
Elkhalifa et al. (2017)	Spontaneous fermentation	S flour	Fermented flour	Stability and clarity of sorghum flour paste, freeze–thaw stability, dispersibility, swelling index, swelling power, starch digestibility, gel consistency and gelling temperatures, starch structural indexes, protein structural indexes, pasting properties
Istianah et al. (2018)	*L. plantarum* + *S. cerevisiae*	S flour WS bread	Dry fermented flour	Flour proximate composition, pasting properties, phenolic content. Bread volume, texture and color
Ge et al. (2020)	Spontaneous fermentation	S flour	Fermented flour	Starch content and resistant starch, starch digestibility, resistant starch molecular weight, molecular functional group of resistant starch, Starch Molecular Chain Length Distribution, Degree of Crystallinity of Sorghum-Resistant Starch, Particle Morphology of Sorghum- Resistant Starch, determination of short chain fatty acid
Karrar et al. (2020)	*L. brevis, L. plantarum*	S sourdough WS dough WS bread	Sourdough	Sourdough bacterial total count, pH and TTA, Dough fermentation properties (rheofermentometer); Bread weight, volume, weight loss, texture, sensory evaluation
Olojede et al. (2020a)	*P. pentosaceus* SA8, *W. confusa* SD8, *P. pentosaceus* LD7, *S. cerevisiae* YC1	GF bread	Sourdough	Sourdough rheological properties; Bread texture, microstructure (SEM), proximate analysis, visual shelf life (mold growth)
Olojede et al. (2022)	*P. pentosaceus* LD7 (PL7), *P. pentosaceus* SA8 (PS8), *W. confusa* SD8 (WS8)	GF bread	Sourdough	Bread volume, weight, baking yield, proximate composition, color, texture and microstructure (SEM); determination of bioactive compounds tannins, total flavonoids, total phenolics), radical scavenging activity (DPPH); sensory evaluation, visual shelf life (mold growth)

V. Final considerations

In recent years, there has been a significant increase in research on sorghum, particularly with regard to its potential health benefits and various strategies to improve its nutritional profile. Many studies have shown how bioprocessing methods such as fermentation and germination can reduce anti-nutritional factors and improve the digestibility of key nutrients, making sorghum a valuable ingredient for health-conscious consumers. However, despite these advances, the incorporation of sorghum flour into a wide range of food formulations, particularly in baking, continues to pose significant technological challenges. Sorghum's inability to form a viscoelastic network of gluten affects dough structure, resulting in lower quality products in terms of texture, volume and overall sensory properties.

In addition, there is a significant gap in the literature on the targeted use of bioprocesses to improve the quality of commodity products, such as composite bread. Although the use of bioprocesses such as sourdough fermentation and controlled sprouting is promising for improving the technological functionality of sorghum flour, specific studies on their effectiveness are limited. In addition, research on the impact of sorghum inclusion and bioprocesses on bread staling, shelf life and other critical quality parameters remains scarce. These areas are particularly important for reducing food waste and improving the sustainability of food production systems.

Addressing these research gaps could significantly advance the potential of sorghum as a sustainable, nutritious grain for broader food applications, contributing to future innovations in food processing and helping to meet global food security and sustainability goals.

Research Objectives and Thesis Structure

The primary objective of this Ph.D. thesis is to address existing knowledge gaps regarding the use of bioprocessing techniques on sorghum flour, with the goal of enhancing its technological functionalities for food applications.

This research pursued three main objectives:

(i) to understand how the molecular changes brought about by different bioprocessing conditions drive diverse technological functionalities;
(ii) to analyze the application of bioprocessed sorghum flour in food formulations;
(iii) to assess the overall quality of the final product in a real-world scenario, focusing on both freshly produced products and their quality throughout shelf life.

To accomplish these objectives, this Ph.D. thesis is organized into three main sections (Figure 4):

- Section 1: Impact of Sprouting and Fermentation on Sorghum Flour Properties (*Case Studies 1* and *2*)
- Section 2: Effects of LAB Fermentation on Wheat-Sorghum Bread Quality (*Case Studies 3* and *4*)
- Section 3: Bread Staling and Its Mitigation (*Case Studies 5* and *6*)

Section 1: Impact of Sprouting and Fermentation on Sorghum Flour Properties

This section explores the impact of sprouting and fermentation on the molecular and technological properties of sorghum flour. In *Case Study 1*, the effects of sprouting drying treatment, and different water contents were evaluated, particularly focusing on the functionality of sorghum starch, which is known to be limited by its interaction with the surrounding matrix. This study aimed to generate insights relevant to practical applications in food products with

varying moisture levels. In *Case Study 2*, this Ph.D. thesis investigated the molecular structure and mobility changes in sorghum flour resulting from fermentation with selected strains of lactic acid bacteria (LAB). The functionality of sorghum flour, as shaped by molecular modifications and interactions, was then examined in terms of its rheological, pasting, and thermal properties, thus enhancing the understanding of its impact on food formulations, especially for bakery applications.

Section 2: Effects of LAB Fermentation on Wheat-Sorghum Bread Quality

The second section of this thesis examines the application of fermented sorghum flour in wheat-sorghum bread and dough formulations. *Case Study 3* assessed the impact of different sorghum batters on key quality parameters affecting bread quality and consumer purchasing decisions. Specifically, the fermentation effects of three LAB strains were evaluated concerning bread's appearance, texture, crumb porosity, color, volatile and sensory attributes. Building on the findings from *Case Studies* 2 and 3, *Case Study 4* analyzed the effects of a single selected LAB strain on the rheological and molecular properties of wheat-sorghum dough. Moreover, the effect of freeze drying on the functionality of sorghum fermented flour was also evaluated. This investigation offered insights into the molecular interactions between wheat and sorghum in dough, which are key determinants of specific bread attributes.

Section 3: Bread Staling and Its Mitigation

The third section addresses the crucial topic of analyzing the quality of wheat sorghum bread over storage. *Case Study 5* investigated the impact of untreated sorghum flour, in various concentrations, on the staling phenomenon of wheat-composite bread. Given the limited research base on this topic, this study aimed to fill critical knowledge gaps regarding staling effects in sorghum-wheat formulations at different structural levels. Leveraging insights from *Case Studies* 2, 3, and 4, and the base knowledge obtained from study 5, in the last Case Study (6), the thesis evaluated how fermentation with a selected LAB strain and the freeze-drying process influenced the staling dynamics in wheat-sorghum composite bread. In both *Case Study 5* and 6, the staling

process was examined at the molecular, mesoscopic, and macroscopic levels, in order to establish a solid foundation for understanding the staling phenomenon and its mitigation in wheat-composite bread formulations.

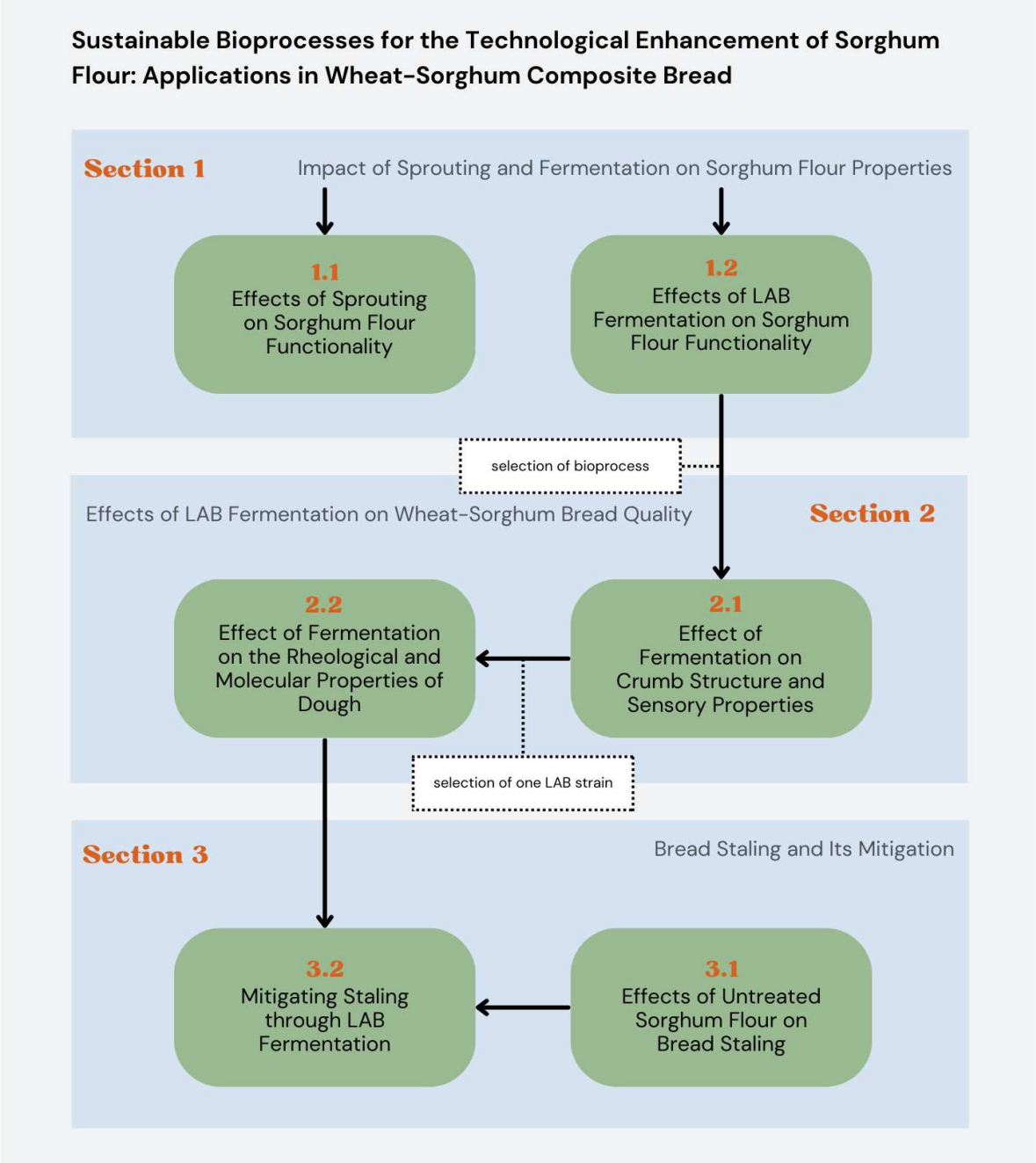

Figure 4. Ph.D. Thesis structure.

Section 1

SECTION 1

Impact of Sprouting and Fermentation on Sorghum Flour Properties

The use of sorghum in food applications faces challenges due to its composition, structure, and the interactions among its macromolecules, especially starch and proteins. Sprouting and fermentation, as sustainable bioprocesses, can significantly modify these characteristics by activating endogenous and exogenous enzymes, thus enhancing sorghum's potential for food applications. This section addresses gaps in the literature regarding sorghum bioprocessing, focusing on the impacts of sprouting and fermentation on sorghum flour's molecular and functional properties. Specifically, it examines how these processes influence sorghum's starch, protein, and other macromolecules, shaping its technological functionality. In *Case Study 1*, the effect of sprouting and drying treatments on sorghum starch functionality is investigated at different water contents, thus giving insights into the potential of application in products with different hydration levels. *Case Study 2*, explores fermentation with selected lactic acid bacteria (LAB) strains, assessing its impact on sorghum molecular status and on its rheological, thermal, and pasting properties. These insights are foundational for optimizing sorghum flour's functionality in bakery products. By identifying conditions that maximize sprouting and fermentation benefits, this section advances sorghum's utilization in the food industry, thus promoting its high-performance for diverse food applications.

1.1 Effects of Sprouting on Sorghum Flour Functionality

Case Study 1

Effect of water content on gelatinization functionality of flour from sprouted sorghum

Introduction and Aim of the Study

Starch gelatinization widely contributes to the functionality of flours and their food applications (Goldstein et al., 2010; Zhu, 2014; Schirmer et al., 2015; Donmez et al., 2021). Indeed, when heated in the presence of excess water, the starch granules undergo a series of structural changes that are reflected from the molecular to the macroscopic level (Schirmer et al., 2015). First, the granules absorb water in the amorphous regions and swell, then water is absorbed by the crystalline regions, leading to the breakdown of amylopectin crystal structures and finally of the granule itself (Zhu, 2014; Donmez et al., 2021). Simultaneously with the melting of starch crystallites and the leaching out and solubilization of amylose, changes in physicochemical properties occur, such as the loss of birefringence and the increase in viscosity (Wang and Copeland, 2012; Zhu, 2014; Schirmer et al., 2015).

The degree of starch gelatinization depends on several factors (heating rate, temperature, water content, presence of other components such as salts, sugars, proteins, lipids, non-starch polysaccharides) (Donmez et al., 2021). Among them, water content is of paramount importance; moreover, the interactions of other components of the food matrix both with water molecules and starch domain affect starch gelatinization (Wang and Copeland, 2012, 2013; Schirmer et al., 2015; Donmez et al., 2021). Usually, increasing levels of water favor high or complete gelatinization degree, increasing the DSC gelatinization enthalpy and granule swelling, and lowering the gelatinization temperatures (Wang and Copeland, 2012; Donmez et al., 2021). Different levels of gelatinization can influence food processing parameters and physico-chemical properties of final products (Schirmer et al., 2015). Therefore, to forecast the effects of starch gelatinization on food

products and processes it is useful to investigate the gelatinization level and water dynamics in starchy products when different water contents are available.

Sorghum starch gelatinization has been reported to be characterized by higher transition temperatures than other grains (Serna-Saldivar and Espinosa-Ramírez, 2019). Moreover, the gelatinization of sorghum starch is limited due to protein-starch granule associations, proteins crosslinking and the presence of tannins. In particular, the presence of a strong protein matrix consisting of around 70% highly hydrophobic kafirins (Xiong et al., 2019), in which the granules are encapsulated, prevents the granules from fully swelling and gelatinizing (Taylor et al., 2006; Khoddami et al., 2023). On the basis of that, sprouting has been studied to modify and improve the nutritional, physicochemical and functional properties of sorghum (Rashwan et al., 2021). While sprouting has been reported to increase both the availability and digestibility of starch and protein, protein solubility, bioavailability of minerals and bioactive compounds, and to reduce the anti-nutritional compounds (Afify et al., 2011, 2012; Lemmens et al., 2019; Saithalavi et al., 2021), the technological properties of the starch fraction have been reported to worsen after sprouting (Li et al., 2017; Marchini et al., 2021b). Upon sprouting, the proteases and amylases of sorghum act reducing protein-starch interactions (Lemmens et al., 2019), but at the same time, amylases can breakdown starch granule structure, thus limiting the ability of the granules to gelatinize (Li et al., 2017; Lemmens et al., 2019). However, some studies have reported that post-sprouting drying conditions (Marchini et al., 2021b) and sprouting time and temperature (Singh et al., 2017; Cardone et al., 2021) tailor the effects of sprouting, including the technological functionality. In particular, the time-temperature combinations of the sprouting and post-sprouting drying treatments influence the degree of grain modification through the modulation of the enzyme activity (Singh et al., 2017; Cardone et al., 2021; Marchini et al., 2021b).

To the best of our knowledge, there are no studies focusing on the gelatinization properties of sprouted sorghum over a wide range of water contents. Such investigations have been carried out in the last 20 years for different starch sources, such as pea starch (Wang and Copeland, 2012), waxy corn, normal corn, potato and pea starches (Tananuwong and Reid, 2004), corn starch (Fu et al., 2014), potato dry matter and starch (Liu et al., 2002), and wheat starch (Orlowska and Randzio, 2010). The present study was undertaken to better understand the physicochemical properties and the gelatinization behavior of water-flour systems of sprouted and unsprouted

sorghum flours. In particular, the role of water content (from 38 to 91%) in the thermal transitions of flour was studied to simulate the conditions of different moisture contents in foods. A combination of physico-chemical techniques, from the macroscopic to the molecular level, was used.

Results here presented have been published in Open Access in:

Chiodetti, M., Tuccio, M. G., & Carini, E. (2024). Effect of water content on gelatinization functionality of flour from sprouted sorghum. Current Research in Food Science, 8(February), 100780. https://doi.org/10.1016/j.crfs.2024.100780

Materials and Methods

Materials

White sorghum grains (*Sorghum bicolor* [L.] Moench) were soaked and sprouted at an industrial sprouting plant (Bühler AG, Uzwil, Switzerland) as reported in our previous study (Marchini et al., 2021a). The soaking and sprouting treatments were made under controlled temperature (25°C) and relative humidity (90%), for 16 and 72 h, respectively. The post-sprouting drying treatments were chosen in order to compare the traditional sun drying conditions applied in some African countries (40°C) (Elkhalifa and Bernhardt, 2010, 2013; Georget et al., 2012) and the temperature applied at an industrial level (50°C) (Marengo et al., 2017; Marti et al., 2017). The drying time was set in order to obtain a comparable final moisture content of sorghum seeds (12.7 ± 0.0 g/100 g w.b.). After sprouting and drying, the sprouted grains (S50, S40), and the unsprouted control sample (US) were milled in order to obtain a whole grain flour with the following particle mass distribution: ≈23% of particles >300 μm; ≈30% of particles between 300 and 200 μm; ≈27% of particles between 200 and 100 μm; and ≈20% of particles <100 μm. Flour milling was performed with a laboratory mill (Labormill, BONA, Italy). For the proximate composition of the above-mentioned flours, we refer to our previous study on the same flours (Marchini et al., 2021b).

Flour-water systems

Sorghum flour-water systems were prepared according to the water-flour ratio reported in Table 2 and considering the moisture content of flours. For simplicity, the water contents will be given below approximated to their respective integers. The hydration range was defined according to the hydration levels also reported in previous studies (Wang and Copeland, 2012; Schirmer et al., 2015), and representative of the conditions of low moisture foods and high moisture foods, in which starch is sufficiently or not sufficiently hydrated (Schirmer et al., 2015).

Table 2. Water:flour ratio and moisture content of each sorghum water-flour system.

water:flour ratio (w:w)	moisture content (% d.b.)
0.42:1	38.0
1:1	56.0
2.3:1	73.3
4:1	82.4
9:1	91.2

Swelling power (Sp)

The swelling power Sp of the samples was measured following the method described by Marchini et al. (2021b), with modifications. The water-flour systems were prepared in 2 mL Eppendorf tubes®, weighing the specific flour and distilled water, followed by vortex for 60 s. After that, the flour-water systems were heated in a water bath at 90 °C for 1 h. The samples were cooled to 30 °C for 30 min and centrifuged at 8000 rpm for 20 min. Once the supernatant was removed, the sediment was weighed. Sp was calculated as the ratio of sediment weight to the dry sample weight. Six replicates, divided in two different days, were carried out for each water-flour system.

Thermal properties

The thermal properties of water-flour systems were measured with a differential scanning calorimeter (DSC, Q200 TA Instruments, USA), calibrated with indium (melting point 156.6 °C, melting enthalpy 28.71 J/g) and mercury (melting point -38.8 °C, melting enthalpy 11.44 J/g). Each water-flour system was prepared by placing the flour and distilled water, accurately weighed, into a stainless-steel pan (Perkin, USA). The system was gently stirred with a needle

until it became homogeneous, taking care not to lose any of the sample. The pans were then hermetically sealed and kept at room temperature overnight before being analyzed. During the test, all the samples were equilibrated at 30°C and then heated to 130°C at 10°C/min. An empty, hermetically sealed pan was used as a reference. Universal Analysis software (version 4.5 A, TA Instruments, USA) was used to determine the onset (Ton, °C), peak (Tpeak, °C) and offset (Toff, °C) gelatinization temperatures and enthalpy change (ΔH, J g^{-1}) of each flow curve. At least 6 replicates, divided in two different days, were analyzed for each sample.

Proton molecular mobility

The ^{1}H molecular mobility of water-flour systems was analyzed through a low-resolution nuclear magnetic resonance (NMR) spectrometer (Minispec, Bruker, Massachusetts, USA) with a frequency of 20 MHz. Samples were prepared by weighing 1 gram of flour and different amounts of water in NMR tubes (10 mm diameter), according to the water-flour ratio reported in Table 2. After that, samples were mixed with a small spatula and then vortexed for 60 sec. To compare the non-gelatinized and the gelatinized samples, each water-flour suspension was analyzed raw (unheated) and after heating (90°C; 1 h) and cooling (30°C; 30 min). All the NMR tubes were sealed with Parafilm® to prevent water loss during heating, cooling and during the analysis.

^{1}H Free Induction Decay (FID) and Carr-Purcel-Meiboom-Gill (CPMG) pulse sequences were used to analyze the sample molecular mobility. During the analysis the temperature was set at 25.0 ± 0.1 °C using an external thermostatic bath (Julabo F30, Julabo Labortechnik GmbH, Seelbach, Germany).

The ^{1}H FID curves were acquired to measure the mobilities and abundances of the least mobile proton populations detectable in the molecular time-frame window of the instrument used (7 to 500 µs). The experiment was performed with 32 scans and 900 data points, with a single 90° pulse, followed by a dwell time of 7 µs, a recycle delay of 1 s, and an acquisition window of 0.5 ms. To quantitatively obtain the proton relaxation times and relative abundances of each population, ^{1}H FID curves were fit with a two-component model (exponential and Gaussian) (Le Grand et al., 2007). The fit was performed with Sigmaplot software, v.10 (Systat Software Inc., USA) according to the following equation [1]:

$$f(t) = y_0 + A^* e^{\left(\frac{t}{TA}\right)} + B^* e^{\left(-\frac{t}{TB}\right)^2} \quad (1)$$

Where y_0 is the FID decay offset, *A* and *B* are the intensities of each relaxation component, and *TA* and *TB* are the apparent relaxation times.

The Carr-Purcel-Meiboom-Gill pulse sequence (Meiboom and Gill, 1958) was used to measure the ^{1}H T_2 relaxation times of more mobile protons than those observed in the ^{1}H FID window. The experiment was performed with 32 scans, a recycle delay (RD) of 1 s, an interpulse spacing (τ) of 0.04 µs and 2500 data points. The ^{1}H CPMG curves were analyzed as quasi-continuous distributions of relaxation times using UpenWin software (Alma Mater Studiorum, Bologna, Italy) (Borgia et al., 1998, 2000). Default values were used for all UPEN parameters, except for the LoXtrap parameter, which was set =1 to avoid extrapolation of relaxation times shorter than the first experimental point.

Four tubes, divided in two different days, were analyzed for each water-flour system, and two ^{1}H FID and ^{1}H CPMG acquisitions were taken from each tube. In total, eight ^{1}H FID and eight ^{1}H CPMG curves were analyzed for each water-flour system.

Statistical Analysis

The data collected were analyzed by one-way analysis of variance (ANOVA) at a significance level of 0.05, followed by Tukey, LSD and Games Howell *post-hoc* tests, according to the homogeneity of sample size and homogeneity of variance among the groups (SPSS software v.27, IBM SPSS Inc., Chicago, IL, USA).

Results and Discussion

Swelling power

In this study, the gelatinization capacity of three different types of sorghum flour, unsprouted (US), sprouted and dried at two different temperatures (S40 and S50), was evaluated at five different water contents. As described above, the gelatinization capacity of starch is influenced by the water content available in the matrix. In fact, the amount of available water that is absorbed by the granules when heated contributes to the degree of gelatinization itself. Swelling power

represents one of the parameters by which the gelatinization degree of a starch or flour can be assessed. The absorption of water and the resulting loss of molecular order in the amorphous and crystalline regions of the granules result in the swelling of the granules (Wang and Copeland, 2012, 2013).

During the Sp analysis, no supernatant was formed after the centrifugation process in the samples with a water content of less than 73%. This indicates that all the added water was completely absorbed by the flour and no amylose leaching occurred (Wang and Copeland, 2012). With water contents ≥73%, the samples showed the formation of supernatant. This shows that up to 73% water content the system was in a water-limited condition, whereas following higher water addition the samples were able to absorb more water until the starch polymers began to leach. The Sp results obtained from the analysis of the different water-flour systems are shown in Figure 5.

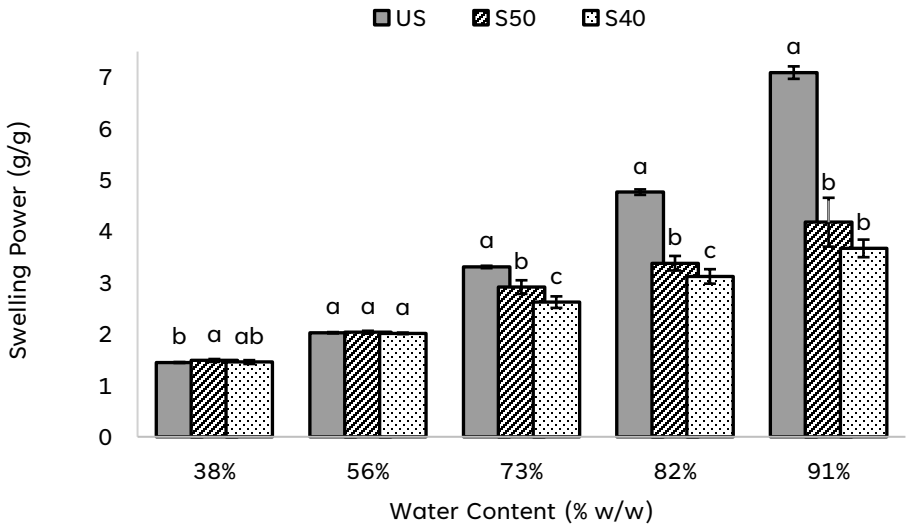

Figure 5. Swelling power of US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) samples at 38, 56, 73, 82, and 91% water contents. Different lowercase letters indicate significant differences between US, S50 and S40 at the same water content ($p < 0.05$).

As can be observed from the graph and easily assumed, the swelling power of the samples was found to increase as the water content increased ($p < 0.05$ in all samples at all water contents). Specifically, going from 38 to 91% of water, the swelling power of US sample showed an increase of ~+390%, the sprouted and 50°C dried sample (S50) of ~+181%, and the sprouted and 40°C dried sample (S40) of ~+152%. The increase in Sp with increasing water content is mainly determined

by the swelling capacity of highly branched amylopectin. In fact, while linear amylose swells and then dissolves in water, amylopectin swells and is not lost (Wang and Copeland, 2012). The increase in Sp as a result of increased water content has been demonstrated by previous studies on starch or flour (Wang and Copeland, 2012; Wang et al., 2014).

Regarding the effect of sprouting and drying treatments on sorghum flour swelling power, the results showed that the Sp in the sprouted samples decreased after sprouting treatment. In fact, as can be seen from Figure 5, the swelling power of US sample resulted higher than that of sprouted samples from 73% water content. The difference between sprouted and unsprouted samples increased with increasing water content. Indeed, the largest difference was found at the highest water content (91%). This indicates that, after heating and cooling, US sample showed a higher increasing ability to retain water with water content increase, if compared to sprouted samples. This may be due to the fact that sprouted samples had a higher amylose content and therefore, proportionally, less amylopectin than US sample (the amylose content and proximate composition of flours are reported in Marchini et al. (2021b). Indeed, many studies have reported that a higher amylose content and a lower amylopectin content negatively influences the swelling of granules and thus the swelling power (Li and Yeh, 2001; Wang et al., 2014). Elkhalifa and Bernhardt (2013) also found that sorghum flour sprouted and dried at 40°C showed a decrease in swelling power compared with unsprouted sorghum flour (Sp at 85 and 100°C). This effect was intensified in Sp at 100°C by increasing the sprouting time. Cardone et al. (2021) also reported that sprouting for 96 h (dried at 50°C x 9h) significantly reduced Sp of sorghum flour. These authors explained this result as a consequence of the degradation of sorghum protein bodies that occurred during germination, which made the starch more available for gelatinization but also more susceptible to α-amylase attacks. However, under conditions of limited amount of water (38% and 56%) the differences among the three samples appeared to taper off. Specifically, at a water content of 56%, the average values of the three samples were comparable indicating that the samples retained the same water as a result of gelatinization process, while, at the lowest water content (38%) the Sp value of S50 was higher than US, but not significantly different to S40. This could be related to the lower or absent leaching of amylose at low water contents, since, as discussed above, no supernatant formation was seen under 73% water content. Another reason

could be an increased starch availability as a result of protein matrix breakdown that occurred upon sprouting, that may be more evident at low water contents.

Comparing the two sprouted samples, it was seen that S50 showed higher Sp than S40 for 73% and 82% water contents. This is indicative of higher starch swelling power when sprouted sorghum is dried at 50°C. The differences highlighted how the drying phase may be of relevance in determining the functionality of sprouted grains. These data could support the hypothesis that greater starch destructuring results in less water retention during gelatinization.

Thermal properties

Based on DSC analysis, a main endothermic peak between ~65 and ~99 °C was identified. This peak has been associated with the breakdown of starch crystalline order (or double-helix structures) that occurs during starch gelatinization (Goldstein et al., 2010). A second endothermic peak between ~96 and ~125 °C was also identified in some thermograms and associated to the melting of amylose-lipid complexes (Zhu, 2014). The heating rate used (10°C/min) may have reduced the resolution of this second minor peak and for this reason, it was not further considered for analysis and discussion in this work. Figure 6 shows the thermograms of the three samples, for each water content, and Table 3 the transition enthalpy and temperatures.

The main endothermic peak, related to the melting of starch granules, was found to increase with increasing water content, in all samples. The dependence of this endothermic peak on water content is reported by numerous studies (Liu et al., 2002; Goldstein et al., 2010; Wang and Copeland, 2012; Wang et al., 2014).

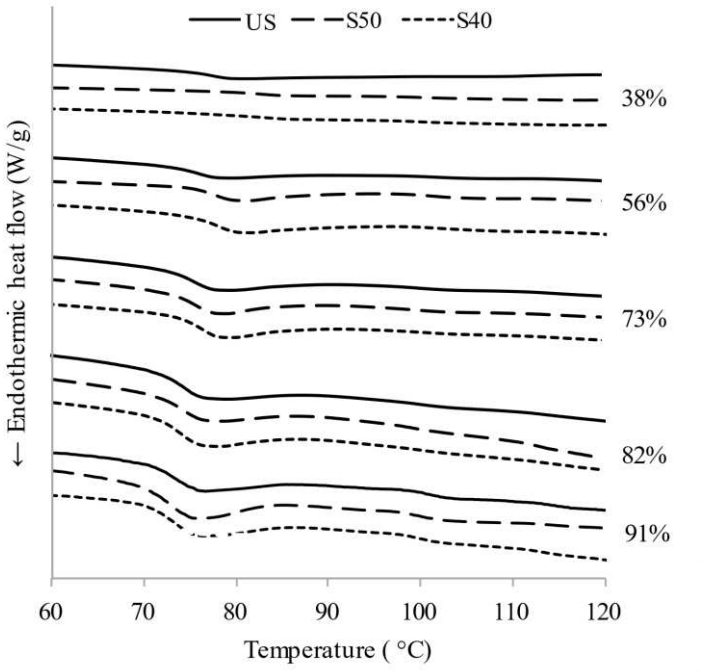

Figure 6. Representative DSC thermograms of US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) samples at different water contents.

Regarding the onset and end-transition temperatures associated with the main endothermic peak (Ton and Toff), they were found to generally decrease with increasing water content (Table 3). Specifically, in the sprouted S50 and S40 samples, the entire peak shifted to lower temperatures, resulting in decreasing Ton, Tpeak, and Toff values. Instead, only the Tpeak and Toff values decreased with increasing water contents in US, indicating that the range of gelatinization temperatures in sprouted samples remained almost constant, while in the unsprouted decreased. Goldstein et al. (2010) and Liu et al. (2002) also associated higher temperatures with low water contents. In fact, at low water contents the granules are not fully hydrated and remain structured, requiring more heat energy for dissociation. In general, the effect of water content on gelatinization temperatures was more pronounced in sprouted samples than in unsprouted samples. In fact, from 38 to 91% water content, the Tpeak decreased by about 10°C in the sprouted samples, and by about 4°C in the US (Table 3. Thermal properties of US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) samples. Different lowercase letters indicate significant differences between

US, S50 and S40 at the same water content ($p < 0.05$). Different capital letters indicate significant differences between 38, 56, 73, 82, and 91% water contents at equal flour used ($p < 0.05$).Table 3). The Ton of US sample also did not change by increasing the water content. This means that varying water content is more crucial in determining flour functionality (in terms of gelatinization temperatures) in sprouted sorghum than in unsprouted sorghum.

Table 3. Thermal properties of US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) samples. Different lowercase letters indicate significant differences between US, S50 and S40 at the same water content ($p < 0.05$). Different capital letters indicate significant differences between 38, 56, 73, 82, and 91% water contents at equal flour used ($p < 0.05$).

	Water content	Sample name		
		US	S50	S40
ΔH (J/g)	*38%*	3.76 ± 0.29 aD	1.06 ± 0.48 bC	0.99 ± 0.11 bC
	56%	4.63 ± 0.60 bC	4.42 ± 0.41 bB	6.05 ± 0.40 aB
	73%	6.38 ± 0.50 aB	6.64 ± 0.61 aA	5.83 ± 0.88 aB
	82%	7.73 ± 0.27 aA	6.93 ± 0.89 aA	6.23 ± 1.21 aAB
	91%	6.90 ± 1.08 aB	6.12 ± 1.73 aAB	8.12 ± 1.08 aA
Ton (°C)	*38%*	67.00 ± 0.98 bAB	74.10 ± 1.17 aA	73.01 ± 1.02 aA
	56%	65.69 ± 1.57 bCB	71.05 ± 1.65 aB	69.22 ± 1.72 aB
	73%	66.44 ± 1.21 bCB	66.11 ± 0.93 bC	69.82 ± 0.59 aB
	82%	65.27 ± 1.15 bC	67.24 ± 0.13 aC	68.77 ±1.17 aB
	91%	68.37 ± 1.23 bA	66.94 ± 0.80 aC	69.02 ± 0.40 aB
Tpeak (°C)	*38%*	79.19 ± 0.63 bA	84.59 ± 0.86 aA	85.40 ± 0.64 aA
	56%	78.44 ± 0.75 bAB	79.52 ± 1.41 aB	80.28 ± 0.65 aB
	73%	77.44 ± 0.28 aB	78.10 ± 0.66 aC	78.01 ± 0.52 aC
	82%	76.78 ± 0.24 aC	76.80 ± 0.24 aD	77.00 ± 0.42 aD
	91%	75.64 ± 0.47 aD	75.28 ± 0.39 aE	75.57 ± 0.37 aE
Toff (°C)	*38%*	97.87 ± 1.38 aA	98.90 ± 1.15 aA	97.69 ± 0.23 aA
	56%	97.40 ± 1.27 aA	98.08 ± 2.49 aA	98.21 ± 1.47 aA
	73%	90.67 ± 1.22 bB	94.59 ± 1.17 aB	89.48 ± 1.37 bB
	82%	90.93 ± 0.99 aB	90.82 ± 1.28 aC	89.35 ± 1.59 aB
	91%	87.20 ± 0.97 aC	86.60 ± 1.42 aD	87.05 ± 1.07 aC

S50 and S40 samples tended to show higher gelatinization temperatures than the US sample (Table 3). These differences emerged clearly at low water contents, while they almost disappeared at high water contents. The reason could be that sprouted samples had higher amylose contents than US (amylose contents are reported in Marchini et al. (2021b): 29±3% (US),

34±4% (S50), 47±0% (S40)). Several studies reported that gelatinization temperatures of starches with high amylose content are generally higher than those with less amylose content (Wu et al., 2013; Wang et al., 2014). However, there is no shortage of studies reporting instead that the presence of long chain amylopectin forming complex crystal structures requires more energy, and thus heat, to dissociate (Lin et al., 2016; Zhang et al., 2020). In addition, it has been reported that even the presence of sugars can increase the gelatinization temperatures, thus possibly explaining higher temperatures in sprouted samples (Wu et al., 2013). Interestingly, at high water contents (82 and 91%), when the samples were more hydrated, differences between samples disappeared, and the gelatinization temperatures resulted the same between US, S50 and S40 (Table 3). Therefore, when the samples are granted abundant hydration, the loss of molecular order occurs at the same temperatures. This is an interesting result in limiting the effect of increasing gelatinization temperatures in sprouted grains.

As reported in Table 3, the ΔH of US and S50 reached its maximum value at a water content of 82%, and then began to decrease slightly. The ΔH of S40, on the other hand, continued to increase after the addition of a higher water content (91%). This phenomenon indicates that, by increasing the water content to the 82% level, maximum gelatinization occurred in US and S50. Similar behavior was observed, even if at different water contents, by Liu et al. (2002) on potato dry matter endotherms. It is worth noting that, although changes in ΔH can be attributed to starch modifications in sorghum flour, other components present in the flour (nonstarch polysaccharides, proteins, lipids, minerals) can affect the ability of starch to gelatinize (Liu et al., 2002; Donmez et al., 2021). In fact, the presence of protein bodies and the hydrophobic nature of sorghum protein matrix, in which starch is trapped, are known to have a limiting effect on starch gelatinization (De Mesa-Stonestreet et al., 2010; Cardone et al., 2021). In sprouted samples, the negative effect of proteins and lipids on starch gelatinization may have been modulated by the enzymatic hydrolysis that proteins and lipids underwent during sprouting. The more prolonged enzyme activity during the drying treatment in S40 might have shown a more pronounced change in protein ability to hinder starch functionality, and thus allowing it to increase the degree of gelatinization even for water contents higher than 82%. These results contrasted with those found for swelling power, where the sample continued to swell even after the water content exceeded 82%. In addition, even when comparing the values of ΔH between US, S40 and S50, they were found to contrast with

swelling power results. In fact, Sp analysis showed generally higher values in US sample than S40 e S50, suggesting better gelatinization properties in US than sprouted samples. The enthalpy change, however, did not show the same trend. By contrast, ΔH resulted higher in US only at 38% water content, while it was higher in S40 at 56% water content and no significant differences were found at other water contents. As reported above, S40 was the sample that exhibited the most pronounced effect of the sprouting treatment on swelling power and ΔH, probably due to more prolonged enzyme activity during drying treatment. In the case of swelling power, however, it worsened, while in the case of ΔH, it partially improved. It should be kept in mind that, in this study, swelling power was calculated as the weight difference related to the retained water after heating, cooling, and removal of supernatant; ΔH, on the other hand, symbolizes the loss of molecular order in the granule structures over a temperature ramp and could provide slightly different information than swelling power. In addition, if the ΔH gelatinization results refer to the specific starch transition, in the case of swelling power the effect of proteins and their ability to bind water and form a gel upon cooling might have influenced the result of Sp.

Proton molecular mobility

The ^{1}H FID analysis of unheated and heated water-flour systems showed the presence of two ^{1}H populations A and B, giving 100% of the total ^{1}H FID signal. The relaxation times and relative abundances of Pop A and Pop B of heated and unheated samples at all water contents are given in Table 4.

^{1}H FID populations of unheated samples relaxed in the range of 0.0133-0.0149 ms (Pop A) and 0.3506-0.8295 ms (Pop B). These findings agree with those previously reported on sorghum flour (Marchini et al., 2021a). Indeed, ^{1}H FID analysis shows the most rigid proton populations with least water contact in water-flour systems. In particular, in unheated systems, the most rigid Pop A has been assigned to the rigid CH protons of crystalline and amorphous structures not in contact with water and of proteins in strong interaction with each other (hydrophobic parts and regions with protein-protein interactions) (Bosmans et al., 2012) which can be reasonably related to the crosslinking of sorghum proteins. On the other hand, the more mobile Pop B has been assigned to the CH protons of amorphous starch and proteins in little contact with confined water (Bosmans et al., 2012).

Table 4. Relative abundances and relaxation times of ^{1}H FID populations A and B of unheated and heated samples. Different lowercase letters indicate significant differences between US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) with same water content and heating state (heated or unheated) ($p < 0.05$). Different capital letters indicate significant differences between 38, 56, 73, 82, and 91% water contents, at equal flour used ($p < 0.05$).

	water content (%)	unheated			heated		
		US	S50	S40	US	S50	S40
Pop A (%)	38%	84.6±0.14bA	83.42±0.84cA	84.9±0.2aA	78.68±0.79aA	73.82±0.71bA	77.98±0.34aA
	56%	75.02±1.55abB	73.89±0.43bB	75.3±0.87aB	54.86±1.07aB	49.08±2.58bB	49.86±1.49bB
	73%	57.7±0.35aC	52.61±0.68cC	56.51±0.78bC	31.67±5.34aC	30.18±1.09aC	28.4±2.31aC
	82%	44.63±1.41aD	41.1±2.65bD	42.76±1.63bD	23.99±4.21aD	22.33±0.93aD	18.21±1.88bD
	91%	26.92±3.17aE	23.26±2.26bE	22.88±2.88bE	14.22±2.27abE	15.05±0.9aE	12.93±1.37bE
Pop B (%)	38%	15.4±0.14bE	16.58±0.84aE	15.1±0.2cE	21.32±0.79bE	26.18±0.71aE	22.02±0.34bE
	56%	24.98±1.55abD	26.11±0.43aD	24.7±0.87bD	45.14±1.07bD	50.92±2.58aD	50.14±1.49aD
	73%	42.3±0.35cC	47.39±0.68aC	43.49±0.78bC	68.33±5.34aC	69.82±1.09aC	71.6±2.31aC
	82%	55.37±1.41bB	58.9±2.65aB	57.24±1.63aB	76.01±4.21bB	77.67±0.93bB	81.79±1.88aB
	91%	73.08±3.17bA	76.74±2.26aA	77.12±2.88aA	85.78±2.27abA	84.95±0.9bA	87.07±1.37aA
tA (ms)	38%	0.0136±0.0001aC	0.0134±0.0002bC	0.0133±0.0001bD	0.0171±0.0001aC	0.017±0.0001aB	0.0161±0.0002bC
	56%	0.0141±0.0001aB	0.0141±0.0001aB	0.0139±0.0001bC	0.0195±0.0002aA	0.0176±0.0002cA	0.0184±0.0008bA
	73%	0.0144±0.0001aA	0.0144±0.0001aA	0.0142±0.0001bB	0.0177±0.0005aB	0.0171±0.0004bB	0.0173±0.0004aB
	82%	0.0146±0.0002aA	0.0145±0.0001aA	0.0145±0.0002aA	0.0169±0.0006aC	0.0168±0.0003aB	0.0172±0.0004aB
	91%	0.0148±0.0006aAB	0.0149±0.0006aA	0.0148±0.0004aA	0.0162±0.0005aD	0.0161±0.0006aC	0.0161±0.0009aC
tB (ms)	38%	0.3531±0.0031aE	0.3506±0.0075aE	0.3529±0.0033aE	0.2966±0.0028bE	0.3153±0.002aE	0.3118±0.0054aE
	56%	0.4267±0.0092aD	0.423±0.0126aD	0.4323±0.0105aD	0.3825±0.0049bD	0.4429±0.0202aD	0.434±0.0053aD
	73%	0.5387±0.0065bC	0.5634±0.0085aC	0.5588±0.0068aC	0.5688±0.0312bC	0.6019±0.0091aC	0.6144±0.0149aC
	82%	0.6153±0.017bB	0.6412±0.0202aB	0.6418±0.0227aB	0.6626±0.0329bB	0.6908±0.0168bB	0.7377±0.0185aB
	91%	0.7701±0.0787aA	0.8072±0.0645aA	0.8295±0.0625aA	0.8048±0.0467bA	0.8097±0.032abA	0.8587±0.0519aA

After heating, ^{1}H FID populations showed relaxation times in the range of 0.0161-0.0195 ms for Pop A and 0.2966-0.8587 for Pop B. Figure 7 reported the Pop A (%) evolution as a function of water content and heating treatment in US, S50 and S40 samples.

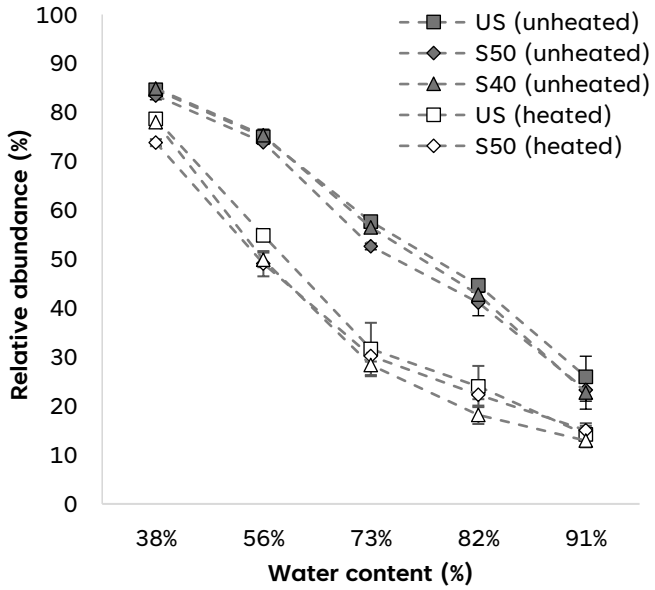

Figure 7. Evolution of Pop A (%) with increasing water content in unheated and heated water-flour systems.

After heating, the ratio of Pop A (%) to Pop B (%) in the samples changed, marking a decrease in abundance of Pop A and an increase in abundance of Pop B, if compared to unheated samples (Figure 7; Table 4). The decrease in Pop A (%) after heating indicates the melting of the crystalline and amorphous structures packed in the starch granules, which occurred during gelatinization. However, the lasting presence of Pop A after heating has been reported to indicate the presence of amorphous structures that remained immobile after heating and the neoformation of amylose crystals after cooling (Bosmans et al., 2012). A decrease in the first population upon heating was also reported by Rondeau-Mouro et al. (2015) in wheat starch models and dough at ~45 % water content. These authors stated that this first component represents a fraction of protons potentially transferable to the other components. In fact, in addition to protons from amylopectin, this population may represent protons from well-organized amylose structures that transfer to the higher mobility population and to the extragranular water phase during amylose leaching that

occurs in the swelling process (Rondeau-Mouro et al., 2015; Rakhshi et al., 2022). As can be observed from Figure 7, the decrease of Pop A (%) abundance from unheated to heated systems, resulted to be dependent on water content, indicating the greatest differences at 56, 73 and 82% water content. At 38% water content, gelatinization of the samples may have not been completed due to limited water content, thus leading to the prevalence of more rigid starch structures even after heating and cooling. At 91%, the high water content markedly reduced the Pop A (%) signal in unheated systems (if compared to lower hydration levels), while in the gelatinized samples the signal decreased to a lesser extent, probably because gelatinization was almost already completed at 82% water content.

Generally, in both unheated and heated samples, the percentage of Pop A decreased (and thus Pop B increased) as water content increased. Probably, in unheated samples, the increasing water content facilitated the water uptake into flour structures, leading to an increase in protons in little contact with water and a decrease of fraction not in contact with water. These results were also found by other authors (Marchini et al., 2021a; Parenti et al., 2021). In heated samples, on the other hand, the decrease of pop A with water increase, can be linked to the extent of starch gelatinization that occurred in water-flour systems. A higher water content can generally be linked to a higher gelatinization, and thus a higher decrease in rigid starch structures. Looking at the mobility of pop A, in the unheated samples, tA tended to increase with increasing water content, while no trend was observed in heated samples. These data highlight the effect of water entry in the channels between the stiffer starch structures in the unheated samples. Instead, a more similar and homogeneous structure in the domain representing the pop A of heated samples at different water contents can be hypothesized. The presence of water in these very constrained zones was also reported by Rondeau-Mouro et al. (2015).

Comparing the three flour samples, S50 showed lower Pop A (%) values than US in unheated systems for all water contents, while S40 resulted higher than US only from 73% water content onward (Figure 7; Table 4). The lower Pop A (%) values in sprouted samples compared to US, may be associated to the breakdown of sorghum rigid protein matrix and modification of starch structures mediated by enzyme hydrolytic activity that occurred during sprouting. These modifications of sorghum matrices may have led to an increase in the signal of the population in little contact with water, assigned to Pop B. Even after heating, the sprouted samples (S40, S50)

tended to show lower values of Pop A (%) than US sample. This effect, however, was not consistent at all water levels (Figure 7; Table 4). Lower Pop A (%) values in heated sprouted samples than US could be due to a different degree of gelatinization of amylopectin crystals and amorphous starch as an effect of sprouting. Another finding to note is the tendency for greater mobility of Pop B in both unheated and heated samples after sprouting (S40 and S50) (Table 4), which may be related to the general breakdown of structures that occurred upon sprouting.

The CPMG analysis of unheated samples (US, S40 and S50) at 38% water content revealed the presence of four ^{1}H T_2 populations, named Pop C, D, E and F, respectively (ascending order of mobility; Figure 8). This result agrees with previous findings on water-sorghum flour systems (Marchini et al., 2021a). According to the literature, the 4 populations can be attributed to CH protons of amorphous starch and proteins in little contact with water and dietary fiber constituents (Pop C); to OH protons of intragranular water and starch, and CH protons of proteins in interaction with confined water (Pop D); and to the exchanging extragranular protons of bran and flour biopolymers in interaction with water (Pop E) (Bosmans et al., 2012; Marchini et al., 2021a). The most mobile Pop F has been attributed to both weakly water-bound OH protons (Marchini et al., 2021a; Parenti et al., 2021) and the lipid fraction of flour (Assifaoui et al., 2006; Hager et al., 2014). In our study, some considerations need to be made. At 38% water content, the protons of two main CPMG populations were represented by those related to exchanging protons of water and intra- and extra-granular starch structures and those of proteins in exchange with confined water or in strong interaction with water (Pop D and E); at the same time, a very small contribution of protons related to Pop F was denoted (Figure 8). Moving from 38% to higher values of water content, the number of populations of unheated samples decreased to 3 populations. Specifically, at 56 and 73% water content, the populations E and F converged into a single new population (Pop EF), given by the merger of the two, which had intermediate relaxation times between these populations (Figure 8). Moving from 56% to 73% water, the mobility of Pop EF increased with increasing water content. Interestingly, for water contents $\geq$ 82%, a new separation of Pop EF occurred, allowing the distinction of Pop E and Pop F. According to Ruan et al. (1999), when all water-binding sites in the flour are saturated, further addition of water forms new, more mobile water layers with increasing distance to water molecules bound to the flour solids (Ruan et al., 1999; Riley et al., 2022). Pop F would then show a progressive increase given the increase

in water content, becoming the main population at water contents of 82 and 91%. The dynamics of Pop F evolution can be explained by the results of Le Botlan et al. (1998), which reported that water contents just above the appearance threshold of "weakly bound" water form a new layer that has a low degree of freedom due to physical reasons that prevent it from rapidly exchanging with the molecules of the "bound" water layers. This water, therefore, has slightly longer relaxation times than "bound" water (Le Botlan et al., 1998) and could explain the lack of complete peak resolution down to a 73% water level in our study. Le Botlan et al. (1998) also reported that when the water content increases, water molecules may begin to exchange with new water molecules in their surroundings and thus acquire greater mobility. This phenomenon could explain the subsequent resolution of the two populations E and F with water contents of 82 and 91%. These data could therefore support that Pop F consists of OH protons of weakly bound water, as reported by other previous studies (Parenti et al., 2021). The more rigid Pop C and Pop D gradually decreased with increasing water content. Population C seemed then to disappear at water contents >82% (Figure 8). Presumably, the relative signal attributable to Pop C became too small compared to the high-mobility components. However, the mobility of Pop C and D was not affected by water content. This behavior is reasonable considering that Pop C and D belong to flour fractions less in contact with water. In contrast, the mobility of Pop E and F was found to be strongly affected by water content. This result agrees with what has already been reported by other authors (Le Botlan et al., 1998; Tananuwong and Reid, 2004; Riley et al., 2022), who stated that proton mobility of starchy products is directly related to water content, especially in case of fractions with high proton mobility, which in this case could be represented by Pop E and F.

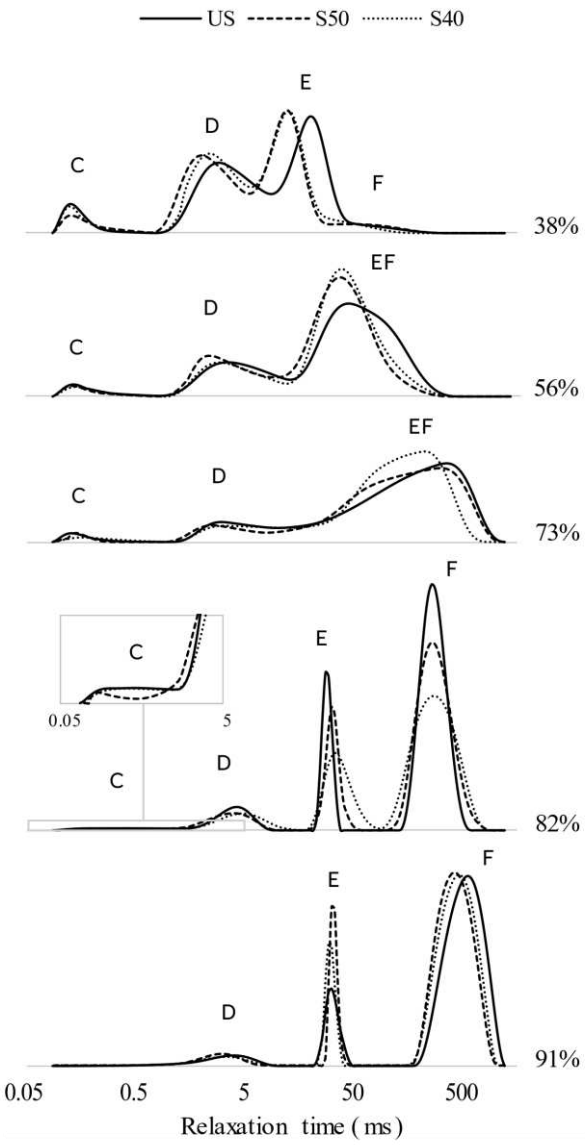

Figure 8. Evolution of representative ^{1}H T_2 CPMG populations of unheated US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) samples at different water contents.

Regarding the molecular differences between US, S40 and S50, different proton relaxation times were observed. Indeed, up to 73% water content, a higher mobility of the D, E and F (or EF) populations was found in the US sample than in the sprouted samples (Figure 8), while at 82% population E was more rigid in US. The general lower mobility in sprouted samples can be explained by the presence of a higher number of bonds established with water within different structural domains of starch and protein. Indeed, sprouting may have led to a greater exposure

of biopolymers binding sites as a consequence of the hydrolysis that occurred upon sprouting. The modification of the properties of starch and protein occurring during sprouting may explain the differences found in the population mobility. In this regard, it was reported that the degradation of amylopectin chains reduces crystalline areas by facilitating water access in the granules (Jakobi et al., 2018), while other authors reported an increased ability of proteins to bind water after sprouting (Elkhalifa and Bernhardt, 2010; Lemmens et al., 2019; Marchini et al., 2021a). However, Rakhshi et al. (2024) found that the heat-induced transformation of model dough systems matched that of their starch-water system counterparts, highlighting how these differences are mainly due to starch properties. The main effect of starch on T_2 relaxation was also reported by Riley et al. (2022). However, Rakhshi et al. (2024) also reported that proteins may also have an impeding effect on starch-water interactions. Sprouting treatment may have changed this hindering effect of proteins and thus the starch-water interactions.

Heating and subsequent cooling of US, S40 and S50 changed the ^1H T_2 CPMG relaxation times distribution of the samples. Figure 9 shows the comparison between the unheated and heated US sample, by way of example. In all samples, a decrease in mobility of Pop E and Pop D after heating and cooling was observed. In fact, during heating, the process of gelatinization occurred, which includes the entry of water, swelling and rupture of starch granules with the melting of crystal structures and mobilization of amylose outside the granules. This in turn may have led to a redistribution of protons characterizing the different populations at the molecular level. Moreover, after cooling, the subsequent gel formation resulted in the decrease of the relaxation times of the extragranular exchanging protons of flour biopolymers interacting with water (Pop E), and in the marked decrease in the abundance of protons with intragranular bonds between water and starch, as well as of the proteins in contact with the confined water (Pop D). The differences in the mobility of Pop D between heated and unheated systems appeared reasonably more pronounced at low water contents (38 and 56%), as insufficient water was available in unheated systems to allow the granules to break and swell. Rondeau-Mouro et al. (2015) reported that the decrease during heating of intragranular protons in starch model systems at ~45% water content was related to granule disintegration and viscosity improvement phenomena. At 56 and 73% water content, a decrease in mobility of Pop E (or EF) was observed. A decreased mobility of Pop E after heating and cooling was also reported by Bosmans et al. (2012) and Hager et al. (2014) in water-

flour systems, where the water content was 47%. Furthermore, in heated systems Pop C disappeared at a lower water level than in the unheated systems. In fact, in heated systems, it was no longer observable from a water content of 82%, while, at 73%, it overlapped with Pop D. This indicates that increasing water content and heating led to a gradual and progressive reduction of CH protons of starch and proteins in little contact with confined water in the sheets, in favor of the high mobility populations in the continuous network between starch, protein and water. In heated systems with a water content of 82%, although two populations (E and F) were visible, they were not fully resolved. Indeed, unlike the unheated systems, where complete separation and resolution of E and F populations occurred at 82%, in the heated systems complete separation of E and F populations occurred at 91% water content. This result indicates the presence of an exchange between weakly bound water molecules and the starch-protein-water network at higher water content levels than in unheated systems. At 91 % water content, the most represented population was Pop F, attributed to the protons of more mobile and weakly bound water expelled from the starch-protein-water network.

Comparing the results of Pop B (FID) with those of Pop C (CPMG), in our work an exact correspondence between the two populations was not found. Pop B was found to increase up to the maximum water content analyzed, while Pop C was found to decrease until it disappeared with increasing water content, both in heated and unheated systems. This result differs from other previous studies, in which these two populations were associated with the same protons, as they relaxed at the same times and had similar behavior before and after heating (Bosmans et al., 2012; Riley et al., 2022). In the cited studies, however, Pop B and C were not evaluated at different water contents. Furthermore, it must be considered that Pop B and C are determined by two different RF sequences, in which proton mobility is assessed at different scales and the relative signal of the populations is returned independently. In fact, the two sequences provide relative, not absolute, data. Compared with low-mobility populations determined by FID analysis, the relative percent signal of Pop B increases with increasing water content because the signal of A decreases, and together they return 100 % of the total FID signal. In the CPMG experiment, on the other hand, Pop C is the more rigid population, whose relative contribution, compared with those with higher mobility, decreases as water content increases.

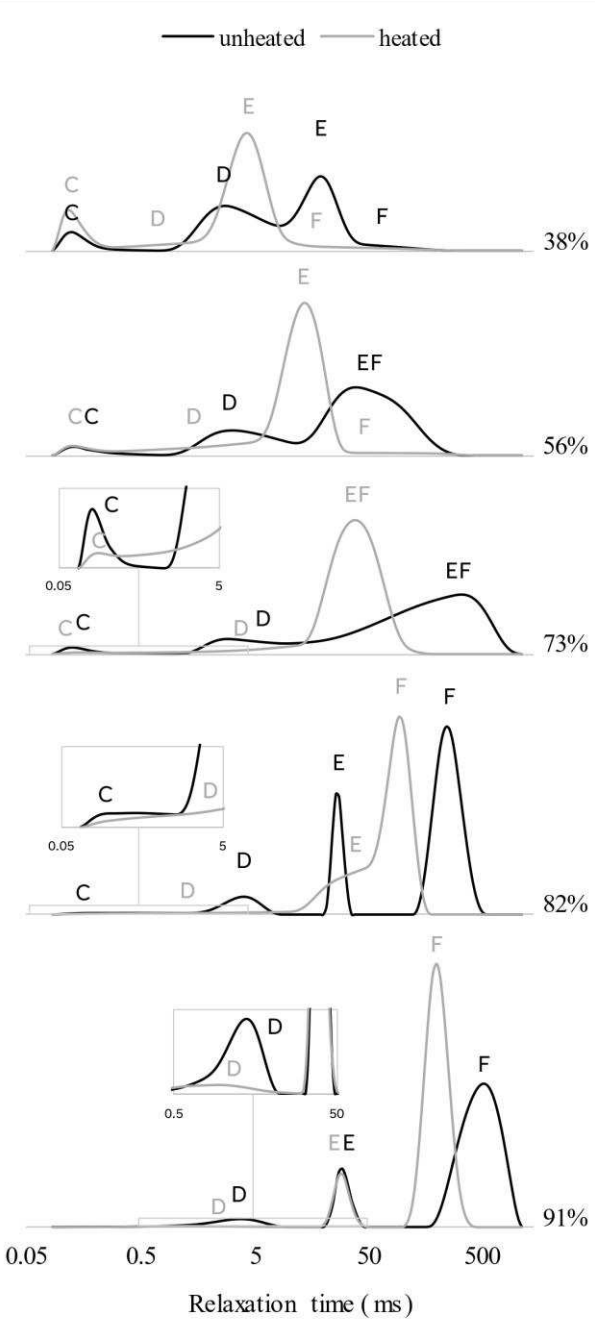

Figure 9. Comparison of representative ^{1}H T_2 CPMG populations of unheated and heated US sample at different water contents.

Looking at the comparison between sprouted samples with the unsprouted samples after heating and cooling (Figure 10), Pop C in the sprouted samples (S40, S50) showed lower relative abundance than in the US. This indicates a post-heating less permanence of the rigid structures

in the sprouted than in the unsprouted, due to the hydrolysis of starch and protein operated by enzymes during sprouting.

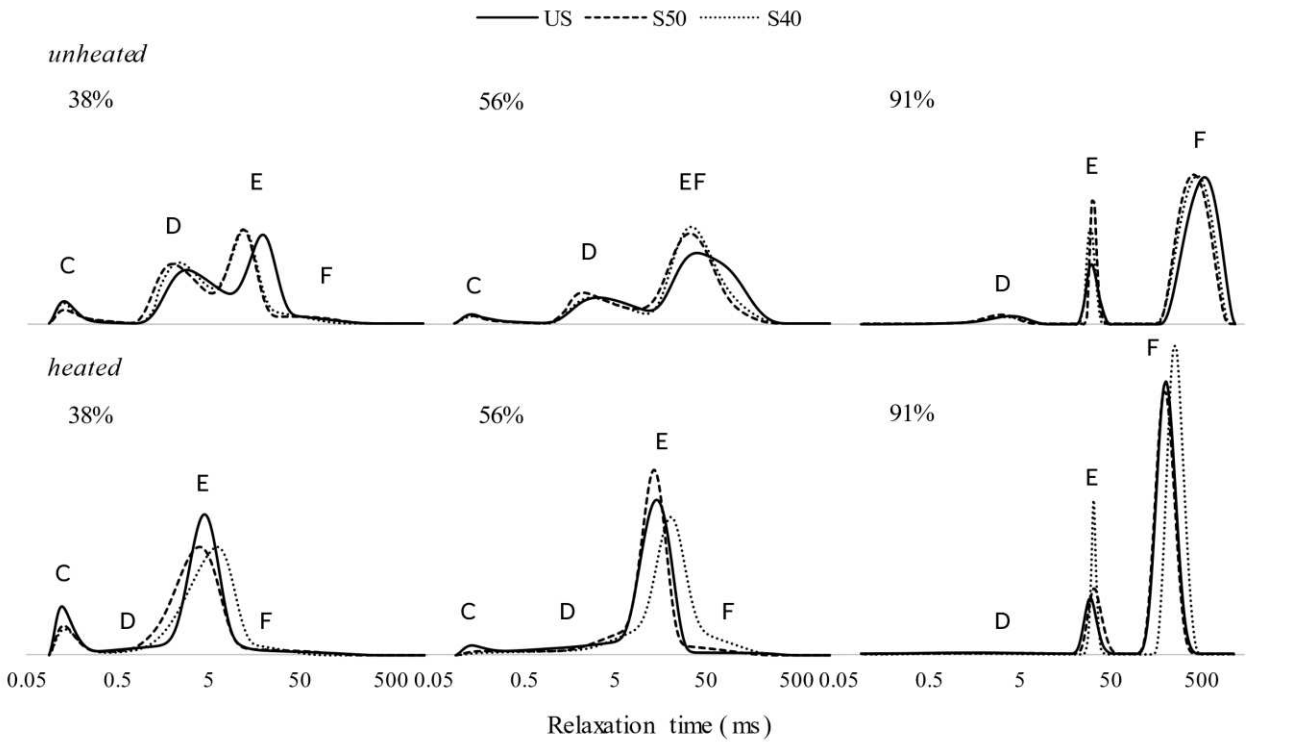

Figure 10. Comparison of representative ^{1}H T_2 CPMG populations of unheated and heated US (flour from unsprouted sorghum), S50 (flour from sprouted and 50°C dried sorghum) and S40 (flour from sprouted and 40°C dried sorghum) samples at selected water content (38, 56 and 91%).

As for the higher mobility populations, however, the mobility of pops E and F (or EF) of sample S40 was higher than that of US and S50 (Figure 10). US and S50, on the other hand, showed similar mobilities. This indicates the presence of weaker and more mobile bonds between water and biopolymers after heating in S40. As previously reported in other studies, this event could be due to the hydrolytic breakdown operated by amylases on starch, which caused a reduction in the swelling power of the sprouted samples (Marchini et al., 2021b). In addition, it may also be related to the different ability of proteins to form a gel of different strength in the newly formed continuous network. At a macroscopical level, a lower gel consistency in sprouted samples was reported by Singh et al. (2017).

Conclusions

In this study, we evaluated the effect of water content and different post-sprouting drying conditions (40°C for 12h; 50°C for 6h) on the macroscopic (swelling power), mesoscopic (thermal properties by DSC) and molecular properties (^{1}H molecular mobility and dynamics by LR ^{1}H NMR) of sorghum flour.

Sprouting and subsequent drying treatment affected all the analyzed properties. The swelling power of the samples was found to be compromised after sprouting at high water contents (≥73%), probably due to the negative effect of the enzyme activity on the ability of amylopectin to swell during gelatinization, but also to changes in the functionality of sorghum proteins. However, low-medium water content proved to be a promising condition for the functionality of sprouted flours, as the swelling power was equal or higher in sprouted than unsprouted. The swelling power of the sample dried at 40°C was the most affected by sprouting treatment. Prolonged enzyme activity may have led to excessive destructuring of starch, making it more accessible but compromising some functional properties. However, as a result of sprouting, sorghum starch resulted more accessible and available to gelatinize, showing higher ΔH values in DSC results. In particular, at medium water content, the gelatinization enthalpy was higher in sprouted grains than unsprouted. The molecular properties of sorghum flour were also affected by sprouting and drying treatment, showing the reduction of the abundances of the most rigid populations in favor of those with higher mobility. The highest mobility of the population related to starch and protein protons in strong interaction with water (after heating and cooling) in the sprouted sample dried at 40°C for 12 h revealed the presence of weaker bonds in the network formed. In general, in all the analysis performed, the sample dried at 40°C for 12h was the one with the values that differed the most from the unsprouted sample, probably due to a more prolonged enzyme activity during the drying phase.

These results may be important when considering the potential application of sprouted sorghum flour in foods with different moisture contents, which help to understand how to improve the use of this flour in Western food products, improving the nutritional profile but without compromising technological quality.

1.2 Effects of LAB Fermentation on Sorghum Flour Functionality

Case Study 2

Effect of LAB fermentation on the molecular and technological properties of sorghum batters

Introduction and Aim of the Study

Many recent studies have examined the possibility of implementing sorghum grain in Western diets (Rumler and Schönlechner, 2021; Ari Akin et al., 2022; Rumler et al., 2022; Khoddami et al., 2023), which may fit into a conscious planning of future food production, considering the effect of climate change on agriculture, crop health, and productivity (Dhankher and Foyer, 2018). However, along with the positive and promising aspects of sorghum, some shortcomings have emerged in the literature. From a nutritional point of view, the poor protein digestibility is the major limitation, which seems to worsen after cooking (Duodu et al., 2002, 2003; De Mesa-Stonestreet et al., 2010). Many critical points also emerge from technological and sensory points of view. Indeed, sorghum is characterized by a strong protein matrix consisting of ~70% highly hydrophobic kafirins (Xiong et al., 2019) with poor ability to hydrate and plasticize (De Mesa-Stonestreet et al., 2010). In addition, starch granules are incorporated into the protein matrix, which limits gelatinization due to protein-starch granule association, protein cross-linking, and the presence of tannins (Taylor et al., 2006; Serna-Saldivar and Espinosa-Ramírez, 2019; Khoddami et al., 2023).

Fermentation is a traditional process able to enhance the technological, sensory, nutritional and safety qualities of food products (Adebiyi et al., 2019; Gobbetti et al., 2019; Garrido-Galand et al., 2021). Recently, interest in food fermentation has increased significantly to harness its potential for the transition towards a more sustainable agri-food system (Jahn et al., 2023). Indeed, through fermentation, food matrix constituents are modified, new compounds are synthesized, and modification of food texture and flavor occurs (Marco et al., 2017; Gobbetti et al., 2019; Garrido-Galand et al., 2021; Jahn et al., 2023). As sorghum represents a staple grain in

many African countries, fermentation has always traditionally been applied on sorghum to produce different foods (Oluwafemi, 2020). For this reason, comparing the literature about sorghum fermentation, many studies have been published mimicking the African households traditional practices, which involve a spontaneous fermentation of sorghum flour (Hugo et al., 2003; Elkhalifa et al., 2004a, 2004b, 2005, 2006, 2017; Correia et al., 2005; Pranoto et al., 2013; Ge et al., 2020). However, the spontaneous fermentation of sorghum flour involves the development of a very complex ecosystem, dependent mainly on the environment and specific conditions, which in turn can be very complex to reproduce in the laboratory or on an industrial scale. If fermentation can contribute to the transition to healthier and more sustainable diets and promote sorghum utilization in western diets, there is a need to move from spontaneous, wild, empirically developed fermentations to rationally designed fermentation processes using intentionally inoculated microorganisms (Marco et al., 2017; Jahn et al., 2023).

Therefore, the aim of this study was to apply selected lactic acid bacteria fermentation on sorghum flour and to characterize the molecular changes of flour biopolymers and their technological implications in terms of rheology, pasting and thermal properties, deepening the knowledge of fermentation-induced changes in sorghum flour and facilitating the understanding of its potential food applications.

Materials and Methods

Materials

Whole heat-treated cereal (HTC) white sorghum (*Sorghum bicolor* (L.) Moench) flour was supplied by MartinoRossi SpA (Cremona, Italy) (carbohydrate 55.7%, fiber 16.4%, protein 10.3%, total fat 3.2%).

Microbiological analysis

Microbial quantification on batters was done by means of plate counting, using different selective and elective media (Table 5). For the analysis, 10 g of each batter were diluted with 90 ml of Ringer's solution (Oxoid) and vigorously mixed. Subsequently, 0.1 ml of the appropriate dilution was spread onto each medium and incubated as indicated in Table 5). For spore-forming bacteria, 10 g of the batter were thermally treated for 10 minutes at 80°C and then pour-plated in a double layer of TSA and incubated under anaerobic conditions (Table 5).

Table 5. Selective and elective media used for microbial quantification of sorghum batters.

Media	Full Name	Producer	Target microorganism	Incubation time	Aerobiosis	Temp. (°C)
M17	-	Oxoid, Ltd., Basingstoke, UK	Cocci	48h	no	37
MRS	De Man, Rogosa and Sharpe	VWR Chemicals, Radnor, PA, USA	Lactic acid bacillus	48h	yes	37
MRS	De Man, Rogosa and Sharpe	VWR Chemicals, Radnor, PA, USA	Lactic acid bacillus	48h	no	30
PCA	Plate Count Agar	VWR Chemicals, Radnor, PA, USA	Total Viable Count (TVC)	24h	yes	30
TSA	Tryptic Soy Agar	VWR Chemicals, Radnor, PA, USA	Spore-forming	48h	no	30
MSA	Mannitol Salt Agar	VWR Chemicals, Radnor, PA, USA	Staphylococcaceae	24h	yes	37
CHR	Chromocult® Coliform Agar	Merck KGaA, Darmstadt, Germany	*Coliforms*	24h	yes	37
YEDC	Yeast Extract Dextrose Chloramphenicol	Oxoid, Ltd., Basingstoke, UK	Yeast and molds	48h	yes	30

Strains revitalization

Three lactic acid bacteria (LAB) strains (Table 6), belonging to the University of Parma Culture Collection (UPCC) and isolated from different food matrices, were chosen based on their well-known fermenting features (Bancalari et al., 2017, 2019; Fuso et al., 2023a). The stock cultures were stored at −80 °C in MRS broth with 12.5% glycerol. Two sub-culturing steps were performed at the conditions indicated in Table 6 to recover the strains from the frozen stock. The growth of each strain was then standardized through three 20-hours propagations steps to reach a concentration of approximately 9 Log CFU/ml.

At the end of the third 20-hour propagation passage, the strains were washed three times with Ringer's solution (Oxoid), before being used as inoculum in sorghum batter.

Table 6. Lactic acid bacteria strains used in this study.

ID	Propriety	Genus	Species	Subspecies	Matrix	T (°C)	O_2	Medium
1932	*UPCC*	*Lactobacillus*	*delbrueckii*	*bulgaricus*	*Curd*	*37*	*No*	*MRS*
4339	*UPCC*	*Lacticaseibacillus*	*casei*		*Cow milk*	*30*	*Yes*	*MRS*
4454	*UPCC*	*Leuconostoc*	*Spp.*		*Sourdough*	*30*	*Yes*	*MRS*

Sorghum flour fermentation

To establish the best fermentation condition, preliminary tests were performed on the selected strains, inoculated in sorghum batter without any sugar addition (0%) and with the addition of 1 and 5% (w/w) of sucrose. Sorghum batters were prepared using 63% (w/w) of water and, based on the sucrose content, respectively 35, 34 and 30% (w/w) of sorghum flour. Three sorghum batters with different sugar content were inoculated with each LAB strain at a concentration of 2% v/w.

After the screening for the best fermentation conditions, fermentation process was held at 25°C for 15 hours and monitored in real-time using the impedometric analysis by means of BacTrac 4300 Microbiological Analyser system® (Sylab, Austria).

To this aim, 18 ml of each inoculated batter were equally distributed in three sterilized BacTrac vials and the total M% variation was recorded, every 10 minutes (Bancalari et al., 2016).

Final fermentation conditions were also determined based on preliminary baking trials (data not shown). Once optimal fermentation conditions were established, sorghum batter fermentation was conducted in triplicate for each strain on separate production days, and the samples obtained were used for the subsequent analyses. At the end of fermentation, the fermented batters were stored at 5°C before analysis (LR ^{1}H NMR, rheology, DSC, pasting properties). For TTA, SDS page, degree of hydrolysis (DH, %), EPS quantification and HPSEC-RID samples were stored at -18°C before analysis.

pH and TTA of sorghum batters

The pH of sorghum fermented batters was measured using a pH meter (Beckman Instrument mod F350, Furlenton, CA, USA) after 15h of fermentation. The batter's total titratable acidity (TTA) was measured according to the method of Jekle et al. (2010) as follows: frozen samples were defrosted at 5°C overnight before being analyzed. Batter samples (5 g) were weighed, and 45 ml of distilled water was added. While stirring, a solution of NaOH 0.1 M was added to the sample solution until the pH 8.5. TTA was expressed in ml of NaOH solution (0.1 M). At least two TTA measurements were performed for each sample batch.

Molecular status of sorghum batters

Molecular weight distribution. The molecular weight distribution of sorghum flour components in fermented batters was determined using High-Performance Size-Exclusion Chromatography coupled with a Refractive Index Detector (HPSEC-RID) following the method of Fuso et al. (2023b), with slight modifications. 300 mg of fermented sorghum batters were added to 1 mL of ultrapure water and kept under stirring for 1h at room temperature. The solutions were centrifuged at 3197 g and 4 °C for 20 minutes, then the supernatant was withdrawn, filtered through a 0.45 µm nylon membrane and transferred into a vial. A 20 µL sample was injected in an Agilent 1260 Infinity II LC system equipped with a refractive index detector (RID) (Agilent, Santa Clara, CA, USA). The separation was performed using ultrapure water as the eluent (flow rate 0.7 mL/min) and a PL aquagel-OH mixed-M column (7.5 × 300 mm, 8 µm) (Agilent, Santa Clara, CA, USA). The column and RID temperatures were set at 30°C and 35°C, respectively. Standard pullulans with molecular weights ranging from 667 to 642,000 Da were used for the calibration curve. The analysis was performed in triplicate for each sample batch.

SDS-PAGE and degree of hydrolysis (%). To determine the molecular weight profile of sorghum proteins after fermentation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and *o*-phthaldialdehyde (OPA) analyses were performed on sorghum batters.

Sorghum batter proteins were solubilized using 5% SDS solution (sample to SDS solution 1:1) and placed in a shaking incubator at room temperature for 30 min. The samples were centrifuged at 4 °C, for 15 min (14,000 rpm). The supernatant was collected and used for further analyses (SDS-PAGE and OPA analysis).

Before performing the SDS-PAGE assay, the protein content of the sorghum batters was determined using the Qubit Protein Assay Kit and the Qubit 2.0 Fluorometer™ (Invitrogen, California, USA) to calculate the amount of sample needed for the subsequent steps (sample volume corresponding to 20 µg of protein). The SDS-PAGE was performed following the method of Prandi et al. (2021), with modifications. A Criterion™ XT 10% Bis-Tris gel (Bio-Rad, Hercules, CA, USA) was used in combination with MES running buffer and Coomassie Brilliant Blue as the staining solution. Once the stain was removed, the gel was scanned using a GS-800™ scanner with Quantity One software (Bio-Rad, Hercules, CA, USA). The degree of hydrolysis (DH; %) of sorghum batter proteins was determined by *o*-phthaldialdehyde (OPA) analysis, according to the method of Sousa et al. (2020), with modifications. Briefly, 32 µL through o-phthaldialdehyde (OPA) analysis, according to the method of Prandi et al. (2021), with modifications. Briefly, 32 µl of diluted extract was mixed with 928 µL of OPA reagent (0.8 mg/mL DDT, 10 mg/mL SDS, 0.05 M borate buffer, 5 mg/mL Tergitol™ 15-S-9, 0.8 mg/mL *o*-phthaldialdehyde). The blank samples were analyzed by replacing the OPA reagent with MQ water. A standard solution of L-isoleucine (2 mg/mL) was used for the calibration curve. Absorbance was measured at 340 nm using a JASCO B-530 UV-Vis spectrophotometer (JASCO, Oklahoma City, OK, USA) after 10 min of incubation at 30°C in the dark. The total number of amino groups was calculated by dividing the total amount of protein (determined by the Kjeldahl method, conversion factor 5.67 (Mariotti et al., 2008)) by the average molecular weight of the amino acids (110 Da). Three replicates were performed for each batch of samples.

EPS quantification. The quantification of exopolysaccharide production by LAB strains in fermented batters was calculated by quantifying the soluble fiber content according to the AOAC official enzymatic-gravimetric method 991.43 for total, soluble, and insoluble dietary fiber in foods (Lee et al., 1992). The analysis was performed in duplicate for each sample batch.

Low-resolution ^{1}H NMR molecular mobility. The proton molecular mobility of sorghum batters was studied using a low resolution ^{1}H NMR spectrometer (the minispec, Bruker Biospin, Milan, Italy) (20 MHz) at 25.0 ± 0.1 °C. The ^{1}H self-diffusion coefficient (D) with a pulsed-field gradient spin-echo (PFGSE) pulse sequence and the T_2 spin-spin relaxation with the Carr-Purcell-Meiboom-Gill (CPMG) sequence were measured for each sample. NMR tubes were filled with sorghum batter (10 mm height), covered with Parafilm® to avoid moisture loss during the analysis,

and placed in a climate chamber (ICH256, Memmert, Schwabach, Germany) at 25°C for 30 min before analysis. The recycle delay (RD) was preliminarily calculated by means of the spin-lattice ^{1}H T_1 sequence (Inversion Recovery) and set for subsequent analyses at 1.2 s. The proton self-diffusion curve was previously calibrated with a CH_3COOH standard solution ($D_{25°C}$ = 1.08 10^{-9} m^2/s) with a gradient pulse amplitude range between 35 and 65 %. The PFGSE sequence was performed with a 40 % gradient pulse amplitude, 16 scans, a gain of 72. After the PFGSE sequence, the T_2 CPMG sequence was applied to the tube, with 32 scans, gain of 72, interpulse spacing of 0.04 and 2500 data points. The CPMG curves were analyzed as a quasi-continuous distribution of relaxation times using UPEN software (Alma Mater Studiorum, Bologna, Italy) and with a discrete exponential model (Sigmaplot v.10, Systat Software Inc., USA) to obtain relaxation times (ms) and proton relative abundances (%), as described by Marchini et al. (2021a). Three tubes were analyzed for each sample batch, and five (PFGSE) and three (CPMG) measurements were performed for each tube. Between one CMPG acquisition and another, 10 min was passed to avoid overheating of the sample.

Technological properties of sorghum batters

The changes in the technological properties of sorghum batters resulting from the molecular modification brought about by LAB fermentation were analyzed in terms of thermal, pasting and rheological properties.

Thermal properties. The thermal properties of the batters were evaluated using a differential scanning calorimeter (DSC Q200, TA Instruments, New Castle, DE, US), calibrated using indium (melting temperature 156.6 °C, $\Delta H = 28.71$ J g^{-1}) and mercury (melting temperature -38.83 °C, $\Delta H = 11.41$ J g^{-1}). 5–10 mg of sorghum batter was weighed into stainless steel pans (PerkinElmer, Waltham, MA, USA) and hermetically closed. An empty pan was used as the reference. The samples were equilibrated at 30 °C and then heated to 130 °C at 5 °C/min, as previously reported by Marchini et al. (2021b). DSC thermograms were analyzed using TA Universal Analysis software (v. 4.5A, TA instruments, New Castle, DE, USA) to obtain the onset (Ton, °C), peak (Tpeak, °C), and offset (Toff, °C) transition temperatures, and enthalpy change (ΔH, J/g) associated with the transition. At least three thermograms were analyzed for each sample batch.

Pasting properties. The pasting properties of sorghum batters were evaluated using a starch stirrer cell (rheometer MCR 102, Anton Paar, Gratz, Austria). The batters were heated and cooled according to the method described by Matignon et al. (2014) with modifications: pre-shear 60 s at 50 °C (160 rpm), holding for 5 min at 50 °C (160 rpm), heating to 95 at 5 °C/min (160 rpm), holding for 5 min at 95 °C (160 rpm), cooling to 25 °C at 5 °C/min (160 rpm), holding 25 °C for 2 min (160 rpm). The measuring cell was covered with a solvent trap to control moisture loss during the entire analysis. The following pasting properties parameters were calculated using RheoCompass software and analyzed: pasting temperature (PT), peak viscosity (PV), holding strength (HS), breakdown (B), final viscosity (FV), setback from peak (SP), and setback from trough (ST). Three replicates were performed for each batch of batter samples.

Rheology. The flow behavior of the sorghum batters was measured using a rheometer (MCR 102, Anton Paar, Gratz, Austria). The analysis of the flow behavior was carried out at 25 °C using a concentric cylinder geometry, with a shear rate between 0.01 and 1000 s^{-1}. Flow curves were elaborated to calculate the consistency coefficient (m) and the flow behavior index (n) using the power-law model, as reported by Marcotte et al. (2001):

$$\sigma = m\,\dot{\gamma}^{\,n} \qquad (1)$$

where σ is the shear stress and $\dot{\gamma}$ is the shear rate.

Three replicates were performed for each batch of batter samples.

Statistical analysis

Data are expressed as the mean ± standard deviation. Differences between sample means were calculated by one-way ANOVA (SPSS Inc., Chicago, IL), followed by LSD *post hoc* test, or by Welch's robust test followed by Games-Howell *post hoc* test in case of non-homogeneous variance. Statistical analysis of microbiological data was performed applying the t-Student test at the 95 % significant level using Microsoft Excel.

Results and Discussion

Microbiological analysis of sorghum flours and batters.

Generally, flour fermentation requires sugar addition, that can be immediately used by the fermenting strains. The amount of sugar addition depends on the kind of flour and the strains

used for fermentation process (Gallo et al., 2020). Being the sorghum sourdough fermentation not deeply explored by the literature, in the present study, two different sugar concentrations were tested and compared to a batter without any added sugar (Gallo et al., 2019).

The lag phase of all the strains, regardless of the sugar concentration used, was less than one hour (Table 7). This result was also observed for the other parameters, such as the growth rate (Rate) and the maximum acidification capacity (Yend) (Table 7). Therefore, from these data, it was observed that the addition of sugar provides no advantages in the growth of the strains used for fermentation, therefore it was decided to continue the experimentation by fermenting the batters without the addition of sugar. Furthermore, given the fast-growing rate of the strains, it was decided to proceed with the fermentation at a temperature lower than the optimal for the tested strains, to allow a slower growth of the fermenting strains. This would be more feasible in the real-case scenario of a bakery dealing with sorghum sourdough products.

Table 7. Mean values of impedometric parameters during sorghum fermentation with different sugar concentrations.

Name	Sucrose	Lag	DS Lag	Rate	DS Rate	yEnd	DS yEnd
Lb. 1932	0%	0.90	0.05	13.34	0.06	42.22	0.065
Lb. 1932	1%	0.90	0.03	13.25	0.09	41.98	0.054
Lb. 1932	5%	0.90	0.06	12.89	0.04	41.44	0.03
Lcb 4339	0%	0.90	0.021	6.40	0.061	42.01	0.04
Lcb 4339	1%	0.90	0.032	6.42	0.056	42.05	0.04
Lcb 4339	5%	0.90	0.076	6.73	0.04	42.09	0.022
L 4454	0%	0.90	0.065	9.98	0.05	35.91	0.054
L 4454	1%	0.90	0.054	9.89	0.03	35.80	0.01
L 4454	5%	0.90	0.045	9.94	0.05	35.63	0.045

The initial microbial load of sorghum flour was evaluated. Total microbial count of batters before inoculum (T0) was, as indicated in Table 8, 3.28 (±0.05) Log CFU/g. Coliforms were present at 2.79 (±0.07) Log CFU/g, *Staphylococcus* spp was detected at a concentration of 2.83 (±0.16) Log CFU/g, while spore-forming bacteria resulted to be 2.29 (±0.26) Log CFU/g. Rod-shaped LAB, neither yeast and molds nor coccoid LAB were revealed (Table 8).

Table 8. Microbial loads and pH of batters before (T0) and after 15 hours of fermentation (T15)

	T0				T15		
	Control	***Lb. 1932***	***L. 4454***	***Lcb 4339***	***Lb. 1932***	***L. 4454***	***Lcb 4339***
Rod shaped LAB (MRS)	0±0	7.47±0.29	7.2±0.38	8.03±0.14	8.98±0.1	8.98±0.03	9.35±0.04
Coccoid shaped LAB (M17)	-	-	-	-	0±0	0±0	0±0
Yeast (YEDC)	0±0	-	-	-	0±0	0±0	0±0
Total microbial count (PCA)	3.28±0.05	-	-	-	8.93±0.12	8.99±0.04	9.38±0.01
Coliform (CHR)	2.79±0.07	-	-	-	5.03±0.03	2.15±0.21	4.03±0.3
Staphylococcus spp (MSA)	2.83±0.16	-	-	-	2.34±0.49	0±0	2.36±0.09
Spores (TSA)	2.29±0.26	-	-	-	1.97±1.12	1.91±0.17	2.41±0.08
pH	6.23±0.01	-	-	-	4.49±0.05	4.33±0.1	4.18±0.11

Plate counts data are reported as mean value of Log CFU/g.

Upon fermentation experiments, the counts of inoculated LAB strains were obtained just after the inoculum and at the end of fermentation, when also contaminants were counted.

The rod-shaped LAB count increased up to 9.35 log CFU/g for all the tested strains, proving their ability to grow on the sorghum batter. Consequently, the total microbial count also increased (Table 8). Coliforms concentration increased to about 3 and 2 Log CFU/g when the fermentation was performed respectively with *Lb*. 1932 and *Lcb*. 4339. On the other end, the coliform count remained almost unchanged after the fermentation with *L*. 4454, which inhibited also the growth of *Staphylococcus* spp during fermentation. The inhibition of coliforms by LAB is known to be due to a general preservation effect obtained from the accumulation of organic acids and alcohols concomitantly with the reduction of the level of free sugars, depletion of oxygen and lowering pH (Hansen, 2002). However, the final pH measured in the fermented batters by *L*. 4454, was neither the lowest nor significantly different, compared to the batters fermented by the other two strains. This is in line with what reported by Abhisingha et al. (2018), who hypothesized that pH lowering might not be the sole factor inhibiting the growth of fecal coliforms, while it is possible that other antimicrobial substances like bacteriocin(s) might be produced by *Leuconostoc* spp (Blocher and Busta, 1985; Charlier et al., 2009; Baril et al., 2012; de Paula et al., 2015).

In Table 9 the results of the impedometric analysis of the fermentations conducted at 25°C for all the strains were reported. As can be seen, two strains, *Lcb.* 4339 and *L*. 4454, adapted more

quickly to the batter environment, showing significantly shorter Lag phases compared to *Lb.* 1932. This result could have been influenced by the fermentation temperature, which was closer to the optimum for *Lcb.* 4339 and *L.* 4454 (mesophilic), while far away from that of *Lb.* 1932 (thermophilic). This trend was observed also for the growth rate (Rate), which was significantly higher for the strain *L.* 4454, likely because of the fermentation temperature. Finally, no significant differences were observed in the final acidification value (yEnd), which was comparable for all three strains. This means that, despite the different growth rates, all the tested strains were able to reach the same final acidification levels in the sorghum batters.

Table 9. Mean values of impedometric parameters for all the strains.

	Lag	DS Lag	Rate	DS Rate	yEnd	Ds yEnd
***Lb.* 1932**	2.70*	0.07	3.95*	0.35	40.70	0.46
***Lcb.* 4339**	0.33	0.14	4.54*	0.46	42.07	0.31
***L.* 4454**	0.42	0.83	6.65*	0.59	36.24	0.42

*Significative differences among the strains for each parameter, t student (α=0.05).

pH and TTA of sorghum batters

After 15 h of fermentation at 25 °C, all fermented samples showed a decrease in pH, from the initial value of 6.23 ± 0.01 (control T0) to different values according to the LAB strain used (Table 8). All the fermented batters showed a statistically lower pH compared to the unfermented counterparts [control 15 (T15), kept at 5 °C for 15 h] (6.19 ± 0.12). The lowest pH value was reached by *Lcb.* 4339 with a pH of 4.18 ± 0.11, followed by *L.* 4454 with pH 4.33 ± 0.1 and *Lb.* 1932 with pH 4.49 ± 0.05. As a consequence of LAB metabolism and organic acid production, fermented batters showed an increase in the total titratable acidity (TTA) of the samples. While the control T15 showed a TTA value of 0.65 ± 0.05 mL NaOH 0.1 M, *Lb.* 1932 showed an increase to 2.63 ± 0.02 mL, whereas the highest values were found in *L.* 4454 and *Lcb.* 4339 with value of 3.13 ± 0.02 mL and 3.08 ± 0.08 mL, respectively.

Molecular status of sorghum batters

High-Performance Size-Exclusion Chromatography (HPSEC) was used to determine the molecular weight distribution of the water-soluble flour components inside sorghum batters after fermentation. Changes in the molecular weight of flour compounds, such as starch, fibers and proteins, have been reported to be important in relation to the modification of rheological and technological properties of flour (Lin et al., 2003; Hammed et al., 2015; Kowalski et al., 2018; Tao et al., 2019) as well as nutrient's digestibility (Martinez et al., 2018; Rieder et al., 2021). The results of HPSEC analysis were reported in Table 10. The low-molecular weight (LMw) fraction did not change after fermentation, showing values of ~0.7 kDa in all samples. In contrast, the relative abundance of this fraction, in terms of peak area (%), decreased upon fermentation, probably because of microbial consumption of compounds belonging to this fraction (monosaccharides and oligosaccharides, amino acids, and small peptides) (Nsogning et al., 2018; Galli et al., 2019; Viesser et al., 2020) as well as an increase in other fractions with different molecular weights (Table 10). In general, in control 15, *Lb.* 1932 and *Lcb.* 4339, the LMw fraction represented the most abundant fraction out of the total peak area (%). In *L*. 4454, by contrast, the most abundant fraction consisted of medium-molecular weight (MMw) compounds (77-265 kDa), highlighting the specific hydrolytic activity of this LAB strain and its different nutritional requirements compared with other LAB strains.

As a result of the biopolymer breakdown performed by the LAB strains on sorghum flour, the molecular weight of MMw1 fraction (7-9 kDa) decreased after fermentation, showing lower values in *L*. 4454 and *Lcb.* 4339 samples than control 15 (Table 10). The relative abundance of this fraction, however, did not change after fermentation. The MMw2 fraction (77-265 kDa) showed the most evident changes after fermentation, both in terms of molecular weight and peak area (%), suggesting structural changes in either starch, soluble fibers, or proteins (Table 10). In particular, the molecular weight of this fraction showed a marked decrease after fermentation in all samples, clearly indicating its depolymerization following LAB growth. The high-molecular weight (HMw) fraction (~1-2 kkDa) also showed significant differences after fermentation, showing a lower molecular weight in fermented samples than in control 15 (Table 10), indicating a breakdown of the chain length of amylopectin, fibers, as well as protein aggregates of high molecular weight.

Table 10. Molecular weight (kDa) and relative peak area (%) of low molecular weight (LMw), medium molecular weight (MMw1 – MMw2) and high molecular weight (HMw) fractions in sorghum batters.

		Control 15	*Lb.* 1932	*L.* 4454	*Lcb.* 4339	*p-value*
Molecular Weight (kDa)	**LMw**	0.69 ± 0.02^{a}	0.72 ± 0.03^{a}	0.74 ± 0.04^{a}	0.67 ± 0.07^{a}	*ns*
	MMw1	9.2 ± 0.8^{a}	9.1 ± 0.9^{a}	6.9 ± 0.8^{b}	6.9 ± 0.5^{b}	**
	MMw2	264.6 ± 21^{a}	115.9 ± 16.1^{b}	80.8 ± 7.3^{c}	77.0 ± 15.6^{c}	***
	HMw	1429 ± 8^{a}	1191 ± 166^{b}	1138 ± 35^{b}	1084 ± 104^{b}	*
Peak area (%)	**LMw**	61 ± 3^{a}	53 ± 6^{ab}	40 ± 9^{c}	48 ± 7^{bc}	*
	MMw1	12 ± 2^{a}	10 ± 3^{a}	10 ± 3^{a}	8 ± 3^{a}	*ns*
	MMw2	17.2 ± 0.2^{d}	28 ± 2^{c}	43 ± 5^{a}	36 ± 4^{b}	***
	HMw	11 ± 1^{a}	8 ± 2^{a}	7 ± 2^{a}	7 ± 1^{a}	*ns*

Different letters indicate significant differences between samples (one-way ANOVA; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Sorghum batter proteins were extracted and characterized in terms of molecular weight (Figure 11). As shown in Figure 11, in the molecular weight range analyzed, no high molecular weight (HMW) aggregate bands were observed in any sample. Electrophoresis was performed under reducing conditions, thus inducing the separation of the subunits originally linked by disulfide bonds. In fact, all bands were below 75 kDa. In all samples, the most intense bands were just below 75 kDa and around 25 and 20 kDa. The band below 75 kDa could refer to the 66 kDa oligomeric band that has been noted in many previous studies on sorghum (Duodu et al., 2002, 2003; Belton et al., 2006; Correia et al., 2010; Marchini et al., 2021b). Clearer bands were observed at approximately 50 kDa, 37 kDa, and 15 kDa. According to previous studies, the low molecular weight bands can be assigned to β- kafirins (16-20 kDa), α-kafirins (23-26 kDa), and γ-kafirins (27-28 kDa) of sorghum (Duodu et al., 2003; Belton et al., 2006; De Mesa-Stonestreet et al., 2010; Marchini et al., 2021b).

As shown in Figure 11, sorghum proteins extracted from fermented and unfermented batters showed similar profiles on electrophoresis gels. The intensity of the bands was similar before and after fermentation, as no new bands appeared, nor did any disappear. This result indicated that the extracted sorghum proteins had similar molecular weights among different samples.

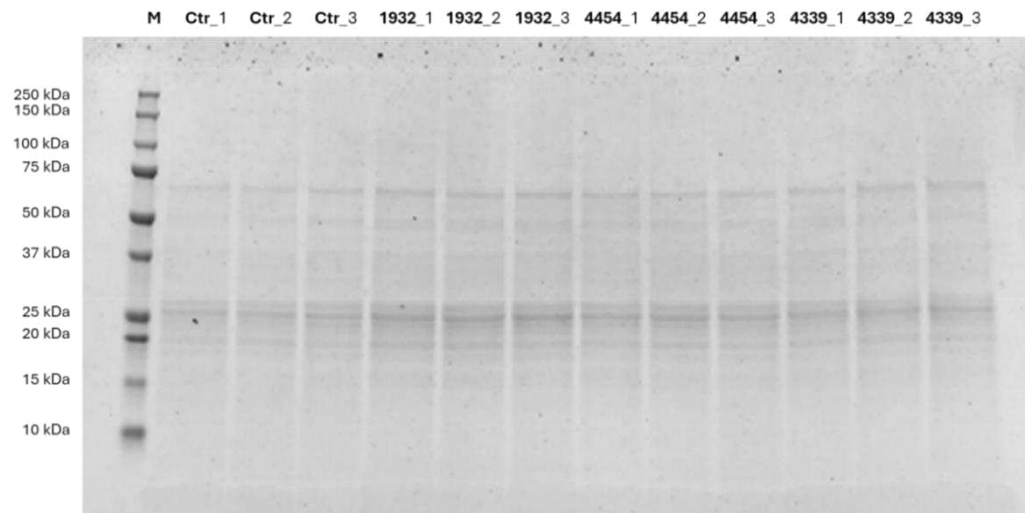

Figure 11. SDS-PAGE gel of sorghum batters. From the left: marker (M); Control 15 (Ctr_1-2-3); fermented samples Lb. 1932 (1932_1-2-3), L. 4454 (4454_1-2-3) and Lcb. 4339 (4339_1-2-3).

To better analyze the degree of hydrolysis of the protein extracts after fermentation, an OPA analysis was also performed. OPA analysis provided information on the ratio of cleaved peptide bonds to the total number of peptide bonds (Prandi et al., 2021). The OPA results did not show significant differences between the samples, showing similar values between the fermented and unfermented samples. Specifically, control 15 sample showed a DH of 0.58 ± 0.02 %, while the fermented samples showed values of 0.57 ± 0.02 %, 0.55 ± 0.03 % and 0.61 ± 0.04 % for *Lb.* 1932, *L.* 4454 and *Lcb.* 4339, respectively. Therefore, it can be assumed that, under the specific fermentation conditions used, LAB strains did not cause any peptide bond cleavage, at least as far as the protein fraction that could be extracted by SDS is concerned). Sorghum proteins may have shown strong resistance to the proteolytic activity of enzymes from selected LAB strains. Sorghum proteins have been shown to be resistant to degradation and digestion (Duodu et al., 2003). Furthermore, heat pretreatment of sorghum flour may have led to protein denaturation, increasing the number of protein-protein bonds and cross-links and the formation of large polymeric complexes (which may not have been extracted before SDS-PAGE analysis), which in turn may have affected the solubility and accessibility of proteins during fermentation (Vu et al., 2017). Moreover, fermentation may have led to the denaturation and inactivation of flour endogenous proteases, which highly contribute to protein hydrolysis (Verni et al., 2019). In addition, fermentation time must also be considered. Indeed, according to other researchers,

protein breakdown is not observed within a few hours of fermentation. For example, a study on the spontaneous fermentation of rice flour showed no differences after 24 hours of fermentation in the molecular weight of proteins analyzed with SDS-PAGE (Park et al., 2020), while there were observed after 48 hours of fermentation (Park et al., 2020). These results are interesting, since the differences in the molecular weight distribution of sorghum flour components observed by HPSEC analysis (Table 10. Molecular weight (kDa) and relative peak area (%) of low molecular weight (LMw), medium molecular weight (MMw1 – MMw2) and high molecular weight (HMw) fractions in sorghum batters.) cannot be attributed to the extractable proteins of sorghum, but most likely to starch, fibers, or to unextracted structural proteins or aggregates.

Due to the well-known importance of exopolysaccharides (EPS) production in the technological functionality of flour for different applications (Lynch et al., 2018; Hundschell and Wagemans, 2019; Wang et al., 2022), the *in situ* production of EPS by the lactic acid bacteria strains tested, was quantified by measuring the differences in the amount of soluble fiber in the samples. Although all the tested strains have been reported to be EPS producers (Fuso et al., 2023a), in the specific fermentation conditions applied in this work, the strains did not show any production of EPS, as the results of soluble fibers did not show any differences between fermented and control samples (Table 11).

Table 11. Soluble fiber content expressed in g/100 g of sorghum batters.

Sample	Soluble fiber (g/100 g)	*p-value ANOVA*
Control 15	0.34 ± 0.24	*ns*
Lb. 1932	0.4 ± 0.26	*ns*
L. 4454	0.28 ± 0.15	*ns*
Lcb. 4339	0.29 ± 0.29	*ns*

It is worth remembering that, in the present study, the fermentation conditions were determined based on preliminary baking tests (data not shown) in order to guarantee enough microbial growth and good performances in the final product. The selected conditions may have not provided the necessary fermentation time or sugar concentration needed for EPS production (Wu

et al., 2023). Moreover, the utilization of EPS as a substrate for microorganism growth after production should be taken into account (Barcelos et al., 2020). Although the quantitative analysis showed no difference in the total soluble fiber content in sorghum batters, the molecular weight of the fiber could still have changed after fermentation and have taken part in the molecular weight changes found in the HPSEC analysis.

In addition to the molecular weight distribution analysis, protein hydrolysis status and *in situ* production of EPS, the protons molecular mobility was also evaluated. Both the self-diffusion coefficient ^{1}H D (PFGSE sequence), related to translational mobility and matter displacement in the system in the absence of a chemical potential (Kovrlija and Rondeau-Mouro, 2017), and the rotational mobility of protons (T_2 CPMG relaxometry), providing information on the relaxation dynamics of protons in a complex system (Riley et al., 2022), were assessed. In particular, relaxation dynamics in starchy systems depend on the nature of molecules, their distribution in different flour compartments, and their interaction/exchange with other components (Kovrlija and Rondeau-Mouro, 2017). Both PFGSE and CPMG sequences provided valuable insights into structural changes of sorghum batters during fermentation. Figure 12a shows the results of the ^{1}H self-diffusion coefficient D. Control 15 exhibited the lowest value among the samples.

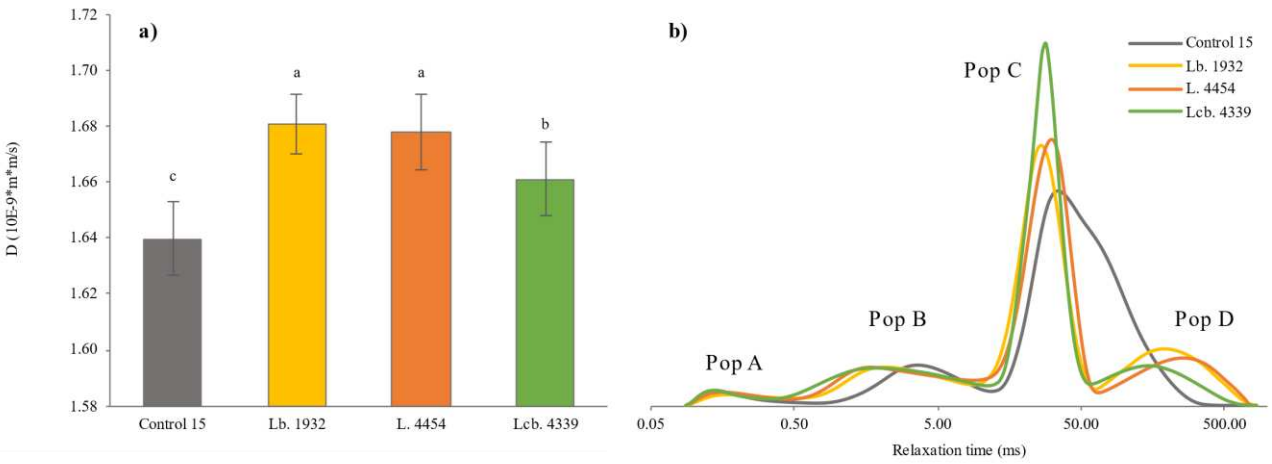

Figure 12. ^{1}H mobility of sorghum batters. (a): ^{1}H diffusion coefficient D values of batter samples. Different letters indicate significant differences between samples (= p < 0.05; ** = p < 0.01; *** = p < 0.001); (b): average CMPG proton relaxation curves of batter samples.*

According to Hopkins et al. (2019) restricted diffusion is determined by a combination of factors, including interactions with the polymer network, steric hindrance, adsorption, van der Waals forces, and ionic interactions. Indeed, lower values of self-diffusion coefficient D in control 15 sample were expected, as matter molecular displacement can be reduced by the presence of structural boundaries and, as showed in HPSEC analysis (Table 10), control 15 contained more compounds of a higher molecular weight than fermented samples, which may have limited self-diffusion. According to Kovrlija and Rondeau-Mouro (2017) the possible presence of starch molecules clustered together creating porous structures may impede proton diffusion in starchy samples. Moreover, the ^{1}H coefficient D represents an average value of modification at different levels, including changes in different biopolymers other than starch (fibers, proteins). Thus, variations in these fractions across different compartments may account for the lower proton diffusion in control 15, if compared to other samples. Additionally, the HPSEC analysis indicated a larger peak area for lower molecular weight fractions in control 15 (Table 10), suggesting a higher concentration of solutes, which has also been reported to affect the proton self-diffusion coefficient D (Götz and Hinrichs, 2008; Kovrlija and Rondeau-Mouro, 2017). Differences in the self-diffusion coefficient D were observed also among the fermented samples, with *Lb*. 1932 and *L*. 4454 showing the highest D values, followed by *Lcb*. 4339. These variations may be due to the specific strain activity affecting the biopolymer and solute concentrations, leading to different molecular weight components that bind water differently and create varying degrees of barriers to proton diffusion. The production of organic acids during fermentation was instead reported to not affect the proton diffusion in dough systems (Hopkins et al., 2019).

The results of T_2 CPMG sequence are reported in Figure 12b (quasi-continuous distributions) and Table 12 (relaxation times and relative abundances). Four CMPG populations were detected in sorghum batters (Pop A, Pop B, Pop C, Pop D) in accordance with previous studies on sorghum flour (Marchini et al., 2021a; Chiodetti et al., 2024). Among the populations, Pop C was the most abundant in all samples. This population refers to the exchanging extragranular protons of starch and flour biopolymers in interaction with water (Bosmans et al., 2012; Marchini et al., 2021a). The second most abundant population was the most mobile Pop D, which has been reported to refer to weakly-bound OH protons of water (Marchini et al., 2021a; Parenti et al., 2021; Chiodetti et al., 2024). The other two CPMG populations, more rigid, refer to CH protons of amorphous starch

and proteins in little contact with water and dietary fiber constituents (Pop A) and to OH protons of intragranular water and starch, and CH protons of proteins in interaction with confined water (Pop B) (Bosmans et al., 2012; Marchini et al., 2021a).

Table 12. Relative percentages and relaxation times of CPMG ^{1}H populations of batter samples

	Control 15	***Lb*. 1932**	***L*. 4454**	***Lcb*. 4339**	*p-value*
popA (%)	4.49 ± 0.29^{c}	8.41 ± 1.1^{b}	8.99 ± 1.31^{ab}	10.08 ± 0.8^{a}	***
popB (%)	12.55 ± 0.73^{b}	13.59 ± 0.75^{a}	13.90 ± 0.78^{a}	13.80 ± 0.54^{a}	***
popC (%)	49.69 ± 4.03^{a}	52.48 ± 1.98^{a}	52.76 ± 3.24^{a}	53.90 ± 2.54^{a}	*ns*
popD (%)	33.08 ± 3.9^{a}	25.52 ± 2.22^{b}	24.09 ± 2.66^{b}	21.67 ± 2.54^{c}	***
tA (ms)	0.32 ± 0.11^{b}	0.93 ± 0.19^{a}	0.87 ± 0.18^{a}	0.83 ± 0.07^{a}	***
tB (ms)	4.43 ± 0.35^{a}	4.31 ± 0.48^{a}	4.18 ± 0.54^{a}	4.09 ± 0.25^{a}	*ns*
tC (ms)	27.74 ± 1.77^{a}	25.58 ± 1.24^{b}	27.05 ± 0.98^{a}	26.18 ± 0.87^{ab}	*
tD (ms)	81.56 ± 8.3^{c}	122.48 ± 12.65^{a}	107.27 ± 17.24^{a}	94.63 ± 17.62^{b}	***

Different letters indicate significant differences between samples (one-way ANOVA; * = p < 0.05; ** = p < 0.01; *** = p < 0.001).

Fermentation changed the molecular relaxation dynamics of different flour molecular domains (Figure 12b; Table 12), with major differences in Pop C and Pop D. As can be seen from Figure 12b, the last two populations (Pop C and Pop D) were partially overlapped in control 15. A high amount of weakly bound water protons (Pop D) and close relaxation times between Pop C and Pop D in control 15 may have resulted in worse resolution and thus overlapping of Pop C and Pop D. Indeed, looking at the comparison between different samples (Table 12), control 15 showed the highest values of Pop D in terms of relative abundance (%), while its relaxation time (ms) was lower than the fermented samples. The mobility of Pop D may also be influenced by solutes and small free components concentration, which were seen to be higher in control 15 than in fermented samples (HPSEC analysis, Table 10). Indeed, Blanco Canalis et al. (2018) attributed the presence of dissolved sugars in the population with highest mobility. A higher presence of dissolved solutes may have lowered the mobility of Pop D. However, the presence of the weakly bound water fraction was greater in the control 15 sample than in the fermented sample. The highest mobility of Pop D was found in *Lb*. 1932 and *L*. 4454 batters. *Lcb*. 4339 showed instead intermediate values

between the control 15 and the other fermented batters. In general, *Lcb*. 4339 showed the lowest contribution of Pop D, both in terms of relative abundance (%) and relaxation time (ms), in favor of populations with lower mobility. The specific strain activity brought about by *Lcb. 4*339 may have led to the establishment of stronger bonds between water and flour biopolymers in more constricted zones or in the network of exchanging protons between water and flour biopolymer. However, even if the relative abundance (%) of Pop C was not significantly higher in *Lcb*. 4339 and *Lb*. 1932 than in the other samples, significantly lower relaxation times of Pop C were found in *Lb*. 1932 and *Lcb*. 4339. Presumably, in *Lb*. 1932 and *Lcb*. 4339 samples, stronger bonds were established between water and flour biopolymers starch, fibers, proteins, that made Pop C more rigid. A lower mobility in fermented samples has previously been associated to the presence of tighter bond between water and intra e extra granular starch, as well as protein, in rice flour (Dou et al., 2023). In general, the differences in proton relaxation dynamics induced by LAB fermentation could be attributed to the modifications of different components, both in terms of molecule accessibility and size. Indeed, microbial enzymatic activity, consumption of nutrients, and the production of organic acids, may have altered the water-binding sites of flour biopolymers and the concentration of solutes. Moreover, Hopkins et al. (2019) affirmed that changes in the composition of flour (damaged starch, amino acid, non-starch polysaccharides) can provide different functional groups and thus affect the ratio of bound/unbound water in the systems. However, few studies have focused on the effect of LAB fermentation on water dynamics in flour using low-resolution NMR techniques. These findings are significant, as the ability of water to bind with different flour components at a molecular level can impact various processing dynamics in food products.

Technological properties of fermented batters

The techno-functional properties of sorghum batters, and their modification as a result of LAB fermentation, were analyzed in terms of thermal, rheological and pasting properties.

The thermal properties of the sorghum batter samples are shown in Table 13. All samples showed a main endothermic peak related to starch gelatinization. As can be seen from Table 13, fermentation with the selected LAB strains resulted in changes in the gelatinization properties of sorghum flour. In particular, a general increase in gelatinization temperatures (Ton, Tpeak, Toff) was observed after fermentation. At the same time, the enthalpy change (ΔH, J/g) associated

with the loss of molecular order during the endothermic transition was higher in fermented samples than in unfermented samples (Table 13). In particular, *Lb*. 1932 was the strain with the highest values of ΔH, followed by *L*. 4454 and *Lcb*. 4339.

Table 13. Thermal parameters of endothermic starch transition detected by DSC analysis.

	Control 15	***Lb*. 1932**	***L*. 4454**	***Lcb*. 4339**	*p-value*
Ton (°C)	72 ± 0.41^{b}	72.27 ± 0.09^{ab}	72.74 ± 0.48^{a}	72.82 ± 0.22^{a}	*
Tpeak (°C)	78.91 ± 0.09^{b}	79.96 ± 0.26^{a}	79.88 ± 0.46^{a}	79.89 ± 0.54^{a}	*
Toff (°C)	89.06 ± 0.67^{b}	91.13 ± 0.15^{a}	91.52 ± 0.31^{a}	90.84 ± 1.3^{a}	*
ΔH (J/g)	1.82 ± 0.17^{c}	2.77 ± 0.29^{a}	2.33 ± 0.34^{ab}	2.22 ± 0.17^{bc}	*

Different letters in the same row indicate significant differences between samples (one-way ANOVA; * = p < 0.05; ** = p < 0.01; *** = p < 0.001).

While the increase in gelatinization temperature after fermentation has been commonly reported on different matrices (potato starch, waxy rice starch, and cassava root starch) (Zhang et al., 2016; Ye et al., 2019; Oyeyinka et al., 2020), the effect of fermentation on ΔH is controversial, reporting both an increase and a decrease. Changes in the thermal properties of flour result from a combination of structural and compositional modifications that occur on flour during fermentation. These changes reflect both the modification of starch and the surrounding matrix, which consists of nonstarch polysaccharides and proteins, leading to direct and indirect changes in the functional properties of flour. Referring to starch modifications, microbial hydrolysis and consumption, as well as the production of organic acids, can lead to the modification of amylopectin structure (different chain length distribution, different crystallinity in terms of abundance and morphology), amylose/amylopectin ratio, and concentration of sugars and oligosaccharides (Zhang et al., 2016; Zhao et al., 2019a; Ma et al., 2022). These modifications have been reported to affect the functional and thermal properties of starch (Lin et al., 2016; Zhang et al., 2020, 2024) and may be responsible for the differences observed between fermented and unfermented batter samples. In particular, a decrease in amorphous portion and an increase in crystallinity due to microbial hydrolysis and organic acid production has been observed after fermentation (Zhang et al., 2024). According to some authors (Zhang et al., 2016, 2024; Zhao et al., 2019b), the relative increase in crystalline regions at the expense of amorphous counterparts

results in an increase in ΔH and gelatinization temperatures as crystals require more energy to dissociate. According to Zhang et al. (2016) and Zhang et al. (2024) this phenomenon occurs mainly in the early stages of fermentation, due to rapid hydrolysis of the amorphous region and increased crystallinity of starch granules. The effect of starch crystallinity on gelatinization temperatures was also reported by Lin et al. (2016), which associated gelatinization temperatures with crystal perfection. Moreover, molecular weight reduction and granule surface erosion due to enzymatic activity has also been reported to affect the temperatures and ΔH of starches (Zhang et al., 2016; Zhao et al., 2019a). However, Lin et al. (2016) and Zhang et al. (2020) reported that a higher presence of amylose and amylopectin medium-short chains increase ΔH and decrease gelatinization temperatures. In our study, however, the temperatures were found to increase. As mentioned above, in addition to direct changes in starch properties, changes in the matrix surrounding starch granules must also be considered. In fact, this matrix composed of protein, fiber, nonstarch polysaccharides can affect the ability of starch to gelatinize and, consequently, DSC results (Zhang et al., 2020). SDS page, DH % and HPSEC analysis results suggested a change in other than extracted proteins flour components. Changes in the structure and function of cell wall fibers and polysaccharides, as well as SDS non-extractable proteins, may have indirectly contributed to the gelatinization capacity of starch as determined by DSC analysis, thus explaining the increase in ΔH that occurred after fermentation.

The pasting properties of sorghum batters were also evaluated to investigate the effect of LAB fermentation on the viscosity of sorghum batters during a heating and cooling ramp. These properties are very important to predict the behavior of sorghum batters during food processing and can be influenced by fermentation. Indeed, over the heating ramp, changes in viscosity depend on the interactions between water and starch, such as water uptake, starch swelling and crystallite melting, exudation of molecular components, granule rupture, amylose content and retrogradation (Kumar and Khatkar, 2017). Pasting properties can also be influenced by proteins due to their ability to form gels after heating and cooling although in grains and pulses proteins are present to a lesser extent than starch (Ragaee and Abdel-Aal, 2006). The changes that occurred in the starch and matrix surrounding the granules during fermentation may have determined the differences found in the pasting properties of sorghum flours, reported in Figure 13 and Table 14.

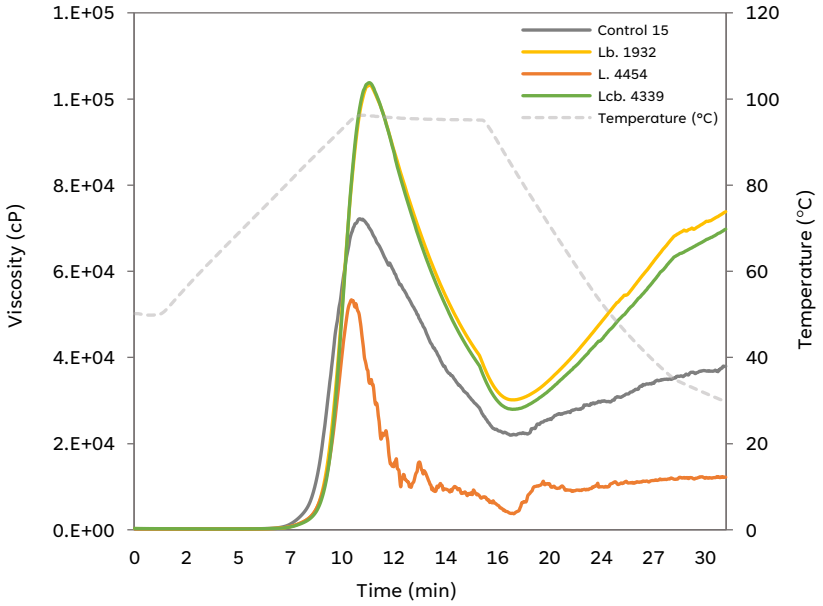

Figure 13. Average pasting curves of sorghum batters.

Table 14. Pasting properties parameters of sorghum batters.

	Control 15	***Lb.* 1932**	***Lcb.* 4339**	*p-value*
PT (°C)	72.31 ± 0.2^{b}	72.29 ± 0.25^{b}	72.91 ± 0.26^{a}	*
PV (cP)	73350 ± 2215^{b}	103584 ± 2594^{a}	104136 ± 1844^{a}	***
HS (cP)	21419 ± 1138^{b}	30142 ± 1185^{a}	27885 ± 2283^{a}	**
B (cP)	51930 ± 1358^{b}	73433 ± 1458^{a}	76260 ± 2785^{a}	***
FV (cP)	37956 ± 3866^{b}	73850 ± 4164^{a}	69787 ± 10283^{a}	**
SP (cP)	35398 ± 2143^{a}	29722 ± 4344^{a}	34353 ± 9653^{a}	*ns*
ST (cP)	16537 ± 2731^{b}	43708 ± 3802^{a}	41902 ± 8166^{a}	**

PV=peak viscosity; PT=pasting temperature; HS= holding strength; B= breakdown; FV=final viscosity; SP=setback from peak; ST=setback from trough.

Different letters in the same row indicate significant differences between samples (one-way ANOVA; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

Among different LAB strains, *L.* 4454 showed CO_2 production during analysis due to its heterofermentative metabolism, and lowered the viscosity values of the batter, which leavened during the analysis (Figure 13).Therefore, the pasting properties results of *L.* 4454 batter were not considered in subsequent comparisons and statistical analysis, as they were more influenced by the presence of air in the matrix rather than the changes in starch and protein fractions. Concerning the other samples, *Lcb.* 4339 batter showed slightly higher PT values than the control, indicating the need for higher temperatures to initiate starch gelatinization (Table 14). The PT values were comparable to the Ton values detected by the DSC analysis, both in absolute terms and in the comparison between the samples. As reported for DSC results the increase in temperatures after fermentation could in fact be due to a relative reduction of amorphous zones, which usually require lower temperatures to gelatinize, either because of microorganisms consumption or acidic environment (Zhang et al., 2024). *Lb.* 1932 and *Lcb.* 4339 samples, on the other hand, showed higher PV values than in the unfermented control. Interestingly, these results agree with DSC data regarding the ΔH (J/g) of *Lb.*1932 sample, while they do not for sample *Lcb.* 4339, which showed the same ΔH (J/g) as the control. Although both PV and ΔH give us information about starch gelatinization capacity, they refer to different properties of flour, thus explaining the differences found between the analyses. According to Ragaee and Abdel-Aal (2006), starch granules with high swelling capacity result in higher peak viscosity. In our case, the possible presence of granules freer from their surrounding matrix may have influenced their availability to swell and gelatinize. Fermented samples also showed an increase in HS and B compared to unfermented samples. During the holding phase, further granule rupture occurs due to high temperature and mechanical shear stress, resulting in a decrease in viscosity (Ragaee and Abdel-Aal, 2006). B refers to shear-induced destruction of starch granules, which ends in HS. According to Xu et al. (2019) a lower HS is related to the higher presence of resistant starch. Therefore, in this case, more resistant starch, which is less soluble, could be present in the control sample, resulting in lower HS. The batter samples showed no differences in setback from peak (SP) but did show differences in setback from trough (ST). Therefore, fermented samples showed higher viscosity recovery from HS levels. This result is associated with strengthening of hydrogen bonding and entanglement between starch chains, recrystallization of amylose which is the measure of the gelling ability or retrogradation ability of starches (Ragaee and Abdel-Aal, 2006; Kumar and Khatkar, 2017). Setback can also be influenced by the levels of amylose in the flour

(Kumar and Khatkar, 2017). Fermented samples also showed a greater ability to form a viscous paste, showing higher FV values (Figure 13; Table 14), confirming a better gel formation after fermentation.

Fermentation changed the viscosity profile and flow behavior of sorghum batters (Figure 14; Figure 15; Table 15). *Lb.* 1932 and *Lcb*. 4339 showed higher viscosity values than the control 15 in the range of shear rate analyzed, while *L*. 4454 showed a higher viscosity than control 15 only at low shear rates (< 10 s^{-1}).

These results are of great interest since, usually, the increase in viscosity after fermentation is reported mainly as a consequence of the production of exopolysaccharides (Xu et al., 2017; Galli et al., 2020). However, in our study, the quantification of soluble fibers did not reveal any EPS production, thus suggesting a different reason behind the changes in rheological behavior. Indeed, the analysis of the molecular status of sorghum batters (HPSEC and LR ^{1}H NMR analysis) revealed a change in the molecular weight distribution and mobility of sorghum flour after fermentation (Table 10; Figure 12; Table 12). Molecular weight changes were most likely related to starch and fiber, rather than protein (Figure 11), but the possible modification of molecular binding sites may have strengthened the bonds in the exchanging network of water starch and proteins as highlighted in CPMG ^{1}H NMR results (Pop C, Figure 12) for *Lb*. 1932 and *Lcb*. 4339.

In our opinion, the increase in viscosity and different flow behaviors may be related to the abovementioned changes in the molecular weight and mobility of flour biopolymers. In particular, higher viscosity values after fermentation could indicate the establishment of more bonds between biopolymer molecules, which has been reported to depend on molar mass and interchain interactions (Torres et al., 2014). Higher consistency coefficients (*m*) in fermented samples (Table 15) could be attributed to increased chain entanglement density (Torres et al., 2014). It has also been reported that the viscosity and flow behavior index (*n*) and its variation with concentration depend strongly on molecular size (Marcotte et al., 2001; Dobrynin et al., 2023). In the whole shear rate range analyzed, the *n* value of all the samples was < 1, thus showing an overall shear thinning behavior (Table 15) (Zhang et al., 2005).

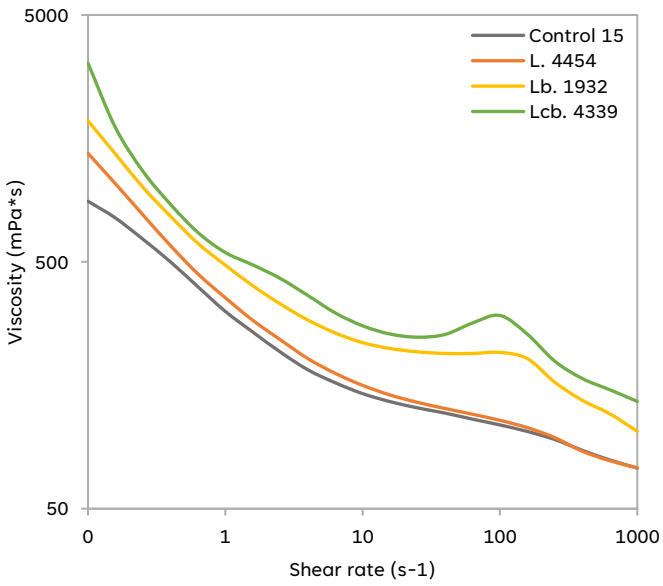

Figure 14. Average viscosity curves of unfermented and fermented sorghum batters.

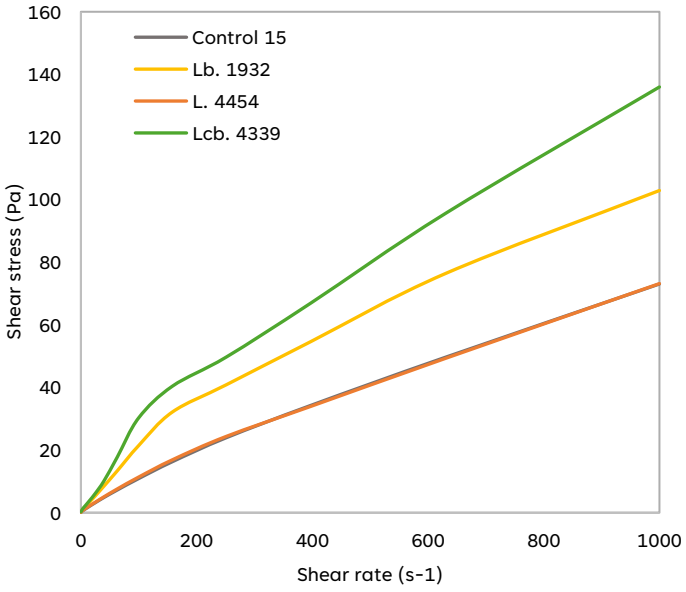

Figure 15. Average flow curves of sorghum batters.

Table 15. Power law model fitting of sorghum batters flow curves. m= consistency index; n= flow behavior index.

	Power law model		
	m **(Pa*s^n)**	***n*** **(-)**	**R^2**
Control 15	0.35	0.74	0.998
Lb**. 1932**	0.58	0.74	0.997
L**. 4454**	0.42	0.71	0.996
Lcb**. 4339**	0.72	0.74	0.997

However, in *Lb*. 1932 and *Lcb*. 4339 samples, a viscosity peak was observed around shear rates of 100 s^{-1}. Indeed, after an initial decrease, the viscosity curves of *Lb*. 1932 and *Lcb*. 4339 began to increase up to a shear rate of 100 s^{-1} and then decreased again for higher shear rates, apparently showing shear thickening behavior around 100 s^{-1} of shear rate. According to the literature, the shear thickening behavior of starch suspensions depends on the concentration of solutes and the particle size and molecular structure of molecules (Crawford et al., 2013; Duan et al., 2024). To investigate deeper into the phenomenon observed, additional tests were performed on batter samples (Figure 16). In particular, a second and third viscosity runs were performed. The second run was performed straight after the first run, without a pre-mixing of the samples, while the third run was performed after remixing the sample. The second run curve did not show any peak (normal shear thinning behavior) (Figure 16), while in the third run curve the peak appeared again (the effect was completely reversible) (Figure 16).

We propose a possible explanation of this phenomenon: medium to high molecular weight polymers align and follow the flow until a certain shear rate, thus showing the typical shear thinning behavior with a decrease of viscosity. In fact, shear thinning or pseudoplastic behavior is the result of molecular alignment, orientation or deformation of macromolecular network in the direction of flow within a substance (Marcotte et al., 2001; Zhang et al., 2005). At a threshold level of shear rate (100 s^{-1}) particles start to collide between each other, they get embedded between themselves and start contrasting the flow (shear thickening). With higher shear rate (>100 s^{-1}), however, particles start to align again, and the viscosity decrease (shear thinning). The particles that are responsible for the peak viscosity formation go on the top of the probe after reaching 1000 s^{-1} shear rate (maximum value of shear rate), thus, an additional run without

premixing (second run) would not be affected by these particles, and thus would not show the peak. However, by remixing again the sample before another viscosity test (third run), the particles would be back in the suspension, and thus show the peak again. The findings align with our hypothesis, indicating that viscosity changes resulting from fermentation are largely dictated by modifications to molecular properties, molecular weight, interactions, and mobility. The consequences of these changes under varying shear conditions are particularly noteworthy, given their potential impact on the processing and utilization of fermented sorghum flour in food products.

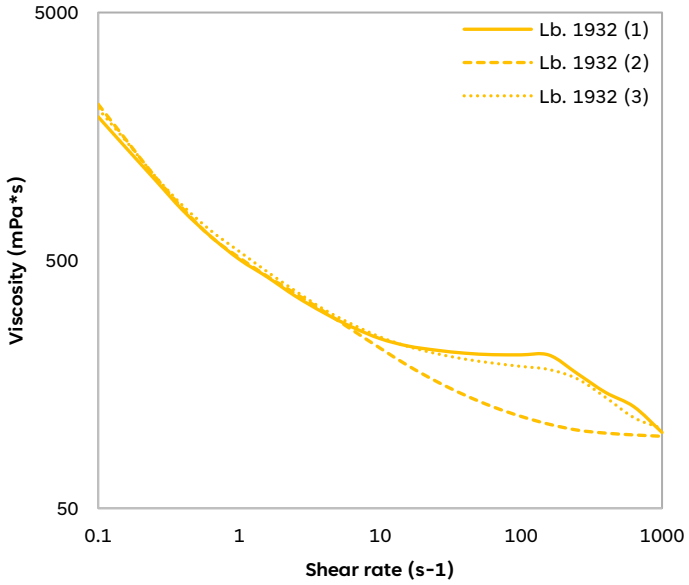

Figure 16. First (1), second (2) (no mixing), and third (3) (remixing) measurement of representative viscosity curves of Lb. 1932 batter sample.

Conclusions

This study demonstrated the significant impact of fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* 1932, *Leuconostoc* spp. 4454, and *Lacticaseibacillus casei* 4339 on the microbiological, molecular, and technological properties of sorghum batters. All LAB strains exhibited robust growth on sorghum, significantly increasing acidification and exerting a suppressive effect on contaminant microbiota. The investigation of molecular structural

modifications and technological results provided a solid understanding of fermentation-induced changes in sorghum flour and its potential applications. At the molecular level, the reduction of low molecular weight fractions and degradation of medium-high molecular weight fractions emphasized the role of microbial activity on the starch and fiber matrix of sorghum. Conversely, the resistance of sorghum proteins to microbial proteolysis indicated that protein structures remain largely unaffected by these LAB fermentation experimental conditions.

These molecular changes were directly reflected in the technological properties of sorghum batters, particularly through enhanced viscosity and improved starch gelatinization. Specifically, the alterations in starch, fiber, and their interactions suggest that LAB fermentation can be strategically applied to improve the functional properties of sorghum-based products, especially by optimizing viscosity and gelatinization behavior. The absence of exopolysaccharide production indicated that the viscosity changes were predominantly driven by biopolymer interactions rather than microbial polymer synthesis. This comprehensive understanding opens pathways to optimize fermentation conditions and tailor sorghum properties for specific food applications. Overall, this study highlights the potential of LAB fermentation as a promising strategy to enhance the functionality of sorghum-based products. Future research should further explore the relationship between molecular and macroscopic properties, optimizing bioprocessing for a wider range of food applications.

Section 2

SECTION 2

Effects of LAB Fermentation on Wheat-Sorghum Bread Quality

Bread is a staple food worldwide, valued for its versatility, accessibility, and nutritional benefits, making it an ideal product for introducing novel ingredients in baking applications. Incorporating sorghum flour into bread represents a strategic opportunity to elevate the presence of this underutilized grain in Western diets. However, the partial substitution of wheat flour with sorghum presents technical challenges, particularly in maintaining the viscoelastic structure essential for producing high-quality bread.

This section of the Ph.D. thesis presents two studies aimed at optimizing wheat-sorghum bread quality through fermentation with lactic acid bacteria (LAB). *Case Study 3* focuses on how fermenting sorghum with different LAB strains influences crucial quality indicators such as crumb structure, color, volatile profile, and sensory attributes. These factors are critical in determining consumer acceptance and the overall appeal of fresh bread. Building on the findings from *Case Study 2* and 3, a specific LAB strain was selected for further exploration in *Case Study 4*, which investigates the combined effects of LAB fermentation and freeze-drying on the rheological and molecular characteristics of wheat-sorghum dough, highlighting how fermentation alters interactions between wheat and sorghum components within the dough matrix.

Together, these studies offer valuable insights into the role of LAB fermentation in modulating dough properties, thereby impacting the final quality of wheat-sorghum bread and paving the way for sorghum's wider adoption in breadmaking.

2.1 Effect of Fermentation on Crumb Structure and Sensory Properties

Case Study 3

Selected LAB fermentation on crumb structure development, appearance, volatile and sensory properties of wheat-sorghum bread

Introduction and Aim of the Study

The addition of sorghum in worldwide daily consumed food, such as bread, would represent a good strategy to promote sorghum utilization in Western diets, as bread represents a daily consumed staple food all over the world. However, sorghum flour inclusion into bread formulations brings to light different shortcomings, as it can negatively impact the technological and sensory properties of bread. These negative effects are in terms of specific volume, texture, and general acceptability (Rumler and Schönlechner, 2021; Ari Akin et al., 2022), and are mainly due to the partial or total substitution of wheat flour with a gluten free flour which plays a role to the detriment of the technological quality of the product (Espinoza-Herrera et al., 2021). Proper bread crumb development is influenced by raw materials and processing conditions throughout various stages, including ingredient mixing, hydration, gluten matrix formation, foam structure creation, and stabilization of the porous structure during baking (Scanlon and Zghal, 2001). Some studies have focused on the application of fermentation on sorghum flour to improve the nutritional, technological, sensory, and safety attributes of food products. However, studies about the effect of fermentation on the technological functionality of sorghum have often referred to the spontaneous fermentation of flour (Hugo et al., 2003; Elkhalifa et al., 2004b, 2004a, 2005, 2006, 2017; Ge et al., 2020), which involves the development of a complex ecosystem and difficult to reproduce, and that highly depend on the place where it was produced. On the other hand, some studies compared both spontaneous fermentation and fermentation with selected lactic acid bacteria (LAB) strains or yeast (Correia et al., 2005; Pranoto et al., 2013), while other studies

investigated the sourdough fermentation of sorghum with selected LAB in the production of gluten-free bread and dough (Schober et al., 2007; Galle et al., 2012; Karrar et al., 2020; Olojede et al., 2020a, 2020b, 2022). However, research on the effects of sorghum fermentation with selected LAB strains in conventional bread, where part of the wheat flour is substituted with sorghum, remains limited (Hugo et al., 2003; Istianah et al., 2018; Karrar et al., 2020). Such research could expand the application of sorghum in more widely consumed products, reaching a broader consumer base.

In a previous study (*Case Study 2*), we investigated the effects of *Lactobacillus delbrueckii* subsp. *bulgaricus* 1932 (*Lb.* 1932), *Lacticaseibacillus casei* 4339 (*Lcb*. 4339), and *Leuconostoc* spp. 4454 (*L*. 4454) on the molecular and technological properties of sorghum flour, with a particular focus on the structural changes in the flour components and their impact on rheological, thermal, and pasting properties. The results indicated that fermenting sorghum with *Lb.* 1932, *Lcb.* 4339 and *L*. 4454 strains could enhance sorghum flour techno-functional properties, suggesting its potential as a viable technology for sorghum-based products. Fermentation with these strains may positively influence bread crumb development, thus affecting key quality attributes such as crumb appearance, loaf volume, texture, and flavor—all of which are important factors in consumer purchasing decisions (Scanlon and Zghal, 2001). Therefore, in this study, we evaluated the effect of fermentation of sorghum flour with *Lactobacillus delbrueckii* subsp. *bulgaricus* 1932, *Lacticaseibacillus casei* 4339, and *Leuconostoc* spp. 4454, on key quality attributes of a wheat-sorghum bread, including crumb development and appearance (crumb porosity and color), texture, volatile profile, and sensory acceptability.

Materials and Methods

Materials

Whole white sorghum HTC (heat-treated cereal) (*Sorghum bicolor* (L.) Moench) flour was supplied by MartinoRossi SpA (Cremona, Italy) (carbohydrate 55.7%, fiber 16.4%, protein 10.3%, total fat 3.2%). 00 type soft wheat flour (*Triticum aestivum* L.) (Italian legislation, 1967; Zhou et al., 2014) was supplied by Molino Agugiaro&Figna Molini SpA (Collecchio, Parma, Italy) (W 190-220, carbohydrate 71%, protein 10%, fiber 2%, fat 0.9%). Fresh yeast (*Saccharomyces cerevisiae*) (AB

Mauri s.p.a., Casteggio, Italy), salt (Italkali s.p.a, Palermo, Italy), sunflower oil (Oleificio Zucchi, Cremona, Italy) and sucrose (Eridania, Bologna, Italy), were purchased from a local market.

Sorghum flour fermentation

Three lactic acid bacteria (LAB) strains, *Lactobacillus delbrueckii* subsp. *bulgaricus* 1932 (*Lb.* 1932), *Leuconostoc* spp. 4454 (*L.* 4454), and *Lacticaseibacillus casei* 4339 (*Lcb.* 4339), were selected from the University of Parma Culture Collection (UPCC) for sorghum flour fermentation based on their known fermentative properties and previous trials (*Case Study 2*). Strain revitalization and microbiological analyses were carried out as described in our previous work (*Case Study 2*). Sorghum batters were prepared using 63% (w/w) of water, 35% (w/w) of flour and inoculated with 2% (v/w) of each LAB strain. Fermentation was conducted at 25°C for 15 hours, as determined by preliminary tests on sorghum batters (*Case Study 2*). After the fermentation period, the batters were either used fresh for breadmaking or subjected to color analysis. The control batter (without inoculum) was kept for 15 h at 5°C, to avoid spontaneous fermentation of the flour. For volatile compound evaluation, samples were frozen at -80°C. Microbial counts, pH measurements, and total titratable acidity (TTA) were performed as reported in our previous study, which used the same matrices (*Case Study 2*). Fermented batters were also assessed for molecular changes and technological properties, as detailed in previous work (*Case Study 2*). All fermentation experiments were conducted in triplicate, with independent batches prepared on three separate days for each LAB strain.

Bread production

Four types of wheat-sorghum bread were prepared: one control bread with unfermented sorghum batter (kept at 5 °C for 15h, without inoculum), and three bread types with fermented sorghum batters (*Lb.* 1932, *L.* 4454, *Lcb.* 4339). Sorghum batters (fermented or unfermented) represented ~25% (w/w) of the total dough weight. This amount was calculated with preliminary baking trials to ensure acceptable loaf quality. Thus, based on the water-to-flour ratio in the fermented batter, the final ratio of wheat to sorghum flour in the bread formulation was 85:15. Bread formulation was determined with preliminary baking trials. On 100 g flour basis, 2% fresh yeast (*Saccharomyces cerevisiae*), 3% sucrose, 2% salt, 3% sunflower oil were added. The amount of water required (58%) for the bread dough was previously calculated by Brabender® Farinograph (Ohg Duisburg, Germany) in order to obtain optimal consistency of 500 BU of wheat-sorghum flour

blends (85:15) (data not shown). The water added to the dough formulation was subtracted from the water that was already present in the sorghum batter.

Breadmaking was performed with a homemaker bread machine (Moulinex Uno, OW3101, Ecully, France) following the steps: kneading (34 min), first rising (25 min; 30°C), kneading (1 min), second rising (25 min; 35°C), kneading (1 min), third rising (45 min; 35°C), baking (58 min; 150°C). As soon as the baking phase ended, the bread was removed from the machine at let cool down at room temperature (~25 °C) for 2.5 hours before being analyzed. Each bread type was produced in triplicate, in three independent batches (three different days).

pH of sorghum batter, bread dough and bread crumb

During breadmaking pH was measured of the freshly fermented sorghum batter, on the bread dough (after mixing, before rising) an on the final bread crumb. Dough and bread pH were measured by directly placing the pH meter probe (Portamess 913 pH, Knick Elektronische Messgeräte, Berlin, Germany) equipped with Double Pore F electrode (Hamilton Company, Bonaduz, Switzerland) in contact with the dough or into a grinded portion of bread crumb. The pH of sorghum batters was already reported in a previous study (*Case Study 2*).

Bread water status: water activity and moisture content

Moisture content (MC, % - g water/100g of sample) and a_w were measured on both bread crust and crumb. Bread crust was taken from the upper part of the loaf, while the crumb was taken from the central part of the loaf. The MC (%) was calculated by drying 1 g of grinded bread (crust or crumb) in a forced air oven (ISCO NSV 9035, ISCO, Milan, Italy) at 105°C until constant weight. The a_w at 25°C was also calculated using an Aqualab 4TE water activity meter (Decagon Devices Inc, Pullman, Washington, USA). At least 3 replicates of MC (%) and a_w were performed for each loaf for both crust and crumb.

Bread crumb structural development

Specific volume. The volume of each bread loaf was calculated according to the AACC official method 10-05 (2001) but using small tapioca pearls instead of rapeseed. Bread specific volume (cm^3/g) was calculated as the ratio between loaf volume (cm^3) and weight (g). The volume of each bread loaf was analyzed at least twice. After measuring the specific volume, the bread was cut in order to tempestive perform the subsequent analyses.

Crumb porosity. Bread crumb porosity was also evaluated through the image analysis of bread central slices. 2 cm-thick slices were scanned with 600 dots for inch (dpi) resolution (Hewlett-Packard, Palo Alto, CA, USA). The images obtained were analyzed using ImageJ software (Media Cybernetics Inc., USA). To maximize the resolution of the crumb structure, images were converted to gray scale and adjusted in terms of luminosity and contrast. The number of objects (pores) and their relative area (mm^2) were obtained from a 6 cm^2 central portion of the slice. Pores with area < 0.01 mm^2 were excluded from the analysis. From the analysis of the dimensional distribution of the pores of each bread slice, four classed were selected to discriminate the differences in crumb porosity: class 1 ($\leq$ 0.1 mm^2), class 2 (0.11-1 mm^2), class 3 (1.01-5 mm^2) and class 4 (> 5 mm^2). The percentage frequency (%) of each pore class and the pore class area (%) were calculated. Six images were analyzed for each bread type.

Texture. Bread texture was analyzed with a Texture Analyzer TA.XT plus (Stable Micro Systems, Goldalming, UK) equipped with a cylindric probe (P/35 Dia Cylinder Aluminum). A texture profile analysis (TPA) was performed on 2x2x2 cm crumb cubes (40% deformation, trigger force = 0.05 N; pre-test speed = 2 mm/s; test speed = 1 mm/s). The double compression curves obtained were analyzed in terms of hardness (N) (maximum height of the first compression peak), cohesiveness (-) (ratio of the areas of the second to the first compression peak) and springiness (-) (ratio of the length of the second to the first compression peak). At least 10 cubes from the central part of the loaf were analyzed for each bread loaf.

Color of sorghum batters and bread

Color of fermented batters and bread (crust and crumb) was determined by a Konica Minolta spectrophotometer (CM 2600d, Minolta Co., Osaka, Japan), with D65 standard illuminant and the standard observer angle set at 10°. Data were obtained through SpectraMagic™ NX software and were expressed according to CIE Lab color space: brightness L* ((-100) black, (+100) white), a* ((-) green, (+) red) and b* ((-) blue, (+) yellow). The crust color was determined by directly placing the crust on the instrument, while the color of the crumb was measured on a compressed slice of bread in order to make the surface smooth and to avoid the pores' presence influence on color acquisition. Color differences between the unfermented (control) and the fermented samples (batter and bread) were also evaluated with the parameter ΔE, following to the formula:

$$\Delta E=\sqrt{(\Delta L^{*2}+\Delta a^{*2}+\Delta b^{*2})} \quad (1)$$

ΔE color differences were considered as not perceived (0 < ΔE > 0.5), barely perceived (1.5 < ΔE > 3.0), perceived (3.0 < ΔE > 6.0), strongly perceived (6.0 < ΔE > 12.0) by the human eye, and as total color difference (ΔE > 12) (Valverde and Moya, 2014). At least ten replicates were performed for each bread loaf and each batch of batter samples.

Volatile compounds analysis of sorghum batters and bread

The volatile profile of sorghum batters and bread was characterized through Head Space Solid Phase Microextraction (HS-SPME) technique, coupled with GC–MS, according to the method of Cirlini et al. (2012), with modifications: 3 g of defrosted sorghum batter or 2 g of sorghum bread, and 5 µl of toluene standard solution (100 µg/ml) were used for the analyses. All the parameters applied for volatile components extraction, in terms of SPME fiber used as time and temperature applied for the sampling step, as those used to set up the chromatographic separation and the subsequent mass spectrometric detection, are the same described by Cirlini et al. (2012).

The identification of sample volatile compounds was carried out by comparing their mass spectra with NIST 14 instrument libraries. The semi-quantification of the detected gas-chromatographic signals was performed using toluene as internal standard. Each identified molecule was associated with an odor type through the molecule database in https://www.flavornet.org/ and https://www.thegoodscentscompany.com/ . The analysis was performed in duplicate for each sample batch.

Bread sensory acceptability

A sensory acceptability test of bread samples was conducted with 54 untrained judges, consisting of 22 men and 32 women, aged between 19 and 55 years. Only healthy individuals with no allergies or dietary restrictions and no aversion to specific food products were selected as panel members. Before sensory testing began, all participants were asked to read and voluntarily sign an informed consent form. A detailed explanation of the method, duration and purpose of the study was provided, along with an assurance that the data would remain anonymous and would not be used for commercial purposes.

Approximately 2.5 hours after baking, a portion of both crumb and crust, measuring 2x2x5 cm, was cut and served to each participant. Four different bread samples (one per type) were

presented simultaneously to each judge, using random numbering and order to minimize bias. All participants were asked to taste and evaluate one sample at a time, providing water to cleanse the palate between different samples. Participants were asked to evaluate each bread sample based on color, overall appearance, odor, consistency, taste, and overall acceptability, using a 9-point hedonic scale. Participants were also invited to leave free comments alongside each score to gain deeper insights into their preferences and perceptions (Rocha et al., 2023).

Numerical scores were analyzed quantitatively to assess the sensory acceptability, while the free comments underwent qualitative analysis. Free comments' text was preprocessed with unitization and tokenization, standardization and cleaning, stop word removal, and stemming, as reported by Anandarajan et al. (2019) and Rocha et al. (2023). Descriptors with similar meanings were also grouped together (Rocha et al., 2023). The frequency of each resultant word was then calculated to identify common themes and descriptors associated with the sensory attributes of the bread. Moreover, a sentiment analysis (Visalli et al., 2020) of unprocessed text was performed based on comment's sentiments, which were categorized as positive (1), neutral (0), or negative (-1).

Statistical analysis

Data were presented as mean ± standard deviation (SD). Differences between sample means were analyzed using one-way ANOVA (SPSS Inc., Chicago, IL), followed by the Least Significant Difference (LSD) *post hoc* test. In cases where variances were not homogeneous, Welch's robust test was applied, followed by the Games-Howell *post hoc* test.

For the volatile compound analysis, principal component analysis (PCA) was performed using SIMCA 18 software (Sartorius, Göttingen, Germany) on both sorghum batters and bread. Two separate models were generated. Prior to model generation, all data were mean-centered and scaled to unit variance (UV). The validity of the models was evaluated based on the R^2 and Q^2 values.

Results and Discussion

Bread crumb structure development

Due to the importance of pH in the determination of gluten network strength and thus crumb development, the pH of sorghum batter, dough and bread was measured, as well as bread specific volume, and reported in Table 16.

Table 16. pH (sorghum batter, bread dough, bread crumb) and specific volume of bread samples (mean ± SD); different lowercase letters in the same column indicate significant differences between samples ($p < 0.05$); different uppercase letters in the same row indicate significant differences between batter, dough and bread. The pH of sorghum batters was taken from our previous study on the same matrices (Case Study 2).

Sample	pH sorghum batter	pH bread dough	pH bread	Bread specific volume (cm^3/g)
Control	6.19 ± 0.12^{aA}	5.54 ± 0.02^{aB}	5.47 ± 0^{aC}	3.03 ± 0.20^{ab}
Lb. 1932	4.49 ± 0.05^{bC}	5.15 ± 0.03^{bA}	5.07 ± 0.02^{bB}	2.99 ± 0.04^{a}
L. 4454	4.33 ± 0.10^{bcC}	5.05 ± 0.01^{cA}	5.02 ± 0.01^{cB}	3.00 ± 0.06^{a}
Lcb. 4339	4.18 ± 0.11^{cB}	5.03 ± 0.01^{cA}	4.96 ± 0.04^{dA}	2.77 ± 0.02^{b}

As previously reported (*Case Study 2*), fermentation decreased the pH of sorghum batters due to the production of organic acids during fermentation. After bread dough kneading, the pH of dough samples with fermented sorghum increased, while the control dough showed a slight decrease, probably due to addition of other ingredients. After proofing and baking, the pH was measured again on a portion of bread crumb and showed slightly lower values compared to the dough. A slight decrease in pH can occur during dough proofing due to both LAB activity, such as the production of organic acids, as well as yeast activity and the production of carbon dioxide. Carbon dioxide dissolves in the aqueous dough phase until it becomes saturated and diffuses into gas cells. The dissolved carbon dioxide reacts with water to form carbonic acid, which imparts the acidic pH to the dough (Miller et al., 1994; Rathnayake et al., 2018).

A representative picture of the wheat-sorghum breads is shown in Figure 17, while the specific volume in Table 16.

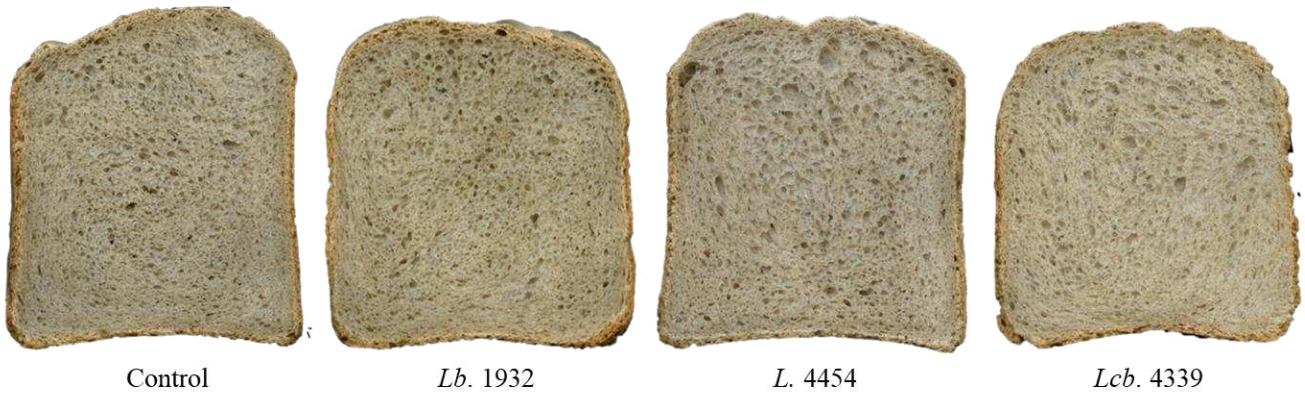

Figure 17. Representative pictures of wheat-sorghum bread slices made with unfermented sorghum batter (control), and sorghum batters fermented with Lactobacillus delbrueckii subsp. bulgaricus 1932 (Lb. 1932), Leuconostoc spp. 4454 (L. 4454), and Lacticaseibacillus casei 4339 (Lcb. 4339).

Loaf volume is considered an important characteristic of bread because it provides a quantitative measure of baking performance and the ability of the dough to retain gas. The desired loaf volume of yeast fermented products is only achieved if the dough provides a favorable environment for yeast growth and gas generation. At the same time, loaf volume also depends on the capacity of gluten matrix to maximize gas retention (Rathnayake et al., 2018). The specific volume did not change after fermentation, except for sample *Lcb.* 4339, which showed a slight but significant decrease compared to the control bread. Different phenomena may explain the decrease in specific volume in *Lcb.* 4339 bread. As shown in Table 16, *Lcb.* 4339 had the lowest pH among samples. The degree of acidity is considered a parameter that affects yeast growth and activity, as well as gluten proteins charge and viscoelasticity (Rezaei et al., 2015, 2016; Rathnayake et al., 2018). Since *Saccharomyces Cerevisiae* is notoriously resistant to low pH, the pH reached during kneading and proofing (~5) should not have inhibited yeast growth during fermentation (Liu et al., 2015; Rezaei et al., 2015). Instead, it may have weakened the gluten network and thus the ability to retain gas during rising and baking. Dough acidification has been reported as a bread volume enhancer, as mild gluten weakening can improve the extensibility of the dough, allowing for higher expansion and the ability to retain gas bubbles. This is due to a reduction in the density of the gluten network and a decrease in resistance to CO_2 release (Su et al., 2019). However, in some cases it is reported that excessive weakening of the gluten network by organic acids can, on the contrary, reduce the beneficial effects on dough expansion and decrease the specific

volume (Su et al., 2019). Rezaei et al. (2016) reported that the reduced entanglement of gluten post fermentation results in dough that flows less, is less extensible and elastic, and breaks or deforms more easily under stress and strain. A competition effect between the yeast (*S. Cerevisiae)* and *Lcb.* 4339 was also considered. However, looking at the results obtained with crumb porosity analysis (Figure 18), this effect was excluded.

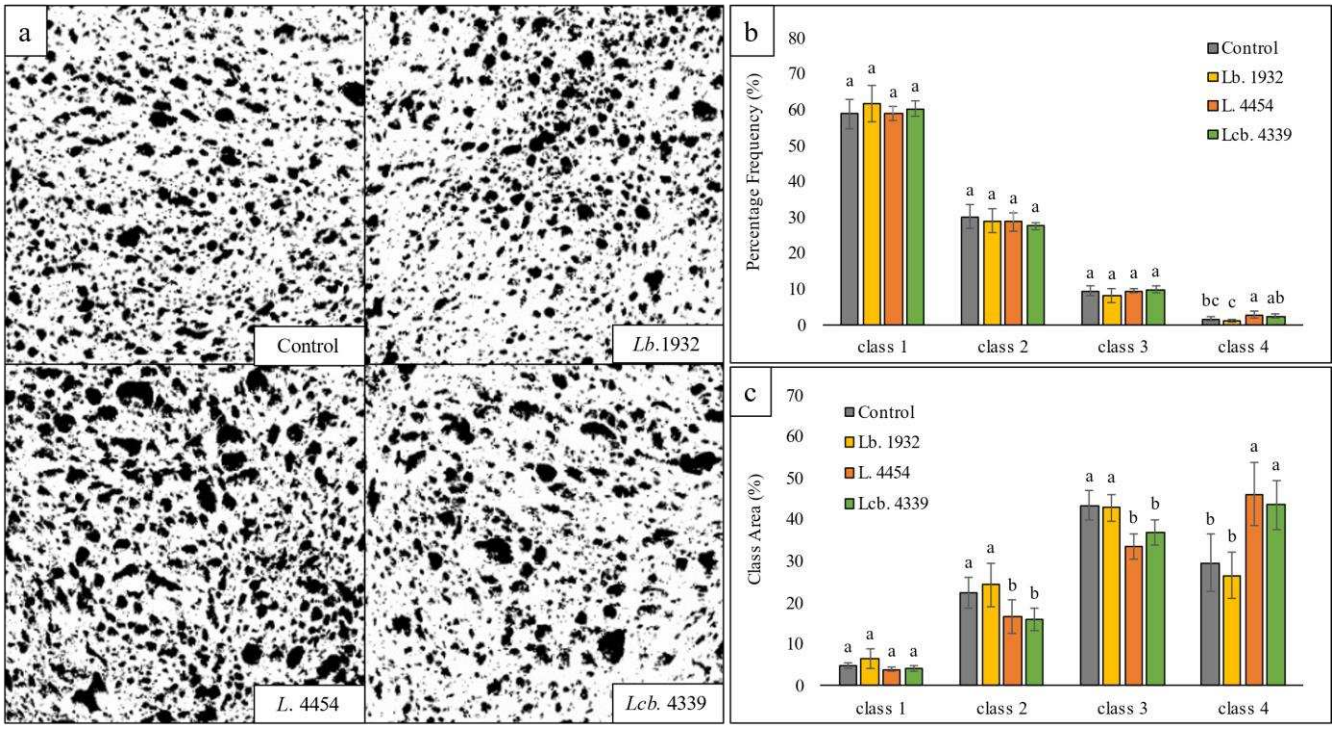

Figure 18. Bread crumb porosity results: a) representative sections of the crumb porosity of bread samples; b) class percentage frequency (%); c) class area (%). Class 1= pores ≤ 0.10 mm²; class 2= pores 0.11-1 mm²; class 3= pores 1.01-5 mm²; and class 4= pores > 5 mm².

Indeed, the percentage frequency distribution (%) of pore classes was similar for the smallest and medium-large pores (0.1 to 5 mm²) across all samples (Figure 18), indicating that distribution of these pores is a common feature, and the bacterial strains used did not significantly affect the formation of pores within this size range. However, slight differences were observed in the percentage frequency of the largest pore class (>5 mm²), which was the highest in sample *L.* 4454, followed by *Lcb.* 4339, the control, and finally *Lb.* 1932. However, most of the differences were observed in the percentage of the area occupied by each pore class (%). In the control and *Lb.*

1932 samples, the greatest area was occupied by class 3 pores (1.01-5 mm²), whereas in *Lcb.* 4339 and *L.* 4454, it was primarily occupied by class 4 pores (> 5 mm²). Both the *Lb.* 1932 and control samples had a larger area occupied by medium-small (class 2) and medium-large (class 3) pores compared to the other samples. In contrast, *Lcb.* 4339 and *L.* 4454 exhibited larger pore sizes, with a greater area occupied by class 4 pores than in the control and *Lb.* 1932 samples. These results suggest that the crumb structure of *Lb.* 1932 and the control was characterized by fewer large pores, leading to a more compact crumb. In contrast, samples *L.* 4454 and *Lcb.* 4339 promoted the formation of larger pores, resulting in a more open crumb structure. The improvement in crumb porosity in samples *L.* 4454 and *Lcb.* 4339 might also be due to the heterofermentative metabolism of *L.* 4454, and the establishment of a mutualistic interaction between *Lcb.* 4339 and the yeast (Gobbetti, 1998). Moreover, the organic acids present in the fermented batters as well as the newly produced during the dough proofing may have promoted yeast activity and thus contributing to the increase in gas production capability (Peres et al., 2005; Su et al., 2019). Therefore, we do not believe that a competition effect between yeast and *Lcb.* 4339 occurred. These results support our hypothesis that, in *Lcb.* 4339 bread an excessive acidic and enzymatic weakening of the gluten matrix (which was already weakened by the presence of non-gluten protein and fiber from the whole-grain sorghum flour), may have led to reduced expansion capacity, promoting a slight collapse of the loaf after proofing. To confirm this, additional tests were conducted with chemically acidified doughs that reached the same pH as the sorghum batter; a decrease in loaf volume was observed (data not shown).

Bread texture was also influenced by sorghum fermentation, as shown in Figure 19. Loaves made with *Lb.* 1932 and *L.* 4454 exhibited greater cohesiveness compared to the control bread. *Lb.* 1932 and *Lcb.* 4339 also showed a higher springiness than the control. However, *Lcb.* 4339 sample was harder than the control bread. When considering bread texture, both the gas phase and the properties of the continuous phase play critical roles. Indeed, bread texture results are not only related to crumb porosity and specific volume but also to factors such as moisture content and the degree of starch gelatinization, which characterize the continuous solid phase (Scanlon and Zghal, 2001). Indeed, according to Scanlon and Zghal (2001), the mechanical properties of bread are influenced by the starch concentration and the extent of starch gelatinization, resulting in variations in crumb porosity at both microscopic and macroscopic levels, thus influencing bread

texture. In our previous study on the same fermented matrices (*Case Study 2*), we observed a higher degree of starch gelatinization in fermented sorghum batters. *Lb.* 1932 and *Lcb.* 4339 samples exhibited higher peak viscosity and final viscosity during heating and cooling ramp in the analysis of batter pasting properties. The increased degree of gelatinization and enhanced interaction with water in the continuous solid phase (starch-water gel) could explain the changes in texture parameters, such as increased cohesiveness and springiness in LAB-fermented bread loaves. The higher hardness of *Lcb.* 4339 than the other samples may instead be related to the reduced specific volume of this sample.

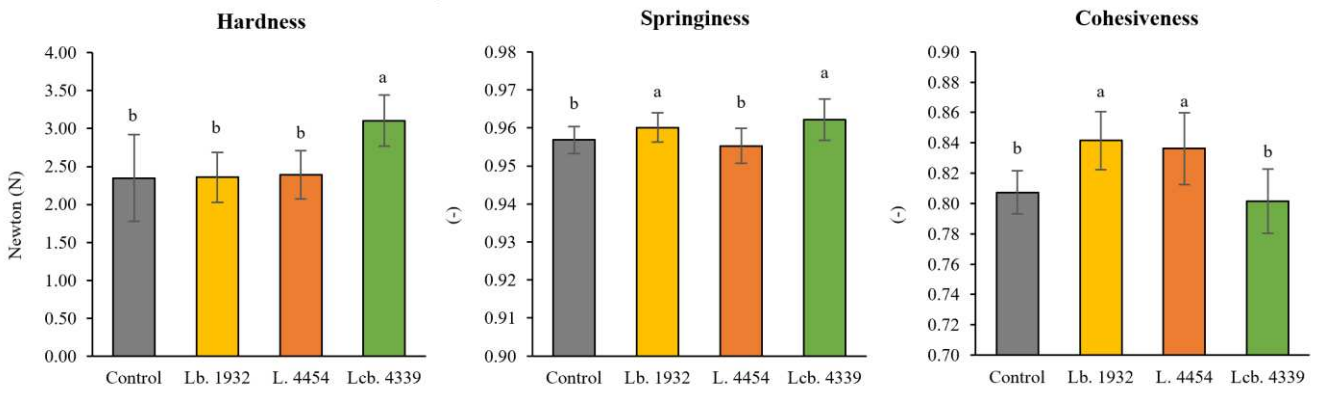

Figure 19. Texture parameters of wheat-sorghum breads. Different letters indicate significant differences between samples ($p < 0.05$).

Bread water status

LAB-fermented loaves showed a higher crumb MC %, measured as g water/100 g sample (Table 17). However, the water activity (a_w) across all crumb samples was comparable, thus without potentially altering bread stability over time (Table 17).

Although the amount of water added to each bread formulation was the same (58%), the dynamics of water loss during baking varied. As we previously reported for the same matrices (*Case Study 2*), all fermented samples exhibited greater interactions with water at different levels (from molecular to mesoscopic), related to changes in the binding sites of different polymers, resulting from the action of microbial enzymes and organic acid hydrolysis. The molecular

modifications caused by LAB fermentation in sorghum and bread dough likely modified the water-binding sites within the flour components. Enhanced interactions with water at various levels might have reduced water losses during baking. Moreover, a higher moisture content in the continuous solid phase of bread may have also influenced bread crumb structure, affecting its cohesiveness and springiness (Figure 19). Regarding the crust, bread made with *Lb.* 1932 had the highest moisture content (MC, %) and a_w (Table 17). In contrast, the crust of *L.* 4454 and *Lcb.* 4339 did not differ significantly from the control. Variations in moisture content in the crust are crucial quality indicators, as they can affect both the crust's crispness and the bread's overall stability over time (Van Nieuwenhuijzen et al., 2008). This is because crust water status may influence the moisture gradient between the crust and crumb, which plays a role in water mobility during bread storage (Curti et al., 2011). Additionally, a higher a_w could impact microbial stability of bread during storage (Tapía et al., 2020).

Table 17. Moisture content (MC, g water/100 g sample) and water activity (a_w) of bread crust and crumb (mean ± SD). Different letters in the same column indicate significant differences between samples ($p < 0.05$).

Sample	MC (%)		a_w	
	crust	crumb	crust	crumb
Control	14.78 ± 1.01^{bc}	40.96 ± 0.62^{b}	0.703 ± 0.010^{bc}	0.958 ± 0.006^{a}
Lb. 1932	16.90 ± 0.79^{a}	41.79 ± 0.25^{a}	0.78 ± 0.025^{a}	0.962 ± 0.009^{a}
L. 4454	15.71 ± 1.06^{b}	42.02 ± 0.58^{a}	0.734 ± 0.035^{b}	0.961 ± 0.005^{a}
Lcb. 4339	14.31 ± 1.14^{c}	41.74 ± 0.69^{ab}	0.688 ± 0.010^{c}	0.957 ± 0.006^{a}

Color properties of sorghum flour and bread

Color is an important indicator of flour quality, as it can potentially affect the appearance, and thus the acceptability, of final products. LAB fermentation affected the color of sorghum batters. An increase in L* (brightness), a* (redness) and b* (yellowness) parameters was observed after fermentation in all fermented samples (Table 18).

As a result of these parameters changes, the fermented batters appeared pinker than the unfermented batters, which had a more grayish color. Total color differences between control and fermented batters were calculated through the ΔE parameter, revealing human eye perceivable

differences between control and *L.* 4454 (ΔE = 3.1), as well as between control and *Lcb.* 4339 (ΔE = 4.1) and barely perceivable differences between control and *Lb.* 1932 (ΔE = 2.6). Interestingly, some color changes that occurred after fermentation were found to be reversible and induced by the pH change. In particular, the parameters L* and a* showed an increasing trend with decreasing pH. The parameter b*, on the other hand, did not show the same trend. Indeed, *Lb.* 1932 (pH 4.49) and *Lcb.* 4339 (pH 4.18) showed the highest b* values.

Table 18. Color of sorghum batters and bread (mean ± SD). Different letters in the same column indicate significant differences between samples ($p < 0.05$).

Sample	Sorghum batter			Bread crust			Bread crumb		
	L*	a*	b*	L*	a*	b*	L*	a*	b*
Control	61.89 ± 0.64^{b}	3.87 ± 0.06^{c}	8.61 ± 0.11^{c}	67.66 ± 3.22^{b}	6.63 ± 0.93^{a}	20.10 ± 1.79^{a}	66.56 ± 3.37^{a}	2.46 ± 0.27^{c}	13.71 ± 0.97^{a}
Lb. 1932	62.75 ± 0.81^{ab}	5.98 ± 0.19^{b}	9.86 ± 0.17b^{a}	70.30 ± 1.97^{a}	6.15 ± 0.88^{ab}	19.83 ± 1.56^{a}	67.36 ± 2.19^{a}	3.17 ± 0.29^{b}	13.62 ± 0.98^{a}
L. 4454	63.30 ± 0.49^{ab}	6.41 ± 0.24b^{a}	9.74 ± 0.28^{b}	70.81 ± 2.13^{a}	5.74 ± 0.81^{b}	19.03 ± 1.68^{a}	66.42 ± 2.68^{a}	3.38 ± 0.32^{a}	14.26 ± 1.45^{a}
Lcb. 4339	63.68 ± 0.55^{a}	6.99 ± 0.32^{a}	10.58 ± 0.51^{a}	70.93 ± 3.60^{a}	5.47 ± 1.26^{b}	18.59 ± 3.52^{a}	65.81 ± 4.15^{a}	3.48 ± 0.44^{a}	14.02 ± 1.25^{a}

Color changes in flour samples as an effect of fermentation have been reported by other authors, on different flour matrices, such as potato (Gong et al., 2020), brown rice (Ilowefah et al., 2015) and sorghum flour (Hugo et al., 2003). In particular, the most reported color change after fermentation was related to the L* parameter, which refers to the sample brightness (100 = white, 0 = black). Gong et al. (2020) reported that the L* increase following fermentation is due to changes and breakdown of color components. In particular, Gong et al. (2020) pointed to lipid content, sugar breakdown, and carotenoids as the main factors affecting the whiteness of flours. Hugo et al. (2003) reported instead that the increase in L* is due to the increasing methylation that occurs in anthocyanin pigments at low pH. Regarding the other color parameters, an increase in a* and b* after fermentation may indicate changes in the chromophoric characteristics of pigments that turn red and yellow as a consequence of low pH and breakdown of flour components. Olojede et al. (2022) found and increase in the red component (a*) of bread (crust and crumb) after the addition of sorghum sourdough. However, the authors did not analyze the

color parameters of sourdough, but attributed the increase in redness of bread to changes and increased content of the phenolic compound that occurred after sorghum fermentation. In our samples, since the parameter b* appeared to be independent of pH changes, its changes were probably related to breakdown of specific color components by *Lb.* 1932 and *Lcb.* 4339 rather than the production of organic acids (and thus, pH drop).

For what concern bread color parameters (Table 18), bread crust showed subtle differences between *Lb.* 1932 bread and control bread, barely perceived by the human eye (ΔE = 2.7). More noticeable differences were observed between the control and *L.* 4454 bread (ΔE = 3.4) and *Lcb.* 4339 bread (ΔE = 3.8). Samples with fermented sorghum tended to develop a lighter crust, as indicated by lower L* values compared to the control. This crust lightening may be due to a reduction in the precursor compounds of the pigments typically formed during the Maillard reaction as the crust reaches high temperatures during baking (Helou et al., 2016). Additionally, the color parameter a*—which was higher in the fermented sorghum—showed a decrease in the crust. This could be due to the thermal degradation of compounds that enhance red coloration or to reduced production of brownish compounds from the Maillard reaction. In fact, the Maillard reaction can be influenced by the concentration of amino acids and reducing sugars, as well as by pH and moisture levels (Ajandouz and Puigserver, 1999; Helou et al., 2016). These compositional differences could have led to the observed color differences in the crust between fermented and unfermented samples. In contrast, the crumb exhibited minimal color differences: while the L* and b* parameters did not show significant variations between the samples, the a* parameter was higher in the fermented samples than in the control, particularly in the *L.* 4454 and *Lcb.* 4339 samples. Indeed, ΔE values were equal to 1.1 between control - *Lb.* 1932 and control - *L.* 4454, and to 1.3 between control - *Lcb.* 4339). These minor differences were expected since fermented batters are mixed with other ingredients in the bread dough. These results in crumb color are consistent with findings reported by Olojede et al. (2022), which reported higher a* values in LAB-fermented bread.

Volatile compounds analysis of sorghum batters and bread

The volatile profiles showed the presence of 32 molecules (1 unidentified) in the headspace of sorghum batters, and 34 molecules (1 unidentified) in sorghum bread. All the detected molecules, with the respective retention time and odor type association were reported in Table 19.

Table 19. Volatile compounds detected in sorghum batters and bread, with odor type description.

Molecule	Odor type	Sample	RT (min)
Alcohols			
ethanol	alcoholic	batter, bread	2.96
2-methyl-1-propanol	ethereal	bread	5.66
isopentyl alcohol	fermented	batter, bread	8.34
1-pentanol (amyl alcohol)	fermented	batter, bread	9.44
1-hexanol	green, herbal	batter, bread	12.15
1-octen-3-ol	earthy, mushroom	batter, bread	14.61
1-heptanol	green	batter	14.77
2-ethyl-1-hexanol	rose, green	batter, bread	15.63
2,3-butanediol	creamy	batter, bread	16.89
1-octanol	waxy	batter, bread	17.29
2,3-butanediol (isomer)	creamy	batter, bread	17.79
furfuryl alcohol/nonanol	bready/floral, waxy	bread/ batter, bread	19.69
phenylethylacohol	floral, rose	batter, bread	25.02
Aldehydes			
hexanal	green	batter, bread	5.14
2-heptenal	green	batter, bread	11.17
nonanal	aldehydic	batter, bread	13.01
2-octenal	fatty	batter, bread	13.91
benzaldehyde	fruity, almond	batter, bread	16.18
2-nonenal	fatty	batter, bread	16.57
Carboxylic Acids			
acetic acid	acidic, sour	batter, bread	14.49
isobutyric acid	acidic	batter, bread	17.43
isovaleric acid	cheesy	batter, bread	19.84
acetic acid derivative	not found	bread	21.41
hexanoic acid	fatty, cheesy	batter, bread	23.67
nonanoic acid	waxy, fatty	batter, bread	30.01
Ketones			
2,3-butanedione (diacetyl)	buttery	batter	3.4
3-hydroxy-2-butanone	buttery, creamy	batter, bread	10.20
6-methyl-5-hepten-2-one	green, citrus	batter	11.55
decalactone	fruity	batter, bread	29.49
Furanic compounds			
2-pentyl furan	fruity, green	batter, bread	8.7
2,5-dihydro-2,5-dimethoxy furan	not found	batter	19.01
2-hexyl furan	not found	bread	22.86
Others			
xylene (isomer)	geranium/characteristic/plastic	bread	6.01
xylene (isomer)	geranium/characteristic/plastic	bread	6.19
xylene (isomer)	geranium/characteristic/plastic	bread	6.36
trimethyl pyrazine	nutty	batter, bread	13.39
ethyl caprylate	waxy	bread	14.12
furfural/1heptanol	bready/green	bread	14.75
1,3-dioxolane	pungent	batter	21.35
not identified	not found	batter, bread	22.06

The quantification of each molecule was reported in Table 20 (batter) and Table 21 (bread). To better understand the effect of LAB strains on the volatiles profile of sorghum batters and bread, two principal component analysis (PCA) were also performed on both the two matrices (Figure 20).

Table 20. Quantitative results of volatile molecules detected in sorghum batters (mean ± SD). Different letters in the same row indicate significant differences between samples (one-way ANOVA; $p < 0.05$).

Molecule	Quantification µg/g			
	Control	*Lb.* 1932	*L.* 4454	*Lcb.* 4339
Alcohols				
ethanol	0.53±0.11^{b}	0.45±0.33^{b}	214.04±17.30^{a}	0.61±0.33^{b}
isopentyl alcohol	0.31±0.30	ND	ND	ND
1-pentanol (amyl alcohol)	5.11±1.96^{c}	7.72±1.22^{bc}	11.34±1.34^{a}	10.75±2.55^{ab}
1-hexanol	23.52±23.83^{c}	77.98±11.8^{b}	198.20±17.21^{a}	84.26±1.14^{b}
1-octen-3-ol	6.05±1.92^{b}	15.86±0.58^{a}	12.02±1.81^{ab}	16.89±5.91^{ab}
1-heptanol	1.31±0.66^{c}	3.01±0.89^{b}	5.26±0.65^{a}	3.76±0.60^{b}
2-ethyl-1-hexanol	0.42±0.06	ND	ND	ND
2,3-butanediol	0.79±0.34^{b}	0.61±0.15^{b}	5.24±1.56^{a}	0.98±0.43^{b}
1-octanol	1.19±0.55^{c}	2.18±0.62^{cb}	5.88±1.00^{a}	3.43±0.34^{b}
2,3-butanediol (isomer)	0.77±0.22^{b}	0.58±0.14^{b}	11.11±2.51^{a}	0.88±0.19^{b}
nonanol	0.33±0.04^{c}	2.37±1.04^{cb}	8.31±1.76^{a}	3.21±0.96^{b}
phenylethylacohol	0.32±0.14^{b}	0.6±0.10^{a}	0.74±0.03^{a}	0.67±0.16^{a}
Aldehydes				
hexanal	10.93±5.3^{a}	15.34±3.86^{a}	1.05±0.11^{b}	2.68±1.25^{b}
2-heptenal	0.69±0.28^{c}	2.56±0.55^{a}	1.27±0.11^{bc}	1.51±0.45^{b}
nonanal	2.50±0.76^{a}	2.61±0.74^{a}	0.64±0.31^{b}	2.05±0.59^{a}
2-octenal	0.36±0.14^{b}	1.15±0.43^{a}	1.05±0.10^{a}	0.89±0.31^{a}
benzaldehyde	3.03±0.10^{c}	5.62±0.50^{b}	14.65±1.90^{a}	4.81±1.96^{bc}
2-nonenal	0.36±0.29	ND	ND	ND
Carboxylic Acids				
acetic acid	ND	36.46±14.10^{b}	294.20±55.82^{a}	29.99±3.03^{b}
isobutyric acid	0.41±0.22^{c}	0.92±0.12^{b}	1.37±0.19^{a}	1.54±0.17^{a}
isovaleric acid	ND	0.96±0.38^{a}	1.45±0.52^{a}	1.92±1.30^{a}
hexanoic acid	1.88±0.03^{c}	15.16±2.63^{bc}	27.71±1.68^{ab}	42.19±17.17^{a}
nonanoic acid	0.45±0.33^{a}	0.48±0.12^{a}	0.62±0.17^{a}	1.16±0.79^{a}
Ketones				
2,3-butanedione (diacetyl)	1.1±0.32^{b}	3.44±1.07^{b}	3.62±0.32^{b}	152.79±11.56^{a}
3-hydroxy-2-butanone	0.64±0.36^{b}	1.93±0.19^{b}	15.09±1.41^{a}	15.2±4.54^{a}
6-methyl-5-hepten-2-one	0.77±0.32^{bc}	1.01±0.23^{ab}	0.22±0.05^{c}	1.54±0.42^{a}
decalactone	1.26±0.29^{a}	1.27±0.13^{a}	1.54±0.16^{a}	1.72±0.38^{a}
Furanic compounds				
2-pentyl furan	0.23±0.01	ND	ND	ND
2,5-dihydro-2,5-dimethoxy furan	0.21±0.06^{b}	1.13±0.22^{a}	0.29±0.04^{b}	0.51±0.25^{b}
Others				
trimethyl pyrazine	0.63±0.35^{a}	0.62±0.25^{a}	0.77±0.03^{a}	0.98±0.22^{a}
1,3-dioxolane	0.53±0.52^{b}	1.04±0.24^{ab}	1.76±0.18^{ab}	2.67±1.39^{a}
not identified	0.86±0.16^{c}	0.93±0.11^{c}	2.11±0.03^{a}	1.27±0.21^{b}

*ND = not detected.

Table 21. Quantitative results of volatile molecules detected in wheat-sorghum bread samples (mean ± SD). Different letters in the same row indicate significant differences between samples (one-way ANOVA; p < 0.05).

Molecule	Quantification µg/g			
	Control - bread	*Lb.* 1932 - bread	*L.* 4454 - bread	*Lcb.* 4339 - bread
Alcohols				
ethanol	1175.09±534.19^{a}	1607.88±448.82^{a}	1759.08±535.44^{a}	1822.21±497.24^{a}
2-methyl-1-propanol	34.67±9.78^{a}	55.44±15.27^{a}	65.99±18.36^{a}	59.96±10.46^{a}
isopentyl alcohol	370.14±87.86^{a}	458.47±94.82^{a}	487.39±131.11^{a}	514.11±105.52^{a}
1-pentanol	7.54±3.75^{a}	6.24±0.60^{a}	6.54±1.31^{a}	4.37±2.13^{a}
1-hexanol	65.57±7.31^{a}	91.98±4.72^{a}	98.97±20.63^{a}	99.57±18.50^{a}
1-octen-3-ol	14.54±1.61^{b}	21.60±4.22^{b}	48.91±16.71^{a}	21.91±3.53^{b}
2-ethyl-1-hexanol	7.74±2.56^{ab}	9.98±2.91^{a}	2.25±0.19^{c}	3.94±2.68^{bc}
2,3-butanediol	32.48±15.66^{b}	64.92±17.42^{ab}	103.74±25.78^{a}	105.08±31.13^{a}
2,3-butanediol (isomer)	10.39±5.16^{b}	20.53±6.01^{b}	47.66±12.13^{a}	37.85±6.76^{a}
furfuryl alcohol/nonanol	9.45±1.61^{a}	8.41±1.75^{a}	14.98±3.65^{a}	26.10±11.83^{a}
phenylethylacohol	79.5±45.09^{a}	89.25±7.81^{a}	128.04±43.91^{a}	125.52±27.66^{a}
Aldehydes				
hexanal	33.79±11.21^{a}	67.11±24.1^{a}	68.69±4.48^{a}	40.84±16.41^{a}
2-heptenal	6.06±2.23^{b}	13.50±4.96^{ab}	16.46±3.26^{a}	18.76±6.00^{a}
nonanal	10.29±3.50^{b}	15.75±1.94^{b}	18.78±4.62^{a}	29.56±10.19^{ab}
2-octenal	5.43±1.63^{c}	8.81±2.98^{bc}	14.41±0.72^{a}	12.85±2.99^{ab}
benzaldehyde	35.72±9.41^{a}	34.17±8.14^{a}	42.32±5.10^{a}	36.17±9.65^{a}
2-nonenal	6.52±3.87^{b}	11.45±2.17^{b}	19.20±4.04^{a}	24.35±4.50^{a}
Carboxylic Acids				
acetic acid	11.8±2.45^{b}	36.26±13.84^{b}	218.32±86.39^{a}	40.34±6.81^{b}
isobutyric acid	17.19±3.06^{b}	37.67±2.55^{a}	47.43±11.8^{a}	46.66±5.57^{a}
isovaleric acid	31.81±11.02^{b}	67.4±9.69^{ab}	94.22±31.78^{a}	81.77±28.33^{a}
acetic acid derivative	2.91±1.13^{b}	4.4±2.16^{b}	5.26±0.96^{ab}	7.99±2.41^{a}
hexanoic acid	11.03±5.35^{a}	15.25±4.59^{a}	16.88±4.62^{a}	21.63±6.74^{a}
nonanoic acid	0.81±0.35^{b}	1.67±0.44^{ab}	2.03±0.57^{a}	2.5±0.66^{a}
Ketones				
3-hydroxy-2-butanone	64.14±13.17^{b}	135.67±13.53^{a}	63.60±6.60^{b}	160.69±18.82^{a}
decalactone	3.96±0.71^{a}	5.84±1.05^{a}	5.21±0.73^{a}	7.82±2.55^{a}
Furanic compounds				
2-pentyl furan	22.54±9.2^{a}	13.76±3.33^{a}	10.24±1.66^{a}	10.67±1.28^{a}
2-hexyl furan	1.35±0.26^{a}	2.46±0.93^{a}	3.32±1.42^{a}	2.63±0.53^{a}
Others				
xylene (isomer)	93.58±69.75^{a}	212.56±129.19^{a}	197.18±214.56^{a}	312.15±67.12^{a}
xylene (isomer)	12.59±3.03^{b}	42.67±28.36^{ab}	35.82±25.67^{b}	89.82±35.12^{a}
xylene (isomer)	72.32±46.55^{a}	202.80±144.44^{a}	165.97±158.01^{a}	275.44±110.67^{a}
trimethyl pyrazine	2.11±0.16^{a}	3.36±1.09^{a}	3.11±1.13^{a}	3.84±0.29^{a}
ethyl caprylate	4.82±3.11^{b}	9.02±3.98^{ab}	15.28±4.27^{a}	14.57±3.61^{a}
furfural/1-heptanol	15.98±0.75^{b}	26.10±3.65^{ab}	34.12±2.78^{a}	33.06±9.37^{ab}
not identified	2.75±0.38^{a}	3.58±0.74^{a}	4.20±0.47^{a}	3.94±0.61^{a}

In general, the volatile profile of the unfermented control batter was characterized by the following classes: alcohols (41.4%), aldehydes (20.7%), carboxylic acids (10.3%), ketones (13.8%), furanic compounds (6.9%), and other compounds (pyrazines, acetals; 6.9%). As expected, with fermentation the volatile contribution of carboxylic acids increased, as a result of LAB activity. As

a consequence, with fermentation, the relative percentages of each class changed, showing 37.0% of alcohols, 18.5% of aldehydes, 18.5% of carboxylic acids, 14.8% of ketones, 3.7% of furanic compounds and 7.4% of other compounds (pyrazines, acetals).

In general, after fermentation the number and concentration of some volatile compounds increased, while others decreased or disappeared (Table 20). For instance, the number of alcohol molecules decreased following fermentation, leading to the disappearance of isopentyl alcohol and 2-ethyl-1-hexanol. The concentration of other alcohols, however, showed an increasing trend in all fermented batters if compared to the unfermented, although not always significant (Table 20). In addition, the aromatic complexity related to carboxylic acids (number of carboxylic acids present) increased with fermentation. Molecules such as acetic and isovaleric acid were produced only in fermented samples. In addition, as expected, the concentration of carboxylic acids already present in unfermented sorghum was found to increase upon fermentation (isobutyric acid, hexanoic acid). Fermentation also resulted in a change in the aldehydic profile of sorghum batters: the concentration of hexanal and nonanal decreased, while 2-nonenal completely disappeared. On the other hand, the concentration of other aldehydes, increased (2-octenal, benzaldehyde, 2-heptenal). The concentration of some ketones also increased following fermentation (2,3-butanedione (diacetyl), 3-hydroxy-2-butanone (acetoin), 6-methyl-5-hepten-2-one). Moreover, fermentation also resulted in the loss of a furanic compound (2-pentyl furan). Some of the detected molecules in control sorghum batter were also detected by Fan et al. (2021) in both raw and cooked sorghum cultivars: 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, hexanal, 2-heptenal, nonanal, 2-octenal, 2-nonenal, 3-hydroxy-2-butanone, 6-methyl-5-hepten-2-one in cooked sorghum, and 1-octen-3-ol, 2-ethyl-1-hexanol, benzaldehyde, 2,3-butanedione and 2-pentyl furan in raw sorghum.

The principal component analysis (PCA) of sorghum batters generated a three-component model ($R^2 = 0.86$; $Q^2 = 0.61$). In the biplot (Figure 20a), only the first two components were reported, since they represented 73.8% of the total variance; loadings were labeled according to the molecules name, while in the loading plot reported in Figure 21 they were labeled according to their odor type, in order to better visualize the odor attributes of samples.

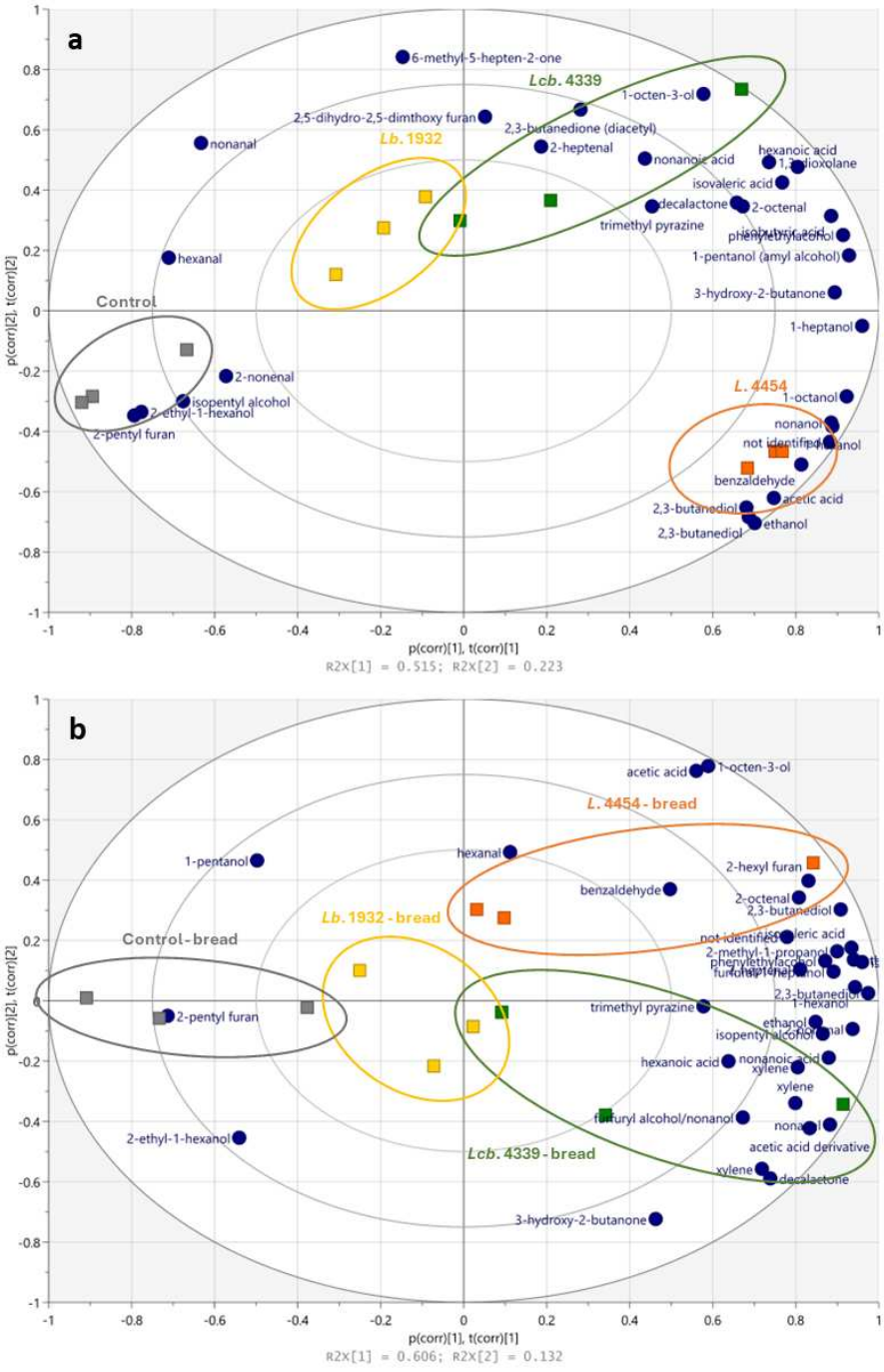

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Figure 20. Biplot of the principal component analysis (PCA) performed on the volatile profile of sorghum batters (a) and sorghum bread (b).

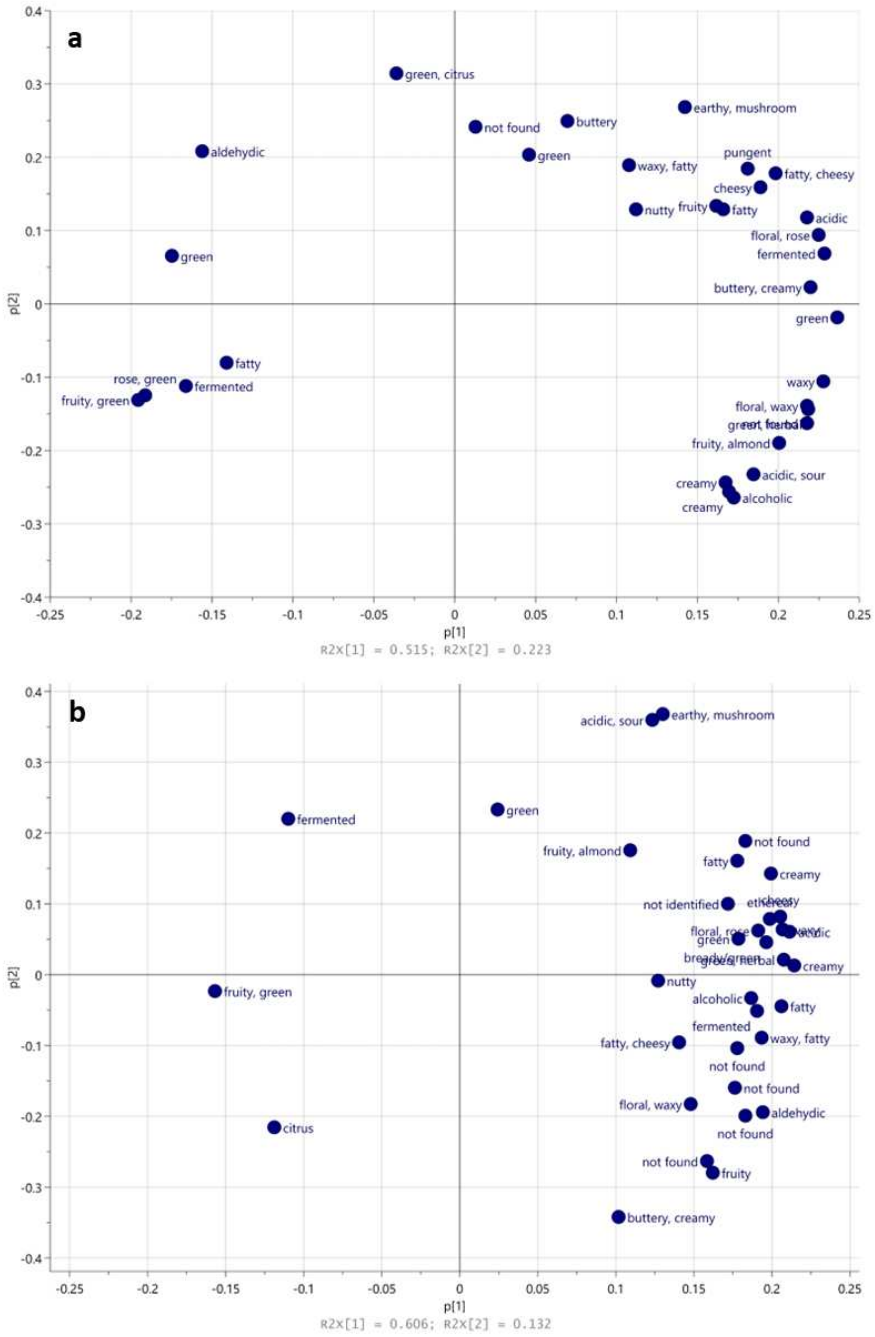

Figure 21. Loading plot of PCA models of sorghum batters (a) and bread (b). Variables have been labeled according to the molecule odor type.

The first principal component p1 (51.5%) showed a scores partition according to fermentation, leading the control batter to the left and the fermented batters to the right. *Lcb.* 4339 and *L.* 4454 were found to be the most divergent samples from the control, while *Lb.* 1932 was close to the center of the plot, as it took intermediate values among the samples. Indeed, *L.* 4454 and *Lcb.*

4339 were the strains that led to the greatest variation in the volatile profile of sorghum batters (Table 20). Looking at the loading's distribution (Figure 20; Figure 21), the aromatic complexity of the fermented samples was higher than the unfermented control. In fact, most of the molecules and their respective odor type associations appeared more concentrated on the right side of the graph, assuming positive values on p1. The second principal component p2 (22.3%) showed instead a partial division of strains according to their homofermentative (*Lb.* 1932, *Lcb.* 4339) (positive values on p2) and heterofermentative (*L.* 4454) (negative values on p2) metabolisms. As a result, *L.* 4454, positioned in the right bottom quadrant, appeared positively correlated with the production of molecules associated with heterofermentative metabolism, such as acetic acid and ethanol, as well as other alcohols such as 2,3-butanediol (creamy), 1-hexanol (green, herbal), nonanol (floral, waxy), and 1-octanol (waxy), and aldehydes such as benzaldehyde (fruity, almond) (Figure 20a; Figure 21). The increased values of acetic acid and ethanol, as of other alcohols and aldehydes after the fermentation with heterofermentative LAB is in accordance with literature (Damiani et al., 1996). The ethanol produced by *L.* 4454 strain was ~405 folds higher than that already present in the control, and ~476 folds and ~352 folds higher than that produced by *Lb.* 1932 and *Lcb.* 4339, respectively (Table 20). Similarly, acetic acid, which was not present in the control, was ~8 folds and ~10 folds higher in *L.* 4454 than in *Lb.* 1932 and *Lcb.* 4339 batters, respectively (Table 20). In addition, fermentation with *L.* 4454 resulted in a greater reduction of some aldehydes such as hexanal and nonanal, which positioned in the opposite quadrant of *L.* 4454, in the PCA biplot. This effect can be linked to the documented ability of heterofermentative strains to reduce aldehydes to other compounds (Kaseleht et al., 2011; Liu et al., 2020).

For what concern *Lcb.* 4339, batters fermented with this strain were distributed within the upper right quadrant, characterized by considerable aromatic complexity. *Lcb.* 4339 appeared positively correlated with the production of some molecules typical of the homofermentative metabolism, such as diacetyl and different carbonyls (Damiani et al., 1996). Indeed, in this quadrant there were positioned molecules associated such as 2,3 butanedione (diacetyl), 1-octen-3-ol, 2-heptenal, nonanoic acid, hexanoic acid, 1,3 dioxolane, isovaleric acid, decalactone, 2-octenal, trimethyl pyrazine, isobutyric acid, phenylethylacohol, 1-pentanol (amyl alcohol). Some of these compounds were also found in wheat sourdough fermented with homofermentative or facultatively heterofermentative strains by Liu et al. (2020) and Kaseleht et al. (2011) and may

derive from both flour lipid oxidation and specific activity and production by LAB strains (Damiani et al., 1996; Pico et al., 2015; Liu et al., 2020). Specifically, the concentration of 2,3 butanedione (diacetyl) in *Lcb.* 4339 batter was ~44 and ~42 folds that found in *Lb.* 1932 and *L.* 4454, while that of hexanoic acid was ~1.5 and ~2.8 folds that found in *Lb.* 1932 and *L.* 4454, respectively (Table 20). Other authors also reported high level of 2,3 butanedione (diacetyl) in rye sourdough by *Lcb. casei* (Kaseleht et al., 2011) or with other facultatively heterofermentative and homofermentative LAB strains on wheat sourdough (Damiani et al., 1996; Liu et al., 2020), and a fermented sorghum-maize slurry (traditional *togwa*) (Mugula et al., 2003). The 2,3 butanedione (diacetyl), is considered an important compound for bread aroma, conferring butter-like notes (Van Kerrebroeck et al., 2018). Also 6-methyl-5-hepten-2-one (green, citrus) seemed to be a molecule characteristic of homofermentative strains (both *Lb.* 1932 and *Lcb.* 4339), positioning at high p2 values. This result is in accordance with Liu et al. (2020), which found higher concentration of this compound in wheat sourdough fermented with homofermentative strains. Only a few alcohols were found in batters started with homofermentative strains, in accordance with Damiani et al. (1996).

The volatile profile of wheat-sorghum bread was instead characterized by 34.4% alcohols, 18.8% aldehydes, 18.8% carboxylic acids, 6.3% furanic compounds and 6.3% ketones, and 15.6% by other compounds (pyrazine, esters, aromatic hydrocarbons) for both control bread and LAB-fermented bread (Table 19; Table 21). Differences in the volatile profile of sorghum bread samples appeared less evident than in sorghum batters. However, this result was expected since sorghum batter represented ~25% of the total dough weight and during bread making, bread dough undergoes various modifications due to yeast activity and baking at high temperatures that may affect the stability of some classes of volatile molecules. Indeed, a combination of enzymatic reactions, fermentation, and thermal reactions have been reported to determine the final flavor of sourdough bread (Ravyts and De Vuyst, 2011). Passing from sorghum batters to bread samples, new molecules were detected: 2-methyl-1-propanol (ethereal), 2-hexyl furan, three xylene isomers (probably *o*-xylene, *p*-xylene and *m*-xylene, with the following odor types: geranium, characteristic, plastic), ethyl caprylate (waxy), furfural (bready), and a derivative molecule of acetic acid (Table 19). These molecules may derive from the addition of other ingredients (wheat flour, oil, yeast) and from the modification and metabolism of baker's yeast during proofing.

Among these molecules, furfural is considered an important compound that determines bread odor and derives from the Maillard reaction (Pico et al., 2015) and was also found in sourdough bread by Siepmann et al. (2019). Moreover, Damiani et al. (1996) reported that the production of iso-alcohols such as 2-methyl-1-propanol is a consequence of yeast fermentation in wheat sourdough. On the other hand, some molecules that were found in sorghum batters were not found in bread samples: 6-methyl-5-hepten-2-one (green, citrus), 2,5-dihydro-2,5-dimthoxy furan, 1,3-dioxolane (pungent), and 2,3-butanedione (diacetyl) (buttery). Yeast and bacteria metabolism, together with degradation at high temperature may have led to the dispersion of these molecules, as previously reported by Siepmann et al. (2019). 2,3-butanedione (diacetyl), for instance, can be reduced by the baker's yeast activity during proofing and being converted to 2,3-butanediol, which is less flavor-active (Van Kerrebroeck et al., 2018) or it can be degraded in Maillard-type reactions (Pico et al., 2015; Van Kerrebroeck et al., 2018). However, ethanol was in general the major volatile compound found in all wheat-sorghum bread, in accordance with literature (Van Kerrebroeck et al., 2018). The differences in ethanol concentration appeared less pronounced, probably due to the yeast activity, which led to the increased ethanol levels in all bread samples (Damiani et al., 1996; Siepmann et al., 2019) (Table 21).

A two components PCA model was obtained (R^2 = 0.74; Q^2 = 0.51) for the volatile profile of wheat-sorghum bread (Figure 20b). Along p1 (60.6 %), a division can be seen between unfermented (left quadrant, negative values) and fermented (right quadrant, positive values). As for the batters, the aromatic profile of LAB-fermented bread was richer than the control bread, with most of the molecules more concentrated on the right side of the graph, assuming positive values on p1. A richer aromatic profile in sourdough bread than control bread was also found by Siepmann et al. (2019) and Van Kerrebroeck et al. (2018). Analogously to the PCA model of the batters, *Lb.* 1932 bread also showed a central spatial distribution, assuming intermediate concentration values among the various molecules (Figure 20b). The spatial centrality of this sample appeared more evident in the bread than in the batters, which did not stand out due to the presence of any compound. Along p2 (13.2%) the division between *Lcb.* 4339 and *L.* 4454 bread, and thus between the homofermentative and heterofermentative metabolism, appeared again evident. Indeed, even if the mixing with other ingredients, the yeast activity and the proofing phase led to minor differences between control bread and LAB-fermented bread, the contribution in the aromatic

profile of *Lcb.* 4339 and *L.* 4454 bread was still evident. Due to its distribution on the PCA biplot *L.* 4454 bread appeared again high for variables such as acetic acid, but also 2-hexyl furan, 2-octenal (fatty), 1-octen-3-ol (earthy, mushroom) (Figure 20b; Table 21). *L.* 4454 was also negatively correlated with 2-ethyl-1-hexanol (rose, green), which was on the opposite quadrant. 2-hexyl furan was however not significantly different in the one-way ANOVA. On the other hand, *Lcb.* 4339 bread was, as for *Lcb.* 4339 batter, high in volatiles with notes like "cheesy", "buttery" or "fatty" given by a higher concentration of molecules such as 3-hydroxy-2-butanone (buttery, cremy), 2-nonenal (fatty), nonanoic acid (waxy, fatty), and hexanoic acid (fatty, cheesy) (Table 21; Figure 20b). Moreover, *Lcb.* 4339 bread was also positively correlated with the production of molecules such as decalactone (fruity), xylene isomers, an acetic acid derivative, nonanal (aldehydic), furfuryl alcohol/nonanol (bread/ floral, waxy), and negatively correlated with 1-pentanol (fermented). However, the differences in the concentration of these molecules were not always significant by the one-way ANOVA (Table 21). The biplot spatial position on control bread indicated instead that control bread was characterized not only by a lower concentration of all the above-mentioned molecules, but also by a higher concentration of 2-pentyl furan (fruity, green), which has been reported to derive from the enzymatic oxidation or autoxidation of flour lipids, and was also reported as the main aromatic compound of a control wheat bread by Siepmann et al. (2019) (Table 21; Figure 20b).

Bread sensory acceptability

The overall appearance, color, odor, taste, consistency, and overall acceptability of bread samples were evaluated. Figure 22 shows the radar plot of all the results obtained. The mean ± standard deviation and ANOVA results were reported in Table 22.

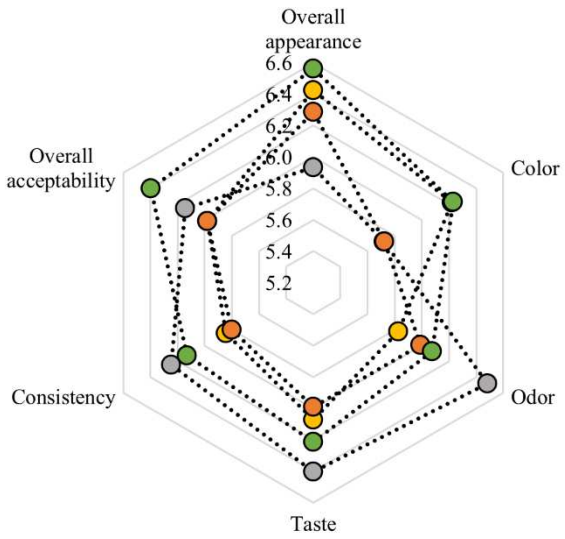

Figure 22. Radar chart of the sensory acceptability test performed on wheat-sorghum bread loaves made with unfermented sorghum batter (control bread, gray), and sorghum batters fermented with Lb. 1932 (yellow), L. 4454 (orange) and Lcb. 4339 (green).

Lcb. 4339 bread achieved the highest score in overall appearance, closely followed by *Lb.* 1932 and *L.* 4454 breads, which were not statistically different from the control. The control sample scored the lowest in this category, indicating that LAB fermentation positively influenced the visual appeal of the bread. According to free comments left by untrained judges on the overall appearance of the samples, the control was often described as "gray", as well as “very compact". “Moldy looking” also appeared only in the free comments left for the control sample. These factors may indeed have affected the aesthetic perception of this sample, making it less attractive. In contrast, differences in color and crumb porosity of *Lcb.* 4339 bread may have increased the overall appearance score of this sample, a very important factor when considering the importance of appearance in purchase decisions. In fact, this sample was the one that showed the greatest color differences in the colorimetric analysis of dough and bread and, together with *L.* 4454 bread, a better development of crumb porosity (Figure 19; Table 17). Fermentation improved the color and crumb porosity of bread, making it more appealing to the panel members than the control.

The results of the color acceptability analysis were not significant, suggesting that color was only part of the consideration of the overall appearance of the product (Table 22; Figure 22). However, when considering the sentiment of the comments left by the judges in both the control and *L.* 4454

breads, most of the sentiments were negative, in *Lb.* 1932 bread they were balanced between negative, positive and neutral, and only in *Lcb.* 4339 bread positive sentiments prevailed. In the free comments left by the judges, "gray" clearly prevailed in the control, followed by "light/pale/faded", but "moldy" and "green" were also found, which were not found in the other samples. In *Lb.* 1932, "light/pale/faded" dominated, followed by "gray" and "neutral". In *L.* 4454 "dark" "homogeneous" prevailed, followed by "gray" and "light/pale/faded". In sample *Lcb.* 4339, "light/pale" and "bright/brilliant" prevailed, followed by "good/nice/pleasant".

With respect to odor, *Lb.* 1932 bread obtained the lowest values compared to the control, while *Lb.* 4339 and *L.* 4454 breads obtained intermediate values, not statistically different from *Lb.* 1932 and the control (Table 22; Figure 22). In this case, in the free comments left by the judges, the words with the highest frequency in the control were " good/nice/pleasant", "yeast" and "bread", while for *Lb.* 1932 bread were "yeast" and "weak/low", followed by "sour" and "strong/intense". Even in the volatile analysis, this sample did not stand out for the presence of specific compounds. This may have made the bread odor weaker than the control, instead highlighting the sour properties imparted by the lactic fermentation, which was perceived as strong and intense by some of the participants.

In terms of taste, no significant differences were found between the samples, which scored quite similarly (Table 22; Figure 22). However, when considering the sentiment of the free comments, for both the control, *Lb.* 1932 and *Lcb.* 4339 breads, the sentiment was mainly positive, while for *L.* 4454 bread it was mainly negative. For what concern the free comments, the control bread was mainly defined as "good/pleasant" followed by "grain/flour", "neutral", "insipid"; *Lb.* 1932 bread as "good/pleasant" followed by "yeasty", "sour", but also "oily/fatty"; *Lcb.* 4339 bread as "good/pleasant" followed by "sour/sourdough"; *L.* 4454 bread, on the other hand, was mainly defined as "insipid/tasteless" followed by "sour" and "good/pleasant".

In terms of consistency, no significant differences were found (Table 22; Figure 22). Interestingly, the texture differences identified by the TPA analysis (Figure 19) were not particularly perceived by the participants. Indeed, the higher hardness of sample *Lcb.* 4339 did not negatively affect the bread texture acceptability rating, as the panel members did not perceive it as harder. This may indicate that fermentation affected the texture and mouthfeel of bread, but not necessarily in a

negative way. Comments were predominantly negative for all bread samples. However, among the free comments left for the control, "soft" prevailed, while for *Lb.* 1932 "hard" followed by "chewy", for *L.* 4454 "chewy" followed by "soft", and for *Lcb.* 4339, "soft" followed by "chewy" and "hard" prevailed.

Regarding the overall acceptability of the bread, no significant differences were found among samples (Table 22; Figure 22). According to the results obtained previously on the flour (*Case Study 2*) and on bread, *Lcb.* 4339 was found to be the sample with the most pronounced effects on the technological properties of flour. During the sensory acceptability test, these modifications were significantly appreciated for their effect on the appearance, probably determined by crumb development and color differences. However, for the odor attribute the control bread tended to show higher values of acceptability, leading to a leveling of the contributions of each attribute. The differences caused by fermentation were probably not strong enough to influence the overall acceptability of bread samples.

Table 22. Sensory acceptability test performed on wheat-sorghum composite breads (mean ± SD). Different letters in the same column indicate significant differences between samples (one-way ANOVA, $p < 0.05$).

Sample	Overall appearance	Color	Odor	Taste	Consistency	Overall acceptability
Control	5.9±1.6b	5.7±1.7a	6.5±1.5a	6.4±1.7a	6.2±1.6a	6.1±1.6a
Lb. 1932	6.4±1.3ab	6.2±1.4a	5.8±1.8b	6.1±1.5a	5.8±1.7a	6±1.6a
L. 4454	6.3±1.4ab	5.7±1.5a	6±1.3ab	6±1.4a	5.8±1.5a	6±1.4a
Lcb. 4339	6.6±1.2a	6.2±1.4a	6.1±1.5ab	6.2±1.6a	6.1±1.6a	6.4±1.4a

Conclusions

The fermentation of sorghum flour with *Lacticaseibacillus casei* 4339 (*Lcb.* 4339), *Leuconostoc* spp. 4454 (*L.* 4454), and *Lactobacillus delbrueckii* subsp. *bulgaricus* 1932 (*Lb.* 1932) had significant effects on the quality attributes of wheat-sorghum bread.

The use of *Lcb.* 4339 and *L.* 4454 resulted in a more open crumb structure, characterized by the formation of larger pores compared to the control and *Lb.* 1932-fermented breads. However, the

increased crumb hardness observed in *Lcb.* 4339-fermented bread can be attributed to its lower specific volume. Nonetheless, both *Lb.* 1932 and *L.* 4454 produced bread loaves with greater cohesiveness, while *Lb.* 1932 and *Lcb.* 4339 contributed to enhanced springiness. Importantly, the differences in texture profile analysis were not perceived by consumers during sensory evaluation, as consistency was scored similarly across all wheat-sorghum breads, irrespective of LAB fermentation. In addition, fermentation with these LAB strains induced notable visual changes, with LAB-fermented breads displaying a lighter crust and increased redness in the crumb. Fermentation with *Lcb.* 4339, in particular, emerged as a promising approach for improving the overall appearance of wheat-sorghum breads, receiving the highest score in terms of overall appearance. Volatile profile analysis revealed that *L.* 4454 fermentation was associated with elevated levels of acetic acid, ethanol, and other key volatile compounds, while *Lcb.* 4339 fermentation was linked to higher concentrations of diacetyl, hexanoic acid and other carbonyls. Interestingly, the least preferred odor during sensory evaluation was found in *Lb.* 1932-fermented bread, which did not exhibit any standout volatile compounds in the profile analysis. This may have resulted in a milder aroma in the *Lb.* 1932-fermented bread compared to the control, while enhancing the sour notes typical of lactic fermentation, though without adding the richer aromatic complexity of *Lcb.* 4339 and *L.* 4454 breads.

These findings suggest that fermentation with these specific LAB strains can effectively enhance the structural and sensory properties of wheat-sorghum bread, representing a promising strategy to increase consumer acceptance and expand its market potential.

2.2 Effect of Fermentation on the Rheological and Molecular Properties of Dough

Case Study 4

Effect of LAB fermentation and freeze drying on the rheological and molecular properties of wheat-sorghum dough

Introduction and Aim of the Study

Understanding dough properties is essential for predicting bread quality, especially when incorporating alternative flours to wheat, such as sorghum. Indeed, wheat flour is commonly used in baking due to its ability to form a strong gluten network, which imparts viscoelastic properties crucial for dough structure and gas retention (Ooms and Delcour, 2019). The absence of gluten in sorghum complicates the formation of the viscoelastic network crucial for high-quality bread, often resulting in products with suboptimal texture and volume (Espinoza-Herrera et al., 2021; Rumler and Schönlechner, 2021; Ari Akin et al., 2022). This limitation is further compounded by the unique structure of sorghum proteins, primarily hydrophobic kafirins, which reduce hydration capacity and hinder interaction with other dough ingredients, posing challenges in gas retention and mechanical resistance during mixing and proofing (De Mesa-Stonestreet et al., 2010; Serna-Saldivar and Espinosa-Ramírez, 2019; Espinoza-Herrera et al., 2021). Consequently, studies on the empirical rheology wheat-sorghum doughs showed decreased water absorption, stability, and extensibility, which in turn can negatively impact the final bread's texture and sensory qualities (Seleem and Omran, 2014; Dube et al., 2021; Rumler and Schönlechner, 2021; Ari Akin et al., 2022). The viscoelastic properties of the wheat-sorghum dough may also be further analyzed through the use of fundamental rheological techniques, such as dynamic oscillation measurements, highly sensitive to changes in chemical composition and physical structure (Song and Zheng, 2007; Iacovino et al., 2024).

To address these challenges, fermentation with lactic acid bacteria (LAB) has emerged as a promising, sustainable method to improving rheology, sensory attributes, and reducing anti-

nutritional factors of grains (Gobbetti et al., 2020). LAB fermentation generates organic acids, enzymes, and other metabolites that modify the functionality of sorghum flour and in turn affect dough properties and structure. Previous studies have shown that fermenting sorghum flour increases water absorption, emulsifying capacity, and texture, enabling sorghum flour to function more effectively in composite doughs (Elkhalifa et al., 2005; Ojha et al., 2018).

In this Ph.D. thesis, *Case Studies* 2 and *3* explored the role of different LAB strains in modifying sorghum's techno-functional properties of sorghum flour and bread. In particular, among the three strains tested, *Lacticaseibacillus casei* 4339 emerged as a promising candidate for improving the techno-functional properties of flour such as viscosity and thermal, and pasting properties (*Case Study 2*), as well as having a positive effect on the color, crumb porosity and overall appearance of wheat sorghum bread (*Case Study 3*).

The use of fresh fermented batters or sourdoughs, however, has certain limitations, including a reduced shelf life. To further address this limitation freeze-drying was explored as a method to extend usability while preserving critical functional properties. Freeze-drying allows for greater flexibility in incorporating fermented sorghum across various baking applications, maintaining the functional benefits of LAB fermentation while enhancing practicality (Taglieri et al., 2021; Gidari - Gounaridou et al., 2023). However, to the best of our knowledge, there is a lack of studies that focus on the rheological and molecular properties of wheat dough with added fermented sorghum flour/dry sourdoughs.

For this reason, building on the findings of *Case Studies* 2 and *3*, this study aims to investigate the combined effects of *Lacticaseibacillus casei* 4339 fermentation and freeze-drying on the rheological and molecular characteristics of wheat-sorghum dough. By examining fermentation and preservation techniques together, this study aims to provide further insights into the role of LAB fermentation in influencing wheat-sorghum doughs properties, deepening the knowledge of fermented sorghum application in the food industry.

Materials and Methods

Materials

Whole white sorghum (*Sorghum bicolor* (L.) Moench) HTC (heat-treated cereal) flour was provided by MartinoRossi SpA (Cremona, Italy) (55.7% carbohydrates, 16.4% fiber, 10.3% protein, and 3.2% total fat); type 00 soft wheat flour (Italian Legislation, 1967) (*Triticum aestivum* L.) was supplied by Agugiaro & Figna Molini SpA (Collecchio, Parma, Italy) (W=190-220, 71% carbohydrates, 10% protein, 2% fiber, and 0.9% fat). Salt (Italkali S.p.A., Palermo, Italy), sunflower oil (Oleificio Zucchi, Cremona, Italy), and sucrose (Eridania, Bologna, Italy) were all purchased from a local market.

Sorghum fermentation and freeze-drying

Sorghum batters were prepared using 63% (w/w) of water, 35% (w/w) of flour and inoculated with 2% (v/w) of *Lacticaseibacillus casei* 4339 (*Lcb. casei* 4339) (University of Parma Culture Collection, UPCC). The strain was selected in previous studies, based on its effect on flour molecular and techno functional properties on sorghum batters and wheat-sorghum composite bread (*Case Study 2* and *3*). Sorghum batter was fermented at 25°C for 15h. Strain revitalization and microbiological analyses were carried out as described in our previous work (*Case Study 2*). After 15h the pH was measured and the fermented batter was used freshly for dough preparation (FF) or freeze dried (25-30 mTorr; -110°C; 48-56 h) (BENCHTOP PRO with Omnitronics™, SP Scientific, Stone Ridge NY, USA). Freeze dried fermented samples (DF) were stored in a glass dryer desiccator at room temperature (25°C) before being used for dough preparation.

Microbiological analyses

LAB counts were carried out on sorghum batters for the inoculum before (T0) and after fermentation (T15). To this aim, batters were serially diluted in Ringer solution (Oxoid, Milan), and 0.1 ml of the appropriate dilution was spread on MRS agar (de Man, Rogosa, Sharpe, Oxoid Ltd., Basingstoke, United Kingdom) plates. At both sampling times, pH was also measured using a pH meter (Beckman Instrument mod F350, Fullerton, CA, USA) and a glass electrode (Hamilton, Bonaduz, Switzerland) at 25°C. The concentration of the inoculated LAB cells was evaluated also after the freeze-drying of the fermented batters by means of plate counting. Ten grams of dried fermented batters were resuspended in 90 ml of Ringer solution and then subjected to ten-fold

serial dilutions. Finally, the appropriate dilution was spread on MRS agar plates, and then incubated at the optimal temperature (Case study 2, Table 5).

Dough preparation

Three wheat-sorghum doughs samples were prepared by mixing wheat and sorghum flour (wheat to sorghum ratio 85:15) and by adding, every 100 g of flour blend, 2 g salt, 3 g of sugar, and 3 g of sunflower oil (no yeast was added). The wheat-to-sorghum flour ratio was determined in a previous study on wheat-sorghum bread with fermented sorghum batters (*Case Study 3*). The ingredients mixing and kneading were performed using a kneading machine (KitchenAid 5KSM5, St. Joseph, Michigan, U.S.A) for 8 minutes at level 1 speed. Sorghum flour was added as unfermented sorghum flour (control), dried fermented sorghum flour (DF) or fresh fermented sorghum flour (FF). The amount of fresh fermented sorghum flour was calculated in order to add the same amount of flour as in dry samples. The total amount of water added (58%) was calculated using a Brabender® Farinograph (Ohg Duisburg, Germany) to achieve an optimal consistency of 500 BU for unfermented wheat-sorghum blends (data not shown). Given the high dependence of the analyses on water content, this standardized addition was used for all experimental doughs, ensuring the same hydration level in the fresh fermented (FF), dried fermented (DF), and control (unfermented sorghum) doughs. The water added in FF dough was then calculated taking into consideration the water that was already present in the fermented batter. Each type of dough was prepared in triplicate, in three independent batches (three different days) of sorghum fermentation.

Dough water activity and pH

For dough pH measurements, 10 g of dough were mixed with 100 mL of distilled water (890-48H Oster/Sunbeam, McMinnville, TN, USA). After that, the mixture was stirred in a beaker for 10 minutes and the pH measurement (Crison Basic 20, Alella, Spain) was recorded. pH measurements were performed in triplicate for each dough batch. Dough water activity was measured at 25 °C with an Aqualab 4 TE (Decagon Devices, Inc. WA, USA). Both dough pH and water activity measurement were performed in triplicate.

Dough Rheology

Rheological measurements of the dough were performed using a stress-controlled rheometer (mod. MCR 302, Anton Paar, Graz, Austria) equipped with a plate/plate sandblasted system (25-mm diameter) and a Peltier convection system (mod. CTD 180, Anton Paar, Graz, Austria) to control the temperature of the samples (25 °C ± 0.1). Disk-shape samples (thickness 5 mm, diameter 25 mm) were gently portioned from the dough using a slicer and a borer. The probe was lowered to a height of 3 mm and the excess sample was removed, after which the normal force was waited for < 2N before the sample was analyzed. The measuring zone was covered with a solvent trap to control moisture loss during the entire analysis.

Amplitude sweep test. For the amplitude sweep test, a constant frequency of 10 rad/s was set, and strain was applied within a range from 0.001% to 100%, divided into 26 measurement points. The parameters calculated from the amplitude sweep test analysis included: the LVR value (corresponding to the strain percentage at the limit of the linear viscoelastic region), the Yield Point value (corresponding to the stress at the upper bound of the LVR), and the Flow Point value (defined as the stress at the crossover point where G' equals G''). Three replicates were performed for each sample batch.

Frequency sweep test. The frequency sweep test was conducted at a constant strain of 0.01%, within the linear viscoelastic (LVE) region determined from preliminary amplitude sweep tests, and over a frequency range from 1 rad/s to 100 rad/s, divided into 13 measurement points. From the analysis performed in the linear viscoelastic region, the frequency dependence of the G' and G'' moduli was evaluated using the power law equation (Tidona et al., 2021):

$$G'(\omega) = k' \cdot \omega^{n'} \quad (1)$$

$$G''(\omega) = k'' \cdot \omega^{n''} \quad (2)$$

Where, k' and k'' represent the value of the modulus G' or G'' at a frequency of 1 rad/s, while n' and n'' indicate the degree of dependence of the viscoelastic properties on frequency variation. Additionally, the tanδ value (G''/G') was calculated. Three replicates were performed for each sample batch.

^{1}H molecular mobility

The proton molecular mobility of sorghum dough was studied using a low resolution ^{1}H NMR spectrometer (the minispec, Bruker Biospin, Milan, Italy) (20 MHz) at 25.0 ± 0.1 °C. The ^{1}H self-diffusion coefficient (D) with a pulsed-field gradient spin-echo (PFGSE) pulse sequence and the T_2 spin-spin relaxation with the Carr-Purcell-Meiboom-Gill (CPMG) sequence were measured for each sample. NMR tubes were filled with sorghum dough (10 mm height), covered with Parafilm® to avoid moisture loss during the analysis. The recycle delay (RD) was preliminarily calculated by means of the spin-lattice ^{1}H T_1 sequence (Inversion Recovery) and set for subsequent analyses at 2 s. The proton self-diffusion curve was previously calibrated with a CH_3COOH standard solution ($D_{25°C}$ = 1.08 10^{-9} m^2/s) with a gradient pulse amplitude range between 35 and 65 %. The PFGSE sequence was performed with a 40 % gradient pulse amplitude, 16 scans, a gain of 72. After the PFGSE sequence, the T_2 CPMG sequence was applied to the tube, with 32 scans, gain of 72, interpulse spacing of 0.04 and 2500 data points. The ^{1}H CPMG curves were analyzed as a quasi-continuous distribution of relaxation times using UPEN software (Alma Mater Studiorum, Bologna, Italy). The ^{1}H CPMG curves were also fitted with a discrete exponential model using Sigmaplot software (v.10, Systat Software Inc., USA) to obtain relaxation times (ms) and proton relative abundances (%) for each ^{1}H population, as described by Marchini et al. (2021a). Three tubes were analyzed for each sample batch, and five (PFGSE) and three (CPMG) measurements were performed for each tube.

Results and Discussion

Microbiological analysis, pH and water activity

The LAB counts in batters proved the ability of *Lcb. casei* 4339 to grow in sorghum batters, increasing from 7.99±0.13 Log CFU/ml to 9.37±0.11 Log CFU/ml during the 15 hours of fermentation. Meanwhile, the pH dropped from 6.23±0.03 to 4.16±0.04 as a consequence of organic acids production upon lactic acid fermentation. Freeze drying impacted negatively on the cultivability of LAB cells, which showed a decrease of over 2 Log CFU/ml compared to fresh fermented samples, with a final concentration of 7.03±0.47 Log CFU/ml. A slight decline in LAB cell cultivability following freeze-drying has been reported in prior research. Although freeze-drying generally shows positive outcomes regarding the revitalization of microorganisms, the low

temperatures involved can lead to substantial damage, largely attributed to the formation of intracellular ice crystals (Stefanello et al., 2018; Taglieri et al., 2021).

The pH and water activity (a_w) of sorghum doughs are presented in Table 23. As expected, the addition of fermented sorghum reduced dough pH in both FF and DF samples. However, freeze-drying modified the pH of the fermented flour, yielding values between the control and FF doughs. This pH decrease may be due to variations in organic acid content following freeze-drying. Although freeze-drying is reported to preserve organic acids better than other drying methods, it may still cause slight acid loss, resulting in a higher pH than FF (Chalmers and Watts, 1972; Gero and Smyrl, 1982; Xie et al., 2022).

In terms of water activity, the control sample exhibited the highest value, while DF and FF samples were similar. Lower water activities in doughs with added sourdough are often attributed to exopolysaccharide (EPS) production. However, no EPS production was observed in the sorghum fermentation with *Lcb.* 4339. Other factors may have contributed to reduced water activity, enhancing water-binding capacity in fermented dough. Organic acids, for instance, reduce molecular motion and promote less mobile water matrices (Hopkins et al., 2019). Additionally, fermentation may alter the structure and functionality of flour biopolymers, including protein charge, enhancing water-binding potential. Enzymatic activity on protein and starch fractions may also generate smaller molecules, such as peptides and oligosaccharides, which enhance water retention capacity (Gámbaro et al., 2006). Moreover, solutes generated by fermentation byproducts can further reduce water activity.

Table 23. water activity (a_w) and pH of Control, Fresh Fermented (FF) and Dried Fermented (DF). Different letters indicate significant differences between samples ($p < 0.05$).

	a_w	pH
Control	0.975 ± 0.003a	5.93 ± 0.05a
FF	0.966 ± 0.006b	4.96 ± 0.11c
DF	0.965 ± 0.005b	5.32 ± 0.07b

LF ^{1}H molecular mobility

^{1}H self-diffusion coefficient

The self-diffusion coefficient ^{1}H D (PFGSE sequence), related to translational mobility and matter displacement in the system in the absence of a chemical potential (Kovrlija and Rondeau-Mouro, 2017) was assessed in order to provide information on the dynamics of protons in a complex system. Table 24 shows the results of self-diffusion coefficient.

Table 24. Relative abundances (%) and relaxation times (ms) of CPMG populations and proton self-diffusion coefficient of Control, Fresh Fermented (FF) and Dried Fermented (DF) samples. Different letters indicate significant differences between samples.

	Control	***FF***	***DF***
tA (ms)	0.50±0.06a	0.49±0.07a	0.46±0.09a
tB (ms)	3.52±0.19a	3.22±0.20b	3.32±0.17b
tC (ms)	15.53±0.40a	14.67±0.47b	14.16±0.25c
tD (ms)	56.29±1.54a	54.76±3.04a	55.43±3.05a
%popA (%)	8.28±0.35a	8.25±0.33a	7.97±0.44b
%popB (%)	25.21±0.37b	27.61±0.50a	27.88±0.72a
%popC (%)	53.62±0.84b	52.70±0.63c	54.17±0.72a
%popD (%)	12.64±0.44a	10.90±0.44b	9.90±0.55c
Self-diffusion coefficient [10^{-9}*m^2/s]	0.621±0.006a	0.611±0.007b	0.584±0.003c

According to Götz and Hinrichs (2008) the diffusive flow processes in solid systems are mainly influenced by the pore system architecture, chemical composition of the solid surface and resulting solid–water phase interactions. Moreover, in starch-based products, according to Kovrlija and Rondeau-Mouro (2017) the possible presence of starch molecules clustered together creating porous structures may impede water diffusion. In this study, wheat-sorghum doughs with fermented sorghum (FF and DF) showed lower ^{1}H diffusion than the control dough. Indeed, the control dough exhibited the highest value in terms of self-diffusion coefficient followed by FF, and DF with lowest value. In a previous analysis of the self-diffusion coefficient on sorghum batters, the results indicated higher self-diffusion in fermented batters mainly due to the matrix

breakdown and thus the decrease of structural boundaries that impede water self-diffusion (*Case Study 2*). However, in this study the addition of sorghum fermented batter to a wheat dough formulation led to the opposite direction, with FF and DF showing lower values than control dough. This apparently contradictory result may derive from different phenomena occurring when fermented sorghum is added to wheat flour. Firstly, the production of compounds with different molecular weights during the fermentation of sorghum flour may interact with wheat biopolymers (starch and protein) during dough kneading in a differed way than the control. Furthermore, a reduction in pH may have altered the functionality of wheat flour biopolymers and their ability to bind water. Moreover, according to Jayaram et al. (2014), the low pH could lead to the formation of gluten aggregates which in turn may impede water diffusion. However, as DF had a higher pH than FF, but a lower proton self-diffusion coefficient, the freeze-drying process may have resulted in additional significant structural alterations. In particular, freeze-drying could facilitate greater water absorption (Jayanthi et al., 2021), thus explaining the lower proton self-diffusion coefficient in comparison with FF. Even at a macroscopic scale the results of a_w showed lower water mobility in doughs with fermented sorghum, however, the differences between DF and FF dough were not detectable.

^1H T_2 mobility

Figure 23 shows the quasi-continuous distributions of the spin-spin relaxation time, T_2, for the three samples at 58% moisture content obtained from CPMG experiments. Four distinct populations (A, B, C and D, from the least to the most mobile proton population, respectively) were found in all samples; their relaxation times (tA, tB, tC and tD) and relative proton abundances (%popA, %popB, %popC and %popD) are reported in Table 24. These populations relaxed in the range of 0.46 – 0.50 ms (A), 3.22 – 3.52 (B), 14.16 – 15.53 (C) and, 54.76 – 56.29 (D), respectively. Population C was found to be the prevalent, encompassing more than 50% of the total detected protons (52.70–54.17%), followed by populations B (25.21– 27.88%), D (9.90 – 12.64%) and A (7.97 – 8.28%). These results in terms of relaxation time and relative abundance agreed with previous studies on sorghum – water based systems at similar moisture content (~58%) (Marchini et al., 2021a; Chiodetti et al., 2024) and wheat dough (Parenti et al., 2021).

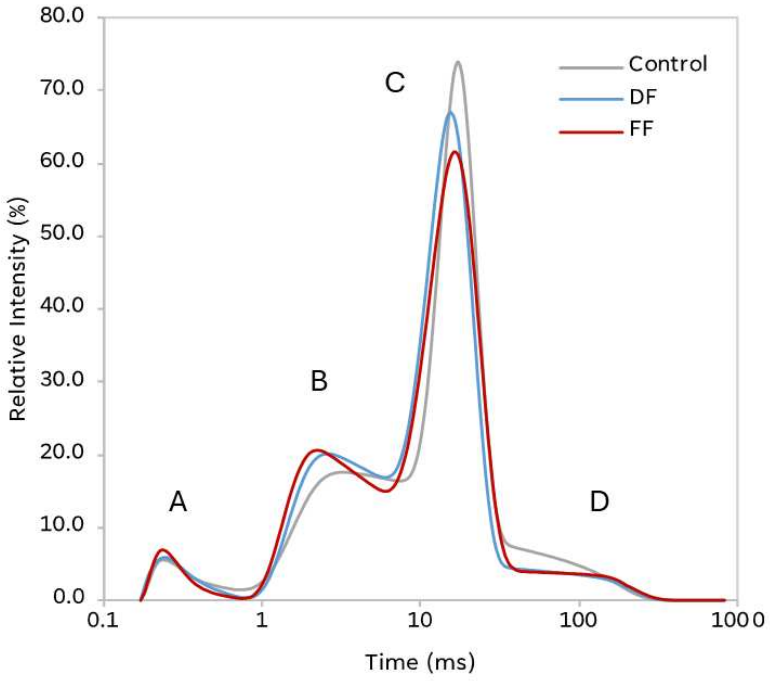

Figure 23. ^{1}H T_2 mobility of wheat - sorghum dough: average CMPG proton relaxation curves of Control, Fresh Fermented (FF) and Dried Fermented (DF).

The mobility of the least mobile ^{1}H T_2 population A (tA) showed no significant differences between samples ($p \geq 0.05$); the population with this range of relaxation times was previously associated with CH protons of amorphous starch (Bosmans et al., 2012). Thus, neither sorghum fermentation nor freeze-drying affected the mobility of the amorphous starch fractions in wheat-sorghum dough.

On the other hand, the mobility of the populations B (tB) and C (tC) showed significant differences ($p < 0.05$) with the control. Populations B was previously attributed to the non-exchanging protons of gluten and the exchanging protons from intragranular water and starch (population B), while population C has been attributed to the protons from extragranular water, surrounding and exchanging with starch protons and to the water protons associated with gluten (Bosmans et al., 2012). The control dough exhibited greater mobility of populations B and C than the fermented samples (FF and DF). Moreover, the mobility of population C also evidenced differences between

FF and DF doughs. In fact, freeze-drying resulted in higher rigidity in the dominant population (lower tC).

For what concern the population D, it has been previously attributed to the fraction of more mobile water in the system (Parenti et al., 2021; Chiodetti et al., 2024). No significant differences in tD values ($p \geq 0.05$) were found in any of the samples. The lack of significance could be due to the very high standard deviation, likely resulting from the wide range of relaxation times that potentially overlapped with other populations.

The relative abundances of CPMG populations showed differences in all the four populations analyzed (%popA, %popB, %popC and %popD). As mentioned above, no differences were found for tA (ms), but the abundance of population A (%popA) showed variations among samples: the control and FF samples highlighted more protons belonging to this population than DF sample. This result suggests that although the interaction between amorphous starch and water did not seem to have changed (same tA), freeze-drying probably reduced the amorphous fraction in sorghum. Regarding population B both fermented samples (FF and DF) showed a significantly higher abundance (%popB) ($p < 0.05$), indicating more exchanging protons in the intragranular zones. DF also showed a higher abundance in population C than the control and FF samples (%popC). Probably, fermentation facilitated water entry into the starch granule (%popB higher in FF and DF) due to the formation of surface pores following starch hydrolysis (Hallén et al., 2004; Yang et al., 2017), while freeze-drying caused more water to be absorbed outside the starch granule and in the protein network formed by wheat gluten proteins and other sorghum proteins (%popC higher in DF). For what concern population D, %popD was lower in FF and DF samples, thus indicating less weakly bound water protons in these samples.

In general, CMPG results evidenced that sorghum fermentation induced structural changes in starch and protein fractions, which affected their molecular interaction with wheat flour components and their interaction with water in the wheat-sorghum dough system. In particular, populations C and D showed a greater abundance of protons within the intragranular and in the extragranular regions. In freshly fermented, water accumulates mainly in the amorphous portion and promote water entry into the starch granule, resulting in increased intragranular water,

whereas freeze-drying seems to increase water in the extragranular space and protein continuous network.

Moreover, an overall increase in water-binding capacity of molecular components, as also evidenced by lower values of a_w and ^{1}H self-diffusion coefficient D was also reflected in CPMG distributions of FF and DF samples. Indeed, the addition of fermented sorghum resulted in the formation of a dough with lower molecular mobility, which is associated with a better ability in binding water by wheat and sorghum biopolymers. The mechanisms may be several. Stronger molecular interactions were probably present in doughs with fermented flour (lower tB and tC), particularly in DF, as also evidenced by self-diffusion coefficient results. According to previous findings (see *Case Study 2*) *Lactocaseibacillus casei* 4339 fermentation of sorghum flour leads to the establishment of stronger bonds between water and flour biopolymers in more constricted zones or in the network of exchanging protons between water and flour biopolymer. Moreover, Yang et al. (2017) and Hallén et al. (2004) reported how the fermentation of flour by lactic acid bacteria led to the hydrolysis of the starch, resulting in smaller, irregularly shaped particles with more holes. These structural changes may have enabled better water absorption capacity of fermented sorghum flour. In addition, a lower pH in DF and FF doughs compared to the control may have affected the mobility of wheat-sorghum doughs, leading to increased rigidity of the system. This phenomenon could be attributed to the formation of gluten aggregates due to lower pH (Jayaram et al., 2014) and to increased number of components with higher ability to bind water. Indeed, the different pH of the doughs may have affected the functionality of wheat biopolymers, resulting in different mobility compared to the control. Moreover, pH can modulate the activity of endogenous (from wheat) and exogenous (microbial) enzymes, which can produce components with higher binding capacity. Indeed, in a previous study on the use of enzymes in bread formulations (Gámbaro et al., 2006), it was proposed that the addition of a mixture of α-amylase and xylanase may produce low molecular weight dextrins with high water retention capacity.

Freeze-drying prompted additional structural alterations that elevated the rigidity of the system. It is plausible to hypothesize that, as previously discussed in the context of proton self-diffusion coefficient, freeze-drying may have enhanced the water absorption capacity of flour components, which could be reflected in the observed increase in rigidity.

Dough rheology

Amplitude sweep

To comprehensively analyze the effect of fermentation and freeze-drying on the viscoelastic properties of wheat-sorghum dough, the variation of the storage modulus (G') and loss modulus (G") of the dough during a strain amplitude sweep was studied (Figure 24).

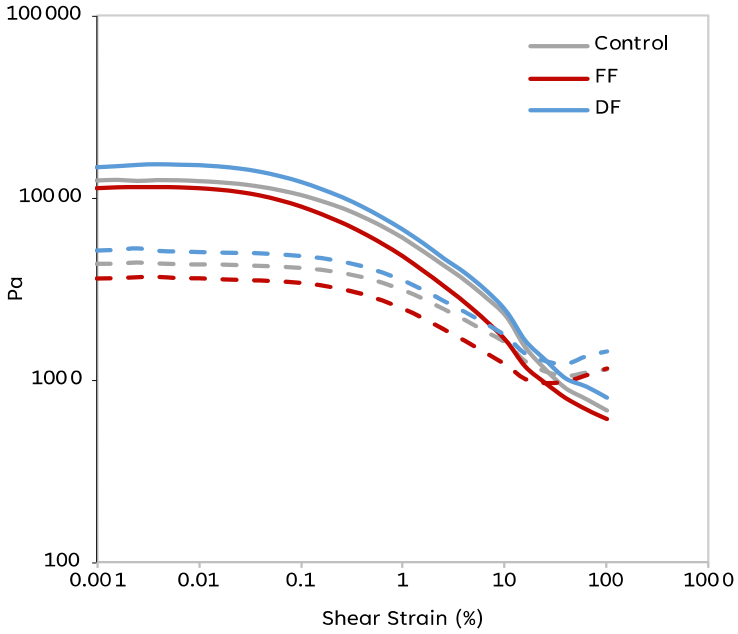

Figure 24. Average amplitude sweep plot for Control, Fresh Fermented (FF) and Dried Fermented (DF).

In the linear viscoelastic region (LVR) all the dough showed higher elastic modulus (G') compared to loss modulus (G"), thus indicating a more solid-like behavior. The yield point (Table 25) was also calculated to quantify the extension of the LVR. Indeed, the yield point corresponds to the stress at the end point of the LVR, this yield value determines the starting point for weakening of gel strength (Yousefi and Razavi, 2015), and where the deformation starts to be irreversible. A higher yield point was found in control dough compared to DF and FF, therefore the samples required different stress to reach the end of the linear viscoelasticity region. In particular, the

control was more capable of withstanding mechanical strain without plastic deformation (Patel et al., 2015). However, the moduli of DF were always found to be higher than control and FF samples, in fact, at the same shear strain (%) DF had the highest moduli. This could be explained by the fact that the DF sample exhibited a greater elastic and solid-like response. For what concern the flow point, that indicates the stress value at the crossover point (G">G'), FF showed the lower values. While DF and control didn't highlight any significant difference. On the contrary, for the Crossover G'=G", that it's the moduli value at the crossover point, no significant differences were found between control and FF, while DF showed the highest value. However, following LVR, the samples exhibit a nonlinear trend, and the G' and G'' moduli may not accurately reflect the true viscoelastic behavior of the sample (Shahbazi et al., 2021).

Table 25. Amplitude sweep output of Control, Fresh Fermented (FF) and Dried Fermented (DF). Different letters indicate significant differences between samples ($p < 0.05$).

	Yield point (Pa)	Flow point (Pa)	Crossover G'=G'' (Pa)
Control	11.84 ± 1.66a	424.11 ± 33.88a	1094.08 ± 57.58b
FF	5.24 ± 1.2b	356.61 ± 18.86b	1048.75 ± 100.14b
DF	4.75 ± 1.09b	459.92 ± 40.67a	1254.11 ± 50.59a

Frequency sweep

Figure 25 displayed the trend of the two modules as a function of frequency: the storage modulus (G') (deformation energy stored in the material after removing the oscillation) and the loss modulus (G'') (mechanical energy lost by the dough during oscillation) (Sullivan et al., 2011). The storage (G') modulus of the doughs was higher than loss (G") modulus at all frequencies and increased with increasing frequency. This is a typical behavior observed in rheological studies of dough (Létang et al., 1999; Miñarro et al., 2012; Mohammed et al., 2012; Aguilar et al., 2015; Maçãs et al., 2023). It could be seen that DF dough had the highest values for both G' and G'', while FF had the lowest moduli values, and control had intermediate values.

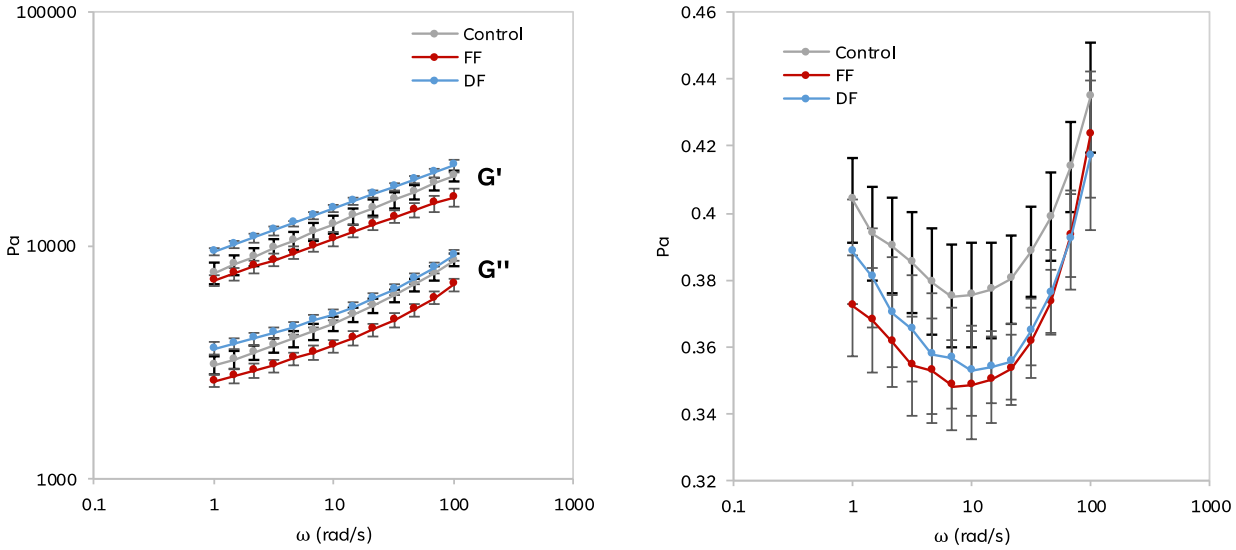

Figure 25. Average frequency sweep plot for Control, Fresh Fermented (FF) and Dried Fermented (DF): a) storage (G') and loss (G") modulus; b) tanδ.

In Table 26 the parameters obtained from the power law fitting (eq. 1 and eq. 2) describing the dependence of moduli on oscillatory frequency, are reported. Power law equations fitted experimental dynamic data with a good level of accuracy, as determination's coefficients (R^2) were always higher than 0.97.

Table 26. Power law parameters (k', n', k" and n") and tanδ values obtained at 1 and 100 rad/s from frequency sweep test for Control, Fresh Fermented (FF) and Dried Fermented (DF). Different letters indicate significant differences between samples.

	Control	***FF***	***DF***
k'	7639.61±780.29b	7049.79±414.58b	9331.94±301.51a
n'	0.209±0.010a	0.181±0.010b	0.187±0.004b
R²	0.99	0.99	0.99
k''	2908.25±243.85b	2493.18±174.10c	3396.53±196.22a
n''	0.221±0.010a	0.199±0.003b	0.195±0.005b
R²	0.98	0.97	0.97
tanδ at 1 rad/s	0.40±0.01a	0.38±0.01b	0.39±0.02ab
tanδ at 100 rad/s	0.44±0.01a	0.42±0.02ab	0.42±0.02b

k' and k" results confirmed the trend mentioned before, with all the samples more represented by storage modulus than loss modulus, and thus indicating a predominant elastic and solid-like behavior of the matrix (k' > k"), in accordance with previous finding on dough (Georgopoulos et al., 2004; Struck et al., 2018; Jin et al., 2020). Moreover, DF dough had the highest values of k' and k", while FF had the lowest value. For k' there were no significant differences between FF and control, while for k" the control sample showed higher values than FF, but still lower than DF. Different k' can be linked to the changes in the strength of the gluten network, which is affected by the dough pH. Indeed, according to Meerts et al. (2018), initially a reduction in pH results in a net positive charge of gluten proteins. This is due to the protonation of certain carboxyl groups present in acidic amino acids. This protonation caused an electrostatic repulsion between gluten chains, leading to a loss of cohesion in the gluten network. Consequently, this resulted in the formation of smaller aggregates and a reduction in the overall strength of the gluten network. Secondly, at low pH, the formation of disulfide bonds (SS), which are essential for the formation of gluten structures, is inhibited due to the reduction in the presence of free thiolate anions (S^-), which are necessary for SH-SS exchange reactions. The lowest pH value observed in the FF sample may explain the lowest moduli and k' and k'' values in this sample. However, despite exhibiting a lower pH than the control sample, DF demonstrated higher moduli and consistency indices. This would therefore indicate that freeze-drying exerts an influence, in addition to that of pH, that leads to a general strengthening of the dough. Similar results after the addition of lyophilized flours were also found by Korus et al. (2022).

From the power law fitting of frequency tests, also n' and n" indices were calculated (Table 26). According to Ferry (1980), when n', which indicates the dependence of viscous modulus (G') on frequency variation, has a value approaching 0, the material behaves like a rubbery material. In our study, n' values (Table 26) ranged between 0.18 and 0.21, consistent with other works on doughs, where the exponent n' was in the range of 0.15-0.28 (Georgopoulos et al., 2004; Upadhyay et al., 2012). In our study, control dough showed significantly higher n' values (0.21) than DF and FF dough (0.19 and 0.18, respectively), which did not differ significantly from each other. Higher values of n' are typical for materials in which there is a large number of components not involved in lattices with other components (Upadhyay et al., 2012). Higher values in the control dough than in the unfermented samples may be justified by the activity that occurred during fermentation.

Indeed, as already reported in the literature, fermentation and pH induced enzyme activity may have led to partial degradation of complex storage proteins into simpler and more soluble products (Yousif and El Tinay, 2001; Taylor and Taylor, 2002), and to the hydrolysis of the starch, resulting in smaller, irregularly shaped particles with more holes (Hallén et al., 2004; Yang et al., 2017). These new compounds presented in doughs with fermented flour, could therefore have participated in the formation of the glutinic network in the dough, thus leading to a lowering of the parameter n'.

Similarly, n", which indicates the dependence of viscous modulus (G") on frequency variation, also shows higher values in control (0.22) than in DF and FF (0.19), which again did not differ significantly ($p \geq 0.05$). It was also noted that in all samples n" was always higher than n'. This finding is consistent with studies on different types of dough, where n" was consistently larger than n' (Kontogiorgos, 2017; Sun et al., 2020). This behavior is in fact typical of gluten network gels (Kontogiorgos, 2017; Sun et al., 2020).

The values of tanδ at 1 rad/s and at 100 rad/s are also presented in Table 26. As expected, since storage (G') and loss (G") moduli increased with angular frequency, the values tanδ at 1 rad/s were lower than tanδ at 100 rad/s. Moreover, as in all cases G' was higher than G", tanδ resulted < 1, thus indicating the prevalence of elastic features over viscous of dough samples. Moreover, the values of tanδ were greater than 0.1 for all samples, indicating the typical weak gel behaviors (Korus et al., 2009). In general, a firm and rigid dough usually shows small tanδ values, while a high tanδ value represents a sticky and slack dough (Jin et al., 2020). Control dough had the highest tanδ values at both low and high frequencies, while FF and DF were not significantly different from each other at either 1 rad/s or 100 rad/s. FF showed the same behavior as control at high frequencies but differed at low frequencies, while DF did not differ from control at low frequencies but showed lower values at high frequencies. The results of tanδ would thus confirm what was said earlier, that fermentation leads to a partial degradation of proteins, starch and other components that subsequently participate in the formation of the starch-protein network. Indeed, according to Zwanzig and Mountain (1965), at low frequencies, high values of tanδ are due to uncross-linked polymers, this would then justify the higher values of control.

The rheological behavior corroborated the findings observed in molecular mobility, where the relaxation time of the C population (tC) indicated a higher rigidity in the DF sample. However, the molecular mobility results (T_2 and ^1H proton self-diffusion coefficient) indicated an intermediate behavior of FF in comparison to DF and control. Nevertheless, this trend, as previously demonstrated, is not reflected in the rheological parameters obtained by frequency sweep. This discrepancy may be attributed to the distinct structural scales assessed by rheometer tests and ^1H low-resolution NMR, along with the higher sensitivity of ^1H NMR in capturing water and starch dynamics, as opposed to the greater sensitivity of dynamic oscillatory rheology to the gluten/protein network.

Conclusions

This study investigated the impact of adding fermented sorghum, either fresh or freeze-dried, on the molecular mobility and rheological properties of wheat-sorghum composite dough. Fermentation with *Lacticaseibacillus casei* 4339 reduced both pH and water activity, likely due to microbial breakdown and the production of water-binding molecules and organic acids. Proton molecular mobility (^1H NMR) analysis indicated that incorporating fermented sorghum flour increased dough rigidity (T_2 and ^1H self-diffusion coefficient), likely due to new compounds formed during fermentation that interact with wheat biopolymers and water. Fermentation and freeze-drying also led to a redistribution of water within intragranular and extragranular zones, with freeze-dried fermented sorghum (DF) facilitating stronger water-polymer interactions that contribute to increased dough rigidity. The lower pH in doughs containing fermented flour likely further modified wheat biopolymer properties. Rheological analysis showed that while fermentation alone can reduce gluten network strength, freeze-drying increases dough rigidity. DF samples exhibited higher storage moduli (G') than fresh fermented sorghum (FF), indicative of a stronger and more elastic structure. This study highlights that combining sorghum fermentation and freeze-drying modify rheological behavior and molecular mobility in wheat-sorghum doughs, providing valuable insights for optimizing sorghum's use in breadmaking. Further research into the combined effects of fermentation and freeze-drying could support functional improvements in bakery applications, facilitating the development of sorghum-based bread formulations.

Section 3

SECTION 3

Bread Staling and its Mitigation

Bread staling is a complex process involving physical quality decay due to structural changes, such as texture hardening, moisture redistribution, and starch retrogradation. Addressing staling in new bread formulations is essential for practical applications and market viability, as it helps extend shelf life, preserve sensory qualities, and reduce food waste. Sorghum flour, with its distinct protein composition and structure, may impact staling dynamics in composite breads. However, the limited research on this topic makes it challenging to fully understand these effects.

To address this gap, *Case Study 5* explores the impact of sorghum flour on bread staling by examining texture, thermal properties, and molecular mobility across different structural levels and sorghum inclusion percentages. *Case Study 6*, in turn, focuses on staling mitigation strategies, particularly through lactic acid bacteria (LAB) fermentation. LAB fermentation, via metabolite production and enzyme activity, may influence the staling of wheat-sorghum composite bread. This study specifically investigates the effects of a selected LAB strain, in combination with freeze-drying, on staling by modulating water mobility and amylopectin retrogradation. Together, these studies provide a comprehensive understanding of how sorghum flour and LAB fermentation impact the staling process in wheat-sorghum composite bread. This research highlights mechanisms that could enhance product stability and quality, supporting the broader application of sorghum in breadmaking and promoting the development of bread with improved shelf life and sensory attributes.

3.1 Effects of Untreated Sorghum Flour on Bread Staling

Case Study 5

The effect of sorghum flour (Sorghum bicolor [L.] Moench) on bread staling: texture, thermal properties, and molecular mobility

Introduction and Aim of the Study

When considering bread formulations, gluten is essential for developing the crumb structure of bread, contributing to proper hydration, foam stabilization, and baking performance (Scanlon and Zghal, 2001). The addition of sorghum proteins to wheat-sorghum bread formulations can affect key quality parameters, including specific volume, texture, and overall acceptability (Rumler and Schönlechner, 2021; Ari Akin et al., 2022). In addition, it has been reported that the protein matrix of sorghum, dominated by highly hydrophobic kafirins, together with protein cross-linking and the presence of tannins, encapsulate starch granules and limit starch swelling and gelatinization (Taylor et al., 2006; De Mesa-Stonestreet et al., 2010; Serna-Saldivar and Espinosa-Ramírez, 2019; Xiong et al., 2019), thus contributing to suboptimal bread properties. Furthermore, one of the key challenges of novel bread formulations in terms of their impact on overall quality is the phenomenon of staling, which results in significant food waste (Dymchenko et al., 2023). Staling, which is associated with loss of softness, flavor, and moisture, is primarily driven by starch retrogradation, particularly recrystallization of amylopectin (Gray and Bemiller, 2003; Curti et al., 2014). This results in firmer, drier crumb structures and an overall decrease in bread quality. Sorghum's unique protein-starch interactions, combined with its hydrophobic protein matrix, may further influence staling dynamics such as water redistribution at different structural levels.

While staling in wheat-based breads has been extensively studied, detailed research on the staling behavior of sorghum-based breads is very limited. Existing studies provide only a partial understanding of how sorghum affects texture and sensory properties during staling (Hugo et al.,

2000; Angioloni and Collar, 2012). A more comprehensive characterization of these processes is critical for optimizing bread quality and shelf life.

This study aims to fill this gap by performing a multi-level analysis of staling in sorghum-wheat composite bread. We will investigate staling dynamics at the molecular, mesoscopic, and macroscopic levels and examine how sorghum affects moisture migration, textural changes, and molecular rearrangements over time. This study will be critical for advancing the use of sorghum in bread production and promoting it as a sustainable, climate-resilient ingredient in global food systems.

Materials and Methods

Bread formulation, production, and storage

Four types of composite bread were produced: STD (100:0 wheat flour – sorghum flour), S10 (90:10 wheat flour – sorghum flour), S20 (80:20 wheat flour – sorghum flour) and S30 (70:30 wheat flour – sorghum flour). Bread formulations [wheat flour (Agugiaro & Figna s.p.a, Collecchio, Italy); sorghum flour (MartinoRossi s.p.a., Gadesco-Pieve Delmona, Italy); sugar (Eridania, Bologna, Italy); salt, (Italkali s.p.a., Palermo, Italy); yeast (AB Mauri Italy s.p.a., Padova, Italy); sunflower seeds oil (Oleificio Zucchi, Cremona, Italy)] are reported in Table 27. Wheat flour composition (as indicated by the producer) was the following: proteins 11.0%, carbohydrates 71.0% (of which sugars 1.5%), fats 0.9%, and fibers 2.0%. Sorghum flour composition (as indicated by the producer) was the following: proteins 10.3%, carbohydrates 55.7% (of which sugars 0.98%), fats 3.2%, and fibers 16.4%.

Bread loaves produced using a home bread-maker (Moulinex Uno B07, Moulinex, France) following these steps: kneading (34 min), first rising (25 min; 30°C), kneading (1 min), second rising (25 min; 35°C), kneading (1 min), third rising (45 min; 35°C), baking (58 min; 150°C). The amount of water to be added to the doughs was determined by determining the water absorption (WA) required to achieve a consistency of 500 UB using the Brabender Farinograph (data not shown).

The bread was cooled at room temperature for two hours before being placed in sealed polyethylene bags. In each bag, 4 mL of ethanol alcohol were sprinkled, and the samples were

stored at 25°C for 8 days. Analysis was conducted at 1, 3, 6, and 8 days after production. Loaves from three batches of production were analyzed for each storage time.

Table 27. Bread formulations.

Ingredients (%)	**STD**	**S10**	**S20**	**S30**
Wheat Flour	100.0	90.0	80.0	70.0
Sorghum Flour	0	10.0	20.0	30.0
Water	58.0	58.2	57.8	57.4
Sugar	4.0	4.0	4.0	4.0
Salt	2.0	2.0	2.0	2.0
Yeast	3.0	3.0	3.0	3.0
Sunflower seed oil	3.0	3.0	3.0	3.0

Fresh bread characterization

Color analysis was determined for the crust and the crumb of each type of bread. The measurements were carried out using a Minolta Colorimeter (CM 2600d, Minolta Co., Osaka, Japan) equipped with a standard illuminant D65 and a 10° position of the standard observer. The results were expressed in accordance with the CIE Lab system. The parameters determined were: L* [0 (black) - 100 (white), brightness], a* (−a* = greenness and +a* = redness) and b* (−b* = blueness and +b* = yellowness). L*, a* and b* values were used to calculate the color difference (ΔE) from STD using the following equation.

$$\sqrt{\left[\left(L^*_{Sample} - L^*_{STD}\right)^2 + \left(a^*_{Sample} - a^*_{STD}\right)^2 + \left(b^*_{Sample} - b^*_{STD}\right)^2\right]} \quad (1)$$

At least ten determinations were performed for each sample.

The specific volume (L/kg) was calculated by dividing the volume of the bread by its weight, according to the standard rapeseed displacement method 10-05 AACC 2001.

Bread staling assessment

Water activity and moisture content

The water activity (a_w) of bread crust and crumb was measured at 25 °C with an Aqualab 4 TE (Decagon Devices, Inc. WA, USA). The moisture content (MC) of the crust and the crumb (from loaf center) (g/100 g) was evaluated following the standard method (AACC, 2001). At least three measurements were taken for each bread loaf both for water activity and moisture content measurement.

Texture Profile Analysis (TPA)

The crumb texture was evaluated using a TA.TX2 instrument (Stable Micro Systems, Goldalming, UK). A Texture Profile Analysis (TPA) test was performed using a cylindrical probe (P/35 Dia Cylinder Aluminium) on at least six cubic sections (2 x 2 x 2 cm^3) of crumb obtained from the central slices of bread loaves. The crumb was compressed to 40% deformation at a speed of 1 mm/s with a trigger point load of 0.05 N. The obtained textural parameters were hardness (peak force of the first compression cycle, N), cohesiveness (ratio of positive area during the second to that of the first compression cycle), and springiness (ratio of the distance from the start of the second area up to the second probe reversal over the distance between the start of the first area and the first probe reversal).

Thermal Analysis (Differential Scanning Calorimetry)

The thermal properties of the bread loaves were analyzed using a differential scanning calorimeter (DSC Q100 TA Instruments, New Castle, DE, USA) calibrated with indium and mercury. To maximize heat transfer during the analysis, the central slice of the loaf was compressed with a 5 kg weight. 5-10 mg of a central crumb slice were taken and placed in stainless steel pans (Perkin Elmer, USA). The pans were then hermetically sealed, quenched, cooled to 80°C, and heated at 5°C/min to 130°C. The DSC thermograms were analyzed using Universal Analysis Software 3.9A from TA Instruments in New Castle, DE. The frozen water content (FW) was calculated by measuring the enthalpy of ice melting peak with the following equation:

$$FW = Entalphy\ of\ ice\ fusion\ x\ \left(\frac{1}{Latent\ heat\ of\ ice\ fusion}\right) x\ \frac{1}{MC}\ x\ 100 \qquad (2)$$

where FW is frozen water at the given experimental conditions (g frozen water/100 g water), Enthalpy Ice Fusion (J/g product), Latent heat of ice fusion is 334 J/g and MC is moisture content (g water/100 g sample). Recrystallized amylopectin (RA) values were obtaining by the calculation

of the enthalpy change (ΔH, J/g) associated with the starch transition peak. Three replicates were analyzed for each sample.

^{1}H Molecular Mobility (LR ^{1}H NMR)

A low resolution nuclear magnetic resonance (LR ^{1}H NMR) spectrometer (20 MHz, MiniSpec, Bruker Biospin, Milan, Italy) operating at 25.0 ± 0.1°C was employed to investigate the molecular mobility of protons in the crumb of bread samples. Crumb samples were collected from the central portion of the loaf, both in fresh and stored samples. 10 mm height samples were then loaded in a 10 mm NMR tube. The tube was sealed with Parafilm® to prevent moisture loss during the analysis. For each tube, a ^{1}H Free Induction Decay (FID) sequence was performed to characterize the decay time of transverse magnetization related to more rigid protons. ^{1}H FIDs were acquired using a single 90° pulse (dwell time: 7 μs, 32 scans, recycle delay: 0.6 s, acquisition window: 0.5 ms, 900 data points). The curves were fitted with a two-component model (exponential and Gaussian; SigmaPlot, version 6, Systat Software Inc., USA).

$$y\,(t) = y_0 + A * exp^{\left[-\left(\frac{t}{T_A}\right)\right]} + B * exp^{\left[-\left(\frac{t}{T_B}\right)^2\right]} \quad (3)$$

Subsequently, a Carr-Purcell-Meiboom-Gill (CPMG) sequence was performed to measure the transverse relaxation time or spin-spin ^{1}H T_2. A recycle delay of 0.6 s, an interpulse spacing of 0.04 ms, 10.000 data points and 32 scans were used. Quasi-continuous distributions of relaxation times

were obtained fitting the experimental curves with a UPENWin software (Alma Mater Studiorum, Bologna, Italy). Three NMR tubes were analyzed for each bread loaf, and three acquisitions were taken from each tube.

Statistical analysis

A two-way ANOVA (SPSS software 27.0, SPSS Inc., Chicago, USA) followed by LSD pairwise comparisons, was used to analyze the main effects of sorghum flour inclusion *(% Sorghum*), storage time (*Storage*) and their interaction *(%Sorghum*Storage*) on the differences among

samples. Moreover, the sum of squares percent (SS, %) was also calculated to assess the contribution (%) of each main factor and the interaction.

Bread color and specific volume data were instead analyzed by one-way ANOVA (SPSS software 27.0, SPSS Inc., Chicago, USA) to analyze the effect of sorghum flour addition *(% Sorghum*). In the case of homoscedastic datasets and homogeneous sample sizes, a Tukey *post-hoc* test with a confidence interval of 95% was used, while in the case of different sample sizes, a *post-hoc* least significant difference (LSD) test was used, with the same confidence interval. Instead, in the case of heteroscedasticity, the Welch test followed by Games-Howell *post-hoc* test with a 95% confidence interval was used.

Results and Discussion

Fresh bread characterization

Color

The color results of crumb and crust of bread samples are shown in Table 28. Regarding the crust, a decrease in the parameters L*, a* and b* as a function of increasing sorghum flour was observed. The parameter L* showed a significant decrease ($p < 0.05$) starting from 20% sorghum flour inclusion. For b* and a*, there was a significant difference in the product already at the first level of sorghum flour inclusion (S10).

In the crumb, L* and b* showed a similar trend as in the crust, while the a* parameter tended to increase with increasing sorghum content. Moreover, for L* and a*, all the samples were significantly different from each other ($p < 0.05$).

The progressive decrease in L*, both in the crust and in the crumb, resulted in a shift from lighter/whiter shades of standard loaves to darker/browner shades of composite bread loaves. An increase in darker/browner shades following sorghum flour inclusion is due to the presence of pigments typical of the tegument of the sorghum caryopsis (Dube et al., 2020); a similar observation could be made for b*, where the decline noted relates to the decrease in the fraction associated with yellow shades, probably higher in wheat flour than sorghum flour.

In the crumb, on the other hand, the progressive and significant increase in a* as a function of the degree of sorghum flour substitution could be due to the presence of phenolic compounds,

such as 3-deoxyanthocyanidins, present in the pericarp of sorghum flour (Xiong et al., 2019; Xu et al., 2021). However, the high temperatures reached in the crust during baking may have degraded some color compounds, thus leading to lower values of a* if compared to wheat bread. The ΔE values reported in Table 28 describe how the human eye perceives the color differences between the three sorghum-containing samples and the STD. All the values of ΔE were >3, and according to Valverde and Moya (2014), it means that the human eye is able to discriminate the crust and crumb color of all the samples compared to the STD.

Specific Volume

As expected, the specific volume showed a decrease ($p < 0.05$) with the inclusion of sorghum flour (Table 28), that was significant already starting from 10% sorghum flour inclusion level. Indeed, the addition of sorghum led to a dilution of the gluten network, mainly due to the lower content of gliadins and glutenins from wheat and the presence of kafirins from sorghum, resulting in a less elastic and extensible gluten network. This phenomenon led to a reduced expansion and development of bread dough during the leavening process, with resulting negative effect on the bread specific volume and crumb structure; the resulting loaves appeared therefore more compact (Hussein and Abd-Alla, 1976; Siddeeg et al., 2017).

Table 28. Color parameters of crust and crumb and specific volume (L/Kg) of STD (100:0 wheat flour – sorghum flour), S10 (90:10 wheat flour – sorghum flour), S20 (80:20 wheat flour – sorghum flour) and S30 (70:30 wheat flour – sorghum flour). Different letters indicate significant differences among samples ($p < 0.05$, one-way ANOVA).

	Crust				*Crumb*				
	L*	**a***	**b***	**ΔE**	**L***	**a***	**b***	**ΔE**	**Sp. Vol. (L/Kg)**
STD	77.3±2.55a	6.93±1.1a	21.42±1.98a		83.92±0.96a	1.34±0.13d	13.66±0.66a		3.09±0.18a
S10	75.86±2.28a	4.26±2.25bc	16.42±4.19b	5.85	79.12±1.73b	2.98±0.86c	13.64±1.49b	5.07	2.63±0.21b
S20	74.63±1.78b	4.72±0.74c	16.29±1.65b	6.19	71.72±2.8c	2.82±0.49b	11.51±0.65c	12.48	2.12±0.26c
S30	71.89±1.97c	5.82±0.82b	16.82±1.85b	7.20	66.76±2.53d	3.26±0.27a	11.21±0.74c	17.44	1.75±0.22d

Bread staling assessment

Table 29 presents the two-way ANOVA results for the main effects *(% Sorghum* and *Storage*) and their interaction effect *(% Sorghum*Storage*), along with percentage SS values for all bread staling parameters studied, which will be discussed in the following paragraphs.

Table 29. F significance level and sum square (SS) percent of the main factors (% Sorghum and Storage) and their interaction (% Sorghum*Storage *(two-way ANOVA, $p < 0.05$).*

Factors	***% Sorghum***		***Storage***		***%Sorghum*Storage***	
	SS%	Sign.	SS%	Sign.	SS%	Sign.
Hardness [N]	78.76	***	15.26	***	5.98	***
Cohesiveness [-]	25.24	***	40.78	***	33.98	***
Springiness [-]	35.40	***	50.59	***	14.01	***
tA (ms)	28.29	***	54.70	***	17.01	***
tB (ms)	73.18	***	17.98	***	8.84	***
%popA (%)	3.97	***	81.34	***	14.69	***
%popB (%)	3.00	***	80.83	***	16.16	***
MC Crust (g H_2O/100 g)	2.97	***	92.39	***	4.64	***
MC Crumb (g H_2O/100 g)	6.46	**	82.38	***	11.16	*
a_w Crust	2.86	**	92.86	***	4.28	***
a_w Crumb	2.20	ns	52.02	***	45.78	ns
FW (g/100g H_2O)	41.23	***	39.90	***	18.87	ns
ΔH amylopectin (J/g)	4.05	**	92.28	***	3.66	ns
tC (ms)	61.97	***	5.41	ns	32.62	***
tD (ms)	52.64	***	22.59	***	24.77	***
tE (ms)	54.36	***	10.74	***	34.90	***
tF (ms)	12.56	***	37.10	***	50.34	***

Texture

Hardness, Cohesiveness and Springiness of wheat-sorghum bread loaves were reported in Figure 26 a, b and c respectively.

For what concern hardness, the two-way ANOVA (Table 29) showed that both *% Sorghum*, *Storage* and their interaction *%Sorghum*Storage* were significant ($p < 0.001$). Indeed, during storage, an expected increasing trend of hardness was observed in all samples. However, it can be seen that the addition of sorghum flour had a greater influence than storage on the hardness results: 75% for *% Sorghum* and 15% for *Storage* (SS %, Table 29). In particular, as indicated by the *post-hoc* of the two-way ANOVA (Table 30) the addition of 20 and 30% of sorghum resulted in a significant increase in hardness. Indeed, as already mentioned for the specific volume, the increased hardness was probably due to the dilution of the gluten network, as a result of the lower content of gliadins and glutenins from wheat and the presence of kafirins and fibers from sorghum, resulting in a less elastic and extensible gluten network (Hussein and Abd-Alla, 1976; Siddeeg et al., 2017). However, no significant difference was observed between S10 and STD ($p \geq 0.05$), indicating that the addition of 10% sorghum did not negatively impact hardness values over time.

Cohesiveness decreased during storage (Figure 26b). For cohesiveness, as opposed to hardness, in the two-way ANOVA, *Storage* had the greatest influence on the results. Moreover, 34% of the result was influenced by the interaction of the two factors *%Sorghum*Storage*. Looking at the *post-hoc* of the two-way ANOVA results (Table 30), significant differences were highlighted between STD and sorghum containing samples, with S10 showing intermediate values and S20-S30 the lowest. No significant differences were observed between S20 and S30 ($p \geq 0.05$).

Storage had the greatest influence on the results of springiness, with 50% of the results influenced by storage time (Figure 26c, Table 29). An increasing trend was observed during the initial days of storage, while a consequent decreasing trend was highlighted after 3 days of storage. Looking at the results of the two-way ANOVA *post-hoc* for *% Sorghum*, the incorporation of 10% sorghum appeared to enhance this textural attribute, as evidenced by the higher springiness values observed in comparison to the STD control (Table 30). In general, the addition of sorghum resulted in significant changes in the texture of bread. In particular, the addition of sorghum at levels of 20% and 30% was detrimental for the bread quality for all the parameters measured. The addition

of 10% sorghum, on the other hand, while generally decreased bread cohesiveness, did not negatively affect bread hardness and appeared to enhance bread springiness.

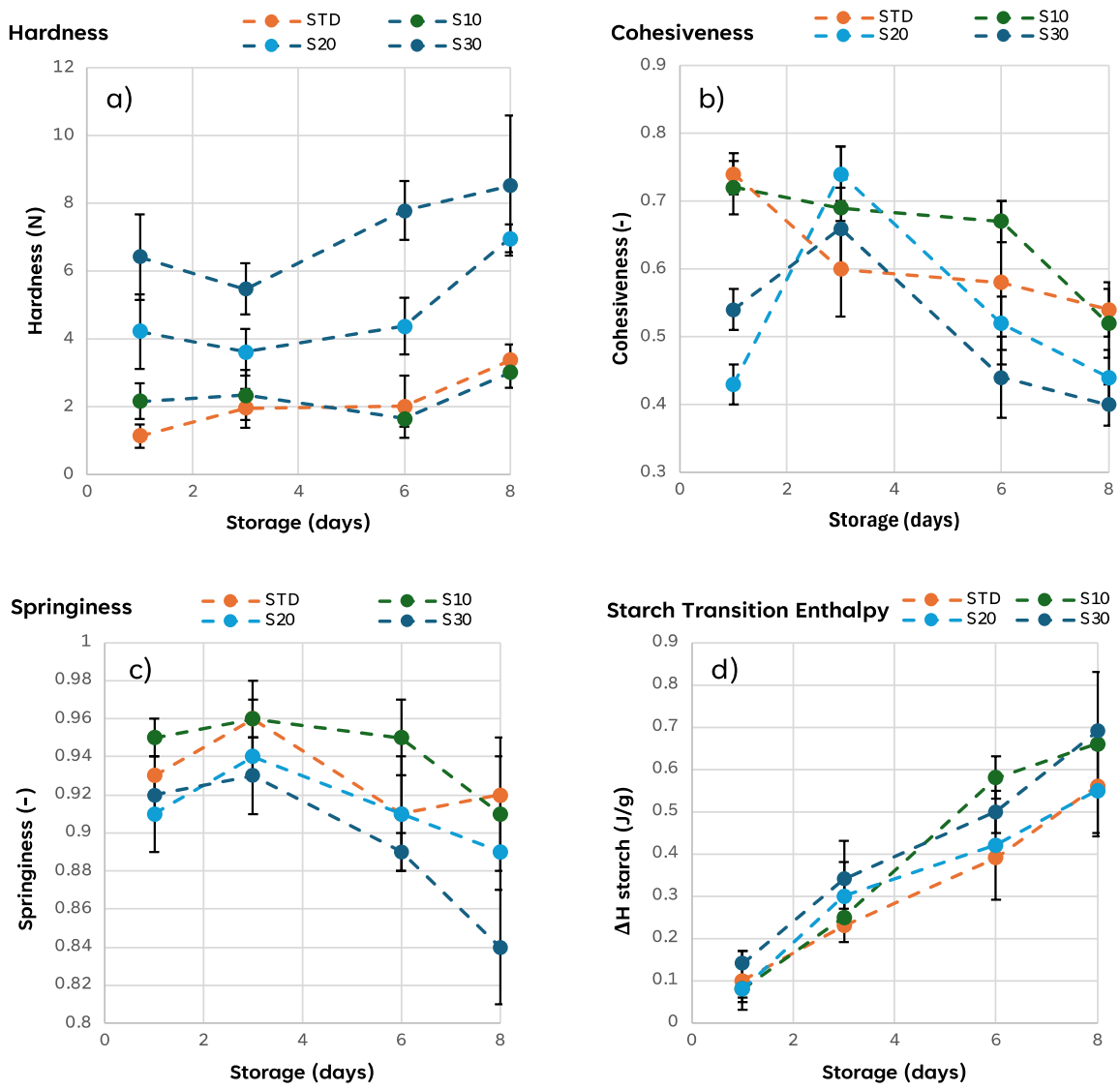

Figure 26. a) Hardness (N); b) Cohesiveness; c) Springiness and d) Starch Transition Enthalpy of STD (100:0 wheat flour – sorghum flour, orange), S10 (90:10 wheat flour – sorghum flour, green), S20 (80:20 wheat flour – sorghum flour, sky blue) and S30 (70:30 wheat flour – sorghum flour, blue).

Table 30. Texture parameters and starch transition enthalpy of STD (100:0 wheat flour – sorghum flour), S10 (90:10 wheat flour – sorghum flour), S20 (80:20 wheat flour – sorghum flour) and S30 (70:30 wheat flour – sorghum flour). Different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples (two-way ANOVA, $p < 0.05$).

	Storage		STD	S10	S20	S30
			c	c	b	a
Hardness (N)	1 days	c	1.14±0.33	2.17±0.52	4.23±1.1	6.42±1.25
	3 days	c	1.96±0.58	2.35±0.74	3.62±0.69	5.47±0.75
	6 days	b	2.01±0.93	1.65±0.24	4.38±0.83	7.79±0.86
	8 days	a	3.37±0.48	3.02±0.45	6.97±0.41	8.53±2.08
			a	b	c	c
Cohesiveness (-)	1 days	a	0.74±0.03	0.72±0.04	0.43±0.03	0.54±0.03
	3 days	b	0.6±0.07	0.69±0.09	0.74±0.04	0.66±0.06
	6 days	c	0.58±0.12	0.67±0.03	0.52±0.04	0.44±0.06
	8 days	d	0.54±0.04	0.52±0.05	0.44±0.04	0.4±0.03
			b	a	d	c
Springiness (-)	1 days	b	0.93±0.01	0.95±0.01	0.91±0.02	0.92±0.01
	3 days	a	0.96±0.01	0.96±0.02	0.94±0.01	0.93±0.02
	6 days	c	0.91±0.03	0.95±0.02	0.91±0.02	0.89±0.01
	8 days	d	0.92±0.03	0.91±0.03	0.89±0.02	0.84±0.03
			b	a	b	a
Amylopectin Fusion Enthalpy (J/g)	1 days	d	0.1±0.07	0.08±0.02	0.08±0.03	0.14±0.03
	3 days	c	0.23±0.04	0.25±0.01	0.3±0.08	0.34±0.09
	6 days	b	0.39±0.1	0.58±0.05	0.42±0.01	0.5±0.05
	8 days	a	0.56±0.12	0.66±0.01	0.55±0.1	0.69±0.14

Moisture content and water activity

Moisture content (MC) and water activity (a_w) of crust and crumb were reported in Table 31, while the SS% and p value of the two-way ANOVA were reported in Table 29.

Looking at two-way ANOVA, both *% Sorghum*, *Storage* and their interaction *%Sorghum*Storage* resulted significant for both MC crust and MC crumb. Looking at the SS% values (Table 29), the greatest influence was due to *Storage* factor (92% and 82%, respectively for MC crust and MC crumb), while *% Sorghum* had smaller influence on the results. In particular, MC crust showed an increasing trend during storage, while MC crumb decreased during storage, both reaching a plateau after 6 days of storage. Even if *% Sorghum* had a smaller influence on the variability among samples, its effect on MC crust was significant ($p < 0.001$). Looking at the *post-hoc* results of the two-way ANOVA (Table 31), the STD sample exhibited higher values of MC crust compared to all sorghum-composite breads. In contrast, no significant differences were observed in MC crumb between STD and sorghum-composite breads. However, MC crumb of S30 was higher than S10 and S20, probably due to the higher compactness of its loaves, that reduced water loss during baking.

With regard to crust water activity (a_w crust), both *% Sorghum*, *Storage* and their interaction *%Sorghum*Storage* resulted significant, but the greatest influence (SS% 92%) was due to the *Storage* factor (Table 29). In particular, during storage, an increasing trend was observed, which reached a plateau after six days (Table 31). This finding aligns with the results of MC crumb and MC crust.

For what concern crumb water activity (a_w crumb), the two-way ANOVA showed no significant effect of *% Sorghum* and the interaction factor *%Sorghum*Storage*, while the factor *Storage* was significant ($p < 0.001$) (Table 29), showing a decreasing trend from day 1 to day 8 (Table 31). In contrast to the findings of MC crumb, MC crust, and a_w crust, where a plateau was reached after six days, the data for crumb water activity demonstrated a significant difference only at day 8.

Table 31. Effects of sorghum inclusion on Moisture Content (g water/ 100 g), Water Activity and Frozen Water (g frozen water/100g water). Different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples (two-way ANOVA, $p < 0.05$).

	Storage		STD	S10	S20	S30
			a	b	b	b
MC Crust (g water / 100 g sample)	1 days	c	21.08±1.67	18.57±2.04	20.54±0.93	19.83±2.43
	3 days	b	24.79±0.98	23.73±0.65	24.45±0.72	23.64±1.48
	6 days	a	26.03±0.87	26.23±1.12	25.91±0.71	26.88±0.65
	8 days	a	26.6±0.65	26.69±0.94	26.34±0.69	26.37±0.49
			ab	b	b	a
MC Crumb (g water / 100 g sample)	1 days	a	41.92±0.59	42.64±0.76	41.73±0.48	42.03±0.16
	3 days	b	41.87±0.21	40.06±1.67	40.93±1.47	41.00±0.39
	6 days	c	39.28±0.46	38.97±2.21	38.17±2.38	39.95±0.45
	8 days	c	38.50±1.29	37.54±1.96	38.46±0.87	40.06±0.98
			a	b	b	a
a_w Crust	1 days	c	0.88±0.01	0.86±0.01	0.86±0.01	0.87±0.01
	3 days	b	0.91±0.00	0.91±0.00	0.91±0.00	0.91±0.01
	6 days	a	0.92±0.00	0.91±0.00	0.92±0.00	0.92±0.00
	8 days	a	0.92±0.01	0.92±0.00	0.92±0.01	0.93±0.00
			a	a	a	a
a_w Crumb	1 days	a	0.960±0.004	0.961±0.008	0.957±0.006	0.963±0.002
	3 days	a	0.964±0.004	0.960±0.003	0.963±0.004	0.963±0.003
	6 days	a	0.959±0.001	0.960±0.007	0.964±0.004	0.960±0.003
	8 days	b	0.955±0.004	0.959±0.002	0.958±0.005	0.956±0.011
			b	b	a	a
FW (g frozen water/100g water)	1 days	a	53.69±2.9	44.62±0.42	57.09±2.63	61.95±8.54
	3 days	a	50.55±1.9	53.27±1.19	54.11±4.83	57.69±4.08
	6 days	a	49.62±5.35	49.5±1.83	51.5±1.2	55.87±1.52
	8 days	b	43.82±8.37	43.06±2.04	50.46±2.75	47.63±3.97

In general, the observed increase in moisture content (MC) and water activity (a_w) within the bread crust, alongside a concurrent decrease in the crumb MC and a_w over time, can be attributed to the well-documented phenomenon of macroscopic water migration from crumb to crust (Vodovotz et al., 2002; Fadda et al., 2014). The smaller variations observed in the crumb compared to the crust were expected, given that crumb samples were taken exclusively from the loaf's center, where water migration is likely slower. Furthermore, the plateau in macroscopic water migration generally observed after six days aligns with existing literature, which reports that most physicochemical changes associated with bread staling occur within the initial days of storage (Curti et al., 2011; Bosmans et al., 2013b).

Thermal Analysis (Differential Scanning Calorimetry)

The major DSC endothermic peak around 0°C was attributed to ice melting (Figure 27) (Baik and Chinachoti, 2001) and it was used to calculate the frozen water content (FW%) of bread samples (Table 31). Ribotta and Le Bail (2007) and Curti et al. (2014) also analyzed the FW (%) in standard wheat bread and found values of ~53% at day 1 and ~43% at day 8, which are in agreement with our results (Table 31). Two-way ANOVA results showed no significant differences ($p \geq 0.05$) in the interaction between the two main factors *%Sorghum*Storage*. However, when *% Sorghum* and *Storage* were considered separately, there were significant differences ($p < 0.001$) with almost equal influence between the two factors (41% and 40%). Indeed, a decreasing trend of FW% was obtained during storage from day 1 to day 8, probably because of the water migration from crumb to crust, as previously reported, and the water incorporation to the crystallin phase of starch where it is no longer able to "freeze" (Hallberg and Chinachoti, 2002; Ribotta and Le Bail, 2007; Curti et al., 2013, 2014). Moreover, for what concern the effect of *% Sorghum*, FW% was found to be significantly higher ($p < 0.05$) in S20 and S30 than in STD and S10, thus suggesting the crucial role of sorghum flour at these inclusion levels in the establishment of different solid-water interactions from STD sample. Specifically, the presence of a higher content of fibers in these samples may have contributed to the formation of weaker solid-water interactions, potentially enhancing the ability of water to freeze and thereby increasing water mobility at the mesoscopic level (Curti et al., 2016).

A second endothermic event was present around 60°C (Figure 27) previously attributed to the melting of amylopectin crystals (Russell, 1983; Baik and Chinachoti, 2000; Curti et al., 2014)

starting from the first day of storage. At day one, the transition could be related to the melting of amylose that had retrograded very quickly or to the gelatinization of starch that had not gelatinized during baking (Li and Gidley, 2022).

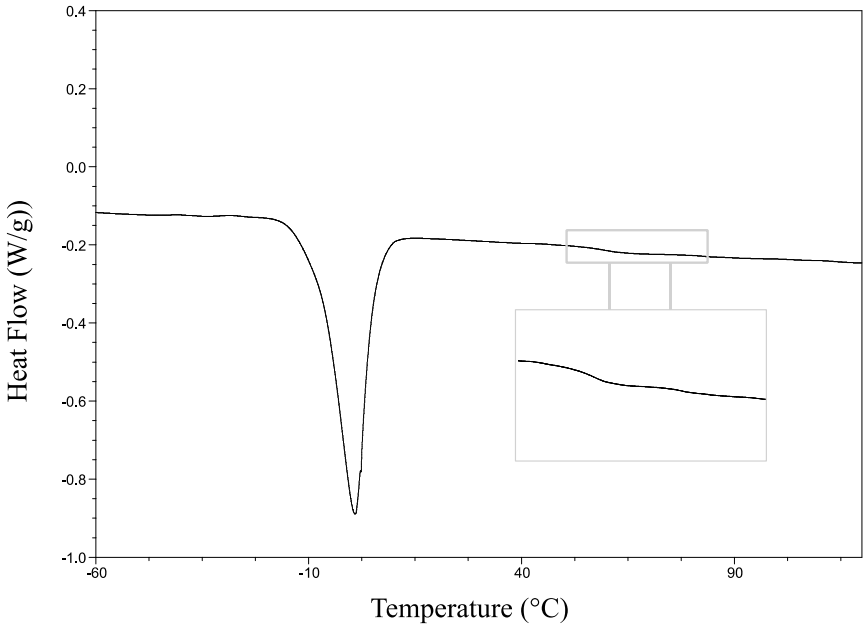

Figure 27. Representative DSC thermograms of STD at day 8 of storage.

The two-way ANOVA results (Table 29) showed that both *Storage* and *% Sorghum* were significant factors, with the 92% of the variance explained by the *Storage*, and only the 4% by *% Sorghum*. The interaction between the two factors was, however, not significant.

RA values increased during storage in all breads, as expected and previously reported by Curti et al. (2014) and Curti et al. (2017b). Furthermore, the *post-hoc* results of the two-way ANOVA (Table 30) showed significant differences in RA values among samples with different sorghum flour addition levels, even if without a clear trend. Indeed, RA higher levels were found in S10 and S30 samples. Although sorghum contains only 55% of starch compared to the 71% of wheat (see paragraph *Bread formulation, production, and storage*), the reduction in starch content with increasing sorghum flour in bread formulation did not result in significant decrease in retrograded amylopectin values. This may indicate that an unclear trend in RA values among the samples

could result from a combination of opposing tendencies: a lower starch content in samples containing sorghum, coupled with a possible accelerated amylopectin retrogradation in these samples. A higher rate of amylopectin retrogradation in sorghum may also probably be affected by the increasing fiber content, which has been reported to affect amylopectin retrogradation rate (Curti et al., 2015), even if the effect is still not fully understood.

^{1}H nuclear magnetic resonance (NMR) mobility

The two ^{1}H FID populations (A and B) resulting from the two-components model fitting are presented in Table 32. Population A was previously associated with the rigid CH protons of starch crystals and amorphous starch and protein not in contact with water (Bosmans et al., 2013a, 2013b). Additionally, this population is associated with amylopectin retrogradation and water migration occurring during storage (Bosmans et al., 2013b). The relaxation time of population A in our samples was ~0.020 ms with a proton abundance of ~63%. Population B has been associated with CH protons of amorphous starch and gluten in regions interacting with confined water (Bosmans et al., 2013a); the relaxation time of population B was ~0.30 ms with a proton abondance of ~37%. These results are consistent with previous findings on bread (Curti et al., 2015; Carini et al., 2017).

The two-way ANOVA output (Table 29) related to tA and tB values showed that, both *% Sorghum*, *Storage* and their interaction *% Sorghum*Storage* resulted significant. Looking at the SS% values, *Storage* factor had the greatest influence on tA (54.70%), while the differences in tB were explained mainly by the factor *% Sorghum* (73.18%).

During storage, both tA (ms) and tB (ms) decreased in all samples (Table 32), thus showing an increase in the rigidity of population A due to the water migration from crumb to crust and to amylopectin crystals formation, and population B due to increased rigidity of the amorphous starch and gluten in regions interacting with confined water (Ottenhof and Farhat, 2004; Bosmans et al., 2013b, 2013a). Moreover, the greatest variation from day 1 to day 8 was found in STD, where tA decreased from 0.028 ms to 0.021 ms, whereas in bread with the highest level of sorghum flour (S30) it decreased from 0.022 to 0.019 ms. This result suggests an important role of sorghum in amylopectin retrogradation and water migration. The presence of sorghum led to a lower mobility of population A, with a proportional decrease in mobility as a function of the percentage

of sorghum (Table 32). The relaxation time tB (ms), however, did not show a specific trend following sorghum flour addition, but in the highest level of sorghum flour inclusion (S30) tB (ms) was found to be higher than STD.

Table 32. ^{1}H FID populations relative abundances (%) and relaxation times (ms) in STD (100:0 wheat flour – sorghum flour), S10 (90:10 wheat flour – sorghum flour), S20 (80:20 wheat flour – sorghum flour) and S30 (70:30 wheat flour – sorghum flour).Different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples (two-way ANOVA, $p < 0.05$).

	Storage		**STD**	**S10**	**S20**	**S30**
			a	b	c	d
	1 days	a	0.027±0.001	0.027±0	0.021±0.001	0.021±0.001
tA	3 days	b	0.022±0.001	0.022±0	0.019±0	0.02±0
(ms)	6 days	c	0.019±0	0.019±0	0.018±0	0.019±0
	8 days	d	0.02±0.001	0.02±0.001	0.018±0.001	0.019±0
			c	b	d	a
	1 days	a	0.318±0.004	0.323±0.003	0.296±0.001	0.321±0.002
tB	3 days	b	0.298±0.016	0.314±0.007	0.292±0.001	0.32±0.002
(ms)	6 days	c	0.3±0.002	0.307±0.002	0.287±0.001	0.314±0.002
	8 days	c	0.3±0.003	0.298±0.003	0.285±0.005	0.321±0.005
			c	b	a	b
	1 days	d	56.58±0.2	60.97±0.42	56.47±0.43	56.67±0.11
%popA	3 days	c	61.23±0.18	60.99±0.36	63±0.31	64.22±0.16
(%)	6 days	b	63.13±0.1	64.31±0.29	65.46±0.42	62.91±0.22
	8 days	a	64.29±0.12	64.94±0.07	67.46±1.28	66.53±0.28
			a	b	c	b
	1 days	a	42.36±0.24	39.03±0.42	43.53±0.43	43.33±0.11
%popB	3 days	b	38.77±0.18	39.01±0.36	37±0.31	35.78±0.16
(%)	6 days	c	36.87±0.1	35.69±0.29	34.54±0.42	37.09±0.22
	8 days	d	35.71±0.12	35.06±0.07	32.55±1.28	33.47±0.28

For what concern the relative abundance of population A and B, the two-way ANOVA results showed that both *Storage*, *% Sorghum* and their interaction *%Sorghum*Storage* were significantly affecting populations A and B relative abundances ($p < 0.001$) (Table 29). However, the 80% of the variance of %popA and %popB was explained by the *Storage* factor and only 4% by the *% Sorghum* and 15% by the interaction of the two factors. In particular, all samples showed an increase of %popA during storage, because the percentage of amylopectin crystals increased, leading to a higher proton abundance in the first FID population (Bosmans et al., 2013b). Consequently, the abundance of population B followed an inverse trend, demonstrating a decline during the storage period. The addition of sorghum resulted in a higher abundance of population A, thereby indicating a greater presence of the crystalline phase in sorghum-composite breads. Consequently, the abundance of population B followed an opposite trend, with the highest abundance observed in the STD. However, the trend was not always constant, with S30 showing lower popA% values than S20, likely due to the interplay of two opposing effects in the sorghum-containing samples, as discussed for RA data.

Four ^{1}H T_2 CPMG populations were found, in accordance with previous findings on bread (Bosmans et al., 2012, 2013b; Curti et al., 2017b), and named C, D, E and F, from the shortest to longest time of relaxation. Their relaxation times are reported in Table 33 while the representative curves at day 1 and day 8 were reported in Figure 28. Population C is usually attributed to the same protons of ^{1}H FID population B (Bosmans et al., 2013b), and thus to nonexchanging protons of amorphous starch and gluten in contact with confined water; population D refers to nonexchanging protons from gluten and exchanging protons from confined water, starch and gluten; population E is attributed to protons from water, starch, and gluten present in the gel network; Population F refers to native flour lipid protons and the lipid fraction in the bread formulation (Bosmans et al., 2013a; Curti et al., 2014; Carini et al., 2017; Curti et al., 2017b).

Table 33. ^{1}H CPMG populations relaxation times (ms) in STD (100:0 wheat flour – sorghum flour), S10 (90:10 wheat flour – sorghum flour), S20 (80:20 wheat flour – sorghum flour) and S30 (70:30 wheat flour – sorghum flour). Different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples (two-way ANOVA, $p < 0.05$).

	Storage		STD	S10	S20	S30
			b	ab	ab	a
	1 days	a	0.18±0.04	0.27±0.05	0.24±0.02	0.29±0.04
tC	3 days	a	0.25±0.02	0.23±0.01	0.24±0.02	0.41±0.57
(ms)	6 days	a	0.16±0.03	0.25±0.03	0.24±0.01	0.27±0.04
	8 days	a		0.25±0.01	0.27±0.03	0.29±0.05
			bc	b	c	a
	1 days	c	1.09±0.31	1.16±0.27	0.94±0.18	1.71±0.25
tD	3 days	bc	1.18±0.15	1.28±0.15	1.26±0.57	
(ms)	6 days	ab	1.51±0.53	1.29±0.24	1.46±0.48	
	8 days	a	1.11±0.13	1.83±0.62		1.79±0.28
			a	a	b	c
	1 days	a	8.61±0.22	8.17±0.7	8.17±0.83	7.7±0.46
tE	3 days	b	8.5±0.26	8.12±0.19	7.76±0.55	7.47±0.29
(ms)	6 days	c	7.3±0.22	7.6±0.29	6.81±0.9	6.76±0.26
	8 days	c	6.95±0.19	7.85±0.15	6.92±0.89	6.93±0.23
			a	b	a	c
	1 days	b	120.74±11.57	132.57±14.04	134.2±14.03	116.9±22.72
tF	3 days	ab	149.94±7.66	124.68±9.89	130.28±15.05	115.4±9.19
(ms)	6 days	ab	124.85±10.42	134.08±11.59	136.12±16.68	125.44±15.45
	8 days	a	143.6±12.34	121.74±7.36	143.23±28.26	121.31±11.7

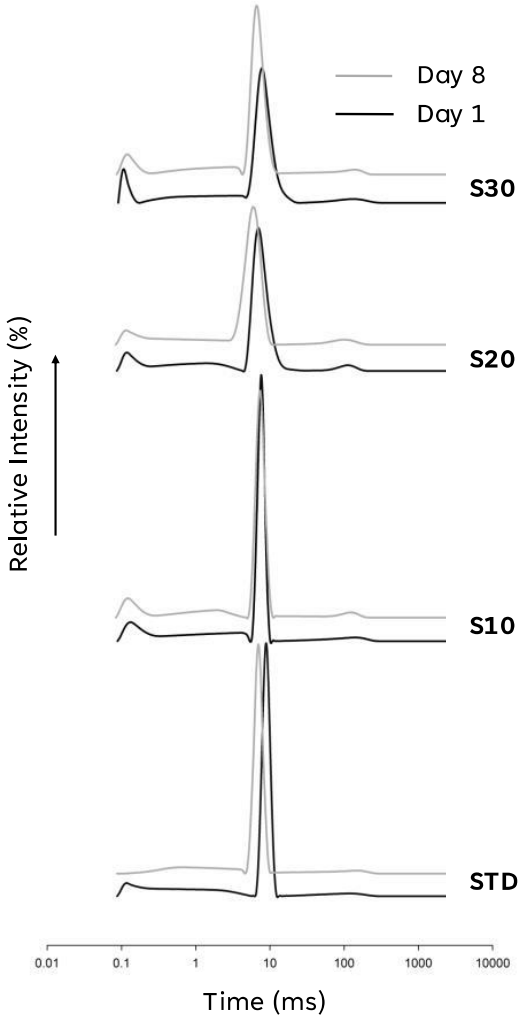

Figure 28. ^{1}H T_2 CPMG distribution of STD (100:0 wheat flour – sorghum flour), S10 (90:10 wheat flour – sorghum flour), S20 (80:20 wheat flour – sorghum flour) and S30 (70:30 wheat flour – sorghum flour) samples at day 1 and 8 of storage.

The two-way ANOVA output (Table 29) related to tC, tD, tE and tF values showed that both *% Sorghum*, *Storage* and their interaction *% Sorghum*Storage* were significant. Looking at SS% results, *% Sorghum* had the greatest influence on the results for all the CPMG T_2 relaxation times (62%, 53% and 54%, respectively for tC, tD and tE), except for the relaxation time of population F, where the variance was mostly influenced by the interaction factor *%Sorghum*Storage*.

Focusing on the *post-hoc* results of the two-way ANOVA (Table 33), no significant differences were highlighted during storage for tC. However, the incorporation of 30% sorghum into the bread formulation was found to increase the mobility if compared to the STD (similarly to tB results). Moreover, the population C in the STD sample at day 8 was not identified. This may be attributed to a shift in the population towards a lower relaxation time, which may not coincide with the acquisition window of the sequence being used (CPMG). For what concern D and E populations, they became closer during storage time. Specifically, the relaxation time of population D (tD) increased during storage, reflecting greater mobility by day 8. For this reason, in some samples taken on specific days (S20 on day 8 and S30 on days 3 and 6), the population D was not identified. This was probably due to an overlap with the predominant population (popE), which relaxed at slightly higher times than popD. The overlapping prevented the identification of two resolute populations, thereby complicating the software's ability to discern population D in the aforementioned samples. Notably, the incorporation of 30% sorghum resulted in significantly higher tD values compared to the standard (STD). Moreover, while population D increased its mobility over time, the relaxation time of the most abundant population E (tE) decreased during storage. Decreasing tE values during storage are in agreement with previous findings, where the decrease in mobility of population E during storage has been attributed to macroscopic and mesoscopic staling phenomena, such as water migration, starch recrystallization and crumb firming (Bosmans et al., 2013a; Curti et al., 2014, 2017b).

In addition, the relaxation time of population E was affected by sorghum flour addition (*% Sorghum*). Indeed, as the percentage of sorghum increased, the relaxation time of population E decreased, suggesting a lower ability to include water in the gel network. According to previous findings (Bosmans et al., 2013b), a more rigid population E can be linked to the increased hardness of bread samples, and thus, in our study, it may be linked to the higher hardness in sorghum-containing samples. In support of this hypothesis, it can be seen that tE values of S10 did not show significant differences with the STD bread, thus indicating no effects in the molecular rigidity of the starch-gluten-water network in these samples until 10% sorghum flour inclusion level. The same trend was observed for hardness, with S10 not significantly different from STD (Table 30).

For what concern population F, the *post-hoc* of the two-way ANOVA (Table 33) showed an increasing mobility during storage (higher tF). Instead, the incorporation of sorghum flour appeared to diminish domain mobility, although there was no significant distinction between S20 and STD.

Conclusions

This study allowed a better understanding of the effect of sorghum flour inclusion in composite bread on the quality of both fresh and stored bread, giving useful insights on the impact of sorghum flour addition on the staling phenomenon. As expected, the addition of sorghum flour led to the modification of some fresh bread quality parameters (specific volume, color) starting from the first level of sorghum inclusion (10%). Sorghum flour biopolymers, such as highly hydrophobic kafirins and fibers, diluted gluten proteins in bread dough and resulted in the development of weaker water-gluten and water-starch interactions in bread. The resulting effects were reflected at different structural levels, from the macroscopic (TPA) to the mesoscopic (FW) and the molecular (^{1}H CPMG). Weaker water-proteins interactions in sorghum-containing bread were associated with lower specific volumes and more compactness of the crumb, which led to higher hardness in composite breads than the control. Moreover, the instauration of weaker interactions of biopolymers with water were also evidenced by the higher levels of frozen water at the mesoscopic level. This phenomenon contributed to the faster increase in hardness over time, thus evidencing that the addition of sorghum seemed to accelerate the staling phenomenon. Moreover, at the molecular level, lower tE values were associated to the increased firmness of bread containing sorghum. For what concern amylopectin retrogradation, no specific trend was evidenced by DSC results (ΔH). However, ^{1}H FID popA% seemed to show an increase in amylopectin crystal formation occurred in sorghum containing samples. Probably, the combined effects of accelerated starch retrogradation in sorghum-containing samples and the lower starch content in sorghum flour made impossible a clear observation of the phenomenon associated with sorghum flour addition.

The inclusion of sorghum flour at a 10% level appeared to mitigate the staling-promoting effects associated with sorghum addition, specifically in terms of bread hardness, springiness, and the molecular mobility of the CPMG proton population E (tE, ms).

Future research should aim to further reduce the adverse effects of sorghum flour incorporation, potentially through treatments such as enzymatic processing, sprouting, or fermentation. These treatments, by enhancing protease and amylolytic activity, could modify the structure and functionality of sorghum biopolymers within wheat-sorghum formulations, thus impacting the staling phenomenon.

3.2 Mitigating Staling through LAB Fermentation

Case Study 6

Effect of Lacticaseibacillus casei fermentation on the staling of wheat-sorghum bread: reduction of water mobility over time and amylopectin retrogradation

Introduction and Aim of the Study

Bread is a staple food consumed worldwide, and its production and consumption play a significant role in global food systems (Mesta-Corral et al., 2024). However, globally, it is estimated that a large proportion of baked goods, particularly bread, are discarded every day (Dymchenko et al., 2023). Bread staling, characterized by the loss of the desirable fresh qualities of bread, such as soft texture, pleasant aroma, and appealing taste (Taglieri et al., 2021), is a major cause of food waste, with substantial economic and environmental implications (Dymchenko et al., 2023). Bread staling is a complex phenomenon that involves a series of physical and chemical changes occurring at the molecular, mesoscopic and macroscopic level in bread over time (Gray & Bemiller, 2003; Fadda, Sanguinetti, Del Caro, Collar, & Piga, 2014; Elena Curti, Carini, Tribuzio, & Vittadini, 2014;). At the mesoscopic level, staling is primarily associated with the recrystallization of starch molecules, particularly amylopectin retrogradation. During baking, starch granules gelatinize and swell, contributing together with gluten matrix to create the soft, elastic structure of fresh bread (Scanlon and Zghal, 2001; van Rooyen et al., 2023). Over time, however, these starch molecules begin to recrystallize, representing one of the main causes leading to a firmer crumb structure and a dry, crumbly texture. Starch retrogradation is influenced by factors such as enzymes, proteins, moisture content, heating rate, storage temperature and relative humidity, and the presence of other ingredients, which affect the crystals and polymorphs formation (van Rooyen et al., 2023). In addition to starch retrogradation, other molecular interactions contribute to bread staling, making the staling process highly variable and complex. Water content, distribution and migration from the crumb to the crust at macroscopic level, and

from gluten to starch at molecular level, plays a significant role (Curti et al., 2014, 2017b; van Rooyen et al., 2023). From a macroscopic perspective, these molecular changes manifest as a loss of freshness, with bread becoming hard, dry, and less palatable over time (Gray and Bemiller, 2003; Fadda et al., 2014; Taglieri et al., 2021).

Various strategies have been explored to extend the shelf life of bread and maintain its desirable qualities for longer periods. Fermentation is a traditional method in bread-making, primarily used for leavening and flavor development, influencing the bread's overall texture, flavor profile and sensory properties and to improve functional/nutritional features (Katina et al., 2006a; Arendt et al., 2007; Gobbetti et al., 2019; Hu et al., 2022; Martín-Garcia et al., 2023). Some studies have highlighted potential of sourdough fermentation in reducing bread staling (Corsetti et al., 2000; Katina et al., 2006b; Fadda et al., 2014; Taglieri et al., 2021; Li et al., 2024), even though the mechanisms are not completely clarified.

Sourdough is reported to be a dough whose typical characteristics are mainly due to its microflora, basically represented by lactic acid bacteria (LAB) and yeasts (Corsetti and Settanni, 2007). In general, sourdough bread has been reported to have a longer shelf life and better sensory properties compared to only yeast-fermented counterparts (Katina et al., 2006a; Katsi et al., 2021). Many of sourdough's key properties stem from the metabolic activities of its LAB, including lactic fermentation, proteolysis, volatile compound synthesis, and its anti-mold and anti-ropiness effects—essential processes during sourdough fermentation (Corsetti and Settanni, 2007). Metabolic products obtained after LAB fermentation, such as organic acids, enzymes, exopolysaccharides (EPS), can also strongly influence the molecular and macroscopic properties of bread (Fadda et al., 2014).

Many studies relate the benefits of LAB fermentation on the staling process to the production of EPS, which have been shown to improve the moisture retention of bread, contributing to a softer crumb and a slower staling rate (Lynch et al., 2018). However, even the production of organic acids (mainly lactic and acetic) can impact both fresh and stored bread quality by affecting the protein and starch fractions and by reducing the pH which results in increased flour protease and amylase activities (Fadda et al., 2014; Taglieri et al., 2021). As reported by León et al. (2002) and Palacios et al. (2004) the enzyme activities play a significant role in the amylopectin

retrogradation and water redistribution among flour macromolecules. In addition, during fermentation, the production of low-molecular-weight compounds, such as phenyl compounds, cyclic dipeptides, hydroxylated fatty acids and peptides, which have antifungal properties, can extend the shelf life of bread (Taglieri et al., 2021). Recent studies have focused on the alternative long-term preservation techniques for sourdough, especially freezing and drying process in general (Taglieri et al., 2021). Even if survival of microflora in dry/lyophilized sourdoughs can be milder compared to fresh sourdough preparations, dry sourdough offers a convenient, ready-to-use option with a longer shelf life than traditional fresh sourdough (Gidari - Gounaridou et al., 2023). A recent study demonstrated that lyophilized chickpea-rice sourdough can serve as an effective alternative for incorporation into dough formulations to improve the flavor, texture and shelf life of gluten-free bread, with analogous potential with the liquid sourdough (Gidari - Gounaridou et al., 2023).

The concomitant occurrence of various phenomena and modifications further contributes to the complexity of the effect of LAB fermentation on bread staling. Furthermore, when wheat flour is replaced with non-conventional flours, which may offer valuable nutritional alternatives, the mechanisms of bread staling and the impact of LAB fermentation become even more intricate, requiring further investigation.

Previous studies have shown that LAB fermentation of sorghum flour can modify water interactions with flour biopolymers, impacting both the technological properties of the flour and the quality of the resulting fresh bread, even in the absence of EPS production (see *Case Studies* 2 and 3). Specifically, among the strains tested, *Lacticaseibacillus casei* 4339 emerged as a strong candidate for enhancing the techno-functional properties of sorghum flour, such as viscosity and pasting properties (*Case Study 2*), and for improving crumb structure, color, and appearance (*Case Study 3*). Consequently, in *Case Study 4*, we analyzed the effects of fermenting sorghum flour with *Lacticaseibacillus casei* 4339 on the rheological and molecular properties of wheat-sorghum doughs, while in the present study, we assess its impact on the staling of wheat-sorghum composite bread, using both fresh fermented sorghum batter (liquid) and freeze-dried fermented sorghum flour. Specifically, we evaluated how the previously reported modified interactions between water and biopolymers on batters (*Case Study 2*), fresh bread (*Case Study 3*), and

wheat-sorghum doughs (*Case Study 4*), affect the dynamics of water migration, crumb hardening, and amylopectin retrogradation, over 8 days of storage.

Materials and Methods

Materials

Whole white sorghum HTC (heat-treated cereal) flour (*Sorghum bicolor* (L.) Moench) was provided by MartinoRossi SpA (Cremona, Italy), containing 55.7% carbohydrates, 16.4% fiber, 10.3% protein, and 3.2% total fat. Type 00 soft wheat flour (Triticum aestivum L.) (Italian legislation, 1967; Zhou et al., 2014) was supplied by Molino Agugiaro & Figna Molini SpA (Collecchio, Parma, Italy), with W=190-220, 71% carbohydrates, 10% protein, 2% fiber, and 0.9% fat. Fresh yeast (*Saccharomyces cerevisiae*) (AB Mauri S.p.A., Casteggio, Italy), salt (Italkali S.p.A., Palermo, Italy), sunflower oil (Oleificio Zucchi, Cremona, Italy), and sucrose (Eridania, Bologna, Italy) were all purchased from a local market.

Sorghum fermentation and freeze-drying

Lacticaseibacillus casei 4339 (*Lcb. casei* 4339) was selected from the University of Parma Culture Collection (UPCC) for sorghum flour fermentation based on preliminary studies on sorghum flour technological properties and fresh wheat-bread quality (*Case Study 2*; *Case Study 3*). Strain revitalization weas carried out as described previously (*Case Study 2*). Sorghum batters were prepared using 63% (w/w) of water, 35% (w/w) of flour and inoculated with 2% (v/w) of *Lcb. casei* 4339 inoculum. Sorghum flour was fermented at 25°C for 15 h. After 15 h, the pH of sorghum batters was measured. The fermented batters were then used for breadmaking, either as fresh liquid batter (FF) or freeze-dried pre-fermented flour (DF); freeze-drying was performed under the following conditions: 25-30 mTorr, -110°C, 48-56 h (BENCHTOP PRO, with Omnitronics, SP Scientific, USA). DF flour was stored in a glass dryer desiccator at room temperature (25°C) before being used for breadmaking. Microbiological analysis on sorghum batters and freeze-dried fermented flour were also performed, as reported in *Case Study 4*.

Bread production and storage

Three types of wheat-sorghum composite bread were prepared. Bread doughs were prepared by mixing wheat and sorghum flour (wheat to sorghum ratio 85:15) and by adding, per 100 g of flour, 2 g of salt, 2 g of fresh yeast, 3 g of sugar, and 3 g of sunflower oil. Sorghum flour was added as

unfermented sorghum flour (control), dried fermented sorghum flour (DF) or fresh fermented sorghum batter (FF). The amount of fresh fermented sorghum batter was calculated to add the same amount of flour as in dry samples. Bread formulation was set in preliminary baking trials. The amount of water added (58%) was calculated using a Brabender® Farinograph (Ohg Duisburg, Germany) to obtain optimal consistency of 500 BU of wheat-sorghum blends (data not shown). The amount of water added in each formulation was the same, as many of the physical parameters analyzed are strictly linked to the amount of water present. The water added in FF bread formulation was then calculated taking into consideration the water that was already present in the fermented batter.

Breadmaking was performed using a homemaker bread machine (Backmeister Big, UNOLD, Germania) with the following program: kneading 1 (13 min, room temperature); proofing 1 (25 min; 22°C); kneading 2 (12 min; 28°C); proofing 2 (40 min; 27°C); proofing 3 (35°C; 29°C); baking (20 min at 104°C and 40 min at 116°C). After baking bread loaves were cooled down at 25°C for 2.5 h. Day-0 loaves were analyzed straight after cooling, while the others were stored in polypropylene bags sprinkled with ethanol on the surface and kept at 25°C for 8 days. Four loaves were produced for each type of bread (Control, FF, DF), and analyzed at day-0, 1, 4 and 8, respectively. The 4 loaves of bread were produced in triplicate, on three different production days (3 independent batches). In total, 36 bread loaves were analyzed.

Although the control bread sample was yeast-fermented, for simplicity, the term "fermented samples" will hereafter refer exclusively to bread samples obtained with LAB fermented sorghum flour.

Bread specific volume, water activity (a_w), moisture content (MC)

Bread loaf volume was determined by the AACC official method for volume measurement by displacement 10-05 (2001) but employing small tapioca pearls instead of rapeseed. The specific volume of the bread (cm^3/g) was then calculated by dividing the loaf volume (cm^3) by its weight (g). Specific volume measurement was performed twice on each bread loaf. Only bread loaves on day-0 were analyzed.

The moisture content (MC, %) and water activity (a_w) of the bread were measured for both the crust and the crumb. The bread crust samples were taken from the top part of the loaf, while

crumb samples were taken from the center. To measure the moisture content, 1 gram of ground bread (either crust or crumb) was dried in a forced-air oven (ISCO NSV 9035, ISCO, Milan, Italy) at 105°C until a constant weight was achieved. The MC (%) was calculated as g of water per 100 g of the sample. The water activity (a_w) at 25°C was measured using an Aqualab 4TE water activity meter (Decagon Devices Inc, Pullman, Washington, USA). For each loaf, three replicates of MC and a_w measurements were performed for both crust and crumb at day 0, 1, 4 and 8 of storage.

Texture

A texture profile analysis (TPA) (Texture Analyzer TA.XT plus, Stable Micro Systems, Goldalming, UK) was performed on 2x2x2 cm crumb cubes using a cylindric probe (P/35 Dia Cylinder Aluminum) (40% deformation, trigger force = 0.05 N; pre-test speed = 2 mm/s; test speed = 1 mm/s). Hardness (N) (maximum height of the first compression peak), cohesiveness (-) (ratio of the areas of the second to the first compression peak) and springiness (-) (ratio of the length of the second to the first compression peak) were calculated from each TPA curve. At least 10 cubes from the central part of the loaf were analyzed for each bread loaf at 0, 1, 4 and 8 days of storage.

Differential scanning calorimetry

Thermal properties of bread were analyzed using a Q200 Differential Scanning Calorimeter (TA Instruments, New Castle, DE, USA), calibrated with indium (melting point: 156.6 °C, melting enthalpy: 28.71 J/g) and mercury (melting point: -38.8 °C, melting enthalpy: 11.4 J/g). A portion of central crumb was compressed with a 5 kg weight to obtain a flat and compact sample to maximize heat transfer within DSC cell during the experiment. Compressed crumb samples (5-10 mg) were then hermetically sealed in stainless steel pans (Perkin Elmer, Waltham, MA, USA), quench cooled to -80 °C and then heated to 130 °C at 5 °C/min.

DSC thermograms were analyzed using a Universal Analysis Software, Version 3.9A (TA Instruments, New Castle, DE, USA) which allowed us to calculate both enthalpies (ΔH; J/g) and transition temperatures [Ton (onset transition temperature), Tpeak (peak transition temperature), Toff (end transition temperature)] of both starch and ice melting transitions. Frozen water content (FW; %) was also calculated from the enthalpy of the endothermic peak around 0 °C, associated

to ice melting transition, using the following equation (Vodovotz et al., 1996; Baik and Chinachoti, 2001):

$$FW = Enthalpy\ Ice\ Fusion * \left(\frac{1}{latent\ heat\ ice\ fusion}\right) * \left(\frac{1}{MC}\right) * 100 \quad (1)$$

Where FW is Frozen water (%, g frozen water/100 g water), Enthalpy Ice Fusion (J/g sample), Latent heat of ice fusion is 334 J/g ice and MC is moisture content of the sample (g water/g sample).

Samples were analyzed in three replicates.

^{1}H molecular mobility and dynamics

^{1}H molecular mobility of bread samples was investigated with a low-resolution NMR spectrometer (20 MHz, the MiniSpec, Bruker Biospin, Milan, Italy) operating at 25.0 ± 0.1 °C.

About 2 g of bread crumb were compressed into a 10-mm NMR tube and sealed with Parafilm® to prevent moisture loss during the experiment. Both ^{1}H FID (Free Induction Decay) and ^{1}H CPMG (Carr-Purcell Meiboom-Gill) sequences were applied, following the method of Curti et al. (2017b) with slight modifications. ^{1}H FID data were acquired using a single 90° pulse, with 32 scans, 0.6 s recycle delay, 0.5 ms acquisition window, 900 data points and 7 μs dwell time. The curves were fitted with a two-component model (exponential and Gaussian; SigmaPlot, version 6, Systat Software Inc., USA), as reported by Curti et al. (2017b). ^{1}H CPMG pulse sequence was applied with 0.6 s recycle delay, 0.04 ms interpulse spacing, 32 scans, and 8000 data points. Curves were analyzed both as quasi-continuous distributions of relaxation times (UPENWin software, Alma Mater Studiorum, Bologna, Italy) and with a discrete exponential model (SigmaPlot, version 6, Systat Software Inc., USA), as reported by Carini et al. (2017).

A 10-minutes interval was allowed between ^{1}H CPMG measurements to prevent overheating of the sample. Three tubes were analyzed for each loaf, and three measurements were made on each tube.

Statistical analysis

Data were expressed as mean ± SD. Bread specific volume was analyzed with one-way ANOVA (SPSS 29 Inc., Chicago, IL), followed by the Least Significant Difference (LSD) *post hoc* test. All staling data were analyzed using a two-way ANOVA (SPSS 29 Inc., Chicago, IL), to assess significant differences ($p < 0.05$) as a result of the main effects of storage time (*st*) and fermentation (*f*) and their interaction effect (*f*st*). Significant factors were then subjected to pairwise comparisons (LSD option).

A Principal Component Analysis was also performed to get an overview of all bread staling dataset (SIMCA 18, Sartorius, Göttingen, Germany). Prior to model generation, all data were mean-centered and scaled to unit variance (UV). The validity of the models was evaluated based on the R^2 and Q^2 values.

Results

Sorghum fermentation and fresh bread properties

pH measurements and LAB counts in sorghum batters, previously documented (see *Case Study 4*), confirmed that *Lcb. casei* 4339 cell counts rose from 7.99±0.13 Log CFU/ml to 9.37±0.11 Log CFU/ml over 15 hours of fermentation, with a corresponding pH drop from 6.23 to 4.16 due to organic acid production. Freeze-drying, however, reduced LAB cultivability by approximately 2 Log CFU/ml, likely due to intracellular ice crystal formation (Stefanello et al., 2018; Taglieri et al., 2021). Nevertheless, Lattanzi et al. (2014) demonstrated that breadmaking with reactivated sourdough remains feasible when supplemented with baker's yeast as an additional starter.

Representative bread samples made with unfermented sorghum flour (control), fresh fermented sorghum batter (FF) and dry fermented sorghum flour (DF) are shown in Figure 29. The specific volumes of bread samples were 2.64 ± 0.06 cm^3/g (control), 2.38 ± 0.09 cm^3/g (FF) and 2.33 ± 0.07 cm^3/g (DF). The specific volume of bread was significantly affected by sorghum fermentation ($p < 0.001$). FF and DF breads, however, showed no significant differences between them, thus demonstrating that the freeze-drying process had no effect on the specific volume of the bread.

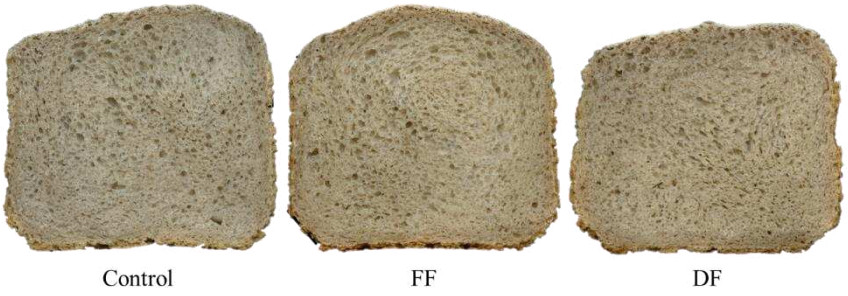

Figure 29. Representative bread samples. Control = wheat-sorghum bread with unfermented sorghum flour; FF = wheat-sorghum bread with fresh fermented sorghum batter; DF = wheat-sorghum bread with dry fermented sorghum flour.

Overview - bread staling parameters

The Principal Component Analysis (PCA) performed on all the staling parameters gave an overview of the staling dynamics of wheat-sorghum bread and helped to understand the relationships between all the physico-chemical changes that occurred at the molecular, mesoscopic and macroscopic levels in the bread samples. A two-component model (R^2 = 0.690; Q^2 = 0.572) was obtained. In the biplot reported in Figure 30, the scores were labeled according to the sample code (Control, FF and DF) and colored according to the storage time (from day 0 to day 8, from yellow to brown).

The first component (p1) seemed to describe the samples according to the storage time, which, as expected, had the strongest influence on the model (56.2% of total variability). Indeed, from the left quadrants to the right quadrants, samples were distributed with increasing storage time. The second component (p2), on the other hand, seemed to describe samples according to the fermentation process. Indeed, control samples were displaced in the bottom quadrants, while fermented samples were displaced in the upper quadrants. Freeze-drying had no clear effect on the spatial distribution of scores. In fact, FF and DF samples were always mixed together. In general, looking at the score's distribution, five groups can be noticed. Group-1 was grouping all samples at day 0, without differentiating between FF, DF and control. Group-2 and -3 divided day-1 samples into control (group-3) and fermented samples FF and DF (group-2). Finally, the last two groups 4 and 5 divided instead the day-4 and 8 samples into control (group-5) and

fermented FF and DF (group-4). Furthermore, as days 4 and 8 grouped together in both fermented and control samples, this indicates that these samples were similar for the measured variables.

In the biplot in Figure 30 it is also possible to observe the loading variables distribution, thus how the variables (measured parameters) correlated with each other and contributed to the model. From the plot it is possible to observe that most of the points were far away from the origin and thus had a strong impact on the model. Whereas points closer to the center, such as a_w-crumb and tF (ms) had a weaker influence in the model generation, and thus on the overall variability between samples.

Moreover, from the biplot was also possible to observe the relationships between scores and loadings (Figure 30). Day-0 samples, were high in variables such as PopB (%), tA (ms), PopC (%), cohesiveness (-), springiness (-), tB (ms), Ton-starch and Tpeak-starch, and low in values of hardness (N), a_w-crust, MC-curst (%), PopA (%) and tD. Moreover, day-0 and day-1 FF and DF samples, situated at the upper left quadrant were the highest in Tpeak-water (°C), Ton-water (°C), MC-crumb (%), FW (%), and the lowest in ΔH-starch (J/g), associate to amylopectin retrogradation after the first day of storage (Li and Gidley, 2022). Day-0 and day-1 control samples were the highest in PopE (%), Tpeak-starch (°C) and Toff-starch (°C), and Toff-water (°C), and the lowest in PopF (%), tC (ms) and PopD (%). On the right quadrants, it is possible to observe that day-4 and day-8 FF e DF samples were high in PopF (%), tC (ms), PopD (%), and the lowest in in PopE (%), Tpeak-starch (°C) and Toff-starch (°C), and Toff-water (°C). On the other hand, day-4 and day-8 control samples were the highest in ΔH-starch (J/g), and the lowest in Tpeak-water (°C), Ton-water (°C), MC-crumb (%) and FW (%).

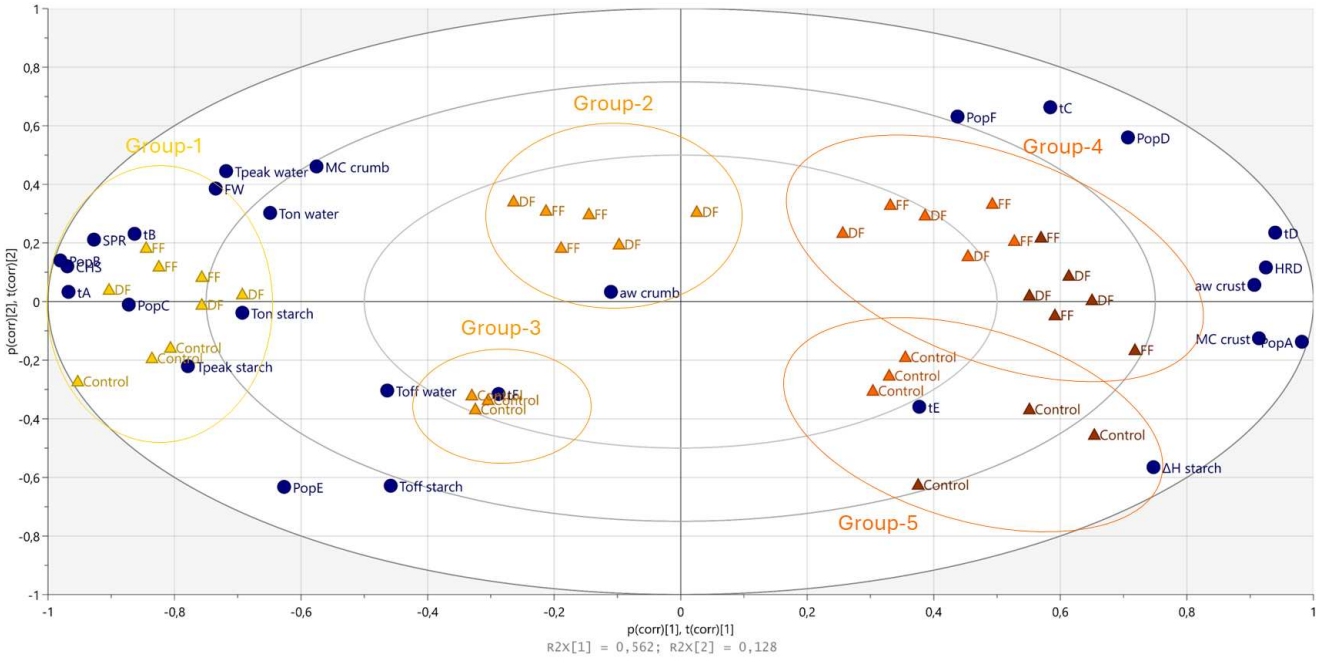

Figure 30. Biplot of PCA performed on staling parameters of wheat-sorghum breads. Score codes: Control = control bread, unfermented; FF = bread with fresh fermented sorghum; DF = bread with dry fermented sorghum. Loading codes: HRD = hardness; CHS = cohesiveness; SPR = springiness; aw crust = crust a_w; aw crumb = crumb a_w; MC crust = crust moisture content; MC crumb = crumb moisture content; FW = frozen water; Ton water = ice melting start temperature; Tpeak water = ice melting peak temperature; Toff water = ice melting end temperature; ΔH starch = starch transition enthalpy; Ton starch = starch transition start temperature; Tpeak starch = starch transition peak temperature; Toff starch = starch transition end temperature; tA = relaxation time FID PopA; tB = relaxation time FID PopB; tC = relaxation time CPMG PopC; tD = relaxation time CPMG PopD; tE = relaxation time CPMG PopE; tF = relaxation time CPMG PopF; PopA = % FID PopA; PopB =% FID PopB; PopC = % CPMG PopC; PopD = % CPMG PopD; PopE = % CPMG PopE; PopF = % CPMG PopF.

Texture

The texture parameters of all bread samples were reported in Figure 31 and Table 34. For what concern hardness, both *st* and *f* factors resulted statistically significant ($p < 0.001$). Over 8 days of storage, bread hardness increased in all samples at all storage points. The hardness levels were however different among samples. Indeed, bread loaves with fermented sorghum (FF and DF) were both harder than the control. However, no differences were found between bread made with fresh fermented sorghum (FF) and bread made with freeze-dried fermented sorghum (DF). The interaction of the two factors *f*st* was however not significant (even though close to significance, $p=0.061$). For what concern both springiness and cohesiveness, only *st* was found to be a significant factor among samples variability. Indeed, over 8 days of storage all samples showed a decrease in both cohesiveness and springiness, without showing any differences among samples as a result of fermentation and drying treatments (*f*). The interaction effect was also not significant, showing the same decreasing trend among different samples (Figure 31, Table 34).

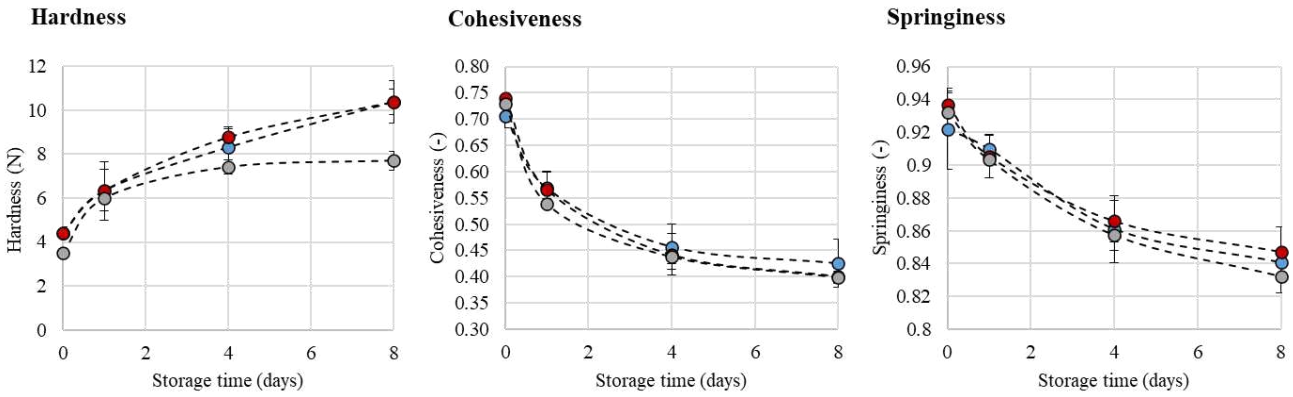

Figure 31. Texture parameters of wheat-sorghum bread, with unfermented sorghum flour (Control, grey), fresh fermented sorghum batter (FF, red), and dry fermented sorghum flour (DF, blue).

*Table 34. Means ± SD of bread staling parameters and two-way ANOVA outputs for f, st and f*st (*= p < 0.05; **= p < 0.01; ***= p < 0.001); different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples.*

	Storage time		Control	FF	DF	*f*	*st*	*f*st*
			b	a	a			
Hardness (N)	day-0	d	3.51±0.07	4.40±0.29	4.42±0.19	***	***	ns
	day-1	c	5.99±0.07	6.33±1.32	6.35±0.95			
	day-4	b	7.41±0.33	8.77±0.49	8.32±0.81			
	day-8	a	7.70±0.44	10.38±0.98	10.38±0.57			
			a	a	a			
Cohesiveness (-)	day-0	d	0.730±0.005	0.740±0.003	0.706±0.023	ns	***	ns
	day-1	c	0.539±0.009	0.566±0.034	0.569±0.030			
	day-4	b	0.438±0.014	0.442±0.039	0.457±0.043			
	day-8	a	0.399±0.011	0.401±0.015	0.426±0.046			
			a	a	a			
Springiness (-)	day-0	d	0.932±0.013	0.937±0.007	0.922±0.025	ns	***	ns
	day-1	c	0.903±0.002	0.905±0.013	0.910±0.009			
	day-4	b	0.857±0.009	0.866±0.013	0.861±0.02			
	day-8	a	0.832±0.010	0.847±0.015	0.841±0.008			
			a	a	a			
a_w-crust	day-0	c	0.793±0.020	0.832±0.013	0.822±0.029	ns	***	ns
	day-1	b	0.852±0.024	0.872±0.012	0.865±0.014			
	day-4	a	0.904±0.003	0.895±0.019	0.898±0.008			
	day-8	a	0.904±0.000	0.902±0.003	0.921±0.005			
			a	a	a			
a_w-crumb	day-0	a	0.961±0.001	0.961±0.005	0.957±0.007	ns	ns	*
	day-1	a	0.965±0.005	0.958±0.001	0.956±0.010			
	day-4	a	0.963±0.002	0.959±0.002	0.957±0.007			
	day-8	a	0.947±0.010	0.962±0.008	0.965±0.007			
			a	a	a			
MC-crust (%)	day-0	c	16.27±0.11	16.57±1.09	17.31±0.72	ns	***	ns
	day-1	b	18.13±0.15	18.63±0.56	18.95±0.66			
	day-4	a	23.08±0.60	22.03±1.16	21.88±1.11			
	day-8	a	23.86±1.62	21.99±1.12	23.44±1.27			
			a	a	a			
MC-crumb (%)	day-0	a	41.93±0.07	42.38±0.36	41.85±0.61	ns	***	ns
	day-1	a	41.79±0.16	42.01±0.45	41.79±0.58			
	day-4	a	41.78±0.17	41.59±0.79	41.66±0.30			
	day-8	b	39.95±0.72	40.51±1.11	41.15±1.09			

FW (%)			a	a	a	ns	***	ns
	day-0	a	59.02±1.62	59.57±2.58	57.31±1.60			
	day-1	ab	54.86±2.12	58.63±0.32	55.59±2.90			
	day-4	b	53.25±2.21	56.29±2.24	55.80±1.89			
	day-8	c	50.23±3.99	50.42±2.79	51.14±2.42			
ΔH-starch (J/g)			a	b	c	***	***	*
	day-0	d	0.39±0.06	0.00±0.00	0.00±0.00			
	day-1	c	0.64±0.08	0.21±0.03	0.21±0.02			
	day-4	b	1.00±0.13	0.72±0.09	0.52±0.08			
	day-8	a	1.26±0.09	0.76±0.07	0.67±0.03			

Water status

The results related to water activity (a_w) and moisture content (MC, %) of crust and crumb, as well as frozen water content (FW, %) as determined by DSC, were reported in Figure 32 and Table 35. The molecular mobility of protons in the different compartments of bread was instead reported in paragraph *^{1}H molecular mobility and dynamics*, concerning the results of ^{1}H molecular mobility.

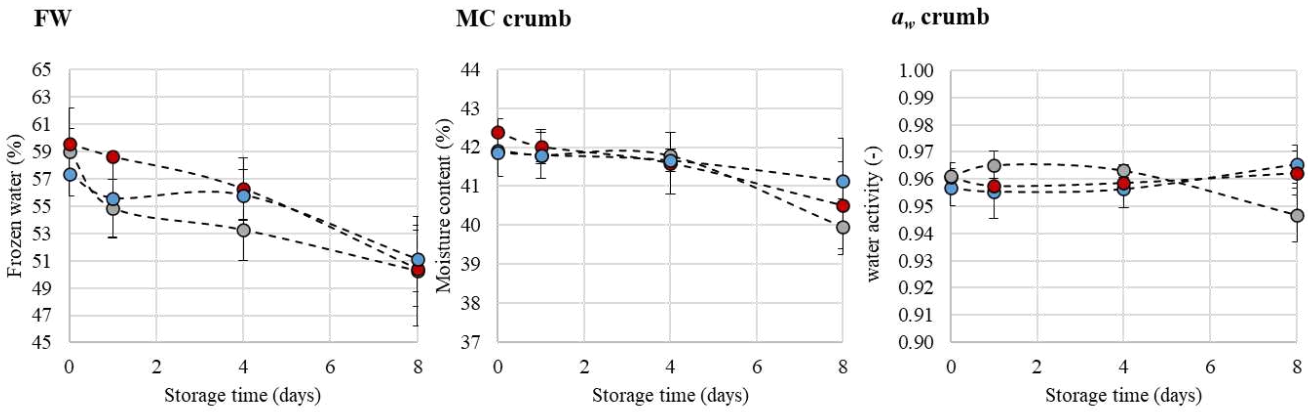

Figure 32. Water status parameters of wheat-sorghum bread, with unfermented sorghum flour (Control, grey), fresh fermented sorghum batter (FF, red), and dry fermented sorghum flour (DF, blue).

*Table 35. Ice melting transition temperatures in wheat-sorghum bread samples (Control., FF, DF) and two-way ANOVA outputs for f, st and f*st (*= p < 0.05; **= p < 0.01; ***= p < 0.001); different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples.*

	Storage time		Control	FF	DF	*f*	*st*	*f*st*
Ton-water (°C)	day-0	a	-17.6±0.85	-17.96±0.55	-17.44±0.86	*	***	ns
	day-1	b	-18.98±0.47	-18.84±0.41	-18.35±0.56			
	day-4	bc	-19.4±0.55	-19.2±0.46	-17.99±0.46			
	day-8	c	-19.64±0.67	-19.46±0.97	-19.01±0.35			
			b	b	a			
Tpeak-water (°C)	day-0	a	1.77±0.03	1.77±0.33	1.88±0.19	*	***	ns
	day-1	ab	1.46±0.08	1.71±0.14	1.67±0.5			
	day-4	b	1.24±0.1	1.56±0.17	1.43±0.22			
	day-8	c	0.88±0.28	1.3±0.07	1.2±0.16			
			b	a	a			
Toff-water (°C)	day-0	a	14.68±0.51	14.4±0.84	15.06±1.08	ns	ns	ns
	day-1	a	14.71±0.41	14.38±0.16	13.66±0.99			
	day-4	a	14.35±0.26	13.87±0.69	13.45±1.47			
	day-8	a	14.64±0.41	13.04±1.03	14.13±0.32			
			a	a	a			

In the PCA model (Figure 30), while FW, MC crumb, Tpeak-water and Ton-water positioned in the opposite quadrant from the day-4 and day-8 control samples (suggesting lower values of water parameters in control samples), the a_w-crumb seemed to be least influent variable in the model generation as it was occupying a central position in the plot. In accordance with that, from the output of two-way ANOVA both the factors *f* and *st* were not significant. However, interestingly, the interaction effect *f*st* was found to be significant (Table 35), thus showing that fermentation changed the water activity dynamics of bread crumb. Indeed, the a_w-crumb in the control bread showed a different trend if compared to the fermented samples FF and DF. While in FF and DF the a_w-crumb was almost constant over storage, in control bread a_w-crumb showed a decreasing trend. In the pairwise comparison of samples at different storage time (Table 36) the differences between DF, FF and control were indeed evident only at day-8 of storage. This result affected the interaction between fermentation and storage time, while keeping the overall differences between samples not significant.

*Table 36. Pairwise comparison of the dependent variable a_w-crumb; based on estimated marginal means. * The mean difference is significant at the 0.05 level. [b] Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).*

Storage time (day)	(I) Sample Name	(J) Sample Name	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
0	Control	DF	0.004	0.005	0.421	-0.006	0.015
		FF	0.000	0.005	0.944	-0.01	0.011
	DF	Control	-0.004	0.005	0.421	-0.015	0.006
		FF	-0.004	0.005	0.462	-0.014	0.007
	FF	Control	0.000	0.005	0.944	-0.011	0.01
		DF	0.004	0.005	0.462	-0.007	0.014
1	Control	DF	0.010	0.005	0.071	-0.001	0.02
		FF	0.008	0.005	0.144	-0.003	0.018
	DF	Control	-0.010	0.005	0.071	-0.02	0.001
		FF	-0.002	0.005	0.706	-0.013	0.009
	FF	Control	-0.008	0.005	0.144	-0.018	0.003
		DF	0.002	0.005	0.706	-0.009	0.013
4	Control	DF	0.007	0.005	0.201	-0.004	0.017
		FF	0.005	0.005	0.374	-0.006	0.015
	DF	Control	-0.007	0.005	0.201	-0.017	0.004
		FF	-0.002	0.005	0.686	-0.013	0.008
	FF	Control	-0.005	0.005	0.374	-0.015	0.006
		DF	0.002	0.005	0.686	-0.008	0.013
8	Control	DF	-0.019*	0.005	0.001	-0.029	-0.008
		FF	-0.015*	0.005	0.006	-0.026	-0.005
	DF	Control	-0.019*	0.005	0.001	0.008	0.029
		FF	0.003	0.005	0.534	-0.007	0.014
	FF	Control	-0.015*	0.005	0.006	0.005	0.026
		DF	-0.003	0.005	0.534	-0.014	0.007

For what concern MC-crumb (%), *st* was found to be a significant factor among samples variability. As expected, either control, FF or DF showed a decreasing trend over time. In this case, *f* and the interaction factor *f*st*, were not significant. However, looking at the pairwise comparison between samples at different storage time, fermented samples were significantly different from the control only at day-8 of storage (Table 37). DF was higher than control, while FF had intermediate values, not significantly different to DF and control.

As expected, the a_w-crust and MC-crust (%) showed instead an increasing trend over time, which resulted significant. Both a_w-crust and MC crust (%) increased from day-0 to day-4 and became

constant between day-4 and day-8. No significant effects of *f* and interaction effect of *f*st* were observed.

*Table 37. Pairwise comparison of the dependent variable MC-crumb; based on estimated marginal means. * The mean difference is significant at the 0.05 level. [b] Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).*

Storage time (day)	(I) Sample Name	(J) Sample Name	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
						Lower Bound	Upper Bound
0	Control	DF	0.073	0.514	0.888	-0.988	1.133
		FF	-0.453	0.514	0.386	-1.514	0.607
	DF	Control	-0.073	0.514	0.888	-1.133	0.988
		FF	-0.526	0.514	0.316	-1.587	0.534
	FF	Control	0.453	0.514	0.386	-0.607	1.514
		DF	0.526	0.514	0.316	-0.534	1.587
1	Control	DF	-0.001	0.514	0.998	-1.062	1.059
		FF	-0.219	0.514	0.673	-1.28	0.841
	DF	Control	0.001	0.514	0.998	-1.059	1.062
		FF	-0.218	0.514	0.675	-1.279	0.842
	FF	Control	0.219	0.514	0.673	-0.841	1.28
		DF	0.218	0.514	0.675	-0.842	1.279
4	Control	DF	0.117	0.514	0.822	-0.943	1.178
		FF	0.188	0.514	0.718	-0.873	1.248
	DF	Control	-0.117	0.514	0.822	-1.178	0.943
		FF	0.071	0.514	0.892	-0.99	1.131
	FF	Control	-0.188	0.514	0.718	-1.248	0.873
		DF	-0.071	0.514	0.892	-1.131	0.99
8	Control	DF	-1.194*	0.514	0.029	-2.254	-0.133
		FF	-0.557	0.514	0.289	-1.617	0.504
	DF	Control	1.194*	0.514	0.029	0.133	2.254
		FF	0.637	0.514	0.227	-0.424	1.697
	FF	Control	0.557	0.514	0.289	-0.504	1.617
		DF	-0.637	0.514	0.227	-1.697	0.424

The two-way ANOVA output related to the FW (%) (Figure 32) showed the significant effect of *st*. Even in this case, as expected, the trend decreased in all samples. No significant effect of *f* and the interaction effect *f*st* were found, indicating that fermentation did not influence bread crumb FW (%) (Table 34).

The results related to the transition temperature of the ice melting peak were reported in Table 35. Both Ton- and Tpeak-water, related to the ice-melting onset and peak temperatures in bread crumb, showed a significant decrease during storage, with a significant effect of the *st* factor ($p < 0.001$). The temperature at the end of transition, in contrast, did not see a significant effect of storage time. As for the effect of fermentation, on the other hand, *f* was also significant ($p < 0.05$), with the fermented samples showing higher temperatures. In contrast, the interaction between the two variables was not significant.

Amylopectin retrogradation

Figure 33 shows the trend of the enthalpy changes associated to the starch transition in bread during 8 days of storage. While amylose retrogradation is responsible for the initial crumb firmness of bread, amylopectin retrogradation has been reported to influence bread firmness and staling after 1 day of storage (Li and Gidley, 2022). For this reason, the transition at day-0 was attributed to starch that did not gelatinize during baking, whereas the transition from day-1 onward is hereafter associated with melting of retrograded amylopectin crystals.

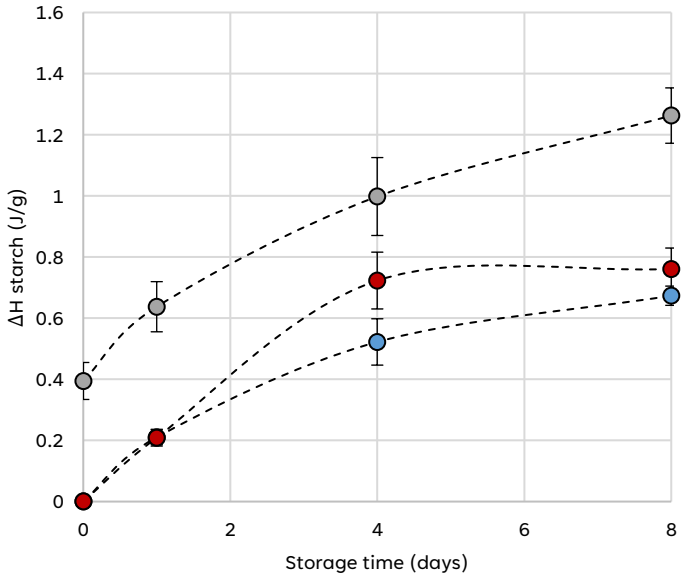

Figure 33. Starch transition enthalpy (ΔH) over 8 days of storage in wheat-sorghum bread samples with unfermented sorghum flour (Control, grey), fresh fermented sorghum batter (FF, red), and dry fermented sorghum flour (DF, blue).

No peak was observed in the fermented samples at day-0 of storage. This indicates that in the fermented samples, all the gelatinizable starch (at the selected moisture content) had indeed gelatinized during baking (Curti et al., 2017b). Therefore, no temperature was assigned to the transition, while ΔH was set to 0 J/g. In the two-way ANOVA analysis of starch transition temperatures, day-0 of all samples was thus excluded. According to the two-way ANOVA, both the main factors *f* and *st* ($p < 0.001$) and their interaction *f*st* ($p < 0.05$) were significant in determining the changes in ΔH-starch. As expected, the increase in starch transition enthalpy during 8 days of storage highlighted the increase in retrograded amylopectin that occurs during storage, with a significant increase for each storage point. The dynamics of amylopectin retrogradation also showed differences among samples, with higher values in the control sample, followed by FF and DF. Fermentation of sorghum flour decreased the amount of retrograded amylopectin compared to the unfermented control sample. Regarding the onset and peak temperatures of the transition (Ton-starch and Tpeak-starch) (Table 38), they were both significantly affected only by *st* ($p < 0.001$) and showed a decreasing trend with time. In contrast, the end-transition temperature (Toff-starch) showed not only a significant decrease over time ($p < 0.05$), but it was also lower in the fermented samples than in the control ($p < 0.001$) (Table 38).

*Table 38. Starch transition temperatures in wheat-sorghum bread samples (Control, FF, DF) and two-way ANOVA outputs for f, st and f*st (*= p < 0.05; **= p < 0.01; ***= p < 0.001); different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples.*

	Storage time		Control	FF	DF	*f*	*st*	*f*st*
Ton-starch (°C)	day-0		60.68±0.15	nd	nd	ns	***	ns
	day-1	a	60.67±0.23	60.41±1.19	61.05±0.4			
	day-4	b	57.8±1.71	56.48±0.64	58.78±1.82			
	day-8	b	58.09±2.07	57.39±0.82	58.4±0.9			
			a	a	a			
Tpeak-starch (°C)	day-0		70.46±1.36	nd	nd	ns	***	ns
	day-1	a	69.58±0.36	69.03±0.84	69.02±0.23			
	day-4	b	67±0.43	67.12±0.17	67.13±0.45			
	day-8	b	68.5±0.53	66.92±1.43	67.63±0.2			
			a	a	a			
Toff-starch (°C)	day-0		82.46±2.57	nd	nd	***	*	ns
	day-1	a	83.77±0.56	80.04±0.42	81.09±1.96			
	day-4	b	81.04±0.86	80.08±0.47	80.51±0.11			
	day-8	b	82.17±1.04	80.33±0.23	79.79±0.38			
			a	b	b			

*nd=not detected

^{1}H molecular mobility and dynamics

The molecular mobility of wheat-sorghum breads was investigated through ^{1}H FID (free induction decay) and ^{1}H CPMG sequences, as reported by Curti et al. (2017b). The results of the discrete exponential fitting for both sequencies were reported in Table 39.

The bi-exponential model fitting of ^{1}H FID curves revealed two ^{1}H populations, designated as PopA (the most rigid) and PopB (more mobile). Previous studies have attributed PopA to immobile crystallized amylose (or amylopectin in stored bread) and/or amorphous starch, as well as the rigid CH protons of gluten. Conversely, PopB has been associated with CH protons of amorphous starch and gluten in regions interacting with confined water (Bosmans et al., 2013b). PopA was found to relax within a range of 0.020-0.032 ms, while PopB exhibited relaxation times between 0.308-0.328 ms, consistent with previous studies (Carini et al., 2017; Curti et al., 2017b).

From the analysis of the ^{1}H CPMG curves, four populations were identified and named, from the most rigid to the most mobile, PopC, PopD, PopE (the most abundant), and PopF, respectively. The UPENWin fitting of ^{1}H CPMG distributions is illustrated in Figure 34 and Figure 35. These four populations have been previously observed in bread staling studies (Bosmans et al., 2013b; Curti et al., 2017a, 2017b). These populations, relaxing in the ranges of 0.309-0.368 ms (PopC), 2.62-4.51 ms (PopD), 7.59-9.03 ms (PopE), 73.19-84.67 ms (PopF), can be attributed to protons of amorphous starch and gluten in little contact with water (PopC), gluten and exchanging protons of confined water, starch and gluten (PopD), mobile exchanging protons of water, starch and gluten in the formed gel network (PopE) and lipid protons (PopF) (Bosmans et al., 2013b; Curti et al., 2017b). PopB and PopC, having similar relaxation times and trends upon storage and across samples, were attributed to the same proton populations, in line with the literature (Bosmans et al., 2013b; Curti et al., 2017b).

*Table 39. CPMG and FID populations relative abundances (%) and relaxation times (ms) in wheat-sorghum bread samples (Control, FF, DF) and two-way ANOVA outputs for f, st and f*st (*= p < 0.05; **= p < 0.01; ***= p < 0.001); different letters in the same column indicate differences between storage days; different letters in the same row indicate differences among samples.*

	Storage time		Control	FF	DF	*f*	*st*	*f*st*
	day-0	a	0.032±0.001	0.031±0.001	0.03±0			
	day-1	b	0.025±0.001	0.024±0	0.024±0.001			
tA (ms)	day-4	c	0.021±0	0.021±0	0.021±0	ns	***	ns
	day-8	d	0.02±0	0.02±0	0.02±0			
			a	a	a			
	day-0	a	0.325±0.001	0.328±0.002	0.325±0.005			
	day-1	b	0.316±0.002	0.319±0.003	0.314±0.001			
tB (ms)	day-4	c	0.311±0	0.316±0.004	0.312±0.005	*	***	ns
	day-8	c	0.308±0.003	0.312±0.003	0.312±0.004			
			b	a	b			
	day-0	c	0.319±0.002	0.328±0.004	0.309±0.012			
	day-1	b	0.316±0.021	0.351±0.008	0.346±0.004			
tC (ms)	day-4	a	0.326±0.008	0.366±0.011	0.368±0.019	***	***	**
	day-8	ab	0.318±0.009	0.359±0.016	0.367±0.012			
			b	a	a			
	day-0	d	2.68±0.1	2.87±0.05	2.62±0.09			
	day-1	c	2.89±0.13	3.59±0.07	3.59±0.19			
tD (ms)	day-4	b	3.7±0.11	4.22±0.17	4.26±0.38	***	***	*
	day-8	a	3.99±0.32	4.45±0.11	4.51±0.08			
			b	a	a			
	day-0	a	9.03±0.16	7.66±0.21	7.59±0.53			
	day-1	ab	8.78±0.23	7.81±0.09	7.98±0.06			
tE (ms)	day-4	b	8.42±0.27	8.25±0.2	8.62±0.44	***	***	***
	day-8	c	8.37±0.16	8.89±0.41	8.99±0.07			
			a	b	b			
	day-0	a	84.67±5.51	78.31±3.88	77.1±3.22			
	day-1	a	80.29±3.65	76.14±2	73.19±1.15			
tF (ms)	day-4	a	78.14±1.47	78.82±1.79	77.78±0.94	**	ns	ns
	day-8	a	78.23±2.81	78.18±1.53	74.07±2.31			
			a	b	b			
	day-0	d	54.81±0.15	54.07±0.57	55.32±1.78			
	day-1	c	59.88±0.27	59.53±0.35	60.73±0.67			
PopA (%)	day-4	b	65.59±0.29	64.33±0.93	65.35±1.48	*	***	ns
	day-8	a	67.83±0.77	66.93±0.65	67.22±0.95			
			a	b	a			
	day-0	a	45.19±0.15	45.93±0.57	44.68±1.78			
	day-1	b	40.12±0.27	40.47±0.35	39.27±0.67			
PopB (%)	day-4	c	34.41±0.29	35.67±0.93	34.65±1.48	*	***	ns
	day-8	d	32.17±0.77	33.07±0.65	32.78±0.95			
			b	a	b			
PopC (%)	day-0	a	16.91±0.39	16.34±0.68	16.7±0.97	ns	***	ns

	day-1	b	15.01±0.28	14.98±0.26	15.62±0.21			
	day-4	c	14.06±0.11	13.61±0.22	14.23±0.57			
	day-8	c	14.1±0.59	13.91±0.62	13.71±0.31			
			a	a	a			
	day-0	c	12.79±0.18	17.16±1.3	16.46±1.7			
	day-1	b	14.31±0.87	26.13±1.81	29.81±3.15			
PopD (%)	day-4	a	20.84±0.21	35.17±3.59	27.68±2.17	***	***	***
	day-8	ab	25.03±4.86	25.71±3.12	26.56±1.28			
			b	a	a			
	day-0	a	67.08±0.49	63.06±1.46	63.54±3.01			
	day-1	b	67.32±1.19	55.5±1.94	50.96±3.22			
PopE (%)	day-4	c	61.8±0.12	47.65±3.24	54.48±1.71	***	***	***
	day-8	bc	57.64±5.51	55.71±2.5	56.18±1.02			
			a	b	b			
	day-0	b	3.22±0.13	3.33±0.07	3.35±0.08			
	day-1	a	3.35±0.09	3.44±0.14	3.47±0.02			
PopF (%)	day-4	a	3.3±0.14	3.59±0.17	3.66±0.21	***	**	ns
	day-8	a	3.22±0.09	3.61±0.13	3.51±0.05			
			b	a	a			

As expected, *st* significantly affected the relaxation times of all ^{1}H FID and ^{1}H CPMG populations ($p < 0.001$), except for PopF, which approached significance ($p=0.058$). The fermentation factor *f* also significantly affected the relaxation times of ^{1}H FID and ^{1}H CPMG populations PopB ($p < 0.05$), PopC, PopD, PopE, and PopF ($p < 0.001$), while did not affect the relaxation time of PopA. Moreover, different trends in relaxation times were observed between different samples over storage (tC, tD, tE), with a significant interaction effect *f*st*.

During storage, PopA, PopB, and PopC became more rigid, showing decreased values for tA, tB, and tC. In contrast, the relaxation time tD increased, and PopD became closer to PopE over time (Figure 34, Table 39). For what concern PopE, tE decreased in the control sample over 8 days of storage, while it slightly increased in FF and DF samples. Overall, the fermented samples FF and DF exhibited higher mobility for PopB (only FF), PopC, and PopD (both FF and DF) compared to the control sample. However, PopE and PopF showed greater mobility in the control sample than in FF and DF samples.

The relative abundances (%) of ^{1}H FID populations PopA and PopB were both influenced by *st* ($p < 0.001$) and *f* ($p < 0.05$). However, no interaction effect *f*st* was noticed. The relative abundance of ^{1}H CPMG PopC (%) was affected only by *st*. PopD and PopE, on the other hand, were significantly

affected ($p < 0.001$) by *st*, *f* and their interaction *f*st*. PopF (%) was affected by both *st* ($p < 0.01$) and *f* ($p < 0.001$).

During storage, the relative abundance (%) of PopA increased, while those of PopB and PopC decreased. In general, FF showed lower values of PopA and higher values of PopB compared to the control. DF did not show significant differences from the control. Regarding the most abundant ^{1}H CPMG populations, PopD and PopE, the relative abundance of PopD increased from day-0 to day-8, while that of PopE decreased, likely due to higher tD values that made PopD and PopE closer. Generally, higher values of PopD and lower values of PopE were observed in FF and DF samples compared to the control. Eventually, the last ^{1}H CPMG population, PopF, showed increasing value over time and was higher in the fermented samples FF and DF than in the control.

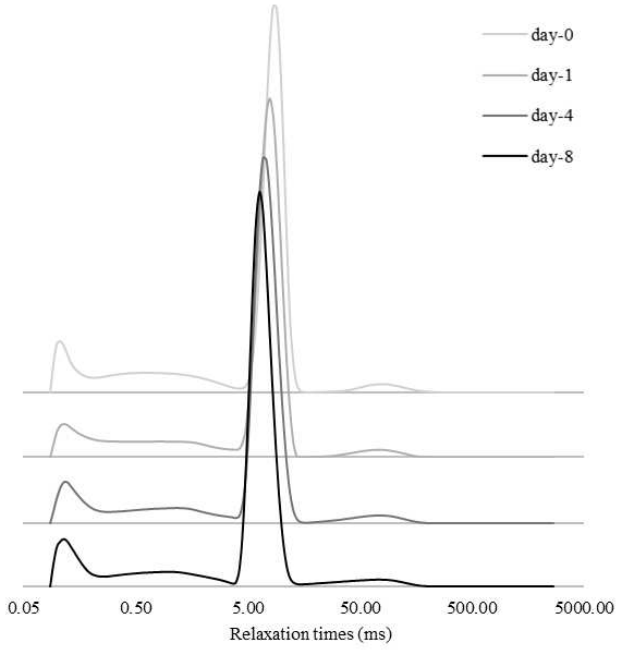

Figure 34. Representative ^{1}H CPMG distributions of control sample at 0, 1, 4 and 8 days of storage.

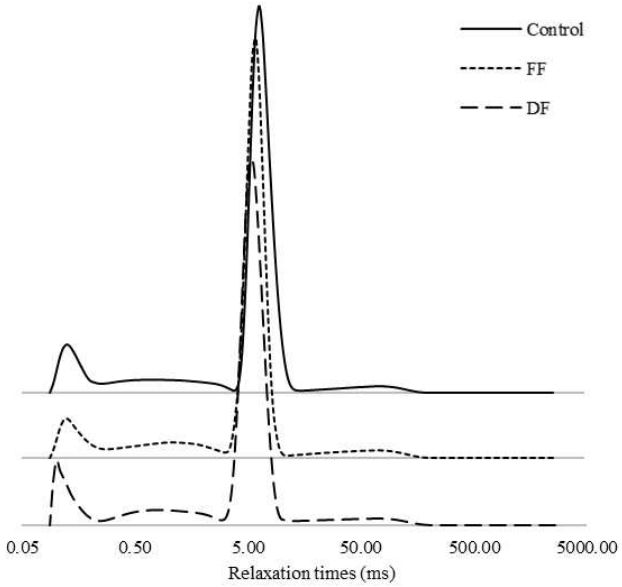

Figure 35. Representative ^{1}H CPMG distributions of control, FF and DF sample at day 8 of storage.

Discussion

The reduction in specific volume found in fermented bread samples was in accordance with a previous study about wheat-sorghum bread with sorghum *Lcb. casei* 4339-fermented batter (*Case Study 3*). In that previous study the lower specific volume had also impacted on the hardness of bread, which however, was not perceived during a sensory acceptability test. Instead, it was the most liked in terms of overall appearance due to crumb porosity and color. As reported previously, the reduction in specific volume of wheat-sorghum bread may be due to the weakening effect of gluten due to the interaction of different factors, such as production of organic acids and reduction of pH, and the pH-activated proteases activity on gluten structure, dilution of gluten proteins with sorghum hydrophobic proteins and fibers (*Case Study 3*). Indeed, DF and FF bread doughs showed lower pH values than the control, as reported in our previous study on the same matrices (*Case Study 4*). In a previous study by Lattanzi et al. (2014), the authors evidenced the negative influence of the freeze-drying on microorganism viability and rheological features of breads, which were firmer and with a lower specific volume than bread from sourdough. However,

in our study the specific volumes of FF and DF breads were not significantly different. Therefore, freeze-drying did not negatively affect the volume of wheat-sorghum fermented bread.

The PCA provided information on water status dynamics, molecular interactions and relaxation dynamics, amylopectin retrogradation and texture parameters, which are considered to be the main causes and effects of bread staling. According to the PCA output, the chemical and physical changes associated with bread staling and loss of freshness underwent the greatest effects in the first days of storage (from 0 to 4) and then slowed down as the storage time increased (between 4 and 8). These results are in agreement with those reported in the literature (Curti et al., 2011; Bosmans et al., 2013b). Moreover, no major differences were found between FF and DF bread, while the differences between control and fermented samples were more evident from day 1 of staling supporting that LAB-fermented sourdough exhibit different staling rate.

Investigating the evolution of water status at different structural levels, including its redistribution from crumb to crust (macroscopic level) and between gluten and starch (molecular level), is crucial for fully understanding the staling process, as water plays a pivotal role in the deterioration of bread quality throughout its shelf life (Curti et al., 2017b). The spatial distribution of sample in the PCA model showed evidently the detrimental effect of storage time on water status but also evidenced the positive effect of fermentation in mitigating its effects on bread. Indeed, the control bread positioned in the opposite quadrant of FW, MC-crumb, Ton-water and T-peak-water, indicating more water loss and water status modification in control bread than fermented bread samples. The two-way ANOVA output a_w-crust and MC-crust, MC-crumb and FW showed that storage time significantly affected the bread water status at the macroscopic and mesoscopic level. The decreased MC-crumb (%) is due to crumb water loss and migration to crust (Baik and Chinachoti, 2000; Bosmans et al., 2013b; Curti et al., 2017b), while the decreased FW (%) has been reported as a result of crumb water loss and water migration from gelatinized starch towards the amorphous and crystalline domains, especially B-type crystals, becoming “unfreezable” (Ribotta and Le Bail, 2007; Fadda et al., 2014; Curti et al., 2017b). In accordance with PCA output both a_w-crust and MC-crust increased until day-4 of storage and became constant at day 8, indicating that most of the water migration that occurred from crumb to the crust in 8 days of storage was almost completed at day. In the last day of storage (day 8) MC-crumb and a_w-crumb were higher in fermented samples than unfermented. In general, higher moisture contents in sourdough bread

or bread with LAB fermented flours have been mainly linked to the production of EPS (Torrieri et al., 2014; Taglieri et al., 2021). However, in our samples we previously found no production of EPS (*Case Study 2*). In contrast, pH and acidity may have influenced the activity of endogenous and exogenous enzymes (microbial enzymes) and may in turn have influenced the MC of samples. Indeed, in a previous study on the use of enzymes in bread formulations, Gámbaro et al. (2006) proposed that the addition of a mixture of α-amylase and xylanase may produce low-molecular-weight dextrin with high water retention capacity, and that could be partly responsible for the lower staling rate. Also, Katina et al. (2006b) reported about bread with starch granules much more swollen after the addition of α-amylase, xylanase and lipase mixtures, as a result of the higher water content and degradation of cell wall components. An increased retention of moisture content, with improved shelf life was also previously found in dried sourdoughs by Hendek Ertop et al. (2018). Moreover, Su et al. (2019) also found that the decline rates of MC in acidified breads during storage were significantly lower than that in the control group. However, they found that the addition of organic acid also lowered MC in bread but would also decrease its decline rates. Therefore, the effect may not only link to the direct and indirect effects of organic acids, but also to the strain specific activity of *Lcb. casei* 4339 and its modification of flour biopolymers interaction with water, which indeed was already reported in our previous studies on fermented sorghum batters (*Case Study 2*) and wheat-sorghum bread (*Case Study 3*). For what concern the ice melting transition temperatures, the decreased onset and peak temperatures over time are in accordance with previous studies. Indeed, according to Ribotta and Le Bail (2007) lower ice-melting transition temperatures during storage may be linked to the development of a possible second transition (glass transition at slightly lower temperatures than the ice-melting; or melting of lower freezing-point ice crystals in freeze-concentrated domains). Moreover, since the temperature at the end of transition, in contrast, did not see a significant effect of storage time, this indicates that the peak widened during storage, and the water began to melt at lower temperatures. Tpeak-water and Ton-water were also higher in fermented samples DF e FF than control, which may be due to the establishment of stronger bonds between water and flour components.

From the PCA output was also evident the role of fermentation on amylopectin retrogradation, with control sample at 4 and 8 days of storage positioning very close to ΔH-starch, thus

suggesting higher values of amylopectin. Amylopectin retrogradation has been reported to be one of the majors happening that determine bread staling in many articles (León et al., 2002; Gray and Bemiller, 2003; Palacios et al., 2004; Ribotta and Le Bail, 2007; Goesaert et al., 2009; Fadda et al., 2014). The reduction of amylopectin retrogradation after fermentation (both in FF and DF) was confirmed by the two-way ANOVA analysis, showing significant effect of fermentation and its interaction with storage time, highlighting the different dynamics of staling in fermented bread. Moreover, among fermented samples, DF had the lowest value. The effect of fermentation on amylopectin retrogradation has been reported as a consequence of both endogenous enzymes from flours which are activated by pH decrease, and microbial exogenous enzymes. With different mechanisms of action, the reduction of amylopectin retrogradation has been documented as a result of α-amylase, maltogenic α-amylase, and xylanase activities (Fadda et al., 2014; Taglieri et al., 2021). For instance, maltogenic α-amylase has been reported to degrade amylopectin outer branches, thus producing amylopectin chains that are too short to crystallize and can prevent the formation of a "permanent" amylopectin network, thus delaying the staling (Goesaert et al., 2009; Fadda et al., 2014). α-amylases can hydrolyze amylopectin and form soluble low-molecular-weight branched-chain polymers, that are less inclined to retrogradation and can affect water availability and mobility (Palacios et al., 2004; Amigo et al., 2016; Taglieri et al., 2021). Moreover, Sun et al. (2022) found that dough fermentation resulted in the increased short chain content and crystallinity of starch with lower molecular weight, which endowed dough with excellent anti-retrogradation ability. Xylanase activity, on the other hand, can interfere with the reorganization of the amylopectin and/or with the redistribution of water in the system, with a consequent retrogradation reduction (Ghoshal et al., 2013; Fadda et al., 2014).

Regarding the macroscopic texture parameters, cohesiveness and springiness seemed to be affected only by storage time, while hardness was affected by both storage time and fermentation. The PCA output showed the great impact of storage time in determining the decrease in cohesiveness, springiness and the increase in hardness, positioning at the most outer points of p1. The decrease in hardness, cohesiveness, and springiness of bread with storage is a well-known effect of bread staling reported in previous studies and is one of the main factors that reduce the acceptability of stale bread (Amigo et al., 2016). However, hardness was also affected by fermentation. This increased hardness in fermented samples has to be related to the reduction

in specific volume found in these samples. Even though the interaction factor resulted to be not significant, it approached significance (p=0.061), and the difference between samples increased upon storage. Many studies conducted in bread and starch gels have attributed the firming of bread that characterizes the staling phenomenon to the retrogradation of starch (León et al., 2002; Palacios et al., 2004; Goesaert et al., 2009). However, based on our results, amylopectin retrogradation did not appear to be the major cause of crumb firming in wheat-sorghum fermented bread. Other studies point to gluten–gluten and gluten–starch interactions as the main causes of bread firming (Martin and Hoseney, 1991; Martin et al., 1991; Palacios et al., 2004). Amigo et al. (2016) also found that when analyzing the effect of α-amylase on crumb firming, the mechanism of action depended on different variables: starch retrogradation, starch gel network rigidity, and starch-protein interactions. Thus, in our samples, the possible effect of the enzymes may have affected starch retrogradation but not resulted in decreased crumb firming, perhaps because there was no decreased rigidity in the starch gel network or starch-protein interaction. The increased hardness may be related to molecular strengthening of starch-protein interactions and thus to molecular changes.

The PCA analysis revealed that both storage time and fermentation affect the proton populations in the ^{1}H FID and ^{1}H CPMG sequences. In the PCA model, the ^{1}H FID populations appeared to be more influenced by storage time, while the ^{1}H CPMG populations were affected by both storage time and fermentation. Changes in the mobility of populations (relaxation times and population abundances) during bread storage have been attributed to specific phenomena characterizing the staling process (Curti et al., 2017a). In our samples, the increased number of rigid protons associated to ^{1}H FID PopA over time was found in all samples. The PopA (%) increase is consistent with previous fundings on bread staling assessment using ^{1}H FID sequence (Bosmans et al., 2013b; Curti et al., 2017b, 2017a). An increase in the relative abundance of PopA (%) can be linked to the general decrease of water in bread crumb, that was lost or that partially migrated to crust and redistributed at a molecular level (Curti et al., 2017b). Moreover, the PopA (%) increase has been linked to amylopectin crystals formation (Bosmans et al., 2013b). A reduced amylopectin retrogradation and crystal formation, and a reduction in crumb water loss in fermented samples (as detected by DSC results for ΔH), may have accounted for the reduced PopA (%) in FF. However, DF was not significantly different from the control sample, even though DF showed the least ΔH-

starch values. This may indicate that a combination of factors may have an influence on PopA and that it's not linearly linked to only amylopectin retrogradation. At the same time, PopB was higher and more mobile in FF that control and DF thus indicating a higher mobility of the fraction associated with CH protons of amorphous starch and gluten in regions interacting with confined water (Bosmans et al., 2013b). Both ^{1}H FID PopB and ^{1}H CPMG PopC also showed a decreasing trend over time, in agreement with previous studies (Bosmans et al., 2013b).

Regarding ^{1}H CPMG PopD and PopE, there was an increase from day 0 to day 8 in PopD and a decrease in PopE over time, as previously reported Curti et al. (2017a). The increase in number of protons belonging to PopD (%) and contemporary decrease of protons belonging to PopE (%) indicated an increased system rigidity, possibly resulting from mobility decrease in the amorphous protons domain over storage (Curti et al., 2017a). PopE also showed a general mobility decrease over time for the control sample, showing decreasing tE values. In the literature, the increased rigidity of PopE over time is associated with the strengthening of the starch network due to amylopectin retrogradation and crumb macroscopic water loss (Bosmans et al., 2013b; Curti et al., 2014, 2017a). According to DSC and water status results, both phenomena occurred more markedly in the control sample than fermented samples. In the fermented samples, on the other hand, tE (ms) increased slightly over time, probably also due to reduced effects of increased amylopectin retrogradation and water loss; however, tE (ms) still tended to maintain lower values than in the control. In other words, the PopE in the fermented samples was generally more rigid than in the control sample. From the literature, a more rigid PopE is associated with the strengthening of the network between gluten-starch and water, which may depend on the type of protein-protein and protein-starch interactions, but also on a different distribution of water at the molecular level between gluten-starch. This may have led to a lower plasticity of gluten in fermented samples, which in turn was associated with higher crumb hardness. Indeed, the linear relationship between tE and crumb hardness has been discussed previously by Bosmans et al. (2013b) to motivate changes during staling, but the relationship could also apply to the effects of fermentation.

The fermented samples also showed a higher abundance of PopD (%) and an increase in its mobility (tD) compared with the control. Therefore, fermentation might have led to changes in the interaction between biopolymers and water at different molecular levels and a different

distribution of water between gluten and starch (Curti et al., 2017a), which resulted in an increase in gluten and exchanging protons of confined water, starch and gluten (PopD) at the expense of mobile exchanging protons of water, starch and gluten in the formed gel network (PopE).

The combination of the effects on PopD and PopE could explain the overall increase in hardness in fermented bread loaves compared to the control bread. The hypothesis behind is that since amylopectin crystals grew less and water loss was less in fermented samples than in the control, the higher rate of increase in hardness in the fermented samples could be due to a general increase in molecular rigidity, potentially also related to greater redistribution of water from gluten to starch, that may have affected the plasticity of gluten, resulting in an increased hardness rate..

Regarding the more mobile ^{1}H CPMG population (PopF), it was mainly attributed to lipid protons in both conventional and gluten-free bread formulations (Bosmans et al., 2013b; Hager et al., 2014; Carini et al., 2017; Curti et al., 2017b; Parenti et al., 2024). In our study, fermented samples showed higher PopF abundance (%), but with lower relaxation times than the control. The fat content was the same in all bread formulations and no major differences were expected following fermentation on fat properties. However, in some recent studies on bread dough and unheated and heated water-flour (Marchini et al., 2021a; Parenti et al., 2021; Chiodetti et al., 2024), a potential attribution of PopF to weakly bound water protons rather than fat has been proposed as a result of behaviors of this population at different water contents. PopF in our study could also be attributed to weakly bound water protons in bread. Indeed, the major differences in PopF among the samples seem to be evident, especially at the last day. In these samples, MC-crumb (%) was also higher at the day-8 of storage.

Conclusions

In this study, we investigated the effect of *Lacticaseibacillus casei* 4339 fermentation on the staling of wheat-sorghum bread using both fresh and freeze-dried fermented sorghum batter. Gluten weakening due to organic acid production and interaction with hydrophobic sorghum proteins resulted in reduced specific volume in fermented samples compared to control. However, freeze-drying did not negatively affect bread volume, which remained comparable to fresh fermented bread.

PCA analysis revealed distinct staling dynamics, with the process being most intense during the first four days and slowing down thereafter. Fermentation delayed water redistribution at macroscopic level possibly due to interactions between microbial enzymes and flour biopolymers that also altered amylopectin structure and reduced amylopectin retrogradation. Despite these benefits, fermentation resulted in increased crumb hardness over time, likely related to reduced specific volume and lower protein-starch-water domain mobility at the molecular level, than the control sample.

Overall, this study demonstrates that LAB fermentation can positively influence bread staling by slowing water loss and amylopectin retrogradation. Freeze-dried fermented sorghum may serve as a convenient and versatile ingredient to extend the shelf life of wheat-sorghum composite bread. Further studies on bread formulation optimization and sensory analysis over time could provide additional insights and improve the practical application of these findings in the development of higher quality, longer-lasting bread products.

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General Conclusions

I. Key Findings

This PhD thesis was structured into three main sections, each addressing specific knowledge gaps in the literature on enhancing sorghum flour's functionality through sprouting and fermentation, its application in wheat-based bread formulations, and the influence of these modifications on bread staling.

In particular, ***Section 1*** focused on the underexplored use of sprouting and fermentation to improve sorghum flour's properties. *Case Study 1* examined how different water contents and post-sprouting drying treatments influence sorghum's functionality, revealing that while extensive sprouting sometimes compromised starch performance, carefully controlled drying treatments enabled tailored functional properties, suited to diverse moisture levels in food products. This study underscored the potential of sprouted sorghum flour to enhance both the nutritional and technological value in food applications with varied moisture contents. Shifting to fermentation, *Case Study 2* investigated lactic acid bacteria (LAB) strains' effects on sorghum flour, finding that although the molecular structure of sorghum components changed, the chosen LAB strains did not modify the protein matrix in the absence of endogenous enzyme activity. However, changes in starch and fiber interactions within the matrix positively impacted technological functionalities such as viscosity, pasting, and thermal properties, positioning fermented sorghum as a valuable ingredient in breadmaking applications.

From **Section 1** to **Section 2** the Ph.D. thesis explored the integration of bioprocessed sorghum into wheat-based bread formulations. In particular, In *Case Study 3* sorghum flour fermented with LAB strains from *Case Study 2* was selected for breadmaking application. Fermented sorghum was incorporated into a wheat bread formulation to assess its impact on quality attributes critical to consumer perception. Findings indicated that fermented sorghum flour did not adversely affect gluten functionality or viscoelastic properties, aside from slight deviations at the lowest pH. However, in this case, the differences were still not perceived during the sensory acceptability

test, and instead, this bread was the most liked in terms of appearance, due to better crumb porosity and color, highlighting fermented sorghum's potential to enhance visual appeal and acceptability in composite breads. In light of the results obtained in *Case Study 3*, *Case Study 4* analyzed the influence of sorghum flour fermentation with the selected strain *Lacticaseibacillus casei* 4339 on the rheological and molecular properties of wheat-sorghum dough. This study provided insights into the impact of sorghum on the gluten matrix, evaluating the use of both fresh and freeze-dried fermented flour. Results demonstrated that fermentation and freeze-drying increased dough rigidity and modified rheological properties, with freeze-dried samples showing increased viscoelastic values, thereby contributing to the knowledge and applicability of using fermented sorghum in bakery applications.

Finally, **Section 3** addressed bread staling, a critical quality attribute, and examined sorghum addition's role and LAB fermentation's potential as a mitigation strategy. *Case Study 5* elucidated how sorghum flour influenced staling dynamics, revealing that weaker water-solid interactions and hydrophobic proteins and fibers from sorghum accelerated water mobility, biopolymer rigidity, and amylopectin retrogradation, leading to faster hardening. In the final *Case Study 6*, insights from earlier studies guided an assessment of the impact of sorghum fermentation with *Lacticaseibacillus casei* 4339 strain on staling in composite wheat bread. This study also assessed freeze-dried fermented flour's effect on staling. Results revealed two distinct influences of fermentation on staling: while fermented sorghum reduced amylopectin retrogradation and prolonged bread freshness by enhancing water mobility, it did not prevent an increase in hardness over time, as demonstrated by the increased rigidity at the molecular level within the protein-starch-water network matrix.

II. Future Perspectives

In summary, this thesis demonstrated that fermentation of sorghum flour with selected LAB strains significantly improved some quality attributes of composite wheat-sorghum bread. Further research should explore additional mechanisms to enhance the functionality of sorghum proteins, potentially reducing the molecular rigidity that contributes to increased bread firmness. Combinations of sprouting and fermentation or the integration of enzymatic treatments may present promising avenues for future research into sorghum's applications. Moreover, as some

instrumental changes in bread quality were not discernible to consumers in sensory evaluations, future studies could benefit from extending sensory testing over storage periods. This would clarify whether changes in staling dynamics affect consumer perception over time, helping to identify key drivers in consumer acceptability during storage.

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About the author

Miriam Chiodetti was born in Civita Castellana (Viterbo, Italy) on April 13, 1996. Miriam obtained a Bachelor's Degree in Food Science and Technology from the University of Tuscia and a Master's Degree in Food Science and Technology from the University of Parma, both with the highest marks. She joined the Food Technology team at the University of Parma as a Ph.D. student in November 2021. Her research mainly focused on sustainable food innovation, in particular on improving the quality of cereal-based products through bioprocessing methods such as sprouting and fermentation.

During her PhD, she presented her research results at various conferences in countries all over Europe. She also collaborated with the Grain Technology Group at the University of Helsinki as a Visiting PhD Researcher in the European HealthFerm project, where she worked on novel fermentation processes for pulses and grains to improve food quality and nutritional value. During her PhD, Miriam also contributed as a scientific reviewer for the International Journal of Food Science & Technology, supporting the peer review process to maintain high standards in food science publications.

Conferences and publications

Contributions to National and International Congresses

Poster contributions

Chiodetti, M., Carini, E. "*Gelatinization properties of sprouted sorghum flour over a wide range of water contents* ". 36th EFFOST International Conference. 7-9 November 2022, Dublin, Ireland.

Chiodetti, M., Carini, E. "*The effect of sorghum flour on bread staling: from the macroscopic to the molecular properties*". 37th EFFOST International Conference. 6-8 November 2023, Valencia, Spain.

Chiodetti, M. "*Bio-sustainable technologies to produce raw materials and food products with improved physico-chemical, nutritional and safety properties*". 26th Workshop on the Developments in the Italian PhD Research on Food Science and Technology, 19-21 September 2022, Asti, Italy.

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Oral contributions

Chiodetti, M., Monica, S., Bancalari, E., Bottari, B., Carini, E. "Effect of selected lactic acid bacteria fermentation on sorghum techno-functional properties". FOODBALT Conference 2023. 11-12 May 2023, Jelgava, Latvia.

Chiodetti, M., Monica, S., Bancalari, E., Bottari, B., Ricci, S., Gigliotti, M., Carini, E. "Effect of lactic acid bacteria fermentation on the technological properties of sorghum composite bread". 37th EFFOST International Conference. 6-8 November 2023, Valencia, Spain.

Chiodetti, M. "*Promoting sorghum for breadmaking through LAB fermentation: from fermented flour to bread staling*". 28th Workshop on the Developments in the Italian PhD Research on Food Science and Technology. 13-15 September 2023, Catania, Italy.

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In progress manuscripts

Chiodetti, M., Monica, S., Gigliotti, M., Bottari, B., Bancalari, E., Fuso, A., Prandi, B., Tedeschi, T., Carini, E. Effect of lactic acid fermentation on the molecular and technological properties of sorghum batters (to be submitted).

Chiodetti, M., Monica, S., Ricci, S., Bottari, B., Bancalari, E., Cirlini, M., Carini, E. Selected LAB fermentation on crumb structure development, appearance, volatile and sensory properties of wheat-sorghum bread (to be submitted).

Chiodetti, M., Gigliotti, M., Monica, S., Bottari, B., Bancalari, E., Carini, E. ^{1}H molecular mobility and rheological properties of wheat - fermented sorghum (fresh and freeze-dried) dough (to be submitted).

Gigliotti, M., Chiodetti, M., Carini, E. The effect of sorghum flour (*Sorghum bicolor* [l.] Moench) on bread staling: texture, thermal properties and ^{1}H molecular mobility (to be submitted).

Chiodetti, M., Monica, S., Bottari, B., Bancalari, E., Carini, E. Effect of fermentation with *Lacticaseibacillus casei* on wheat-sorghum bread staling – reduction of water mobility over time and amylopectin retrogradation (to be submitted).

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Participation at European projects

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