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TESIS DOCTORAL:

Desarrollo y caracterización de cervezas artesanales elaboradas con excedente de pan como sustituto parcial de malta.

Presentada por Carlos Martín Lobera para optar al grado de Doctor por la Universidad de Valladolid

Dirigida por:
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DOCTORAL PROGRAMME IN AGROFOOD SCIENCE AND ENGINEERING AND BIOSYSTEMS

PhD THESIS

Development and characterization of craft beers brewed with surplus bread as a partial malt substitute.

Submitted by Carlos Martín Lobera in fulfilment of the requirements for the doctoral degree (PhD) at the University of Valladolid

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“Permanence, perseverance and persistence in spite of all obstacles, discouragement, and impossibilities…, distinguish the strong soul from the weak.”

Thomas Carlyle

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RESUMEN

En los últimos años, el desperdicio alimentario se ha convertido en un reto prioritario para la sostenibilidad de los sistemas agroalimentarios, y el pan duro o de desecho constituye una de las fracciones más relevantes dentro de los residuos alimentarios en numerosos países. De forma paralela, la industria cervecera depende de la malta de cebada como materia prima principal, cuyo procesado implica un consumo significativo de recursos. En este contexto, la valorización de pan duro como materia prima cervecera representa una estrategia de interés para avanzar hacia modelos de producción más eficientes, reduciendo residuos y favoreciendo el aprovechamiento de recursos dentro de un enfoque de economía circular. La presente tesis doctoral evalúa la viabilidad tecnológica, nutricional y sensorial de emplear pan duro —con especial atención al pan integral— como sustituto parcial de malta en distintos estilos de cerveza, así como el efecto de esta estrategia sobre la calidad del producto durante el almacenamiento.

Para ello, se diseñó y validó un método de elaboración específico que permite incorporar pan duro, hasta un 50% como sustituto parcial de malta en la formulación cervecera, garantizando condiciones controladas de procesado y reproducibles entre elaboraciones. El trabajo experimental se desarrolló en tres fases interconectadas, durante algo más de 4 años de investigación. En primer lugar, se realizó un estudio comparativo utilizando una cerveza tipo pale ale como modelo, en el que se sustituyó el 50% de la malta por diferentes tipos de pan duro (blanco, integral, centeno y maíz), con el objetivo de seleccionar el tipo de pan más adecuado. Posteriormente, en base a la evidencia obtenida, se seleccionó el pan integral y se aplicó una sustitución fija del 50% de la malta en tres estilos claramente diferenciados (American lager, India pale ale y Bavarian weiss), elaborando en cada caso una cerveza control (100% malta) y su equivalente con pan. En estas cervezas se caracterizó el comportamiento fermentativo y se analizaron parámetros fisicoquímicos, color y turbidez, contenido fenólico, capacidad antioxidante, perfil volátil y calidad sensorial. Finalmente, para evaluar la estabilidad a largo plazo, las cervezas elaboradas con y sin pan integral se almacenaron durante 12 meses a 15 °C, monitorizando al inicio y al final del periodo la evolución de parámetros fisicoquímicos, microbiológicos, fenólicos, antioxidantes y sensoriales.

En conjunto, los resultados mostraron que la sustitución parcial de malta por pan duro es técnicamente viable a nivel de proceso y permite obtener cervezas con características coherentes con las expectativas de cada estilo, manteniendo un rendimiento fermentativo comparable al de las cervezas elaboradas únicamente con malta. El pan integral se consolidó como la alternativa más favorable al combinar un comportamiento tecnológico adecuado con mejoras en el perfil de compuestos bioactivos: las cervezas elaboradas con pan presentaron incrementos consistentes del contenido en polifenoles totales y, en consecuencia, una capacidad antioxidante igual o superior a la de los controles. Los efectos sobre el color y la turbidez fueron dependientes del estilo, observándose variaciones atribuibles tanto a la naturaleza del pan como a las particularidades de cada formulación. El análisis del perfil volátil confirmó que la sustitución parcial por pan no compromete la complejidad aromática, y la evaluación

sensorial evidenció que la calidad organoléptica se mantiene y, en determinados atributos, puede verse reforzada. Durante el almacenamiento, las cervezas con pan integral mostraron una mayor retención de compuestos fenólicos y de actividad antioxidante, junto con una evolución fisicoquímica compatible con la estabilidad del producto y sin comprometer la seguridad microbiológica. En conclusión, el empleo de pan duro —especialmente pan integral— como sustituto parcial de malta constituye una alternativa sólida para reducir el uso de malta, valorizar residuos de panificación y desarrollar cervezas con un perfil nutricional y de estabilidad mejorado, ofreciendo una vía aplicable para impulsar la sostenibilidad en la producción cervecera.

ABSTRACT

In recent years, food waste has become a major challenge for the sustainability of agri-food systems, and stale or discarded bread represents one of the most significant fractions of food waste in many countries. At the same time, the brewing industry relies on barley malt as its main raw material, the production and processing of which requires considerable resources. In this context, the recovery of stale bread as a raw material for beer production is a promising strategy to support more efficient models, reduce waste, and promote resource recovery within a circular economy framework. This doctoral thesis evaluates the technological, nutritional and sensory feasibility of using stale bread—particularly whole wheat bread—as a partial substitute for malt in different beer styles, and examines the impact of this strategy on product quality during storage.

To this end, a specific brewing method was designed and validated to enable the incorporation of stale bread—up to 50%—as a partial malt substitute in the brewing recipe, ensuring controlled processing conditions and reproducibility across batches. The experimental work was carried out in three interconnected phases. First, a comparative study was conducted using a pale ale as a model beer, in which 50% of the malt was replaced with different types of stale bread (white, whole wheat, rye and corn) to identify the most suitable bread type. Subsequently, based on the results obtained, whole wheat bread was selected and a fixed substitution level of 50% of the malt was applied in three clearly differentiated beer styles (American lager, India pale ale, and Bavarian weiss), producing in each case a control beer (100% malt) and its corresponding bread beer. For these beers, fermentation performance was characterized, and physicochemical parameters, color, turbidity, phenolic content, antioxidant capacity, volatile profile, and sensory quality were analyzed. Finally, to evaluate long-term stability, beers brewed with and without whole wheat bread were stored for 12 months at 15 °C, and the evolution of physicochemical, microbiological, phenolic, antioxidant, and sensory parameters was monitored at the beginning and at the end of the storage period.

Overall, the results showed that partial substitution of malt with stale bread is technically feasible at the process level and allows the production of beers consistent with the expected characteristics of each style, while maintaining fermentation performance comparable to that of beers brewed exclusively with malt. Whole wheat bread emerged as the most favorable option, combining appropriate technological performance with improvements in the bioactive profile: bread beers showed consistent increases in total polyphenol content and, consequently, antioxidant capacity equal to or greater than that of the controls. The effects on color and turbidity were style-dependent, reflecting both the nature of the bread and the specific formulation of each beer. Volatile analysis confirmed that partial substitution with bread does not compromise aromatic complexity, and sensory evaluation indicated that overall organoleptic quality is maintained and, for certain attributes, may even be improved. During storage, beers brewed with whole wheat bread exhibited greater retention of phenolic compounds and antioxidant activity, alongside a physicochemical evolution compatible with product stability and without compromising microbiological safety. In conclusion, the use of stale bread—especially whole wheat bread—as a partial substitute for malt constitutes a robust alternative to reduce malt usage, valorize bread waste, and produce beers with an improved nutritional profile and enhanced stability, providing an applicable pathway to strengthen sustainability in brewing.

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I. INTRODUCTION

I. INTRODUCTION

1.1. Food waste, bread surplus and circular economy in agro-food systems

Global food systems are currently under intense pressure to provide affordable, safe and nutritious food while reducing their environmental footprint. Nevertheless, a substantial proportion of food produced worldwide is never consumed. Recent international reports estimate that around 14% of food is lost along the supply chain before reaching retail, and a further 17% is wasted at the retail and consumer levels, totalling more than 900 million tonnes of food waste every year (FAO, 2022; UNEP, 2021). This situation has important environmental, economic and ethical implications, and it has led to the adoption of the circular economy as a guiding paradigm for future food systems.

Within this framework, the valorisation of food waste and by-products has become a strategic priority. Bread is particularly relevant in this context because it is one of the most widely consumed staple foods in many countries and, at the same time, one of the products most frequently discarded at the retail and household. Due to its high moisture content and soft structure, bread is highly perishable and very susceptible to staling and microbial spoilage, which leads to large volumes of unsold or leftover loaves every day (Martin-Lobera et al., 2022). Estimating the exact amount of wasted bread is challenging, but available data indicate that hundreds of tons may be discarded daily worldwide, representing a loss of valuable nutrients and embedded resources.

From a compositional point of view, bread is a starch-rich matrix that also contains relevant amounts of protein and minor bioactive compounds. Typical bread residues contain more than 70% starch and up to 14% protein on a dry matter basis, together with minerals, lipids and fibre fractions (Martin-Lobera et al., 2022; Kumar et al., 2023). Unlike lignocellulosic feedstocks, which usually require severe physical or chemical pre-treatments, bread can be readily hydrolysed by amylases, amyloglucosidases and proteases, yielding fermentable sugars and nitrogen sources that are easily accessible for microorganisms. This makes surplus bread an attractive substrate for different biorefinery routes, including the production of biofuels, organic acids, enzymes, biomass or functional food ingredients (Kumar et al., 2023).

In recent years, bread waste has been increasingly viewed not only as a disposal problem but as a valuable secondary resource that can be reintegrated into the food chain. The concept of circular biorefineries proposes using bread as a feedstock for higher value-added applications, prioritising food and feed uses over energy recovery whenever possible (Kumar et al., 2023). In this context, brewing has emerged as a particularly promising avenue for bread valorisation. Because beer production is traditionally based on cereal-derived carbohydrates and relies on enzymatic starch hydrolysis followed by alcoholic fermentation, the replacement of part of the malted grain with bread offers a technically compatible strategy to reduce food waste while maintaining product quality. This approach has attracted growing interest from both craft and industrial breweries, aligning with broader sustainability goals and consumer demand for environmentally responsible products (McDonagh et al., 2024; Dall'Acqua et al., 2025; Martin-Lobera et al., 2022).

1.2. Beer production, craft beer sector and quality attributes

Beer is one of the oldest and most widely consumed alcoholic beverages in the world, traditionally produced from malted cereals, hops, yeast and water. Conventional brewing can be divided into a sequence of technological stages that include malting, mashing, wort boiling, fermentation, maturation, stabilization and packaging (Shopska et al., 2022; Díaz-Sánchez et al., 2022). During malting, controlled germination and kilning of barley or other cereals activate endogenous enzymes and modify the endosperm structure, preparing the grain for efficient starch and protein hydrolysis during mashing (Thesseling et al., 2019). In the mashing step, milled malt and possible adjuncts are mixed with hot water under specific temperature rests, allowing amylolytic and proteolytic enzymes to convert starch into fermentable sugars and proteins into peptides and amino acids. The resulting sweet wort is then separated from the solid grain fraction, boiled with hops to isomerise α-acids and extract hop-derived aroma compounds, clarified and cooled before yeast pitching (Shopska et al., 2022; Beer Production by Fermentation Process, 2023). Alcoholic fermentation converts fermentable sugars into ethanol and carbon dioxide while generating a complex profile of secondary metabolites that largely define beer flavor and aroma. Finally, maturation, filtration and packaging complete the process and ensure product stability (Díaz-Sánchez et al., 2022; Shopska et al., 2022).

Each ingredient plays a specific and essential role in determining beer quality. Malted barley is traditionally the main source of fermentable extract and contributes to color, body, foam stability and flavor-active Maillard products (Thesseling et al., 2019; Díaz-Sánchez et al., 2022). Hops supply bitterness through iso-α-acids, impart characteristic herbal, citrus or resinous aromas and provide antioxidant and antimicrobial compounds. Yeast not only performs alcoholic fermentation but also produces higher alcohols, esters, organic acids and sulphur compounds that shape the sensory profile. Water composition influences mash pH, enzymatic activity, hop utilisation and mouthfeel. In addition to these four traditional ingredients, modern breweries frequently employ adjuncts such as unmalted cereals, sugars, fruits or spices to adjust extract composition, flavor, color, cost or nutritional profile (Shopska et al., 2022). Within this group of adjuncts, cereal-based by-products and side streams from the food industry have attracted increasing attention as potential raw materials that can improve sustainability without compromising quality.

Over the last two decades, the beer sector has experienced a profound transformation due to the rapid expansion of the craft beer movement. Although the precise legal definition of "craft beer" varies among countries, common features include small-scale and independent production, emphasis on traditional brewing techniques and a strong focus on product differentiation through distinctive flavors, ingredients and styles (Lerro et al., 2020; Faganel, 2023; Garavaglia, 2025). Craft breweries have contributed to diversifying the beer market by reinterpreting classic styles and creating novel ones, often experimenting with non-conventional grains, fruits, herbs, barrel ageing and mixed fermentations. Despite signs of market saturation and regional slowdowns, the craft beer segment remains a dynamic and innovative part of the global beer industry, with a substantial economic impact and an important role in local and regional identities (Faganel, 2023; The Brainy Insights, 2025).

This evolution has also reshaped the way beer quality is understood. From a technological perspective, key quality attributes include appropriate alcohol content and real extract, adequate attenuation, correct pH, color and turbidity, foam stability and microbiological safety (Bamforth, 2017; Díaz-Sánchez et al., 2022). These parameters ensure process efficiency, regulatory compliance and product stability. From a nutritional and functional standpoint, beer contains a variety of micronutrients and secondary metabolites derived mainly from malt and hops, such as minerals, B-group vitamins and polyphenols. These polyphenols contribute to antioxidant capacity and may play a role in the potential health effects of moderate beer consumption (Thesseling et al., 2019; Lerro et al., 2020). In parallel, there is growing interest in enhancing the content of bioactive compounds or incorporating ingredients perceived as more wholesome, such as whole grains or fibre-rich materials, while controlling alcohol content and caloric value.

Sensory quality remains central to consumer acceptance. Appearance (color, clarity, foam), aroma, flavor, mouthfeel and overall balance are the main factors that determine taste and preference, especially in the craft beer segment, where consumers often seek more intense, complex and differentiated flavor profiles (Lerro et al., 2020; Barbagallo et al., 2025). Studies show that craft beer is generally perceived as higher-quality, more genuine and more natural than conventional lager, and consumers are willing to pay a premium for products that offer distinctive sensory experiences and align with values such as authenticity and sustainability (Lerro et al., 2020; Bimbo et al., 2023). Consequently, any technological innovation in brewing, including the use of alternative raw materials or food by-products, should be evaluated not only in terms of process feasibility and analytical parameters, but also in relation to its impact on the sensory profile and consumer acceptance.

In this context, the replacement of a portion of malt with surplus bread represents an attractive strategy because it directly addresses food waste reduction while potentially modifying beer quality attributes in a controlled manner. Surplus bread provides readily fermentable carbohydrates, proteins and minor components that can influence extract, color, flavor and the content of phenolic compounds. However, its incorporation into brewing formulations must be carefully optimised to maintain desirable physicochemical, nutritional and sensory properties across different beer styles and during storage. Addressing these challenges is one of the central motivations of the research presented in this doctoral thesis, which systematically investigates the use of bread—particularly whole wheat bread—as a malt substitute and its effects on beer quality in pale ale, lager, India pale ale and Bavarian weiss beers, as well as on their long-term stability.

1.3. Polyphenols, antioxidants and nutritional aspects of beer and bread

Beyond its role as an alcoholic beverage, beer is a source of several nutrients and bioactive compounds derived mainly from malt and hops. On a typical serving basis, beer provides carbohydrates, a moderate amount of alcohol, small quantities of proteins and amino acids, minerals such as potassium, magnesium and silicon, and B-group vitamins originating from yeast and malt (Estruch, 2021; Yang & Gao, 2021). However, one of the most relevant groups of compounds from a nutritional and functional perspective are polyphenols. These secondary metabolites include phenolic acids, flavonoids, tannins and other related structures that are extracted from the malted grains and hops during mashing and wort boiling (Martínez-Gómez et al., 2020; Šibalić et al., 2021). Polyphenols contribute to beer flavor, color and colloidal stability, and they are also responsible for a significant fraction of its in vitro antioxidant capacity (Nardini, 2023; Habschied et al., 2021).

The polyphenol profile of beer is strongly influenced by the type of cereal and malt used, kilning conditions, hopping regime and process variables. Darker malts and specialty malts typically increase the content of Maillard-derived compounds and melanoidins, which can exhibit antioxidant activity and metal-chelating properties (Martínez-Gómez et al., 2020). In addition, hop-derived polyphenols and iso-α-acids contribute to bitterness and may modulate oxidative stability and foam properties (Krofta et al., 2008; Mikyška et al., 2022). As a result, beers differ widely in their total polyphenol content (TPC) and antioxidant capacity, with some styles, such as darker ales or strongly hopped beers, generally showing higher values than pale lagers (Zhao et al., 2013; Piazzon et al., 2010; Bertuzzi et al., 2020; Nardini & Foddai, 2020; Mitić et al., 2014).

Bread also constitutes an important dietary source of carbohydrates, proteins and bioactive compounds, particularly when whole grain flour is used. In whole wheat bread, the inclusion of bran and germ fractions increases the content of dietary fibre, minerals, vitamins and phenolic compounds compared with refined white bread (Adebo & Medina-Meza, 2020; Zhang et al., 2023). Many of these phenolics are bound to cell wall components and are released to some extent during processing and digestion, contributing to the overall antioxidant capacity (Zhang et al., 2023). Whole-grain products and fibre-rich foods have been associated with potential health benefits, including reduced risk of cardiovascular disease, type 2 diabetes and certain cancers, largely attributed to their content of fibre and phenolic compounds (Dahl & Stewart, 2015; Stephen et al., 2017). In contrast, refined bread, made from white flour, contains lower levels of fibre and phenolics, which may result in a reduced contribution to overall antioxidant intake (Adebo & Medina-Meza, 2020).

When surplus bread is incorporated into brewing formulations, these compositional differences become highly relevant. The type of bread (white vs. whole wheat vs. other cereal breads), the proportion of substitution and the technological conditions can influence the transfer of phenolic compounds and other bioactive substances from bread to wort and beer. In this context, the first study included in this doctoral thesis evaluated the use of different types of bread (white, whole wheat, rye and corn) to replace 50% of the malt in a pale ale beer. The results showed that beers brewed with whole wheat bread displayed significantly higher total polyphenol content and antioxidant capacity than the control beer and beers made with other types of bread, while maintaining appropriate physicochemical parameters and acceptable sensory quality (Martin-Lobera

et al., 2022). Similar increases in TPC and antioxidant activity have been reported in other bread-enriched or phenolic-rich beers, confirming that formulation can be used to modulate the bioactive profile of beer (Habschied et al., 2020; Nardini & Foddai, 2020; Piazzon et al., 2010). This provided a first indication that the careful choice of bread type can be used to enhance the nutritional-related properties of beer, particularly in terms of phenolic content and antioxidant potential.

These findings were further explored in the subsequent studies of the thesis, where whole wheat bread was selected as the most suitable bread type and used at a 50% substitution level in different beer styles. By analyzing beers such as American lager, India pale ale and Bavarian weiss, and by assessing their physicochemical, nutritional and sensory attributes, both at bottling and after long-term storage. The research aimed to determine to what extent whole wheat bread can systematically improve the polyphenol content and antioxidant capacity of beers without compromising quality across a range of styles, including 12 months of storage (Martin-Lobera et al., 2025a, 2025b). In this way, the nutritional and functional dimension of bread-enriched beers becomes a central focus of the present doctoral thesis directly aligned with current trends towards more sustainable and health-oriented alcoholic beverages (Nardini, 2023; Yang & Gao, 2021).

1.4. Valorisation of surplus bread: current strategies and focus on brewing

Bread surplus has attracted growing attention as a target for valorisation within circular bioeconomy strategies. Traditionally, unsold or surplus bread has been used to low-value uses, mainly as animal feed or, in many cases, simply discarded or sent to landfill. Both options represent a loss of resources embedded in bread production—such as cereals, water and energy—and contribute to environmental pressures associated with waste management (Brancoli et al., 2020; Ben Rejeb et al., 2022). Recent estimates indicate that bread is among the most wasted food products in industrialised countries, with hundreds of tonnes discarded daily worldwide, largely due to its short shelf life and consumer preference for freshly baked products (Katajajuuri et al., 2014; Martin-Lobera et al., 2022). Within the broader framework of food waste hierarchies, prevention and reuse for human consumption are prioritised over animal feed, material recycling and energy recovery, which has stimulated research into higher value applications for surplus bread (European Commission, 2020; Kumar et al., 2023; Hafyan et al., 2024).

To address this challenge, a wide range of technological approaches has been explored to transform surplus bread into higher value products. Non-food biorefinery routes include the production of bioethanol, biogas, succinic acid and other organic acids, microbial biomass, enzymes and platform chemicals using bread as a readily fermentable substrate (Leung et al., 2012; Pietrzak & Kawa-Rygielska, 2014; Cerda et al., 2016; Narisetty et al., 2021). Bread residues contain more than 70% starch and up to 14% protein on a dry matter basis, and treatment with amylases, amyloglucosidases and proteases enables efficient release of fermentable sugars and nitrogen sources, which makes bread a more accessible substrate than lignocellulosic feedstocks requiring harsh pre-treatments (Narvhus & Sørhaug, 2006; Martin-Lobera et al., 2022; Kumar et al., 2023). Other studies have proposed the use of hydrolysed bread waste as a growth medium for baker's yeast or other microorganisms, further broadening the portfolio of possible biotechnological applications (Benabda et al., 2018).

In parallel to these non-food biorefinery routes, several strategies have aimed at reintroducing surplus bread into the human food chain. One approach has been its use as an ingredient in reformulated bakery products, such as crackers, biscuits or new bread types, by incorporating dried and milled bread into dough formulations (Narvhus & Sørhaug, 2006; Verni et al., 2020; Ben Rejeb et al., 2022). This can partially close the loop within bakery operations, although the proportion of replacement is often limited by dough rheology, loaf volume and sensory constraints. Other developments include the incorporation of bread crumbs or flours into extruded snacks, as a way of reducing bakery waste and creating new cereal-based products, or their use in batters, coatings and meat products, where they may contribute to texture, color and flavor (Samray et al., 2019; Ben Rejeb et al., 2022). While these applications help to reduce waste and can add value, they usually require significant reformulation and are often constrained to specific product categories or local supply chains, which limits the overall volume of surplus bread that can be valorized.

In this context, brewing has emerged as a particularly promising avenue for bread valorisation. From a technological perspective, there is a strong compatibility between bread and traditional brewing raw materials: both are based on cereal-derived starch that can be enzymatically hydrolyzed into fermentable sugars, and bread also contributes proteins and other nitrogenous compounds that can support yeast growth (Martin-Lobera et al., 2022; Kumar et al., 2023). The fact that bread has already undergone baking implies that most of the starch is gelatinized, which may facilitate enzymatic access during mashing when combined with malt. Early studies demonstrated that waste bread can be used to produce ethanol and other fermentation products, and more recent work has specifically examined its use as a partial substitute for malted barley in brewing (Almeida et al., 2018; Brancoli et al., 2020; Narisetty et al., 2021). Environmental assessments suggest that diverting surplus bread to brewing reduces the global warming potential compared with conventional waste management options, particularly when high substitution levels are achieved and decomposition in landfills is avoided (Brancoli et al., 2020; McDonagh et al., 2024).

Several recent studies have explored the practical feasibility of brewing with surplus bread. Almeida et al. (2018) reported that a craft beer brewed with waste bread had approximately 20% lower carbon footprint than a control beer produced with 100% malt. Brancoli et al. (2020) evaluated different treatment options for surplus bread and highlighted beer production as an environmentally preferable route within a portfolio of valorisation pathways. Narisetty et al. (2021) studied the replacement of malt with bread and concluded that, under their conditions, up to 25% of the malt could be substituted without compromising fermentability due to the need for endogenous malt enzymes. Similarly, brewing trials using bakery leftovers as malt substitutes have also been reported (Dymchenko et al., 2023), and more recently, McDonagh et al. (2024) showed that substituting up to 60% of malted barley with waste breadcrumbs can still yield beers with sufficient alcohol content, while reducing the overall carbon footprint of beer production. Dall'Acqua et al. (2025) evaluated the impact of using wasted bread as a brewing adjunct in ale beer, finding no major differences between control and bread beers in most physicochemical and sensory parameters. Together, these works provide increasing evidence that bread can be used as a malt substitute from both technological and environmental perspectives, although the optimal substitution level and the influence of bread type and beer style are still under active investigation.

Beyond technical feasibility, brewing with surplus bread also offers strong symbolic and communication potential in the context of sustainability. A single batch of beer can upcycle considerable amounts of bread that would otherwise be discarded, and this message is easily understood and valued by consumers who are increasingly concerned about food waste and environmental impact (Almeida et al., 2018; Connolly, 2019; Ben Rejeb et al., 2022). For craft breweries in particular, the use of surplus bread aligns well with their emphasis on local sourcing, innovation and storytelling, providing a tangible example of circular economy in practice and differentiating their products in a competitive market. However, for this valorisation route to be viable at industrial scale, it is essential to demonstrate that partial malt substitution with bread can be implemented without compromising key quality attributes of beer—physicochemical, nutritional and sensory—across different styles and over shelf-life.

Addressing these questions constitutes the core focus of the present doctoral thesis. In the first study, different types of stale bread (wheat, whole wheat, rye and corn) were tested as partial malt substitutes in pale ale, showing that up to 50% of malt can be replaced and that beers brewed with whole wheat bread displayed higher total polyphenol content and antioxidant capacity than the control (Martin-Lobera et al., 2022). Subsequent studies used whole wheat bread, at the same substitution level, to produce American lager, India pale ale and Bavarian weiss beers, and later assessed their long-term stability during storage, revealing that bread-enriched beers can maintain or even improve nutritional-related and sensory properties without compromising physicochemical, microbiological or colloidal stability (Martin-Lobera et al., 2025a; 2025b). These contributions position surplus whole wheat bread not only as a sustainable raw material for brewing, but also as a functional ingredient capable of improving beer quality within a circular economy framework.

1.5. Brewing with bread as a malt substitute: from proof-of-concept to multi-style beers

For many years, the idea of using bread as a brewing adjunct remained at the level of isolated initiatives or small-scale experiments, often motivated by cost reduction or the availability of surplus bread streams (Almeida et al., 2018; Connolly, 2019). In most of these early approaches, bread was incorporated at relatively low substitution levels and with limited analytical or sensory characterization, making it difficult to draw firm conclusions about its technological feasibility and impact on beer quality (Brancoli et al., 2020; Narisetty et al., 2021). Moreover, formulations were typically limited to a single beer style, and the characteristics of the bread itself (type of flour, presence of whole grains, baking conditions) were not systematically considered. As a result, the scientific basis for using bread as a partial malt substitute has historically been fragmented and largely empirical (Martin-Lobera et al., 2022).

In the context of circular economy and food waste reduction, there is a need to move beyond anecdotal experiences and develop a more rigorous understanding of how different types and levels of bread inclusion affect brewing performance and beer properties. Bread differs from malt not only in its processing history, but also in terms of starch structure, protein state, fibre content and the presence of additional ingredients such as fats, sugars or improvers (Narvhus & Sørhaug, 2006; Kumar et al., 2023). These differences may influence mash viscosity, extract yield, fermentability, foam stability

and flavor (Villacreces et al., 2022; Habschied et al., 2021). At the same time, bread—especially when made from whole grains—can contribute additional phenolic compounds and other bioactives that are of interest from a nutritional perspective (Piazzon et al., 2010; Nardini, 2023). Therefore, systematic studies are required to determine the conditions under which bread can replace a substantial fraction of malt without impairing, and potentially even improving, the physicochemical, nutritional and sensory quality of beer (Martin-Lobera et al., 2022; Dall'Acqua et al., 2025).

The first experimental study included in this doctoral thesis was designed precisely as a proof-of-concept to address some of these questions. In that work, different types of bread (white, whole wheat, rye and maize) were used to replace 50% of the malt grist in a pale ale formulation, while keeping the overall brewing process as close as possible to conventional practice. The beers obtained were evaluated in terms of general physicochemical parameters (alcohol content, original and real extract, pH, color, turbidity), total polyphenol content, antioxidant capacity and sensory properties. The results demonstrated that such a high level of malt substitution is technologically feasible and can yield beers that meet typical quality requirements. Importantly, beers brewed with whole wheat bread showed significantly higher polyphenol content and antioxidant capacity than the control beer and those produced with other bread types, while maintaining an overall acceptable sensory profile (Martin-Lobera et al., 2022). This study thus provided a first evidence-based indication that the choice of bread type is critical and that whole wheat bread is particularly promising as a brewing adjunct.

Based on these findings, the second study of the thesis moved from a single-style proof-of-concept to a broader evaluation across different beer styles. In this case, whole wheat bread was selected as the most suitable bread type and was used to replace 50% of the malt in three distinct beer styles: American lager, India pale ale (IPA) and Bavarian weiss. These styles were chosen because they differ significantly in terms of raw material composition, hopping regime, yeast strain and sensory expectations, thereby providing a robust test of the versatility of bread-based formulations. The beers were characterized by their physicochemical properties, color and turbidity, total polyphenol content, antioxidant capacity, volatile profile obtained by HS-SPME-GC-MS and sensory attributes. The results indicated that the incorporation of whole wheat bread at this substitution level is compatible with all three styles, preserving key technological parameters and, in several cases, enhancing the content of phenolic compounds and antioxidant capacity relative to the corresponding control beers without bread (Martin-Lobera et al., 2025a). Similar observations have been reported in other recent works where waste bread was used as a brewing adjunct, confirming that relatively high substitution levels can be reached while maintaining beer quality (McDonagh et al., 2024; Dall'Acqua et al., 2025).

Together, these two-studies trace progression from the initial demonstration that substantial malt replacement with bread is possible in a single pale ale beer to a more comprehensive assessment of whole wheat bread as a functional adjunct in multiple beer styles. They show that surplus bread, when carefully selected and incorporated, can be used not only to reduce food waste but also to modulate the nutritional-related and sensory properties of beer in a controlled way (Almeida et al., 2018; Brancoli et al., 2020; Martin-Lobera et al., 2022, 2025a). At the same time, they raise new questions regarding how such formulations behave during storage and whether the observed advantages in terms of polyphenol content, antioxidant capacity and sensory profile are maintained

over the product's shelf-life. These aspects are addressed in the third experimental study of the thesis, which focuses on the long-term stability of bread-enriched beers (Martin-Lobera et al., 2025b).

1.6. Long-term stability of beer: physicochemical, microbiological and sensory dimensions

Beer is an inherently unstable product whose properties change over time as a result of physical, chemical and microbiological processes. From a technological perspective, beer stability is often described along two main dimensions: ageing stability, Which refers to sensory changes that occur during storage, and haze (or colloidal) stability, related to the appearance of turbidity or sediment formation (Bamforth, 2011; Vanderhaegen et al., 2006). Ageing stability is mainly determined by oxidative and non-oxidative reactions that modify the concentration of active flavor compounds, while haze stability is associated with colloidal phenomena involving proteins, polyphenols, polysaccharides and other macromolecules (Wang et al., 2021; Jongberg et al., 2020).

During storage, beer tends a gradual loss of freshness characterized by decreased hop aroma, changes in bitterness quality and the development of "stale" or "oxidised" notes. These sensory changes are related to the formation and accumulation of carbonyl compounds (such as aldehydes), the degradation of hop-derived volatiles and transformations in higher alcohols and esters (Vanderhaegen et al., 2006; Baert et al., 2012). Temperature and oxygen exposure are recognised as key external drivers of these processes: higher storage temperatures and the presence of dissolved or headspace oxygen accelerate the formation of staling compounds and drastically shorten shelf-life (Lehnhardt et al., 2018; Ditrych et al., 2024). Consequently, both industrial and craft breweries pay close attention to packaging conditions, oxygen pick-up and cold-chain management in order to preserve flavor stability. Typical commercial life of packaged beers range from a few months to one year, depending on style, packaging material and storage conditions (Bamforth, 2011; Klimczak et al., 2024).

Colloidal stability is another crucial aspect of beer quality. Non-biological haze is mainly caused by the formation of insoluble complexes between haze-active proteins and polyphenols, as well as by residual starch, β-glucans and other high-molecular-weight carbohydrates (Wang et al., 2021; Jongberg et al., 2020). Biological haze, in contrast, is caused by suspended yeast or contaminating microorganisms. Excessive haze formation may negatively affect visual appearance and consumer perception, particularly in styles expected to be bright and brilliant, although in some modern beer categories (e.g., hazy IPAs or certain wheat beers) a certain degree of turbidity is desirable (Oxford Companion to Beer, 2011; Mastanjević et al., 2018). In any case, controlling the balance between haze-active and haze-protective components, together with appropriate filtration, stabilization or clarification strategies, is essential to ensure that the intended visual profile is maintained throughout the declared shelf-life (Bamforth, 2011; Królak et al., 2023).

Polyphenols play an ambivalent role in this context. On one hand, they contribute significantly to antioxidant capacity and can help delay oxidative reactions that compromise beer flavor and nutritional quality (Aron & Shellhammer, 2010; Habschied et al., 2021). On the other hand, their ability to interact with proteins and form insoluble complexes makes them central actors in colloidal instability and haze formation (Aron

& Shellhammer, 2010; Wang et al., 2021). Historically, many breweries have prioritised physical stability by partially removing polyphenols through fining agents or filtration, sometimes at the expense of antioxidant potential (Martínez-Periñán et al., 2011; De Francesco et al., 2020). More recent work has emphasized the need to balance these effects, recognising that raw material choice, kilning conditions, hopping regime and process design can be used to modulate both the phenolic profile and the resulting stability characteristics (Carvalho et al., 2022; Klimczak et al., 2024). Studies on beers enriched with phenolic-rich ingredients, such as fruits, teas or novel cereal fractions, show that storage is often accompanied by a gradual decrease in total phenolic content and antioxidant capacity, although the magnitude and kinetics of these changes depend strongly on the ingredient and processing conditions (Habschied et al., 2020; da Silva et al., 2021).

Microbiological stability is also a prerequisite for long-term beer quality. Modern brewing practices—comprising hygienic design, pasteurisation or microfiltration, and the antimicrobial effect of ethanol, hop iso-α-acids and low pH—normally ensure that beers remain free from spoilage over their intended shelf-life (Suzuki, 2011; Xu, 2020). Nevertheless, certain lactic acid bacteria (Lactobacillus, Pediococcus) and wild yeasts can survive and proliferate under favorable conditions, causing off-flavors, acidification, turbidity and gushing (Xu, 2020; Suiker et al., 2022). Any change in raw material composition, including the introduction of by-products such as surplus bread, must therefore be evaluated not only in terms of physicochemical and sensory attributes but also with regard to its impact on microbiological stability and potential contamination risks.

Despite the extensive literature on beer ageing and stability, most studies on innovative formulations focus on the characterization of beers at the time of packaging, paying comparatively less attention to their evolution during storage. This is particularly true for beers brewed with alternative raw materials and food-industry side streams, where research often stops at demonstrating process feasibility and initial improvements in nutritional or sensory properties (Habschied et al., 2021; De Francesco et al., 2020). Consequently, there is limited information on whether such advantages are maintained, attenuated or even reversed over typical shelf-life periods, and on how they interact with standard stability challenges such as oxidation, haze formation and sensory ageing.

The third experimental study of this doctoral thesis addresses these gaps by evaluating the long-term stability of beers brewed with and without whole wheat bread as a partial malt substitute. In that work, American lager, India pale ale and Bavarian weiss beers produced with 50% substitution of malt by whole wheat bread are compared with their respective control beers over 12 months of storage at 4 °C and 20 °C. Physicochemical parameters (alcohol content, pH, color, turbidity, dissolved CO_2), total polyphenol content and antioxidant capacity, microbiological counts and sensory attributes are monitored at several time points in order to obtain a comprehensive picture of how bread enrichment influences beer behaviour during shelf-life. The results, discussed in detail in the corresponding chapter, show that whole wheat bread can contribute to preserving or even improving certain stability-related attributes—particularly phenolic content, antioxidant capacity and sensory quality—while maintaining acceptable colloidal and microbiological stability over the evaluated period (Martin-Lobera et al., 2025b).

1.7. Research gap and aims of this doctoral thesis

The growing interest in circular economy strategies for the agro-food sector has highlighted surplus bread as a significant and underexploited resource. Although numerous technological routes have been proposed for its valorisation, including animal feed, bioethanol, organic acids and various biorefinery products, relatively few approaches reintegrate surplus bread into high-value foods. Brewing stands out as a particularly attractive option due to the technological compatibility between bread and malt-based processes and the strong communication potential associated with reusing food waste into a widely consumed beverage. However, despite increasing attention from industry, the scientific evidence supporting the systematic use of surplus bread as a brewing adjunct remains limited and fragmented.

Existing studies tend to use relatively low substitution levels, focus on a single type of bread and beer style, and provide only basic analytical characterization. Important technological aspects—such as the impact of bread on extract yield, color, turbidity and foam stability—are not always fully addressed, and nutritional-related parameters such as total polyphenol content and antioxidant capacity are frequently overlooked. In addition, the sensory implications of replacing malt with bread have seldom been evaluated in detail, even though consumer acceptance is a critical factor for the successful introduction of such products. As a result, there is still insufficient knowledge about how different bread types and inclusion levels affect the overall quality of beer and about the conditions under which surplus bread can be used to partially replace malt without compromising product performance.

A further limitation of the current literature concerns the temporal dimension of beer quality. Most studies on bread-enriched beers focus on the characterization of freshly bottled products, with little or no consideration of their evolution during storage. Given that beer is typically distributed and consumed over several months, it is essential to understand how bread-based formulations behave over time. Key questions remain largely unanswered, including whether the initial advantages observed in terms of phenolic content and antioxidant capacity are maintained throughout shelf-life, how bread incorporation influences physicochemical and colloidal stability, and whether it affects microbiological safety or accelerates sensory deterioration. Addressing these issues is particularly relevant for craft and industrial breweries that must balance innovation, sustainability and product reliability.

In this context, the overarching aim of the present doctoral thesis is to provide a comprehensive and experimentally grounded assessment of surplus bread, and specifically whole wheat bread, as a partial malt substitute in beer production. The thesis seeks to explore not only the technological feasibility of using bread at relatively high substitution levels, but also its impact on the physicochemical, nutritional, sensory and stability-related attributes of beer across different styles and storage conditions.

II. OBJETIVOS

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En consecuencia, el objetivo general de esta tesis doctoral es investigar la viabilidad tecnológica, nutricional y sensorial del uso de pan excedentario, en particular pan integral, como sustituto parcial de la malta en diferentes estilos de cerveza, y evaluar el impacto de esta estrategia sobre la calidad y la estabilidad de la cerveza a lo largo del tiempo.

Este objetivo general se aborda mediante los siguientes objetivos específicos:

1. Evaluar el efecto de sustituir el 50 % de la malta por distintos tipos de pan (blanco, integral, de centeno y de maíz) en una cerveza tipo pale ale, centrándose en los parámetros fisicoquímicos, el contenido total de polifenoles, la capacidad antioxidante y las propiedades sensoriales.
2. Evaluar el uso de pan integral como sustituto del 50 % de la malta en diferentes estilos de cerveza (American lager, India pale ale y Bavarian weiss), caracterizando las cervezas resultantes en términos de atributos fisicoquímicos, color y turbidez, contenido fenólico, capacidad antioxidante y perfil volátil.
3. Determinar la estabilidad a largo plazo de cervezas elaboradas con y sin pan integral como sustituto parcial de la malta en los estilos American lager, India pale ale y Bavarian weiss, mediante el seguimiento de los cambios fisicoquímicos, microbiológicos, fenólicos, antioxidantes y sensoriales durante 12 meses de almacenamiento a 15 °C

A continuación, se resume el logro de los objetivos específicos.

El Objetivo 1 se aborda en el Capítulo 1, que corresponde al primer estudio experimental, «*Bread as a Valuable Raw Material in Craft Ale Beer Brewing*». En este estudio se empleó una única formulación de pale ale como cerveza modelo y se sustituyó el 50 % de la molienda de malta por cuatro tipos diferentes de pan excedentario (blanco, integral, de centeno y de maíz). Se incluyó, a efectos comparativos, una cerveza control elaborada con un 100 % de malta de cebada. Todas las cervezas se produjeron en condiciones de elaboración representativas de la producción de cerveza artesana tipo ale. Los productos resultantes se caracterizaron en términos de parámetros fisicoquímicos básicos, contenido total de polifenoles, capacidad antioxidante, perfil volátil y propiedades sensoriales, proporcionando una comparación directa de las implicaciones tecnológicas y nutricionales asociadas a cada tipo de pan como sustituto parcial de la malta en pale ale.

El Objetivo 2 se aborda en el Capítulo 2, que incluye el segundo estudio experimental, «*Brewing with Whole Wheat Bread to Produce Different Beer Styles*». A partir de la evidencia obtenida en el Capítulo 1, se seleccionó el pan integral como el tipo de pan más adecuado y se empleó a un nivel de sustitución fijo del 50 % de la molienda de malta en tres estilos de cerveza claramente diferenciados: American lager, India pale ale y Bavarian weiss. Para cada estilo se elaboró una cerveza control (100 % malta) y una cerveza con pan (50 % malta + 50 % pan integral). Las cervezas se caracterizaron en cuanto a atributos fisicoquímicos, color y turbidez, contenido fenólico y capacidad antioxidante. Además, se analizó el perfil volátil, lo que permitió evaluar el impacto del pan integral sobre las características tecnológicas, nutricionales y fisicoquímicas de cada estilo.

El Objetivo 3 se aborda en el Capítulo 3, que presenta el tercer estudio experimental, «*Whole Wheat Bread Improves the Nutritional Composition and Quality of Beer During Long-Term Storage*». En este caso, las mismas cervezas American lager, India pale ale y Bavarian weiss elaboradas con y sin pan integral en el Capítulo 2 se almacenaron durante 12 meses a 15 °C. Al inicio y al final del periodo de almacenamiento se tomaron muestras de cerveza para monitorizar los parámetros fisicoquímicos, la estabilidad microbiológica, el contenido total de polifenoles, la capacidad antioxidante y los atributos sensoriales. Este diseño permitió evaluar la estabilidad a largo plazo de las cervezas enriquecidas con pan integral en comparación con sus correspondientes cervezas control, así como determinar cómo la adición de pan influye en la evolución de la calidad y de las propiedades relacionadas con la composición nutricional a lo largo del tiempo.

En conjunto, los tres capítulos conforman una progresión coherente: desde una evaluación inicial de diferentes tipos de pan en un único estilo de cerveza, pasando por la validación del pan integral en varios estilos de cerveza, hasta la evaluación de la estabilidad a largo plazo de estas cervezas durante un almacenamiento prolongado a 15 °C.

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Accordingly, the general objective of this doctoral thesis is:

To investigate the technological, nutritional and sensory feasibility of using surplus bread, particularly whole wheat bread, as a partial malt substitute in different beer styles, and to evaluate the impact of this strategy on beer quality and stability over time.

This general objective is addressed through the following specific objectives:

1. To evaluate the effect of replacing 50% of the malt with different types of bread (white, whole wheat, rye and corn) in a pale ale beer, focusing on physicochemical parameters, total polyphenol content, antioxidant capacity and sensory properties.
2. To assess the use of whole wheat bread as a 50% malt substitute in different beer styles (American lager, India pale ale and Bavarian weiss), characterising the resulting beers in terms of physicochemical attributes, color and turbidity, phenolic content, antioxidant capacity and volatile profile.
3. To determine the long-term stability of beers brewed with and without whole wheat bread as a partial malt substitute in American lager, India pale ale and Bavarian weiss styles, by monitoring physicochemical, microbiological, phenolic, antioxidant and sensory changes over 12 months of storage at 15 °C.

The achievement of the specific objectives is summarised below.

Objective 1 is addressed in Chapter 1, which corresponds to the first experimental study, *"Bread as a Valuable Raw Material in Craft Ale Beer Brewing"*. In this study, a single pale ale formulation was used as a model beer and 50% of the malt grist was replaced by four different types of surplus bread (white, whole wheat, rye and corn). A control beer brewed with 100% malted barley was included for comparison. All beers were produced under brewing conditions representative of craft ale production. The resulting products were characterized in terms of basic physicochemical parameters, total polyphenol content, antioxidant capacity, volatile profile and sensory properties, providing a direct comparison of the technological and nutritional-related implications of each bread type as a partial malt substitute in pale ale.

Objective 2 is addressed in Chapter 2, which includes the second experimental study, *"Brewing with Whole Wheat Bread to Produce Different Beer Styles"*. Based on the evidence obtained in Chapter 1, whole wheat bread was selected as the most suitable bread type and was used at a fixed substitution level of 50% of the malt grist in three clearly differentiated beer styles: American lager, India pale ale and Bavarian weiss. For each style, a control beer (100% malt) and a bread beer (50% malt + 50% whole wheat bread) were produced. The beers were characterized with respect to physicochemical attributes, color and turbidity, phenolic content and antioxidant capacity. In addition, the volatile profile was analyzed, allowing the impact of whole wheat bread on the technological, nutritional-related and physicochemical characteristics of each style to be assessed.

Objective 3 is addressed in Chapter 3, which presents the third experimental

study, *"Whole Wheat Bread Improves the Nutritional Composition and Quality of Beer During Long-Term Storage"*. In this case, the same American lager, India pale ale and Bavarian weiss beers brewed with and without whole wheat bread in Chapter 2 were stored for 12 months at 15 °C. At the beginning and at the end of the storage period, beer samples were collected to monitor physicochemical parameters, microbiological stability, total polyphenol content, antioxidant capacity and sensory attributes. This design made it possible to evaluate the long-term stability of beers enriched with whole wheat bread compared with their corresponding control beers and to determine how bread addition influences the evolution of quality and nutritional-related properties over time.

Together, the three chapters provide a coherent progression: from an initial screening of different bread types in a single beer style, through the validation of whole wheat bread in several beer styles, to the assessment of the long-term stability of these beers during extended storage at 15 °C.

II. OBJECTIVES

III. MATERIALS AND METHODS

3.1 Materials

Brewing raw materials, surplus bread and analytical reagents used in the three experimental chapters are described below, avoiding repetition where common to more than one study.

3.1.1 Brewing raw materials

All beers were brewed in the pilot brewery of the University of Valladolid (Campus of Palencia, Spain) using commercial malted grains, adjuncts, hops and brewing yeasts.

Malted barley and wheat were supplied by Weyermann (Bamberg, Germany) and Castle Malting (Beloeil, Belgium), and were used in different combinations depending on the beer style and chapter:

- In **Chapter 1** (pale ale beers with different types of bread), the grist was composed of Pilsen malt (EBC 3) and Munich Type I malt (EBC 12) from Weyermann®, together with Biscuit malt (EBC 45) from Castle Malting, and Cara Rye malt (EBC 150) from Weyermann.
- In **Chapters 2 and 3** (American lager, India pale ale and Bavarian weiss beers brewed with or without whole wheat bread), the malt and adjunct formulations followed the recipes in Table 1 of the corresponding articles and included Pale Ale malt (EBC 7), Pilsner malt (EBC 3), Munich Type I malt (EBC 12), Pale wheat malt (EBC 5), corn flakes (EBC 3.5) and rice flakes (EBC 2.5). Pale Ale malt, corn and rice flakes were obtained from Castle Malting, whereas Pilsner, Munich Type I, Pale wheat and Cara amber malt (EBC 60) were sourced from Weyermann.

Surplus bread was supplied by "La Tahona de Sahagún" (Palencia, Spain), a local bakery. In **Chapter 1**, four types of bread were used as partial malt substitutes in pale ale beers: white wheat bread, whole wheat bread, rye bread and corn bread. In **Chapters 2 and 3**, only stale whole wheat bread (approximately three days after baking) from the same bakery was used, replacing 50% of the total malt mass on a 1:1 weight basis in American lager, IPA and Bavarian weiss formulations. In all cases, bread loaves were milled immediately before mashing in a two-roll mill with a 1 mm gap, using the same equipment and settings as for the malts.

All beers were brewed with bottled mineral water (Monte Pinos, Carbónicas Navalpotro S.A., Almazán, Spain), used both for mashing and sparging in every chapter.

Pelleted hops were purchased from Laguilhoat (Fuenlabrada, Spain). In **Chapter 1**, Centennial (9.6% α-acids), Cascade (6.7% α-acids) and Simcoe (13.6% α-acids) hops were employed in the American pale ale recipe. In **Chapters 2 and 3**, Saaz (3.8% α-acids), Cascade (6.8% α-acids), Citra (12.7% α-acids) and Magnum (13.1% α-acids) hops were used to formulate American lager, IPA and Bavarian weiss beers according to the specified bitterness targets.

All fermentations were carried out with commercial dried brewing yeasts supplied by Fermentis (Marcq-en-Baroeul, France). In **Chapter 1**, SafAle S-04 was used for primary fermentation and SafAle F-2 for bottle refermentation of pale ale beers. In **Chapters 2 and 3**, SafLager S-23, SafAle US-05 and SafAle WB/W-06 were used for primary fermentation of American lager, IPA and Bavarian weiss beers, respectively, whereas SafAle F-2 was used for bottle conditioning in all styles.

3.1.2 Reagents and standards

The same analytical-grade reagents and standards were used across the three experimental chapters for the determination of total polyphenol content, antioxidant capacity and soluble protein, with an additional medium for microbiological analyses in the long-term storage study.

Methanol, gallic acid and Folin–Ciocalteu reagent were obtained from Merck Millipore (Madrid, Spain). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Sodium hydroxide (0.01 M), sodium chloride (NaCl), anhydrous D(+)-glucose for ACS analysis, Coomassie blue G-250 (CBBG) and Bradford reagent were purchased from Panreac (Castellar del Vallés, Spain). All solutions were prepared using analytical-grade reagents and distilled water.

In **Chapter 3**, microbiological stability during storage was additionally evaluated using Raka–Ray agar, which was acquired from Scharlab (Sentmenat, Spain).

3.2 Methods

3.2.1 Experimental design

All experimental work was carried out in the pilot brewery of the University of Valladolid (Palencia, Spain) and is presented in Chapters 1–3 of this thesis.

In Chapter 1, a control pale ale beer was brewed using the malts and hops that were most frequently repeated in the consulted recipes, i.e., those for American pale ale, one of the most-consumed types (Brewers Association, 2021). a single pale ale formulation was used as a model beer.

Also, four brews were made according to the control pale ale beer recipe, using different kinds of bread (wheat, rye, whole wheat, and corn) to replace, in each case, 50% of the malt weight with the same amount of stale bread weight. The pale ale beers produced were named using abbreviations, as follows: control (ALE); wheat bread (WHIB); rye bread (RYEB); whole wheat bread, also known as brown wheat (BROB); and corn bread (CORB).

In Chapter 2, three different styles of craft beer were elaborated due to their being the most widely consumed beers in the world: American lager for lager beer and, in the case of ale beers, IPA (Indian pale ale) and Bavarian weiss beer. All beers were brewed in duplicate, in individual 10 L batches, and labeled with abbreviations as follows: 100% malt beers were designated as American lager (LA), Indian pale ale (IPA), and Bavarian weiss ale (W); bread beers were codified the same way as the controls with the addition of the letter "B" at the end.

In Chapter 3, the same American lager, IPA and Bavarian weiss beers brewed with and without whole wheat bread (as described in Chapter 2) were produced again in duplicate 10 L batches and stored for 12 months at 15 °C to assess long-term stability. Beer samples were designated using the following codes: LA and LAB for the lager control beer (100% malt) and the lager brewed with whole wheat bread (50% malt + 50% whole wheat bread), respectively; IPA and IPAB for the India pale ale control beer and the corresponding whole wheat bread beer; and W and WB for the Bavarian weiss

control beer and the Bavarian weiss brewed with whole wheat bread.

Samples were taken immediately after maturation (fresh beer, S0) and after 12 months of storage (S12) to evaluate physicochemical, microbiological, bioactive and sensory changes over time.

3.2.2 Brewing process

For Chapter 1, all beer batches were brewed in duplicate, obtaining 5 L of beer in each case and labelling each one by the abbreviation previously mentioned, followed by the number 1 or 2 depending on the duplicate. The malt and bread were milled at the University of Valladolid cellar (University of Valladolid Campus, Palencia, Spain) just before mashing. The total amount of malt used was 1046 g per brew; in detail, the following amounts of malt were added to 6 L of mineral water: Pilsen (45% by weight), Munich type I (40% by weight), Biscuit (10% by weight), Cara Rye (5% by weight), mineral water, pelleted hops (Centennial, Cascade, and Simcoe), and yeast.

The resulting ground malt was mixed with preheated mineral water at 40 ± 1 ◦C for 20 min for mashing into a stainless macerator tank. In all cases, mashing took two hours, at 67 ± 1 ◦C with manual stirring, to convert the starches from the malt and bread into sugar. Finally, the mash temperature was raised to 78 ± 1 ◦C for a 10 min period, at a rate of 1 ◦C per min (mash out), to deactivate the enzymes; then, the mixtures remained at room temperature for 24 h.

Mashing was followed by wort separation and sparging until the desired amount of wort was collected. The sparging was performed with natural mineral water at 80 ± 1 ◦C until the final volume of 5 L was completed, in order to achieve a greater extraction of fermentable sugars.

For the lupulization step, wort boiling at 100 ◦C required 60 min, following the time scheme shown in Table 3.2.1, and resulted in a total bitterness of 30 IBU (international bitterness units).

Table 3.2.1. Characteristics of the brewing pelleted hops used, the hops' addition time during boiling, and the amount of hops used in each treatment (5 L) (Chapter 1).

Variety of Hops	IBU	Alpha Acids (%)	Boil Min.	Weight (g)
Centennial	15	9.6	1	5.79
Cascade	10	6.7	30	7.86
Simcoe	5	13.6	59	3.34

All wort was fermented using the commercial yeast SafAle S-04, at 21 ± 1 ◦C, in 6 L tanks for 10 days. After beers reached the final attenuation degree, the temperature was gradually reduced to 4 ◦C in a cooling chamber; the beer remained for a week in the tanks to facilitate lees removal and beer maturation. After the removal of lees, all the containers were tempered to 21 ◦C, and the carbonation phase was performed with 33 mL volume glass bottles, using dextrose and SafAle F-2 yeast, to obtain 2.0 bar of internal CO_2 pressure. All bottles rested for 14 days to finish fermentation at 21 ± 1 ◦C. Finally, beer bottles matured in a refrigerated chamber at 4 ◦C for 2 weeks.

In Chapters 2 and 3, beers (100% malt grains and flakes) were brewed as a control in each style, and total malt amounts added to 10 L of mineral water were 2.381 Kg per

American lager, 2.650 Kg per IPA, and 2.170 Kg per Bavarian ale brew. Also, bread beers were made according to the control ones in each cited style, replacing 50% of the weight malt with the same weight of stale whole wheat bread (Table 3.2.2).

Table 3.2.2. Brewing raw materials used in the elaboration of craft beers (Chapters 2 and 3).

Raw Material	American Lager Beer	IPA Beer	Bavarian Weiss Beer
Grain malt and flakes (% by weight)	Pale Ale EBC 7 (Castle Malting, Beloeil, Belgium) (85%)	Pilsner EBC 3 (Weyermann, Bamberg, Germany) (81%)	Pilsner EBC 3 (Weyermann, Bamberg, Germany) (50%)
	Corn flakes EBC 3.5 (Castle Malting, Beloeil, Belgium) (10%)	Munich Type I EBC 12 (Weyermann, Bamberg, Germany) (15%)	Pale Wheat malt EBC 5 (Weyermann, Bamberg, Germany) (50%)
	Rice flakes EBC 2.5 (Castle Malting, Beloeil, Belgium) (5%)	Cara amber EBC 60 (Weyermann, Bamberg, Germany) (4%)	
Pelleted hops	Saaz 3.80% a.a. (Laguilhoat, Fuenlabrada, Spain)	Cascade 6.80% a.a. (Laguilhoat, Fuenlabrada, Spain)	Magnum 13.10% a.a. (Laguilhoat, Fuenlabrada, Spain)
		Citra 12.70% a.a.. (Laguilhoat, Fuenlabrada, Spain)	
Bread	Whole wheat	Whole wheat	Whole wheat
Yeast	Saflager S-23 (Fermentis, Marcq-en-Baroeul, France)	Safale US-05 (Fermentis, Marcq-en-Baroeul, France)	Safbrew W-06 (Fermentis, Marcq-en-Baroeul, France)
	Safale F-2 (Fermentis, Marcq-en-Baroeul, France)	Safale F-2 (Fermentis, Marcq-en-Baroeul, France)	Safale F-2 (Fermentis, Marcq-en-Baroeul, France)
Water	Monte Pinos (Carbónicas Navalpotro S.A, Almazán, Spain)		

The yeast strains Saflager S-23, Safale US-05, and Safbrew W-06 were used for the fermentation process in tanks, while the Safale F-2 strain was employed for bottle fermentation; whole wheat bread was acquired in a stale condition (three days after production) from "La Tahona de Sahagún" (Palencia, Spain), and milling was carried out immediately after its arrival at the laboratory.

The detailed brewing process, which was optimized by our team in an early stage and recently published (Martín-Lobera et al, 2022) is shown in Figure 3.2.1.

First, malts and stale bread were ground separately, in a two-roll mill spaced 1 mm, just before mashing. Second, the mineral water was preheated to a temperature of 40 ◦C, and the resulting ground malt or ground malt plus bread at 50% by weight were poured into buckets and stirred for 20 min. Next, the temperature was increased at different steps, depending on each recipe, as described in Figure 1, and kept for 24 h for extended maceration at room temperature in order to prolong enzymatic activity and improve starch and protein hydrolysis, thereby enhancing fermentable sugar availability. Then, after lautering wort was transferred to the kettle, the remaining bagasse was sparged using hot mineral water at 80 ◦C until the final volume of 10 L was completed. All batch wort were boiled at 100 ◦C for 60 min. Saaz pelleted hop was added at the start of boiling to obtain 18 International Bitterness Units (IBU) for the American lager recipe. In the case of Indian pale ale, two different pelleted hops were used: Cascade hop was infused to contribute 35 IBU at the beginning of boiling, and Citra hop was added in the middle of this procedure to achieve the remaining 15 IBU. For the Bavarian ale recipe, Magnum pelleted hop was introduced at the start of boiling to achieve 15 IBU. After boiling, the hot trub was removed, and the wort was rapidly cooled using a freezing chamber at −25 ◦C to reach the appropriate fermentation temperature (Figure 3.2.1). Before yeast

inoculation, all wort batches were oxygenated by manual agitation for 2 min to ensure proper yeast activity.

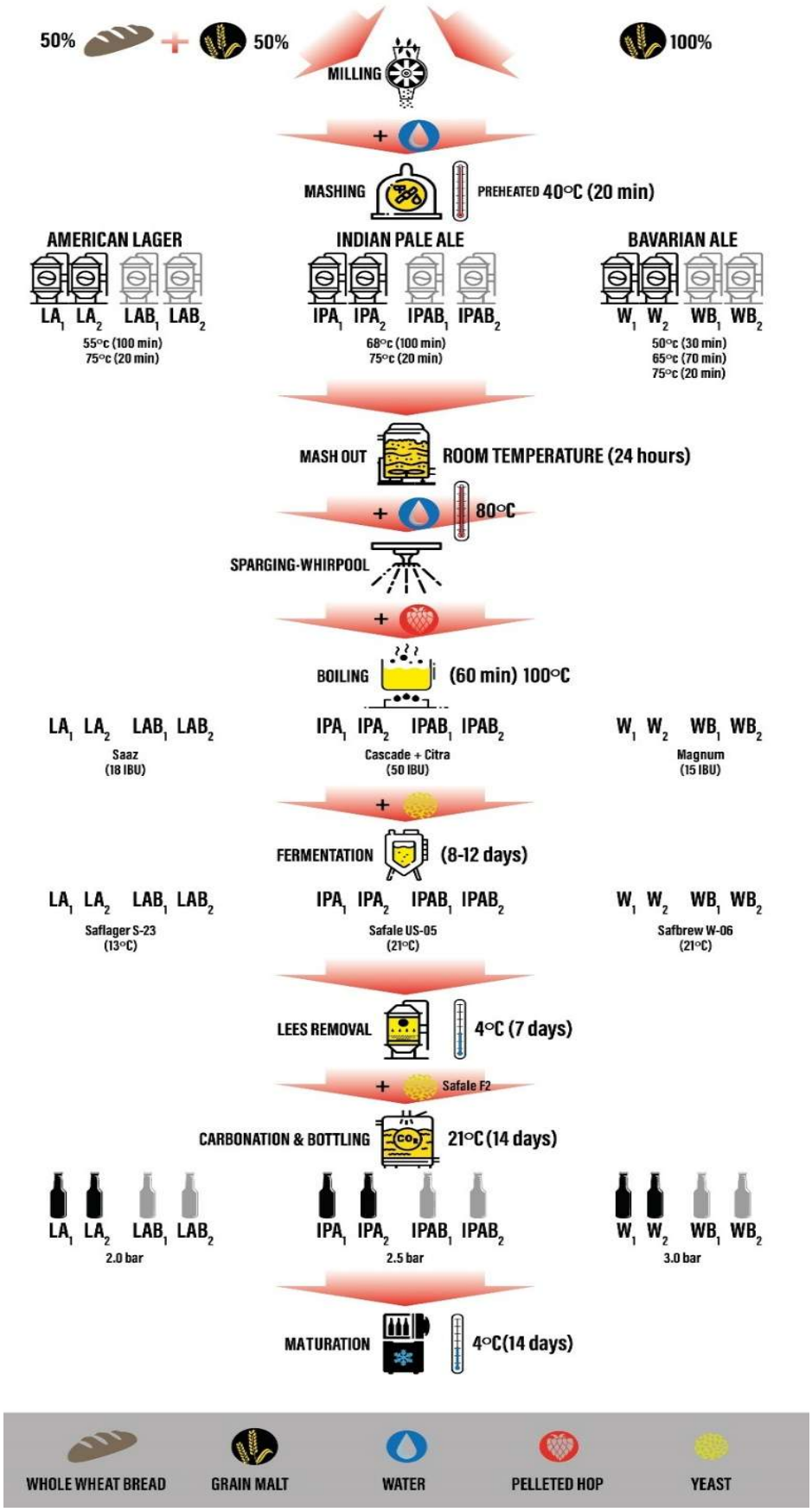

Figure 3.2.1. Brewing process diagram.

Different commercial yeasts were used for primary fermentation, applied at a concentration of 0.5 g per liter, according to Figure 1. All dry yeast strains were rehydrated prior to inoculation. Rehydration was performed in sterile water at 25 ◦C, using a yeast-to-water ratio of 1:10 (w/v), in accordance with the manufacturer's guidelines. The suspension was allowed to rest undisturbed for 15 min before being pitched into the wort. Primary fermentation was conducted in 10 L stainless steel tanks for 8 to 12 days. The tanks were equipped with an overpressure valve to allow safe CO_2 release. Fermentation temperature was consistently maintained by placing the tanks in a climate-controlled laboratory room, set to the specific conditions required for each beer style (13 ◦C for lager and 21 ◦C for ale fermentations). After beers reached the final attenuation degree, the temperature was gradually reduced to 4 ◦C in a cooling chamber; the beer remained in the tanks for a week to facilitate the next stage of lees removal and beer maturation. After lees removal, all containers were tempered to 21 ◦C. Carbonation was then carried out in 330 mL glass bottles using dextrose and Safale F-2 yeast. Dextrose was added at 4 g per liter of beer per bar of target pressure, aiming to reach internal CO_2 levels of 2.0 bar for lager, 2.5 bar for IPA, and 3.0 bar for Bavarian beer. Then, all bottles rested for 14 days to finish fermentation at 21 ◦C and final pressure was verified using crown-cap aphrometers. Finally, all beer bottles matured in a refrigerated chamber at 4 ◦C for 2 weeks.

In Chapter 3, immediately after the 2-week cold maturation period, an initial set of samples from each batch was taken and analyzed (fresh beers, S0). The remaining bottles were then stored upright in a controlled-temperature chamber at 15 °C for 12 months, in darkness and without agitation, to simulate realistic cellar-type storage. At the end of this period, a second set of samples was taken from each batch (S12) for comparative analyses.

3.2.3 Physicochemical analysis

Physicochemical properties were determined using the same basic protocol in all three chapters. Unless otherwise stated, all measurements were performed in triplicate.

For turbidity, pH, acidity, alcohol by volume, dry/real extract and color, unfiltered beer samples were used, except for spectrophotometric measurements (color, bioactive compounds and protein), which required prior centrifugation and filtration.

- Turbidity was measured with a turbidimeter (HI 98703, Hanna Instruments, Eibar, Spain). Samples were poured into transparent glass cuvettes with lids and turbidity was recorded in nephelometric turbidity units (NTU).
- pH was determined with a calibrated pH-meter (sensiON™+ pH3, HACH-LANGE, Hospitalet, Spain).
- Titratable acidity was assessed by continuous pH monitoring during titration with NaOH until pH 7.0 was reached; results were expressed as % lactic acid.
- Alcohol by volume (ABV) was measured using an ebulliometer (GAB system, model 1010006, Moja, Spain) calibrated with distilled water. The boiling temperatures of water and the beer sample were compared and ABV was read from the instrument scale with 0.1% precision. In Chapters 2–3, ebulliometric readings were validated by comparison with ABV values calculated from original and final gravity according to EBC equations.
- Dry extract / real extract was measured gravimetrically using a thermobalance

(Gibertini Eurotherm, Novate Milanese, Italy). Degassed beer (1.000 ± 0.005 g) was dried at 105 °C to constant weight; the residue was expressed as % (m/m) dry or real extract.

- Color (EBC) was determined by measuring absorbance at 430 nm in a 1 cm path length cuvette (Genesys 20 UV–Vis, ThermoFisher Scientific, Madrid, Spain) using distilled water as blank. The absorbance values were multiplied by 25 to obtain color in EBC units.

3.2.4 Spectrophotometric analysis of bioactive compounds

3.2.4.1 Total polyphenol content (TPC)

The total polyphenol content was determined by the Folin–Ciocalteu method by measuring absorbance at 760 nm (Magalhães et al. 2010), using the spectrophotometer mentioned above. A calibration line was performed using different concentrations (0.0–30 mg L^{-1}) of standard solutions of gallic acid. The concentration of total phenols is expressed as mg of GAE mL^{-1} of the sample.

3.2.4.2 Antioxidant activity (DPPH assay)

The antioxidant capacity of the beer samples was determined according to the method described by Abderrahim et al. (2013). Beer samples, once filtered and diluted (the 50 μL sample or the blank control), were introduced and mixed with 1000 μL of DPPH (60 μMol L^{-1} dissolved in methanol 1: 1/10 mMol L^{-1} Tris-HCl buffer pH 7.5) in a 5 mL volumetric flask. At 0 min, and after 20 min of incubation at room temperature in the laboratory (21 ± 2 °C), a small volume was introduced into 10 mm quartz cuvettes, and absorbance was measured at 520 nm with the spectrophotometer mentioned above. The antioxidant capacity of the beer, expressed in μMol DPPH mL^{-1}, was calculated using the following mathematical formula:

$$\mu\text{Mol (DPPH mL}^{-1}) = ((A_0 - A_t)/A_0) \times ((V_t\,[DPPH] \times FD)/\text{mL})$$

where A_0: control absorbance (DPPH diluted in methanol); A_t: sample absorbance; V_t: total reaction volume in liters; $[DPPH]$: $DPPH$ concentration; FD: dilution factor; and mL: sample milliliters used in the reaction.

3.2.4.3 Protein content

In Chapters 2 and 3, soluble protein content was measured using a method based on the Bradford test (Bradford, 1976) was followed, according to which the quantification of proteins is based on the union of the Coomassie blue dye G-250 (Bradford reagent) with the proteins available from the analyzed beer samples. In these analyses, 3140 μL of distilled water and 200 μL of the Bradford reagent were added to 60 μL of the beer sample in a test tube. A calibration line from 1 to 40 μL was also constructed using serum albumin (0.1 μg μL^{-1}). Finally, samples were measured at 595 nm.

3.2.5 Headspace gas chromatography–mass spectrometry (HS-GC–MS)

Volatile compounds were analyzed by HS-GC–MS in Chapters 1 and 2. Sample preparation followed the headspace method described by Liu et al. (2005), were analyzed by HS-GC-MS (headspace gas chromatography coupled to a mass spectrometer) in a QP2010 Shimazdu device with an AOC 5000 autosampler (Shimadzu Europa GmbH, Duisburg, Germany) and an HP-5MS column (30 m long, 0.25 mm internal diameter, and 25 μm of film). Two mL of each previously filtered beer were placed in a 10 mL HS vial with NaCl 20% (w/v), and heated up to 80 ◦C at 250 rpm for 15 min; this was performed prior to the 100 μl HS injection of the sample in splitless mode. The pressure was set at 110 kPa and Helium was used as a carrier gas. The interface temperature was 250 ◦C and the injector temperature was 120 ◦C. The oven followed the following program: an initial temperature of 40 °C for 2 min, a ramp-up of 10 °C/min to 140 °C, and a second ramp-up of 7 °C/min to 250 °C. Data were acquired in full scan mode in a m/z range of 30–350, and peak identification was determined by comparison with the NIST08 and WILEY229 libraries.

3.2.6 Microbiological analysis (Chapter 3)

Two microbiological methods were employed to assess potential contamination in the beer samples:

- Lactobacillus spp. Count: Lactobacillus spp. was quantified through surface plating on selective media. In this technique, a small volume of sample was spread evenly on the surface of agar plates specific to Lactobacillus spp., which were then incubated under conditions optimal for the growth of these bacteria. Colony-forming units (CFU) were counted after incubation, with a detection limit of <100 CFU/mL. This approach enables a reliable assessment of Lactobacillus spp. presence and growth, which is critical given their role in beer spoilage (Bokulich et al., 2013).
- Enterobacteriaceae Detection: Enterobacteriaceae presence was determined via membrane filtration. In this method, the beer samples were filtered through 0.45-micron Millipore membranes (Merck Millipore, Darmstadt, Germany). The membranes were subsequently placed on selective agar plates, designed for Enterobacteriaceae growth and incubated to facilitate colony formation. Colonies were then counted, with a detection limit of <10 CFU/mL, Membrane filtration is a widely used method in the brewing industry for detecting low levels of Enterobacteriaceae, enhancing quality control measures. This technique involves passing beer samples through a membrane filter that captures bacteria, which are then cultured on selective media to identify contaminants. Studies have demonstrated the effectiveness of membrane filtration in detecting microbial trace contaminations in beer, including Enterobacteriaceae, thereby ensuring product safety and quality (Turvey et al.,2017).

3.2.7 Sensory analysis (Chapter 1 and 3)

Eight professional beer tasters (five men and three women) were trained following ISO 8586:2012 standards. The training process included six two-hour tasting sessions, in which panelists learned to identify various beer descriptors using a presence–absence scale and a discontinuous three-point scale. During the first two sessions, tasters focused on descriptor identification through a free choice profile technique paired with a control identification test. In the remaining four sessions, a standardized tasting sheet was

developed, incorporating the most relevant descriptors selected through a qualitative focus group technique. Criteria for potential removal were set, with panelists scoring below 70% on the control test subject to elimination. This process resulted in the exclusion of two tasters, yielding a final panel of six highly qualified judges.

The sensory evaluation took place in a single session at 5:00 p.m. Before beginning, tasters were briefed on the project objectives and the specific beers to be evaluated. Each beer was tested by duplicate, with twelve samples presented in total. After the first six samples, a short break was given. Each beer was served at 6 °C in glasses conforming to ISO 3591:1977 standards, with a unique three-digit random code assigned to each sample, and a randomized order of presentation for each taster. The analysis was conducted in a controlled tasting room that met UNE-EN ISO 8589:2010 guidelines. Panelists spent approximately two minutes on each sample, using a descriptive approach aligned with the tasting sheet developed during training. This sheet included descriptors to facilitate the sensory profiling of the beers across three distinct categories:

- Visual: Intensity, tonality, limpidity, froth color, and CO_2 bubbles.
- Aroma: Maltiness, cereal malt, ripe fruit malt, hoppy, exotic fruit, citric fruit, herbaceous, yeast, tropical fruit yeast, spicy yeast, bread yeast, toasted, coffee, licorice, and caramel, along with defect descriptors such as oxidized, cider, vinegar, musty, stable, and soapy.
- Taste: Acidity, CO_2, bitterness, body, and persistence.

These descriptors encompassed most of the sensory categories, including several primary terms represented in the beer sensory wheel.

3.2.8 Statistical analysis

All analytical measurements were performed in triplicate.

In Chapter 1, data analysis was performed to establish the differences between the averaged values for physicochemical measurements, total polyphenol content, and antioxidant capacity; analysis was conducted using an analysis of variance (one-factor ANOVA) and Tukey´s significant difference test (HSD), with statistical significance being set at a p-value < 0.05. Principal component analysis (PCA) was used for HS-GC–MS, sensory analysis, and product characterization for the sensory data analysis.

In Chapter 2, Statistical analysis was carried out using Xlstat v.2023.3.1 statistical software (Addinsoft, Paris, France). Data analysis was conducted to identify differences between the mean values for physicochemical and spectrophotometric measurements; analysis was performed using an analysis of variance (one-factor ANOVA) and Tukey's significant difference test (HSD), with statistical significance being set at a p-value < 0.05. Principal component analysis (PCA) was used for HS-GC-MS and physicochemical analyses.

In Chapter 3, All analyses were conducted by triplicate. Statistical analyses were conducted utilizing XLSTAT v.2023.3.1 software (Addinsoft, Paris, France). For each sampling time (S0 and S12), all physicochemical variables were first assessed with a two-way MANOVA (fixed factors: Style and Bread); multivariate significance was evaluated using Wilks' lambda (Λ), which ranges from 0 to 1 ($\Lambda \approx 0$ indicates strong group differences; $\Lambda \approx 1$ indicates no multivariate effect). This statistic tests the null hypothesis that there are no significant multivariate differences among the beer types. Statistical

significance was determined based on the associated p-value < 0.05, considered significant. Each variable was then analyzed with a two-way ANOVA (Style, Bread, Style × Bread) and, within the same sampling time, re-analyzed by a one-way ANOVA (12 beer batches as levels). Pair-wise differences were located with Tukey's HSD ($p < 0.05$). Additionally, the R Pearson correlation coefficients, calculated at $p < 0.05$, were used to determine relationships among the physicochemical and sensory variables examined.

Together, these methods provided a coherent, comparable framework to evaluate the technological performance, nutritional-related properties and sensory quality of beers brewed with surplus bread across different styles and storage conditions.

IV. PUBLICATIONS INCLUDED IN THIS DOCTORAL THESIS

CHAPTER 1

Bread as a Valuable Raw Material in Craft Ale Beer Brewing.

Martin-Lobera, C.; Aranda, F.; Lozano-Martinez, P.; Caballero, I.; Blanco, C.A. *Foods* **2022**, 11, 3013. https://doi.org/10.3390/foods11193013.

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Article

Bread as a Valuable Raw Material in Craft Ale Beer Brewing

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Abstract: One of the ingredients used for brewing is barley, which should be malted; it is considered the most polluting agricultural input. On the other hand, food wastage is today a widespread problem that causes significant environmental damage and also generates large economic losses worldwide. One of the most wasted food products is bread; it is estimated that hundreds of tons of bread are wasted every day worldwide. In this study, the brewing of ale beers with bread was carried out. For this purpose, up to 50% of the malt weight was replaced by different types of bread: wheat bread, whole wheat bread, rye bread, and corn bread. A physicochemical and sensory comparison was made with 100% malt ale beer. All beers brewed with bread had an alcoholic strength similar to that of the control beer, except the corn beer. Beers brewed with whole grain bread showed a higher antioxidant capacity and a higher total polyphenol content. The sensory analysis presented different profiles depending on the type of bread; in general, the addition of bread created a greater olfactory intensity in nose. Thus, it was found that it is possible to brew beer with bread substituting up to 50% of the malt. In addition, it was also shown that the beer brewed with whole wheat bread had similar characteristics to the control beer, even improving some beneficial health properties, representing a great advantage for the brewing industry all over the world.

Keywords: brew; beer; surplus bread; antioxidants; polyphenols; organoleptic properties; volatile composition

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1. Introduction

Beer is a classic alcoholic beverage, and it is one of the most internationally popular drinks [1]. It is a beverage usually made from malted cereal grain (such as barley), yeast, hops, and water. Sometimes, adjuncts and food additives are also included. There are different types of beer, each of which has specific organoleptic properties encompassing gustative, visual, and aromatic perceptions. These properties are affected by the raw materials, the fermentation of the wort, and the technological conditions used in production and packaging [1,2].

Concern about environmental issues and the impact that companies have on them has increased. This also affects beer consumers, creating new challenges for the brewing industry. Problems related to water consumption, energy efficiency, waste generation, emissions management, and the environmental impact of the brewing process have become important topics of discussion and are receiving increased attention from large and small breweries [3].

The beer brewing process has several outputs, such as brewer's spent grain, hot trub, and residual brewer's yeast, which may cause negative environmental effects [4]. Producing and selling beer requires various types of inputs, such as raw materials, machinery, packaging materials, and transportation [3]. These impacts differ depending on the stage of the beer product life cycle. The total process can be divided into five different stages: the production and transportation of raw materials, beer production, wastewater treatment

in the brewery, the production and transportation of packaging, and the distribution of the final product to customers [5]. The production and transport of these ingredients have been identified as key contributors to the life cycle environmental impact of beer [6,7].

Food waste generates substantial economic losses globally [8], and bread, in particular, is the most commonly wasted food product in developed countries.

The production of bread is estimated to generate about 100 million tons per year, 65% of which is consumed in Europe [9]. During the storage of bread, a complex physicochemical process defined as staling occurs, mainly driven by the loss of moisture and retrogradation of starch [10]. Moreover, bread composition makes it susceptible to microbial attack, which is why preservatives that inhibit spore, mold, and/or yeast growth are used to reduce spoilage and ensure safety [11]. Determining the precise amount of bread wasted during its life cycle is a difficult task, but it is estimated that hundreds of tons are wasted daily worldwide [9]. However, the limited shelf-life is only one of the reasons why this large amount of bread is wasted.

Bread is a starchy food and an important source of easily extractable fermentable sugars, which is in direct contrast to lignocellulosic feedstocks, where harsh physical, chemical, and/or enzymatic pre-treatment processes are required for the release of fermentable sugars. During the last decade, several initiatives focused on finding alternatives for recycling bread waste have taken place: bread has been proposed as a substrate to produce chemical products for pharmaceutical companies, the food industry, biofuels, and enzymes [12–14]; as a substrate for the biomass production of *Saccharomyces cerevisiae* [15]; and in the production of ingredients for food processing [16]. Bread residues contain a high concentration of starch (over 70% dry matter) and protein (up to 14% dry matter) [9], and treatment with amylases, amyloglucosidases, and proteases easily leads to the release of available compounds for microbial growth [17].

Craft beer is characterized by the use of high-quality ingredients together with non-traditional ones (which, apart from reducing costs, adds special flavor and sensorial characteristics), as shown in our previous article [18].

Recently, some small breweries have started to use surplus bread to make their beers, replacing part of the malted barley that was originally used as a source of sugar for fermentation [19,20]. Studies already carried out have shown that the proportion of malt that can be replaced is limited [8], as malt contains enzymes necessary to break down bread starch into fermentable sugars, meaning that it is not practical to replace more than 25% of the malt. In this work, an attempt is made to valorize and recycle bread waste and brew ale beers using different types of bread as a partial substitute for malt. For this purpose, ale beer was brewed by replacing up to 50% of the malt weight with different types of bread: wheat bread, whole wheat bread, rye bread, and corn bread. Subsequently, a physicochemical comparison (including antioxidant activity and total polyphenol content) with 100% malt ale beer was made. A sensory comparison (visual and taste) between the different beers brewed was also developed.

2. Materials and Methods

2.1. Raw Materials

The brewing ingredients used for craft beer elaboration are shown in Table 1:

Table 1. Main Brewing raw materials used in the elaboration of craft beers.

Grain Malt	Pilsen EBC 3 (Weyermann, Bamberg, Germany)
	Munich Type I EBC 12 (Weyermann, Bamberg, Germany)
	Cara Rye EBC 150 (Weyermann, Bamberg, Germany)
	Biscuit EBC 45 (Castle Malting, Verviers, Belgium)

Table 1. *Cont.*

Hops Pellets	Centennial 9.6% a.a. (Laguilhoat, Fuenlabrada, Spain)
	Cascade 6.7% a.a (Laguilhoat, Fuenlabrada, Spain)
	Simcoe 13.6% a.a. (Laguilhoat, Fuenlabrada, Spain)
Bread	White wheat bread
	Whole wheat bread
	Corn bread
	Rye bread
Yeast	SafAle S-04 (Fermentis, Marcq-en-Baroeul, France)
	SafAle F-2 (Fermentis, Marcq-en-Baroeul, France)
Water	Monte Pinos (Carbónicas Navalpotro S.A, Almazán, Spain)

The yeast strain SafAle S-04 was used for the fermentation process in tanks, and the SafAle F-2 strain for fermentation in bottles; bread was purchased from La Tahona de Sahagún (Palencia, Spain), a local bread producer.

2.2. Reagents and Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was acquired from Sigma-Aldrich Química S.A., Madrid Spain. Gallic acid, methanol, and Folin–Ciocalteu reagent were acquired from Merck Millipore, Madrid, Spain. Sodium hydroxide (NaOH) 0.01 N, sodium chloride (NaCl), D(+)-glucose anhydrous for ACS analysis, Coomassie blue G.250 (CBBG), and Bradford reagent were acquired from Panreac, Castellar del Vallés, Spain. All solutions were prepared using analytical grade reagents and distilled water.

2.3. Pale Ale Beer Production in the Pilot Brewery

A control pale ale beer was brewed using the malts and hops that were most frequently repeated in the consulted recipes, i.e., those for American pale ale, one of the most-consumed types [21]. The total amount of malt used was 1046 g per brew; in detail, the following amounts of malt were added to 6 L of mineral water: Pilsen (45% by weight), Munich type I (40% by weight), Biscuit (10% by weight), Cara Rye (5% by weight), mineral water, pelleted hops (Centennial, Cascade, and Simcoe), and yeast.

Also, four brews were made according to the control pale ale beer recipe, using different kinds of bread (wheat, rye, whole wheat, and corn) to replace, in each case, 50% of the malt weight with the same amount of stale bread weight.

The pale ale beers produced were named using abbreviations, as follows: control (ALE); wheat bread (WHIB); rye bread (RYEB); whole wheat bread, also known as brown wheat (BROB); and corn bread (CORB). All elaborations were brewed in duplicate, obtaining 5 L of beer in each case and labeling each one by the abbreviation previously mentioned, followed by the number 1 or 2 depending on the duplicate.

The malt and bread were milled at the University of Valladolid cellar (University of Valladolid Campus, Palencia, Spain) just before mashing. The resulting ground malt was mixed with preheated mineral water at 40 ± 1 °C for 20 min for mashing into a stainless macerator tank. In all cases, mashing took two hours, at 67 ± 1 °C with manual stirring, to convert the starches from the malt and bread into sugar. Finally, the mash temperature was raised to 78 ± 1 °C for a 10 min period, at a rate of 1 °C per min (mash out), to deactivate the enzymes; then, the mixtures remained at room temperature for 24 h.

Mashing was followed by wort separation and sparging until the desired amount of wort was collected. The sparging was performed with natural mineral water at 80 ± 1 °C until the final volume of 5 L was completed, in order to achieve a greater extraction of fermentable sugars.

For the lupulization step, wort boiling at 100 °C required 60 min, following the time scheme shown in Table 2, and resulted in a total bitterness of 30 IBU (international bitterness units):

Table 2. Characteristics of the brewing pelleted hops used, the hops' addition time during boiling, and the amount of hops used in each treatment (5 L).

Variety of Hops	IBU	Alpha Acids (%)	Boil Min.	Weight (g)
Centennial	15	9.6	1	5.79
Cascade	10	6.7	30	7.86
Simcoe	5	13.6	59	3.34

All wort was fermented using the commercial yeast SafAle S-04, at 21 ± 1 °C, in 6 L tanks for 10 days. After beers reached the final attenuation degree, the temperature was gradually reduced to 4 °C in a cooling chamber; the beer remained for a week in the tanks to facilitate lees removal and beer maturation.

After the removal of lees, all the containers were tempered to 21 °C, and the carbonation phase was performed with 33 mL volume glass bottles, using dextrose and SafAle F-2 yeast, to obtain 2.0 bar of internal CO_2 pressure. All bottles rested for 14 days to finish fermentation at 21 ± 1 °C.

Finally, beer bottles matured in a refrigerated chamber at 4 °C for 2 weeks.

2.4. Physicochemical Analysis

All analyses were performed in triplicate. Unfiltered samples were extracted, excepting the color method, in which samples were previously centrifugated in a centrifuge (Bunsen, model KOCH 1460, Humanes de Madrid, Spain) at 4000 rpm for 5 min, as well as filtered by a vacuum filter (model: Kitasato) and Millipore filters (Merck Millipore, Darmstadt, Germany) of 0.45 microns.

- Turbidity
- Beer turbidity was measured with a turbidimeter (Hanna Instruments, HI 98703 model, Eibar, Spain), and each sample was placed in a transparent glass container with a lid. Each of these containers was placed in the turbidimeter to obtain turbidity values in NTU (nephelometric turbidity units).
- pH: pH was measured with a pH-meter (HACH-LANGE, calibrated sensiON™ + pH3 model, Hospitalet, Spain).
- Acidity: pH-meter measurements were taken in continuous function. An acid–base titration was performed until a pH of 7 was reached. The results were expressed in terms of lactic acid percentage.
- Alcohol By Volume (ABV): an ebulliometer (GAB system, 1010006 model, Moja, Spain) was used. It was calibrated with a standard (distilled water). The boiling temperatures of the standard (water) and the test sample (beer) were compared, and the volumetric alcohol content was calculated with a precision of 0.1 ABV using a ruler scale.
- Color (EBC): color was measured on a spectrophotometer (ThermoFisher Scientific, model 20 Genesys UV-Vis, Madrid, Spain). A beer sample of 3 mL, previously filtered, was introduced into a standard glass cuvette of 1 cm. The absorbance at 430 nm was measured, and distilled water was used as a blank. The obtained value was transformed to the European Brewery Convention (EBC) scale, multiplying the value by 25.
- Dry Extract: dry extract was measured with a thermobalance (Gibertini Eurotherm brand, Novate Milanese, Italy). An identical weight of each beer sample (1 g) was placed on the balance. The water contained in the sample was evaporated, and the remaining solid (dry extract) was weighed. The percentage of dry extract can be obtained directly via the difference with the total sample introduced.

2.5. Total Polyphenol Content and Antioxidant Capacity in Craft Beers

Samples were previously centrifugated in a centrifuge (Bunsen, model KOCH 1460, Humanes de Madrid, Spain) at 4000 rpm for 5 min, as well as filtered by a vacuum filter (model: Kitasato) and Millipore filters (Merck Millipore, Madrid, Spain) of 0.45 microns.

- Total polyphenol content (TPC).
- The total polyphenol content was determined by the Folin–Ciocalteu method by measuring absorbance at 760 nm [22] using the spectrophotometer mentioned above. A calibration line was performed using different concentrations (0.0–30 mg/L) of standard solutions of gallic acid, resulting in the following equation: Y = 0.0243x + 0.0209, R = 0.9959. The concentration of total phenols is expressed as mg of GAE (gallic acid equivalents) per mL^{-1} of the sample.
- Antioxidant capacity (DPPH)
- The antioxidant capacity of the different beers was measured using the method described by Abderrahim et al. [23]. Beer samples, once filtered and diluted (the 50 µL sample or the blank control), were introduced and mixed with 1000 µL of DPPH (60 µMol L^{-1} dissolved in methanol 1: 1/10 mMol L^{-1} Tris-HCl buffer pH 7.5) in a 5 mL volumetric flask. At 0 min, and after 20 min of incubation at room temperature in the laboratory (21 ± 2 °C), a small volume was introduced into 10 mm quartz cuvettes, and absorbance was measured at 520 nm with the spectrophotometer mentioned above. The antioxidant capacity of the beer, expressed in µMol DPPH mL^{-1}, was calculated using the following equation:

$$\mu\text{Mol (DPPH mL}^{-1}) = ((A_0 - At)/A_0) \times ((Vt\ [DPPH] \times FD)/mL) \quad (1)$$

where A_0: control absorbance (DPPH diluted in methanol); At: sample absorbance; Vt: total reaction volume in liters; [DPPH]: DPPH concentration; FD: dilution factor; and mL: sample milliliters used in the reaction.

2.6. Headspace Gas Chromatography–Mass Spectrometry Analysis (HS-GC–MS)

The preparation of the samples was carried out following the general method described by Liu and colleagues [24].

Samples were analyzed by HS/GC–MS (headspace gas chromatography coupled to a mass spectrometer) in a QP2010 Shimazdu device with an AOC 5000 autosampler and an HP-5MS column (30 m long, 0.25 mm internal diameter, and 25 µm of film).

Samples of 2 mL of each beer were previously filtered, placed in a 10 mL HS vial with NaCl 20% (*w*/*v*), and heated up to 80 °C at 250 rpm for 15 min; this was performed prior to the 100 ul HS injection of the sample in splitless mode.

Helium was used as a carrier gas, and the pressure was set at 110 kPa. The injector temperature was 120 °C, and the interface temperature was 250 °C. The oven followed the following program: an initial temperature of 40 °C for 2 min, a ramp-up of 10 °C/min to 140 °C, and a second ramp-up of 7 °C/min to 250 °C. Data were acquired in full scan mode in a m/z range of 30–350, and peak identification was made by comparison with the NIST08 and WILEY229 libraries.

2.7. Descriptive Sensory Analysis

2.7.1. Panel of Judges

A panel of 8 professional beer tasters, 5 men and 3 women, was trained according to the ISO 8586:2012 standard at the premises of the company Cibus In para Agroalimentación S.L. Tasters were trained in the recognition of beer descriptors through six tasting sessions of 2 h each; they used a presence–absence scale and also rated quantification on a discontinuous 3-point scale. The first two sessions included identification training, using a free-choice profile technique and an identification test control. In the last four sessions, a tasting sheet was established that included the most important descriptors, using a focus group qualitative technique. The criteria for the possible elimination of a judge were considered

and were specified for tasters who scored lower than 70% on the control identification test. Thus, a total of 2 tasters were eliminated, with 6 very well qualified tasters remaining.

2.7.2. Sensory Evaluation Session

Sensory analysis was carried out during a single session at five o´clock in the afternoon. Before starting the evaluation session, tasters were informed of the project objective and the kinds of beers to be tasted. All beers were examined in duplicate; then, ten beers were served, with a break after the tasting of the first five. Sufficient amounts of each sample at 6 °C were served in glasses standardized according to ISO 3591:1977. Each sample was coded with random numbers of 3 digits; samples were served in a different order for each taster. Tests were carried out in a tasting room that met the recommended guidelines of the UNE-EN ISO 8589:2010. Tasters spent approximately two minutes with each sample, using a descriptive method with the same tasting sheet used in training, which contained descriptors that allowed them to characterize the beer sensory profile: visual (intensity, tonality, limpidity, froth color, and CO_2 bubbles); aroma (maltiness, cereal malt, ripe fruit malt, hoppy, exotic fruit hop, citric fruit hop, herbaceous hop, yeast, tropical fruit yeast, spicy yeast, bread yeast, toasted, coffee, licorice, and caramel, as well as defects, such as: oxidized, cider, vinegar, musty, stable, and soapy); and taste (acidity, CO_2, bitterness, body, and persistence). These descriptors comprised most of the classes and some of the first-tier terms reported in the beer sensory wheel.

2.8. Statistical Analysis

Statistical analysis was carried out using Xlstat v.2021.1 statistical software (Addinsoft, Paris, France).

Data analysis was performed to establish the differences between the averaged values for physicochemical measurements, total polyphenol content, and antioxidant capacity; analysis was conducted using an analysis of variance (one-factor ANOVA) and Tukey's significant difference test (HSD), with statistical significance being set at a p-value < 0.05.

Principal component analysis (PCA) was used for HS-GC–MS, sensory analysis, and product characterization for the sensory data analysis.

3. Results and Discussion

3.1. Physicochemical Analysis

3.1.1. Turbidity

White bread beers yielded similar values for turbidity compared to the control beer (see Table 3). With the exception of whole wheat bread beers, all beers made with bread presented lower values of turbidity than the control beer (ALE), with this result being statistically significant in the case of the beers made with corn bread and rye bread.

Table 3. Values of the beer physicochemical properties (mean ± S.D.).

Beer/Analysis	Turbidity	Color (EBC)	pH	Acidity (% Lactic Acid)	ABV (%)	Dry Extract (%)
ALE 1	932.33 ± 29.57 ^{C}	25.46 ± 0.03 ^{A}	3.83 ± 0.06 ^{ABC}	0.03 ± 0.01 ^{A}	4.33 ± 0.06 ^{AB}	5.71 ± 0.03 ^{A}
ALE 2	975.67 ±15.63 ^{B}	24.06 ± 0.01 ^{B}	3.74 ± 0.01 ^{CD}	0.03 ± 0.01 ^{A}	4.33 ± 0.07 ^{AB}	5.53 ± 0.11 ^{AB}
WHIB 1	907.67 ± 8.33 ^{C}	17.82 ± 0.04 ^{E}	3.93 ± 0.01 ^{A}	0.03 ± 0.01 ^{A}	4.21 ± 0.07 ^{B}	5.35 ± 0.05 ^{BC}
WHIB 2	935.00 ± 3.61 ^{C}	16.33 ± 0.04 ^{F}	3.88 ± 0.04 ^{AB}	0.03 ± 0.01 ^{A}	4.37 ± 0.15 ^{AB}	5.03 ± 0.05 ^{DE}
BROB 1	1061.00 ± 3.46 ^{A}	19.40 ± 0.11 ^{C}	3.85 ± 0.04 ^{ABC}	0.02 ± 0.01 ^{A}	4.21 ± 0.10 ^{B}	5.03 ± 0.08 ^{DE}
BROB 2	1027.67 ± 6.51 ^{A}	21.00 ± 0.21 ^{D}	3.77 ± 0.01 ^{BC}	0.03 ± 0.01 ^{A}	4.58 ± 0.07 ^{A}	4.94 ± 0.03 ^{E}
CORB 1	743.67 ± 0.58 ^{E}	11.74 ± 0.05 ^{I}	3.81 ± 0.02 ^{ABC}	0.02 ± 0.01 ^{A}	3.39 ± 0.12 ^{C}	3.96 ± 0.06 ^{G}

Table 3. *Cont.*

Beer/Analysis	Turbidity	Color (EBC)	pH	Acidity (% Lactic Acid)	ABV (%)	Dry Extract (%)
CORB 2	689.67 ± 8.39 ^{F}	11.75 ± 0.02 ^{I}	3.85 ± 0.02 ^{ABC}	0.02 ± 0.01 ^{A}	3.49 ± 0.25 ^{C}	4.31 ± 0.16 ^{F}
RYE 1	834.67 ± 7.51 ^{D}	13.24 ± 0.08 ^{G}	3.64 ± 0.11 ^{D}	0.02 ± 0.01 ^{A}	4.25 ± 0.08 ^{AB}	5.01 ± 0.03 ^{DE}
RYE 2	708.00 ± 19.31 ^{EF}	15.11 ± 0.10 ^{H}	3.85 ± 0.02 ^{ABC}	0.02± 0.01 ^{A}	4.47 ± 0.06 ^{AB}	5.23 ± 0.06 ^{CD}

$^{A–G}$ Means without any common letter within the same column are significantly different ($p < 0.05$).

Whole wheat bread and white bread had the highest amounts of crumb, causing a higher content of suspended particles. On the other hand, the beer made with corn bread, as well as the one made with rye bread, had a very compact crumb, and the crust was much thicker, which favored the maceration process, releasing fewer solids into the wort and, therefore, generating less turbidity in the finished beers.

3.1.2. Color (EBC Scale)

The results obtained herein show that the control beers were a darker color (a higher value on the EBC scale), while the beers brewed with bread exhibited paler colors. This may be because the bread dough (crumb and crust) added less EBC color value than toasted malt. Considering the bread-made beers, the bread crust color influenced the wort color and the finished beer darkness. Whole wheat bread was the darkest among the beers with added bread, due to its crust.

It can be concluded, after this analysis, that bread is capable of imparting color to beer, although to a lesser extent than malt.

3.1.3. pH and Acidity

The control beers presented a pH range between 3.83 and 3.74, which was quite close to the expected value for these types of beers [25]. If we take this standard range as a reference, all beers made with bread showed similar pH values, without presenting significant differences between treatments with or without bread (Table 3).

As for acidity, no significant differences were observed in any of the treatments when making the 100% malt beer compared to the beers partially replacing malt with bread.

3.1.4. Alcoholic Strength

With regard to alcoholic strength, it can be observed that the control beers reached a ABV of 4.33, a very similar value to that achieved by the beers made with white bread, whole wheat bread, and rye bread. This indicates that the sugars obtained from bread were as fermentable as those from malt; in addition, the lower enzyme content present in the wort, because of the 50% malt replacement with bread, did not affect the transformation into fermentable sugars, resulting in a similar alcoholic content. It must be noted, however, that there was a lower alcoholic strength achieved in the brewing of corn bread, which only attained a value of 3.39–3.49 ABV; this was a statistically significant difference from the other treatments.

3.1.5. Dry Extract

The control beers were the ones with the highest dry extract values (5.71–5.53%); all bread beers presented lower values, especially the beers brewed with cornbread (4.31–3.96%), which presented the lowest values. This difference could be explained because the malt, in the absence of bread, was able to transfer more organic compounds into the wort during maceration.

3.2. Total Polyphenol Content (TPC) and Antioxidant Capacity in Craft Bread Beers

Phenolic compounds are generally considered one of the most important antioxidant sources in beer [26]. Phenolic compounds in beer are of great interest to brewers, as they

directly affect beer quality. In addition to their positive effect on oxidation prevention, they can negatively influence colloidal and foam stability and, thus, shorten the shelf life of beer [25].

Comparative results between beers with respect to TPC and antioxidant capacity are shown in Figure 1.

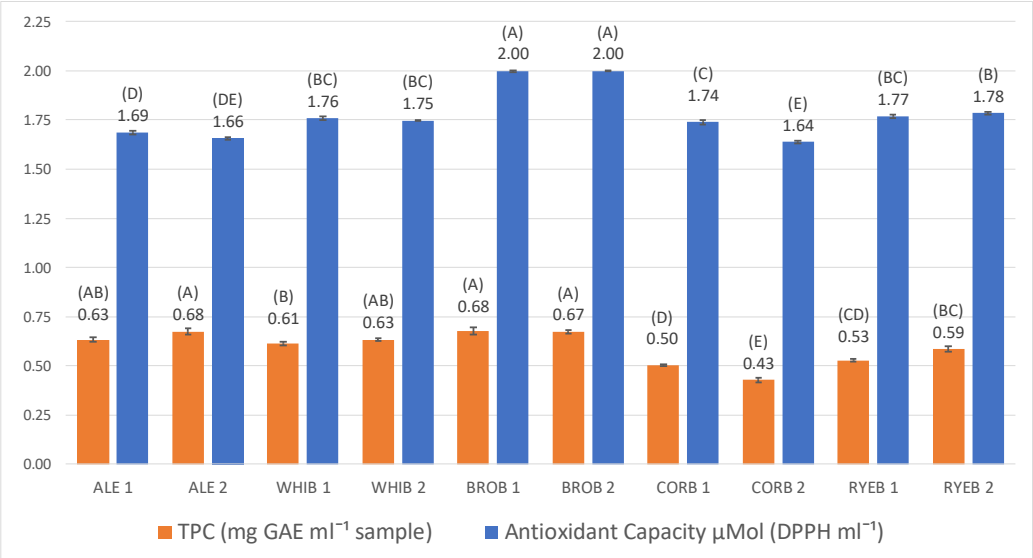


Figure 1. Antioxidant capacity and total polyphenol content in the craft bread beers studied. A–E means without any common letter within the same column are significantly different ($p < 0.05$).

Figure 1 shows that beers made with whole wheat bread and white bread presented similar TPC values compared to control beers. Only in two treatments, namely those made with rye bread and corn bread, were TPC values lower and statistically significant compared to the others (Figure 1). The results obtained are in agreement with another study, in which four styles of craft beer were analyzed, and the total polyphenol content ranged from 448.57 to 531.30 mg GAE L^{-1} [27].

Regarding industrial beers, there are many references concerning TPC analysis; for instance, lager and pilsner beers ranged between 464 and 579 mg GAE L^{-1} [25]. In another research study, the TPC values of 34 lager beer samples were studied, and results ranged between 152.01 mg GAE L^{-1} and 339.12 mg GAE L^{-1} [28]. In another work, Indian pale lager beers were analyzed, obtaining values ranging from 160 to 620 mg GAE L^{-1} [29]. In addition, there are many studies that analyzed TPC in ale beers, such as wheat ale beers, which had a value of 403.1 mg GAE L^{-1} [30]; American pale ale beers, which showed a value of 540 mg GAE L^{-1} [31]; and blond ale beers, which had values ranging from 125 to 544.3 mg GAE L^{-1} [32]. In addition, there were two other works that investigated ale beers, obtaining values ranging from 382.7 to 563 mg GAE L^{-1} [33,34]. Finally, another research study examined dark ale beers, resulting in values ranging from 448.1 to 542.4 mg GAE L^{-1} [35]. It is worth mentioning that the choice of raw materials and the brewing processes used for different kinds of beers have a major influence on the polyphenol content of the final product [34].

All TPC values determined in this study (Figure 1) were in the same range as the cited references; higher values were found in the control beers, whole wheat beers, and white wheat beers, which also attained darker colors than the other bread beers. This positive correlation between TPC and EBC color was also found in other studies [30,34].

In addition, the results showed that beers brewed with corn bread had lower TPC values, which agrees with several studies carried out with beers brewed with corn malt [36,37].

With regard to the antioxidant capacity (Figure 1), beers made with whole wheat bread presented significantly higher levels of this parameter compared to the other brews studied, including the control beers. The results also showed that all beers made with bread, as a

partial substitute for malt, had a higher antioxidant capacity than the control beer, except the corn bread beers. This is in accordance with a previous study of commercial beers [35], where somewhat lower values for antioxidant capacity were indicated, ranging from 0.56 to 1.66 μMol DPPH mL^{-1}.

Replacing 50% of malt with bread increases the antioxidant capacity and, in general, the polyphenol content of beers, improving properties that would benefit the health of the consumers [38].

3.3. GC–MS Detection

The identification of the main volatile compounds present in beers was performed using library data, as described in Section 2.6. Relative quantification was performed as a percentage, relating the area under each peak to the sum of all peak areas. Thus, 14 volatile compounds were identified, but only 7 were included in the volatile profile due to their higher relative areas and discriminating power. The results for the seven main volatile compounds according to the retention times and peak area (%) are shown in Table 4.

Table 4. Comparison of retention times and peak areas (%) of volatile compositions in craft bread beers.

Beer/Compound	Isoamyl Acetate	Phenyl Carboxylate	Linalool	Phenylethyl Alcohol	Ethyl Octanoate	Citronellol	Decanoic Acid Ethyl Ester
Retention Time (min.)	4.83	8.64	8.72	8.94	10.28	10.75	13.24
ALE 1	32.45	3.49	4.59	18.61	12.05	2.40	9.09
ALE 2	28.39	5.33	4.64	21.28	15.08	2.26	5.97
WHIB 1	29.03	7.67	5.31	31.99	6.37	2.54	0.73
WHIB 2	33.33	7.69	5.20	26.98	6.46	1.87	0.89
BROB 1	25.94	3.69	4.17	15.35	16.39	2.05	4.12
BROB 2	23.50	5.07	5.19	14.03	18.58	2.97	5.60
CORN 1	18.20	8.63	9.72	33.87	3.41	5.25	0.37
CORN 2	21.40	10.29	8.29	30.86	5.71	3.79	0.71
RYE 1	28.84	7.80	6.55	28.78	4.40	2.93	0.54
RYE 2	28.11	8.46	5.94	27.43	6.52	2.08	0.62

As an example, the chromatogram of the whole wheat bread beer is shown in Figure 2.

These main volatile compounds were also identified by other authors using the GC–MS technique [39–41]; those results showed high amounts of isoamylacetate, phenylethyl alcohol, and different ethyl compounds, plus other minority compounds, such as acid ethyl esters [41], linalool, and citronellol [39].

PCA was applied to evaluate the data trends. Two principal components were extracted that explained 91.52% of the total variance. F1 explained up to 68.70% of the total variance, and F2 explained another 22.81%. By representing F1 versus F2, a scatter plot of the analyzed beers (biplot) was obtained (Figure 3). Representing our variables with respect to these two factors, it appears that the "isoamyl acetate" variable is totally independent of any other studied variables and is associated with a negative value for Factor 1 and a positive value for Factor 2.

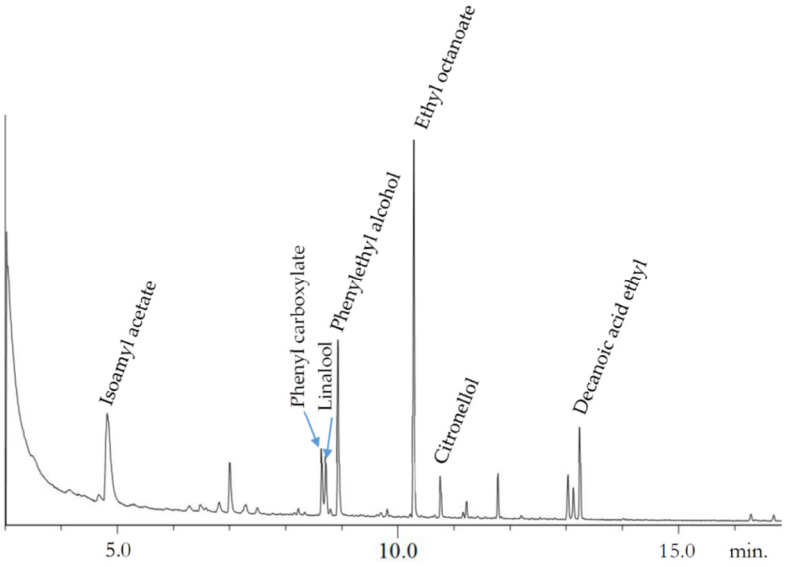


Figure 2. GC–MS chromatogram of whole wheat bread beer.

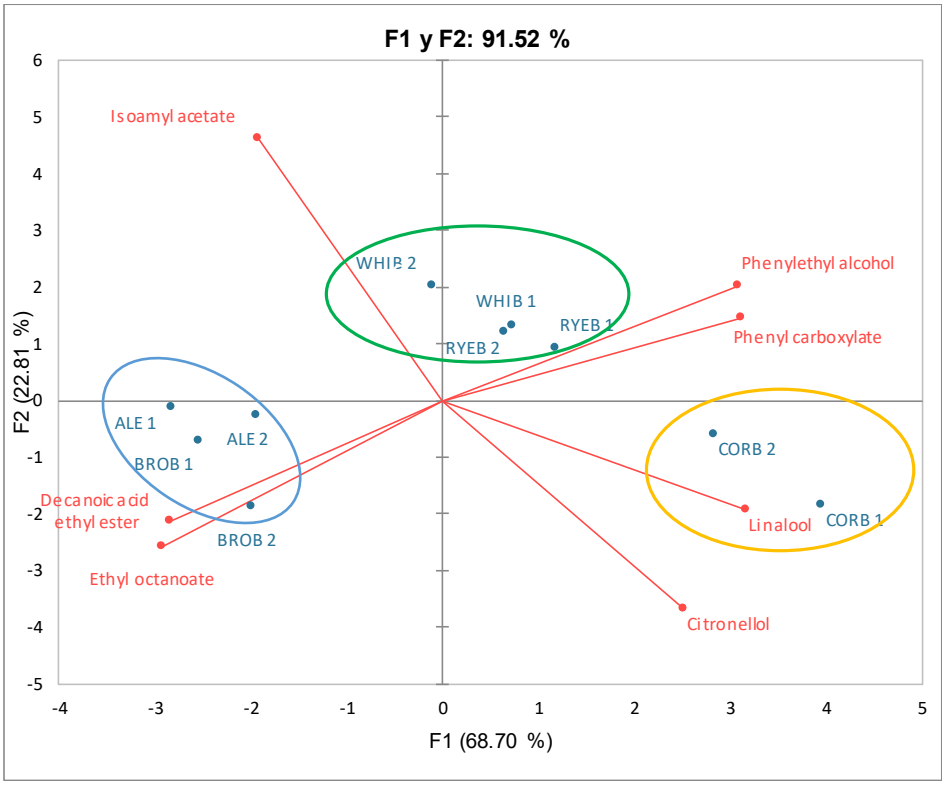


Figure 3. Principal component analysis of volatile compounds in analyzed bread beers.

Factor 1 had negative loadings for isoamyl acetate, ethyl octanoate, and decanoic acid ethyl ester, as well as high positive loadings for citronellol, linalool, phenylethyl alcohol, and phenyl carboxylate. Factor 2 had positive loadings for isoamyl acetate, phenylethyl alcohol, and phenyl carboxylate, as well as negative loadings for ethyl octanoate, decanoic acid ethyl ester, citronellol, and linalool.

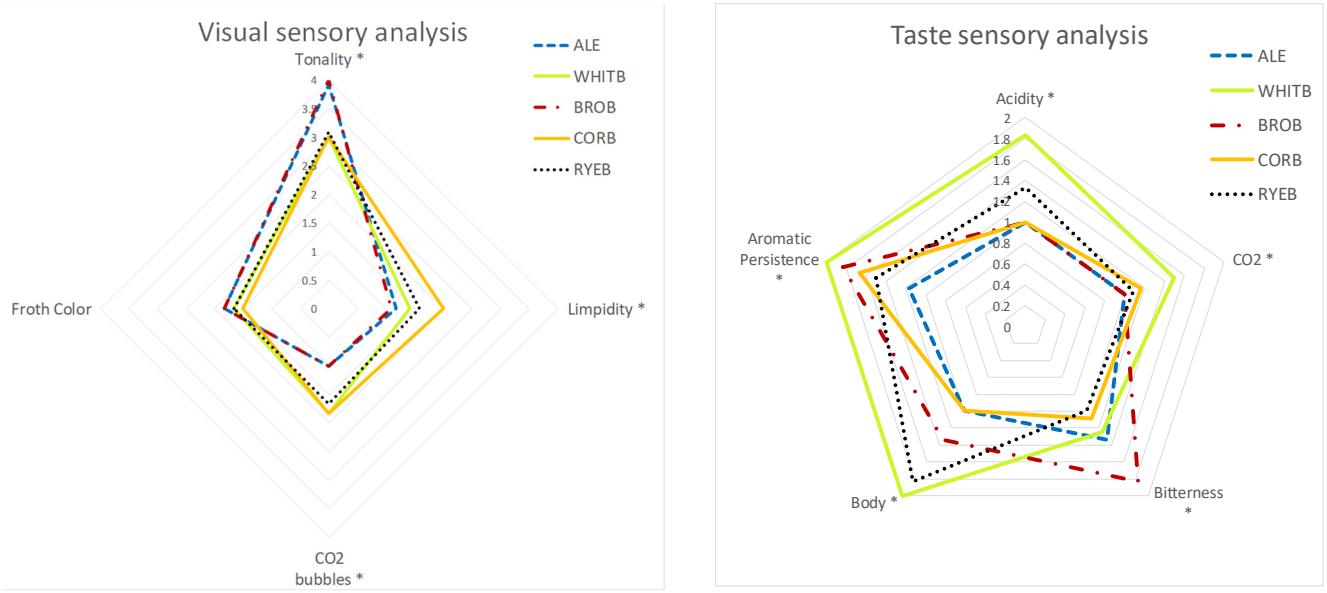
Based on the results of the PCA and considering the studied beer samples, RYEB and WHIB were grouped together. This group of beers was the most neutral beers, which contained phenylethyl alcohol, phenyl carboxylate, and isoamyl acetate. On the other hand, BROB and ALE were also grouped together, and they appear in the lower left quadrant. This group of beers was characterized by the presence of ethyl octanoate and decanoic acid

ethyl ester. Finally, in the lower right quadrant, the beer CORB was associated with the presence of citronellol and linalool (Figure 3).

3.4. Descriptive Sensory Analysis

3.4.1. Visual and Taste Sensory Analysis

The beer color analysis showed that the beer brewed with whole wheat bread attained the same color profile as the control beer, presenting a greater tonality; these results match those obtained experimentally for TPC (Figure 4a).



(**a**) (**b**)

Figure 4. (**a**) Visual radar graphic; (**b**) Taste radar graphic. * Statistically significant differences (p-value ≤ 0.05).

These results were in accordance with those of the physicochemical analysis, where control beers and whole wheat bread beers presented higher EBC color values and more turbidity than the other brewed beers.

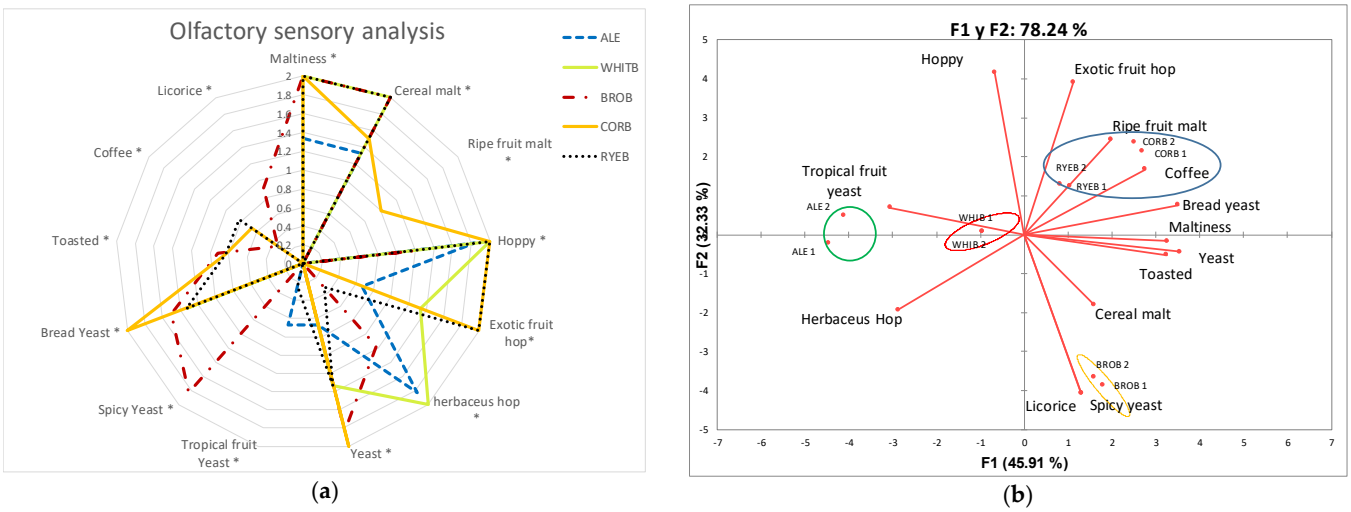
Regarding the taste phase, all beers made with bread generally exhibited a profile with greater intensity in taste descriptors than the control beer. In particular, this was true for white wheat bread beer in terms of body, acidity, and CO_2. In addition, the beer made with whole wheat bread presented greater bitterness, and this characteristic could be related to the increased content of polyphenols, providing the beer with a greater flavor intensity and longer persistence (Figure 4b).

The results obtained in the taste analysis show that the sensory profile varies considerably according to the beer analyzed. The beer brewed with white bread was the one that presented the highest values for acidity, CO_2, body, and aromatic persistence.

On the other hand, the beer brewed with corn bread was the most similar to the control beer, with very similar values for acidity, body, and bitterness; however, this beer had a considerably higher aromatic persistence than the control beer (Figure 4b).

3.4.2. Olfactory Sensory Analysis

Results from the olfactory descriptive analysis of the different beers showed that, in general, the beers brewed with bread had a more complex and intense odor profile than the control beer brewed with 100% malt (Figure 5a). However, all beers analyzed showed significant differences in terms of their main aromatic notes and some tasty flavors.



(**a**) (**b**)

Figure 5. (**a**) Olfactory radar graphic; (**b**) PCA olfactory analysis. * Statistically significant differences (p-value ≤ 0.05).

In order to corroborate the results obtained in the physicochemical analyses, a principal component analysis of the sensory variables was performed, obtaining two factors that explained 78.24% of the variance associated with the variables. In this PCA plot, F1 explained 45.91% of the total variance, and F2 explained another 32.33%. Representing our variables with respect to these two factors, it appears that the "herbaceous hop" variable was totally independent of the rest of the variables studied and was associated with negative values for Factor 1 and Factor 2 (Figure 5b).

Factor 1 had positive loadings for the majority of analyzed attributes: fruity exotic hop, ripe fruit malt, coffee, bread yeast, maltiness, yeast, toasted, cereal malt, spicy yeast, and licorice, as well as high negative loadings for hoppy, tropical fruit yeast, and herbaceous hop. Objects close together had similar characteristics; therefore, maltiness, yeast, and toasted variables appear to be highly positively correlated. Factor 2 had negative loadings for maltiness, yeast, toasted, cereal malt, spicy yeast, licorice, and herbaceous hop, as well as high positive loadings for fruity exotic hop, ripe fruit malt, coffee, bread yeast, fruity tropical yeast, and hoppy.

Based on the PCA results and considering the studied beer samples, CORB and RYEB were grouped together (Figure 5b). This group of beers showed exotic fruit hop, ripe fruit malt, coffee, and bread yeast flavors. In addition, the control beers were mainly associated with tropical fruit yeast flavor and were very close to beers brewed with white bread. Finally, in the lower right quadrant, the beer BROB was associated with the presence of licorice and spicy yeast flavors.

4. Conclusions

Brewing from bread is a viable alternative to traditional beer fermentation, although a significant fraction of malt is necessary. The percentage of malt that can be replaced by bread is up to 50%, meaning very important savings for the beer industry. All beers made by partially replacing malt with stale bread, except in the case of corn bread, achieved the same extraction of sugars, eventually reaching a similar alcoholic strength and physicochemical profile compared to the control beer, especially in the case of whole wheat bread beer.

Bread releases fewer particles into the brewing wort than malt, resulting in less cloudy and, in general, less intensely colored beers.

In particular, beers brewed with corn bread were of lower intensity, exhibiting lower values than the rest of the beers in terms of color, turbidity, alcoholic strength, dry extract, and total polyphenols; as such, corn bread beer can be defined as a lighter and weaker beer. In general, bread beers presented characteristic attributes in the different phases of the tasting, allowing them to be classified into different sensory profiles.

Beers brewed with whole wheat bread resulted in a product with physicochemical characteristics similar to that obtained using only malt, surpassing the rest of the elaborations made with bread. In addition, the total polyphenol content and antioxidant capacity make it a beer with healthier properties and better sensorial characteristics, providing a higher level of bitterness and greater persistence in the mouth. All of these results mean a great advancement and advantage for the brewing industry all over the world.

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CHAPTER 2

Brewing with Whole Wheat Bread to Produce Different Beer Styles.

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Brewing with Whole Wheat Bread to Produce Different Beer Styles

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Abstract: Beer is one of the most widely consumed alcoholic beverages and is rich in nutrients. Meanwhile, bread waste is a major contributor to global food waste. This study investigated substituting up to 50% of malt with whole wheat bread in American lager, Indian pale ale, and Bavarian weiss ale to reduce bread waste and enhance beer's nutritional profile. The study assessed physicochemical properties, bioactive compounds, and volatile profiles of bread-based beers versus traditional malt-based brews. Results showed that bread beers maintained key properties while increasing bioactive compounds, especially in Bavarian weiss, which had higher total polyphenol content (1.04 mg GAE mL^{-1} compared to 0.507 mg GAE mL^{-1}). Antioxidant activity in weiss beer also increased (2.007–2.057 µMol DPPH mL^{-1} relative to 0.68–1.75 µMol DPPH mL^{-1} in 100% malt weiss). PCA analysis highlighted a distinct bioactive profile in bread beers, with elevated phenylethyl alcohol and ethyl octanoate. Substituting malt with bread was feasible, producing beers of comparable quality and potential health benefits. These findings support bread as a sustainable, cost-effective malt alternative, reducing waste and enhancing beer within a circular economy framework.

Keywords: whole wheat bread; craft beer; lager; weiss; IPA; physicochemical profile

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1. Introduction

Nowadays, food waste is a global problem. Recent studies estimate that 14% of food produced is lost in the supply chain before reaching retail stores [1], while 17% of available food is wasted at marketing levels and consumption [2]. The latest world estimates showed that approximately 931 million tonnes of food waste were generated in 2019, with 61% from households, 26% from food services, and 13% from retail [2].

The excessive production of food to meet the demand of a growing global population, coupled with wasteful consumer behavior, has led to significant food waste, posing a major challenge for sustainability and resource efficiency.

Bread is a very important food in the human diet due to its nutritional composition. For example, 100 g of bread typically contains about 59.8 g of starch, 22.3 g of moisture, 1.56 g of total organic nitrogen, and 8.9 g of protein [3]. It is produced in large quantities to satisfy high consumer demand, mainly in North America and Europe [4]. However, bread has a short shelf life of 3–6 days at room temperature, mainly due to its high nutrient content, making it susceptible to rotting and hardening [5].

This, along with consumer preference for freshly baked goods, has led to bread piling up from bakeries to retailers and homes [6].

The waste hierarchy, developed in the 1970s to prioritize different waste management strategies, has evolved in recent years and has been adapted to food waste. This pyramid prioritizes prevention actions first, followed by routes aimed at the reuse of surplus food suitable for human consumption, the reuse of food not intended for human consumption as animal feed, the recycling of materials in products of high added value (without carrying out complete degradation), nutrient recycling, energy recovery, and, as a last option, the elimination of food waste [7]. Regarding environmental impact, previous studies have examined different management options. For the most part, the results supported the same waste hierarchy. For example, Brancoli et al. [8] evaluated the relative environmental impacts of different treatment options for surplus bread and observed that a reduction at source and the use of surplus bread in different recovery pathways (animal feed, donation, beer and ethanol production) are environmentally preferable options compared to waste (e.g., anaerobic digestion and incineration).

Beer is among the most popular alcoholic beverages in the world [9]. It is typically produced using malted cereal grains (e.g., barley), yeast, hops, and water; however, adjuncts and additives are sometimes used by the brewing companies. Beer is a beverage rich in nutrients such as carbohydrates, amino acids, minerals, vitamins, and polyphenols, which result from a multi-step brewing and fermentation process [10].

Furthermore, it is a beverage rich in antioxidants, such as phenolic compounds, melanoidins, SO_2, and vitamins [11]. Phenolic compounds are a group of chemical substances characterized by the presence of at least one phenolic unit. Several studies have shown that phenols contribute more than 50% to the antioxidant activity of beer [12,13]. Several studies have shown that these compounds, in addition to playing a key role in antioxidant activity (AOX), also affect the sensory stability of beer [14]. Certain polyphenols and their oxidation products are sensory active, affecting beer astringency and bitterness [15,16], and even beer color, aroma, and flavor stability [11,17].

Brewing beer from bread is a viable alternative to traditional beer fermentation, although a significant fraction of malt is required, as malt contains necessary enzymes to break down bread starch into fermentable sugars. Despite the potential use of this bread waste for beer brewing, there are currently few studies on the matter.

Almeida et al. [18] brewed a craft beer with waste bread and concluded that the resulting beer had a 20% lower carbon footprint compared to the control craft beer. Subsequently, Brancoli et al. [8] investigated the use of leftover bread as a substitute for malted barley in brewing (25–28% by weight) and determined the GWP savings. It was concluded that the GWP decreased by 0.46 kg CO_2 eq. per kg of wasted bread used in brewing. This calculation was obtained without including the reduction in emissions due to the reduced decomposition of bread in landfills. In 2021, Narisetty et al. [19] showed that a maximum of 25% bread can replace barley due to the need for enzymes. Three years later, McDonagh et al. [20] studied the feasibility of using waste bread to brew beer, investigating the impact on alcohol content and the environmental implications of this substitution. The results showed that beer brewed with up to 60% malted barley by weight replaced by bread had sufficient fermentability to produce the required volume of alcohol. They also concluded that the annual carbon footprint was reduced by 7.13% in carbon dioxide equivalent compared to the industrial process.

Recently, in 2025, Dall'Acqua et al. [21] evaluated the possibility of replacing barley malt with wasted bread in ale beer, as well as its impact on the resulting beverage, both during its production and in the final product. The results showed no differences between the control beers and those brewed with bread in most physicochemical and sensory analyses.

In a previous study carried out with different types of bread (wheat, whole wheat, rye, and corn bread), it was demonstrated that up to 50% of malt could be successfully

replaced with stale bread, which represents a very important saving for the brewing industry. Furthermore, it was found that beer made with whole wheat bread had a similar physicochemical profile to beer made with 100% malt. In addition, a higher total content of polyphenols and antioxidant capacity was observed, presenting it as a beer with healthier properties and better sensory characteristics than conventional beer [22].

This study is based on our prior research, investigating optimal outcomes achieved in brewing whole wheat bread beers, with the primary objective of evaluating the performance of our brewing process design across different beer styles, specifically, American lager, Indian pale ale (IPA), and Bavarian weiss ale, by comparing the physicochemical characteristics of 100% malt brews versus whole wheat bread-based beers.

2. Materials and Methods

2.1. Reagents and Standards

Methanol, gallic acid, and Folin–Ciocalteu reagent were purchased from Merck Millipore (Madrid, Spain). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was acquired from Sigma-Aldrich Química S.A. (Madrid Spain). Sodium chloride (NaCl), sodium hydroxide 0.01 Mol L^{-1}, Coomassie blue G.250 (CBBG), D (+)-glucose anhydrous for ACS analysis, and Bradford reagent were obtained from Panreac (Castellar del Vallés, Spain). All solutions were prepared with analytical-grade reagents and distilled water.

2.2. Raw Materials

Three different styles of craft beer were elaborated due to their being the most widely consumed beers in the world: American lager for lager beer and, in the case of ale beers, IPA (Indian pale ale) and Bavarian weiss beer. All beers were brewed using 100% malt as control samples. In the experimental beers, 50% of the total malt was replaced with the same weight of whole wheat bread (1:1 substitution). This replacement was done proportionally according to the original recipe, meaning all types of malt were reduced equally, keeping the same proportions as in the control. According to the style, the raw materials used in beer recipes for this study are shown in Table 1.

The yeast strains Saflager S-23, Safale US-05, and Safbrew W-06 were used for the fermentation process in tanks, while the Safale F-2 strain was employed for bottle fermentation; whole wheat bread was acquired in a stale condition (three days after production) from "La Tahona de Sahagún" (Palencia, Spain), a local bread producer, and milling was carried out immediately after its arrival at the laboratory.

2.3. Brewing Procedure

All beers were brewed at the University of Valladolid pilot cellar (University of Valladolid Campus, Palencia Spain). Beers (100% malt grains and flakes) were brewed as a control in each style, and total malt amounts added to 10 L of mineral water were 2.381 Kg per American lager, 2.650 Kg per IPA, and 2.170 Kg per Bavarian ale brew. Also, bread beers were made according to the control ones in each cited style, replacing 50% of the weight malt with the same weight of stale whole wheat bread. All beers were brewed in duplicate, in individual 10 L batches, and labeled with abbreviations as follows: 100% malt beers were designated as American lager (LA), Indian pale ale (IPA), and Bavarian weiss ale (W); bread beers were codified the same way as the controls with the addition of the letter "B" at the end.

Table 1. Brewing raw materials used in the elaboration of craft beers.

Raw Material	American Lager Beer	IPA Beer	Bavarian Weiss Beer
Grain malt and flakes (% by weight)	Pale Ale EBC 7 (Castle Malting, Beloeil, Belgium) (85%)	Pilsner EBC 3 (Weyermann, Bamberg, Germany) (81%)	Pilsner EBC 3 (Weyermann, Bamberg, Germany) (50%)
	Corn flakes EBC 3.5 (Castle Malting, Beloeil, Belgium) (10%)	Munich Type I EBC 12 (Weyermann, Bamberg, Germany) (15%)	Pale Wheat malt EBC 5 (Weyermann, Bamberg, Germany) (50%)
	Rice flakes EBC 2.5 (Castle Malting, Beloeil, Belgium) (5%)	Cara amber EBC 60 (Weyermann, Bamberg, Germany) (4%)	
Pelleted hops	Saaz 3.80% a.a. (Laguilhoat, Fuenlabrada, Spain)	Cascade 6.80% a.a. (Laguilhoat, Fuenlabrada, Spain)	Magnum 13.10% a.a. (Laguilhoat, Fuenlabrada, Spain)
		Citra 12.70% a.a. (Laguilhoat, Fuenlabrada, Spain)	
Bread	Whole wheat	Whole wheat	Whole wheat
Yeast	Saflager S-23 (Fermentis, Marcq-en-Baroeul, France)	Safale US-05 (Fermentis, Marcq-en-Baroeul, France)	Safbrew W-06 (Fermentis, Marcq-en-Baroeul, France)
	Safale F-2 (Fermentis, Marcq-en-Baroeul, France)	Safale F-2 (Fermentis, Marcq-en-Baroeul, France)	Safale F-2 (Fermentis, Marcq-en-Baroeul, France)
Water	Monte Pinos (Carbónicas Navalpotro S.A, Almazán, Spain)		

The detailed brewing process, which was optimized by our team in an early stage and recently published [22], is shown in Figure 1.

First, malts and stale bread were ground separately, in a two-roll mill spaced 1 mm, just before mashing. Second, the mineral water was preheated to a temperature of 40 °C, and the resulting ground malt or ground malt plus bread at 50% by weight were poured into buckets and stirred for 20 min. Next, the temperature was increased at different steps, depending on each recipe, as described in Figure 1, and kept for 24 h for extended maceration at room temperature in order to prolong enzymatic activity and improve starch and protein hydrolysis, thereby enhancing fermentable sugar availability. Then, after lautering wort was transferred to the kettle, the remaining bagasse was sparged using hot mineral water at 80 °C until the final volume of 10 L was completed. All batch wort were boiled at 100 °C for 60 min. Saaz pelleted hop was added at the start of boiling to obtain 18 International Bitterness Units (IBU) for the American lager recipe. In the case of Indian pale ale, two different pelleted hops were used: Cascade hop was infused to contribute 35 IBU at the beginning of boiling, and Citra hop was added in the middle of this procedure to achieve the remaining 15 IBU. For the Bavarian ale recipe, Magnum pelleted hop was introduced at the start of boiling to achieve 15 IBU. After boiling, the hot trub was removed, and the wort was rapidly cooled using a freezing chamber at −25 °C to reach the appropriate fermentation temperature (Figure 1). Before yeast inoculation, all wort batches were oxygenated by manual agitation for 2 min to ensure proper yeast activity.

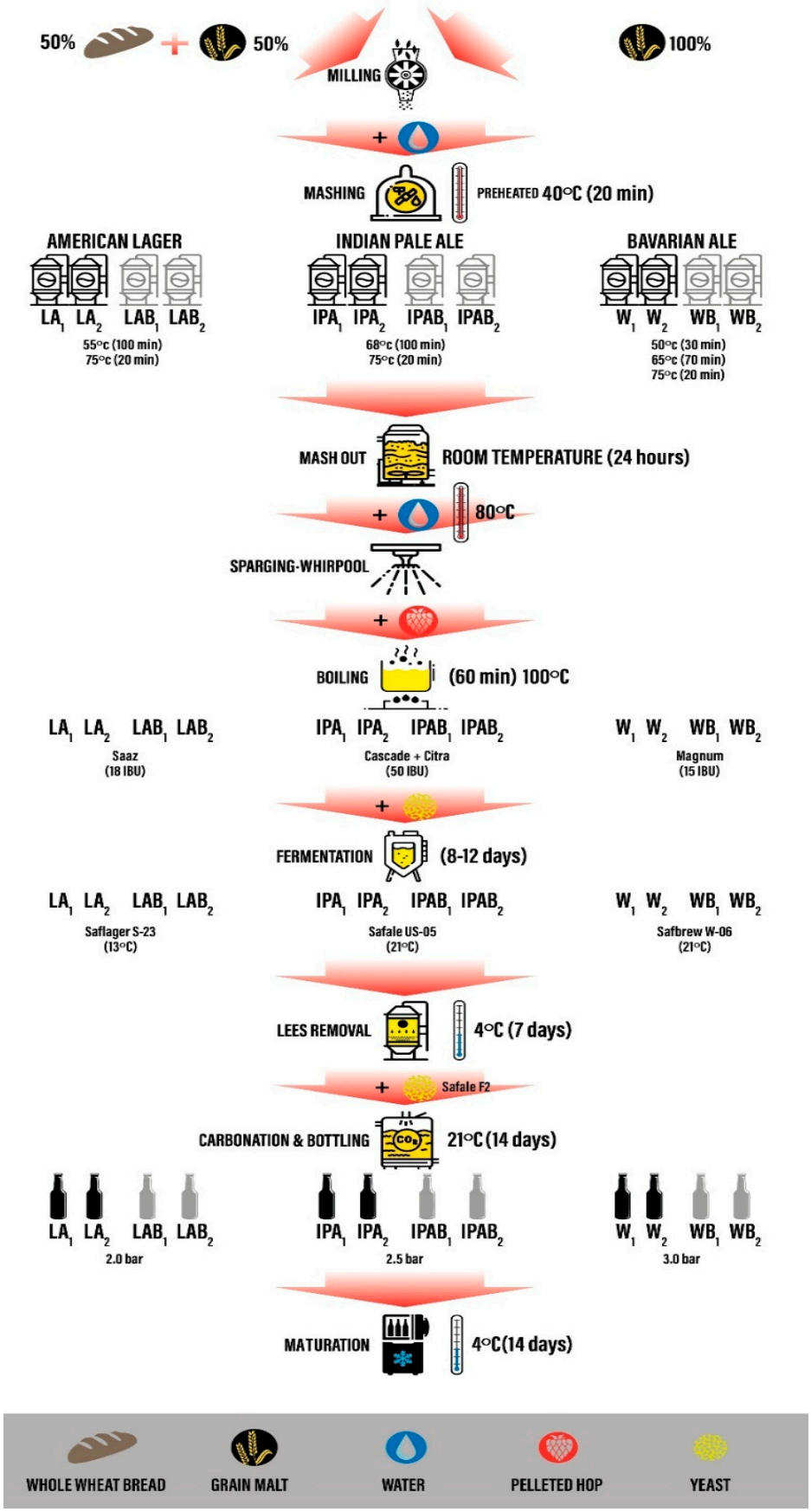

Figure 1. Brewing process diagram.

Different commercial yeasts were used for primary fermentation, applied at a concentration of 0.5 g per liter, according to Figure 1. All dry yeast strains were rehydrated prior to inoculation. Rehydration was performed in sterile water at 25 °C, using a yeast-to-water ratio of 1:10 (*w*/*v*), in accordance with the manufacturer's guidelines. The suspension was allowed to rest undisturbed for 15 min before being pitched into the wort. Primary fermentation was conducted in 10 L stainless steel tanks for 8 to 12 days. The tanks were equipped with an overpressure valve to allow safe CO_2 release. Fermentation temperature was consistently maintained by placing the tanks in a climate-controlled laboratory room, set to the specific conditions required for each beer style (13 °C for lager and 21 °C for ale fermentations). After beers reached the final attenuation degree, the temperature was gradually reduced to 4 °C in a cooling chamber; the beer remained in the tanks for a week to facilitate the next stage of lees removal and beer maturation. After lees removal, all containers were tempered to 21 °C. Carbonation was then carried out in 330 mL glass bottles using dextrose and Safale F-2 yeast. Dextrose was added at 4 g per liter of beer per bar of target pressure, aiming to reach internal CO_2 levels of 2.0 bar for lager, 2.5 bar for IPA, and 3.0 bar for Bavarian beer. Then, all bottles rested for 14 days to finish fermentation at 21 °C and final pressure was verified using crown-cap aphrometers. Finally, all beer bottles matured in a refrigerated chamber at 4 °C for 2 weeks.

2.4. Physicochemical Analysis

Unfiltered samples were extracted, excepting those used for color analysis, in which samples were previously centrifugated in a centrifuge (Bunsen, model KOCH 1460, Humanes de Madrid, Spain) at 4000 rpm for 5 min, as well as filtered by a vacuum filter (model: Kitasato) and Millipore filters (Merck Millipore, Darmstadt, Germany) of 0.45 microns.

- pH was measured with a pH-meter (HACH-LANGE, calibrated sensiON™⁺ pH3 model, Hospitalet, Spain).
- Acidity: pH-meter measurements were taken continuously. An acid–base titration was performed until a pH of 7 was reached. Then, the results were expressed in terms of lactic acid percentage.
- Turbidity was measured with a turbidimeter (Hanna Instruments, HI 98703 model, Eibar, Spain). The different samples were placed in a transparent glass container with a lid, and then each container was placed in the turbidity meter to obtain NTU turbidity values (Nephelometric Turbidity Units).
- Alcohol By Volume (ABV) was measured with an ebulliometer (GAB system, 1010006 model, Moja, Spain). First, it was calibrated with distilled water as a standard. Subsequently, the boiling temperatures of the standard (water) and the test sample (beer) were compared, and the volumetric alcohol content was calculated to the nearest 0.1 ABV using a ruler scale. Full attenuation of each batch was verified by three consecutive daily density readings at 20 ± 0.1 °C (stable FG = 1.010–1.012 SG) using a calibrated densimeter. The ebulliometric values were then validated by comparing the resulting % ABV with those calculated from the original and final gravities according to the EBC reference equation.
- Real Extract was determined in accordance with the principles of Analytica-EBC (Real Extract of Beer) by direct gravimetry. After degassing each beer at 20 ± 0.1 °C, an accurately weighed 1.000 ± 0.005 g aliquot was transferred to a pre-dried, tared pan in a thermobalance (Gibertini Eurotherm, Novate Milanese, Italy). The sample was dried at 105 °C until the rate of mass loss was <0.1 mg per 30 s, guaranteeing constant weight. The residue represents the real extract, expressed as % (m/m).

2.5. Spectrophotometric Analysis

Samples were first decarbonated by magnetic stirring for 30 min, and then centrifuged in a centrifuge (Bunsen, model KOCH 1460, Humanes de Madrid, Spain) at 4000 rpm for 5 min, as well as filtered by a vacuum filter (model: Kitasato) and Millipore filters (Merck Millipore, Madrid, Spain) of 0.45 microns.

All filtered beers were measured on a spectrophotometer (ThermoFisher Scientific, model 20 Genesys UV-Vis, Madrid, Spain).

- Color (EBC): Beer color was determined according to the standard method of the European Brewery Convention (EBC).
- Total polyphenol content (TPC): The total polyphenol content was determined by the Folin–Ciocalteu method by measuring absorbance at 760 nm [23], using the spectrophotometer mentioned above. A calibration line was performed using different concentrations (0.0–30 mg L^{-1}) of standard solutions of gallic acid, resulting in the following equation: Y = 0.0243x + 0.0209, R = 0.9959. The concentration of total phenols is expressed as mg of GAE mL^{-1} of the sample.
- Antioxidant capacity (DPPH): The antioxidant capacity of the beer samples was determined according to the method described by Abderrahim et al. [24]. Beer samples, once filtered and diluted (the 50 µL sample or the blank control), were introduced and mixed with 1000 µL of DPPH (60 µMol L^{-1} dissolved in methanol 1: 1/10 mMol L^{-1} Tris-HCl buffer pH 7.5) in a 5 mL volumetric flask. At 0 min, and after 20 min of incubation at room temperature in the laboratory (21 ± 2 °C), a small volume was introduced into 10 mm quartz cuvettes, and absorbance was measured at 520 nm with the spectrophotometer mentioned above. The antioxidant capacity of the beer, expressed in µMol DPPH mL^{-1}, was calculated using the following mathematical formula:

$$\mu\text{Mol}\left(\text{DPPH mL}^{-1}\right) = ((A_0 - A_t)/A_0) \times ((V_t\,[DPPH] \times FD)/\text{mL})$$

  where A_0: control absorbance (DPPH diluted in methanol); A_t: sample absorbance; V_t: total reaction volume in liters; [*DPPH*]: *DPPH* concentration; *FD*: dilution factor; and mL: sample milliliters used in the reaction.
- Protein content: A method based on the Bradford test [25] was followed, according to which the quantification of proteins is based on the union of the Coomassie blue dye G-250 (Bradford reagent) with the proteins available from the analyzed beer samples. In these analyses, 3140 µL of distilled water and 200 µL of the Bradford reagent were added to 60 µL of the beer sample in a test tube. A calibration line from 1 to 40 µL was also constructed using serum albumin (0.1 µg μL^{-1}). Finally, samples were measured at 595 nm.

2.6. Headspace Gas Chromatography–Mass Spectrometry Analysis (HS-GC-MS)

Sample preparation was performed according to the general method described by Liu et al. [26].

Samples were analyzed by HS-GC-MS (headspace gas chromatography coupled to a mass spectrometer) in a QP2010 Shimazdu device with an AOC 5000 autosampler (Shimadzu Europa GmbH, Duisburg, Germany) and an HP-5MS column (30 m long, 0.25 mm internal diameter, and 25 µm of film).

Two mL of each previously filtered beer were placed in a 10 mL HS vial with NaCl 20% (*w*/*v*), and heated up to 80 °C at 250 rpm for 15 min; this was performed prior to the 100 µl HS injection of the sample in splitless mode.

The pressure was set at 110 kPa and Helium was used as a carrier gas. The interface temperature was 250 °C and the injector temperature was 120 °C. The oven followed the

following program: an initial temperature of 40 °C for 2 min, a ramp-up of 10 °C/min to 140 °C, and a second ramp-up of 7 °C/min to 250 °C. Data were acquired in full scan mode in a m/z range of 30–350, and peak identification was determined by comparison with the NIST08 and WILEY229 libraries.

2.7. Statistical Analysis

All analyses were performed in triplicate. Statistical analysis was carried out using Xlstat v.2023.3.1 statistical software (Addinsoft, Paris, France).

Data analysis was conducted to identify differences between the mean values for physicochemical and spectrophotometric measurements; analysis was performed using an analysis of variance (one-factor ANOVA) and Tukey's significant difference test (HSD), with statistical significance being set at a p-value < 0.05.

Principal component analysis (PCA) was used for HS-GC-MS and physicochemical analyses.

3. Results and Discussion

3.1. Physicochemical Analysis

The main physicochemical properties of beer are shown in Table 2.

Table 2. Values of physicochemical properties in craft beers (mean ± S.D).

Sample	Turbidity (NTU)	pH	Acidity (% Lactic Acid)	ABV (%)	Real Extract (%)
LA1	620.33 ± 52.29 ^{CD}	3.92 ± 0.01 ^{D}	0.03 ± 0.02 ^{A}	5.52 ± 0.10 ^{B}	5.65 ± 0.24 ^{DE}
LA2	684.33 ± 16.01 ^{D}	3.94 ± 0.01 ^{D}	0.04 ± 0.01 ^{A}	5.56 ± 0.04 ^{B}	5.65 ± 0.13 ^{DE}
LAB1	542.00 ± 18.73 ^{DE}	4.16 ± 0.01 ^{BC}	0.04 ± 0.01 ^{A}	5.25 ± 0.10 ^{B}	6.15 ± 0.21 ^{BC}
LAB2	462.00 ± 9.00 ^{E}	4.12 ± 0.01 ^{C}	0.04 ± 0.01 ^{A}	5.43 ± 0.15 ^{B}	5.73 ± 0.30 ^{CD}
IPA1	675.67 ± 26.5 ^{D}	4.42 ± 0.02 ^{A}	0.04 ± 0.01 ^{A}	6.98 ± 0.13 ^{A}	6.74 ± 0.16 ^{A}
IPA2	627.33 ± 29.19 ^{CD}	4.31 ± 0.09 ^{AB}	0.04 ± 0.01 ^{A}	7.00 ± 0.10 ^{A}	6.48 ± 0.09 ^{AB}
IPAB1	620.00 ± 5.29 ^{CD}	4.31 ± 0.02 ^{AB}	0.04 ± 0.01 ^{A}	6.70 ± 0.22 ^{A}	5.93 ± 0.13 ^{CD}
IPAB2	671.67 ± 37.07 ^{D}	4.20 ± 0.04 ^{BC}	0.04 ± 0.01 ^{A}	6.72 ± 0.19 ^{A}	5.99 ± 0.15 ^{CD}
W1	1023.67 ± 39.70 ^{A}	3.85 ± 0.03 ^{D}	0.05 ± 0.01 ^{A}	5.37 ± 0.15 ^{B}	5.46 ± 0.11 ^{DEF}
W2	1017.33 ± 30.35 ^{A}	3.84 ± 0.02 ^{D}	0.05 ± 0.01 ^{A}	5.23 ± 0.15 ^{B}	5.21 ± 0.16 ^{EF}
WB1	807.67 ± 27.50 ^{B}	3.78 ± 0.01 ^{D}	0.05 ± 0.01 ^{A}	5.22 ± 0.10 ^{B}	5.17 ± 0.12 ^{EF}
WB2	867.67 ± 16.44 ^{B}	3.82 ± 0.01 ^{D}	0.05 ± 0.01 ^{A}	5.40 ± 0.10 ^{B}	5.06 ± 0.05 ^{F}

$^{A–F}$ Means without any common letter within the same column are significantly different ($p < 0.05$).

Using whole wheat bread as a partial malt substitute in brewing did not result in significant changes in turbidity, except for hazy beers like Bavarian weiss styles, where a decreasing trend and significant differences were observed when malt was replaced by whole wheat bread versus the control. In fact, high turbidity is a distinguishing feature of wheat beers due to their high molecular weight proteins and polysaccharides [27].

These results are consistent with our previous research, which showed that bread decreased turbidity when used as a starchy source in pale ale beers [22], perhaps due to the reduction in malt content caused by its partial replacement.

The pH values ranged from 3.78 to 4.42. No significant differences in pH were observed between bread beers and 100% malt beers among ale styles. Indian pale ales had higher values (4.20–4.42) compared to the other beers; this may be attributed to the use of a greater amount of hops compared to the other styles.

Significant differences were observed between the control American lager (LA) and the American lager bread beer (LAB), where the pH increased with the addition of the whole wheat bread. Habschied et al. [28] studied 26 samples of different beer styles and

reported pH values of 3.9–4.12 for lagers and 3.62–4.64 for Indian pale ales, which are in range with the values obtained in this study.

As for the acidity, no statistically significant differences were found in the samples, regardless of beer style or raw material used. However, the weiss beers showed higher acidity; this was also observed in the study of Pai et al. [29], where the sample of lager beers with the lowest pH was also the most acidic. Moreover, pH and acidity are considered crucial criteria in the brewing industry, as they influence sensory parameters such as color, taste, and the biological and chemical stability of the beer [29,30]. The results obtained in this research showed that samples presented lower acidity in general compared to Silva et al. [30], who studied craft beers, where lagers had an average acidity of 0.18%, Indian pales ales 0.28%, and weiss beer 0.17%.

All brewing styles, independent of the raw material, achieved the alcoholic strength recommended by the Brewers Association Beer Style Guidelines [31], and Indian pale ales showed the highest ABV, ranging from 6.70–7.0%. Byeon et al. [32], who studied wheat malt beers, reported an alcohol content of 4.83–5.37%; these results are similar to those obtained in our investigation for weiss and weiss bread beers. Another study reported ranges of alcohol content in the samples used of 3.2–6.7% for American lagers, which agrees with our findings [33]. All these results confirm that the alcoholic content was consistent with the expected parameters, regardless of the partial substitution of malt with whole wheat bread in each beer style.

In general, all beers showed no significant differences in real extract content, except for Indian pale ales, which presented higher values (6.48–6.74%). This trend may be related to the formulation of IPA recipes, which typically involve higher malt concentrations and dry hopping, both of which may contribute to an increased level of non-fermentable substances in the final beer. A similar observation was reported in previous research on bread beers, which showed lower real extract values compared to beers brewed with 100% malt. This trend may be attributed to the fact that, in the absence of bread, malt can release more organic and fermentable compounds into the wort during mashing [22].

Finally, the physicochemical parameters analyzed in our investigation were consistent with the recent findings in 2025 of Dall'Acqua et al. [21], who studied wheat craft beers brewed with wasted wheat bread as a partial starchy adjunct. They reported no significant differences in ethanol concentration between control and bread-enriched beers. Likewise, both studies observed similar pH values, reduced turbidity, and a slight decrease in real extract when bread was used as a partial malt substitute.

3.2. Spectrophotometric Properties

3.2.1. Color (EBC)

The beer color results were heterogeneous. American lagers had the lowest EBC (9.58–11.58), displaying their characteristic golden tone. Significant differences were observed in lager and weiss beers when the control beers versus bread beer samples were compared, although this effect was divergent, increasing in lager bread beers and decreasing in weiss bread beers (Figure 2). The higher hue observed in the control weiss beers may be attributable to their substantially higher residual turbidity, which can scatter light and artificially raise the absorbance at 430 nm, thereby increasing the calculated EBC value.

These results indicated that bread contributes to a darker color when pale malts are used, like in the American lagers, but that the opposite effect is observed when compared to roasted malts, being unable to achieve the same EBC as these kinds of malts. This could be due to the pigments of whole wheat bread in the crumb and especially in its brown crust, which influences the wort and finished beer darkness.

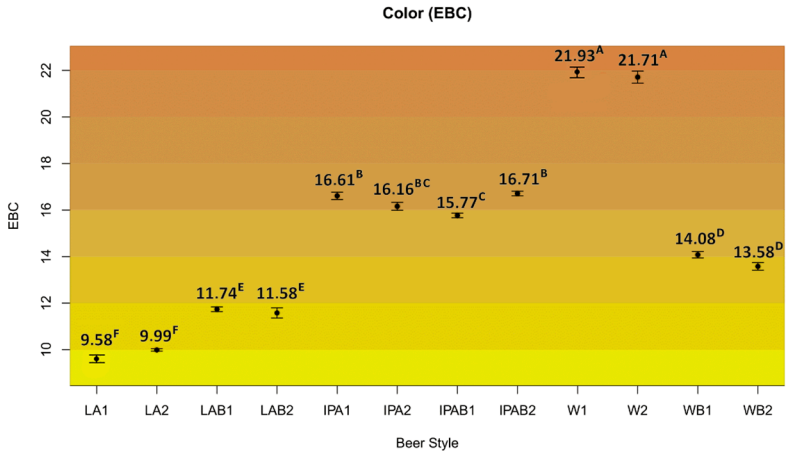

Figure 2. Color measurements in the craft bread beers studied. Different uppercase letters mean significant differences ($p < 0.05$).

3.2.2. Total Polyphenol Content (TPC)

Polyphenolic compounds in beer typically derive from hops (30%) and malts (70%) or develop during the chemical reactions that take place in the brewing process [34]. Important characteristics, such as astringency, body, mouth fullness, and flavor, are influenced by polyphenols. The presence of these components is of great interest to the industry because they prevent oxidation and affect colloidal and foam stability [15,35]. Furthermore, the presence of phenolic compounds is attributed to positive health impacts when beer consumption is moderated [36].

The TPC result for the samples, as shown in Figure 3, ranges from 0.649 to 1.04 mg GAE mL^{-1}. A significant increase in the total polyphenol content was observed in bread beers, regardless of the style. The highest result was found in the sample IPAB1 with 1.04 mg GAE mL^{-1}, and the lowest in W1 with 0.649 mg GAE mL^{-1}.

The findings in our study for lager and weiss beers, particularly those brewed with bread, were higher than the values reported by Bertuzzi et al. [36], who analyzed TPC in 80 samples of craft and industrial beers, showing the average values of 0.507 mg GAE mL^{-1} for craft lagers and 0.403 mg GAE mL^{-1} for wheat craft beers.

Nardini and Foddai [37] reported TPC ranging from 0.274 to 0.321 mg GAE mL^{-1} for lagers and 0.383 to 0.446 mg GAE mL^{-1} for ale-style beers. Habschied et al. [35] examined commercial lager brands, reporting TPC results from 0.076 to 0.117 mg GAE mL^{-1}, and Piazzon et al. [38] reported an average value of 0.452 mg GAE mL^{-1} for lager, 0.484 mg GAE mL^{-1} for Pilsner, 0.504 mg GAE mL^{-1} for wheat, and 0.563 mg GAE mL^{-1} for ale beers. Silva et al. [30] determined that the TPC for Indian IPA craft beers ranged between 0.514 and 0.936 mg GAE mL^{-1}. Additionally, Iannone et al. [39] reported a lower TPC for craft IPA. All previously reported values are lower than the TPC determined in this study, particularly in comparison with whole wheat bread beers.

Although the TPC ratio largely depends on the beer style, we discovered an important fact. All the values obtained in whole wheat beers were higher than the controls. So, the use of whole wheat bread significantly increased the total polyphenol content. The fermentation process that wheat undergoes during bread making, as well as maceration and fermentation during beer production, allows the polyphenols present in wheat to become bioavailable [34,40,41]. This could explain the increase in these parameters.

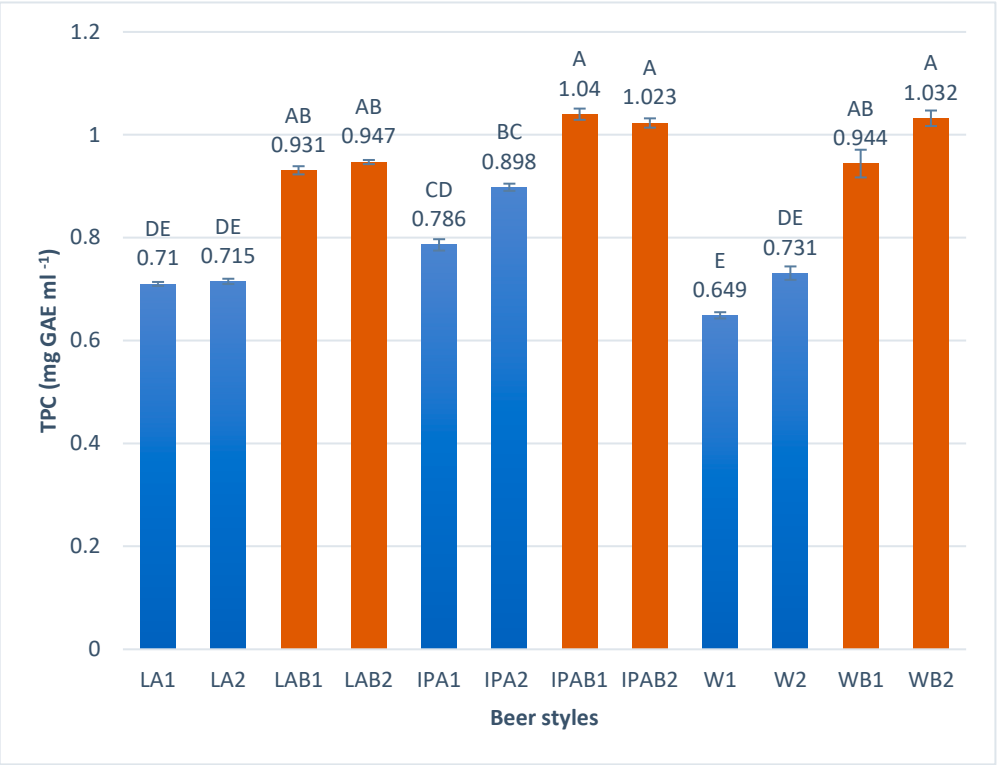

Figure 3. Total polyphenol content in the craft bread beers studied. Different capital letters indicate significant differences ($p < 0.05$).

3.2.3. Antioxidant Activity by DPPH

The results presented in Figure 4 show the differences in antioxidant activity among the samples analyzed.

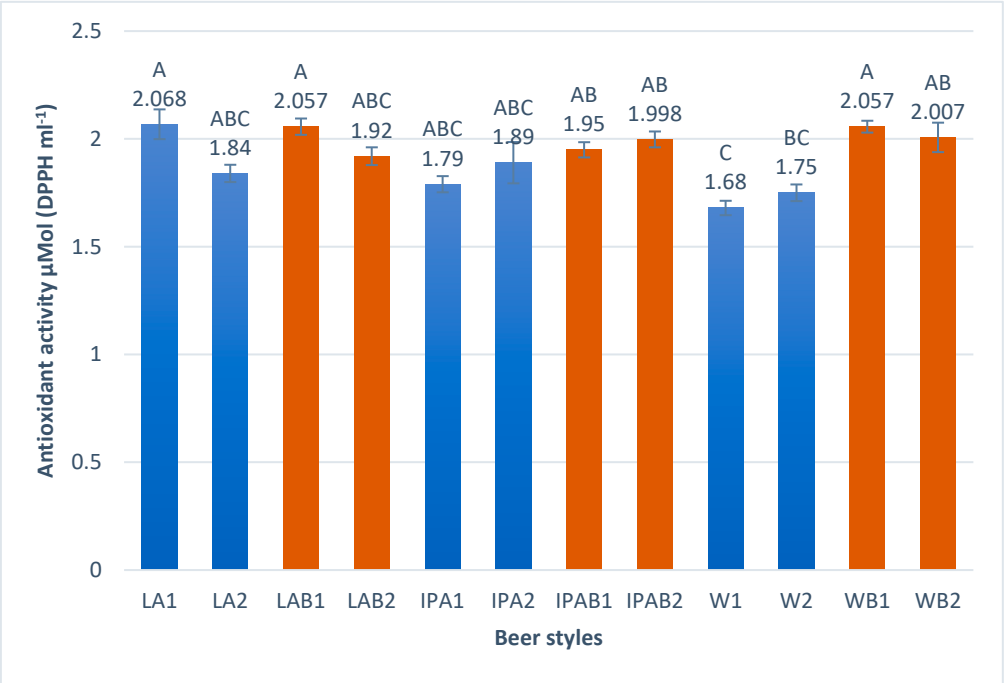

Figure 4. Antioxidant activity in the craft bread beers studied. Different capital letters indicate significant differences ($p < 0.05$).

Despite replacing malt with whole wheat bread by up to 50%, the resulting beers did not lose antioxidant capacity; on the contrary, they showed slightly higher results compared to the control beers. This effect is particularly noticeable for weiss beers, where beers brewed with bread ranged from 2.007–2.057 µMol DPPHmL^{-1} compared to 1.68–1.75 µMol DPPHmL^{-1} for the 100% malt beers. Mitić et al. [42] studied commercial beers, in which lower values for antioxidant capacity than those we obtained were reported, ranging from 0.56 to 1.66 µMol DPPHmL^{-1}.

3.2.4. Protein Content

Figure 5 shows the protein content obtained for the different samples; the values range from 0.564 for control IPA beers to 1.949 mg mL^{-1} in bread lager beers. The results showed that the whole wheat bread beers had protein levels comparable to the 100% malt beers, with a slight increase observed in the bread brews for IPA and weiss styles. Hu et al. [43] reported a higher protein content in wheat craft beer in comparison to 100% malt control. Protein plays a key role in the foam stability, mouthfeel, and overall quality of the beer [44]. It is also involved in the formation of haze in beer, which affects its sensory characteristics [45].

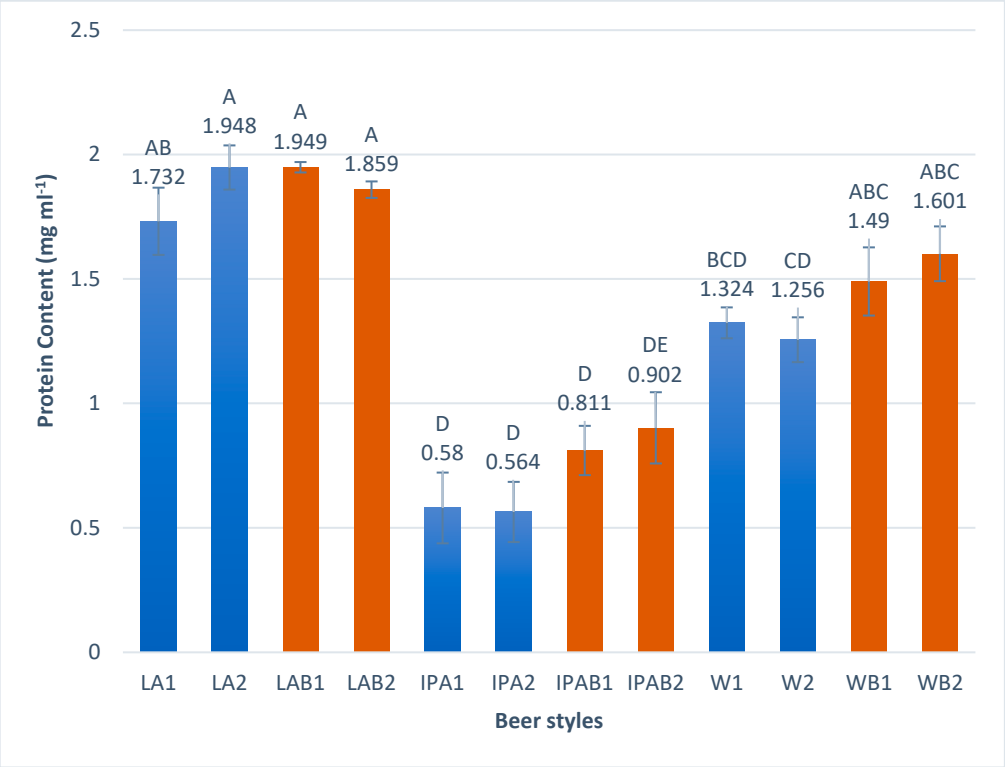

Figure 5. Protein content in the craft bread beers studied. Different capital letters indicate significant differences ($p < 0.05$).

To analyze the differences in color and bioactive compounds, like TPC, Antioxidant Capacity, and Protein Content, the Principal Component Analysis (PCA) could effectively screen feature components to distinguish different samples by reducing data dimensionality, thereby identifying more understandable features and accelerating the processing of valuable sample information [46]. PCA provided a comprehensive visualization of the relationship between the samples and their variables, capturing 95.59% of the total variance through two principal components: F1 at 67.26% and F2 at 28.33% (Figure 6). The samples of this study clustered into three groups: the first group (second quadrant) includes the control weiss and IPA control beers, the second group (first and fourth quadrants) com-

prises the samples made with whole wheat bread, and the third group (third quadrant) comprises the control lagers. These groupings suggest close relationships among samples within each group, indicating similar characteristics.

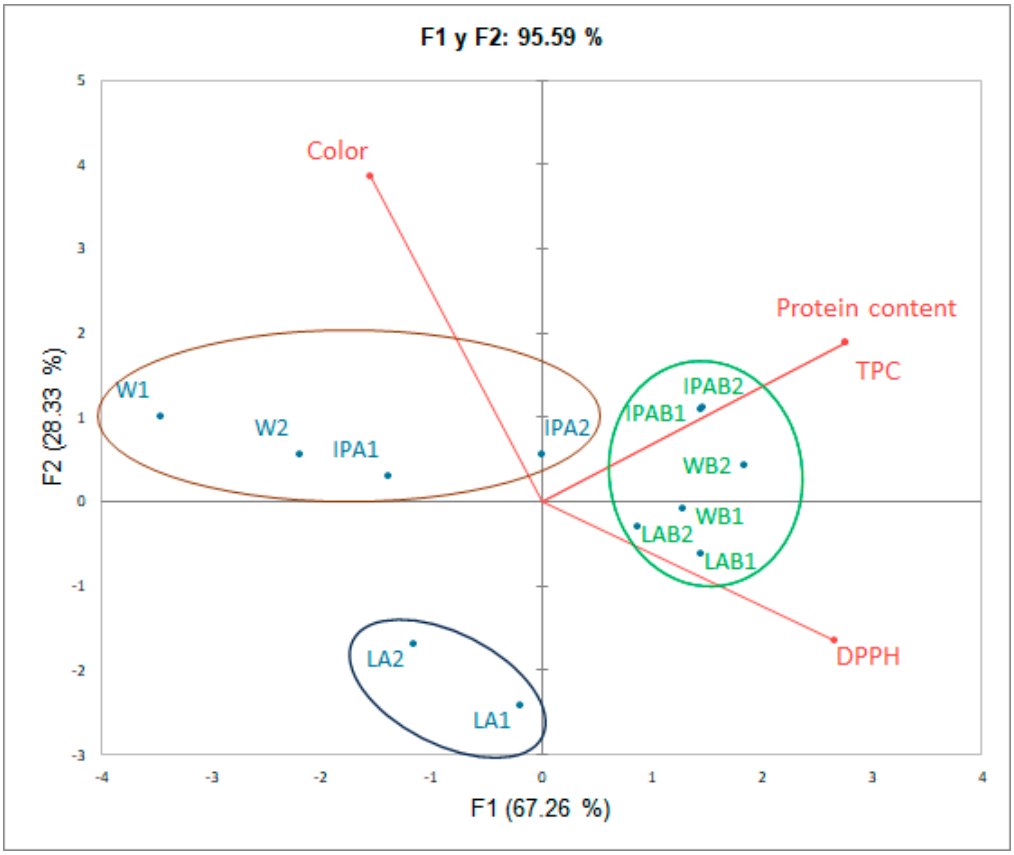

Figure 6. Principal Component Analysis of the bioactive compound profile in craft beers.

The first principal component (F1) explains most of the variance, highlighting the importance of protein content, TPC, and DPPH. Samples brewed with bread (LAB, IPAB, WB) exhibit higher values for these variables, suggesting enhanced nutritional and antioxidant profiles. The co-occurrence of TPC and DPPH vectors in the first component (F1) indicates a close relationship between these variables, as shown in previous studies, which have reported a significant correlation between these parameters [37], attributing the antioxidant activity of beer mainly to its phenolic compound content [47]. These associations were likewise apparent in the findings of our study, where the samples made with whole wheat bread (LAB, IPAB, and WB) showed a tendency toward higher antioxidant activity, while presenting higher values of TPC. The color variable, which correlates positively with F2, further differentiates the samples, particularly the lager control beers, which exhibited the lowest color values, and the weiss control beers, which displayed the highest.

3.3. Headspace Gas Chromatography-Mass Spectrometry (HS-GC-MS) Detection

The main volatile compounds present in beers were identified and relative quantification was expressed as a percentage (%) by comparing the area under each individual peak to the total chromatographic peak areas. Among the 11 volatile compounds identified, only eight were included in the volatile profile due to their higher relative areas. Furthermore, no off-odor volatile compounds (e.g., vicinal diketones or staling aldehydes) were detected in our HS-GC-MS analyses, with concentrations below the detection limits of the method. The results for these eight predominant volatile compounds, including their retention times and peak area percentages (%), are presented in Table 3.

Table 3. Relative main peak areas (%) of volatile compositions in craft bread beers.

Sample	Isoamyl Acetate	Butyl Alcohol	Hexanoic Acid Ethyl Ester	Glycine Benzoil	Phenylethyl Alcohol	Ethyl Octanoate	Phenethyl Acetate	Decanoic Acid Ethyl Ester
Retention Time (min.)	5.33	7.39	7.55	9.23	9.53	10.87	11.84	13.92
LA1	54.00	3.09	8.78	1.19	12.18	15.79	1.78	3.19
LA2	52.90	2.89	8.99	1.56	13.77	14.31	1.63	3.96
LAB1	24.19	2.17	4.93	5.70	47.54	12.57	1.89	0.99
LAB2	22.98	1.80	5.01	5.45	49.42	12.87	1.80	0.67
IPA1	41.19	4.70	5.42	5.60	32.85	5.60	2.58	2.06
IPA2	40.97	3.94	6.05	6.50	29.54	7.84	2.78	2.38
IPAB1	25.73	2.21	3.07	6.18	47.69	10.75	2.06	2.31
IPAB2	27.72	1.89	4.12	5.34	46.97	10.86	1.33	1.77
W1	29.94	2.29	4.95	4.54	49.12	6.75	1.78	0.64
W2	28.23	3.02	4.00	4.87	52.43	5.55	1.34	0.56
WB1	26.68	1.67	1.52	1.75	62.03	4.47	1.52	0.36
WB2	26.14	1.08	1.81	2.33	63.15	3.98	1.17	0.34

The difference in flavor beer compounds may be caused by the raw materials used, technological parameters, and yeast [48,49]. Beer flavor is affected by various volatile organic compounds, including alcohols, esters, aldehydes, ketones, and phenols [50]. These compounds were also identified by other researchers using the GC–MS technique [27,51], specifically in Chinese craft IPA and Pilsner lager beer [52], showing significant levels of isoamyl acetate, phenylethyl alcohol, various ethyl compounds, and minor concentrations of compounds like acid ethyl esters [27].

Principal Component Analysis (PCA) was used to analyze data trends, focusing on the key compounds. Isoamyl acetate, phenylethyl alcohol, ethyl octanoate, and hexanoic acid ethyl ester were then selected as key volatile compounds to group the different beers, as they each contributed more than 8% of the relative areas. Two principal components were extracted, explaining 97.78% of the total variance. F1 accounted for 82.20% of the total variance, while F2 explained an additional 15.57%. A scatter plot (biplot) of the analyzed beers was generated by plotting F1 against F2 (see Figure 7). Notably, the "isoamyl acetate" variable was found to be independent of other variables, showing negative values for both Factor 1 and Factor 2, as was the case for phenylethyl alcohol, which exhibited positive values in both factors.

The other two main volatile compounds, hexanoic acid ethyl ester and ethyl octanoate, displayed negative loadings in F1 and positive values in F2.

Based on the PCA results and the beer samples studied (Figure 7), bread beers and weiss beers were grouped together. These beers were all made with wheat as the raw material, either in malt or whole bread form, and were characterized by higher levels of phenylethyl alcohol, particularly in weiss bread beers. This led to pronounced fruit and floral aromas like rose and honey, with a taste profile including apricot notes, typical of wheat beers. These results were consistent with a recent investigation among Chinese beers, which detected significant values in phenylethyl alcohol contents, making them significant compounds for beers [52]. Moreover, a recent study conducted in 2024 by Coelho et al. [53] on beers brewed with a 50% replacement of malt by stale bread also detected 2-phenylethanol, an alcohol known to contribute a rose-like fragrance. Also, in accordance with our earlier investigation, beers brewed using whole wheat bread exhibited a distinctive presence of ethyl octanoate [22], particularly observed across lager and IPA styles.

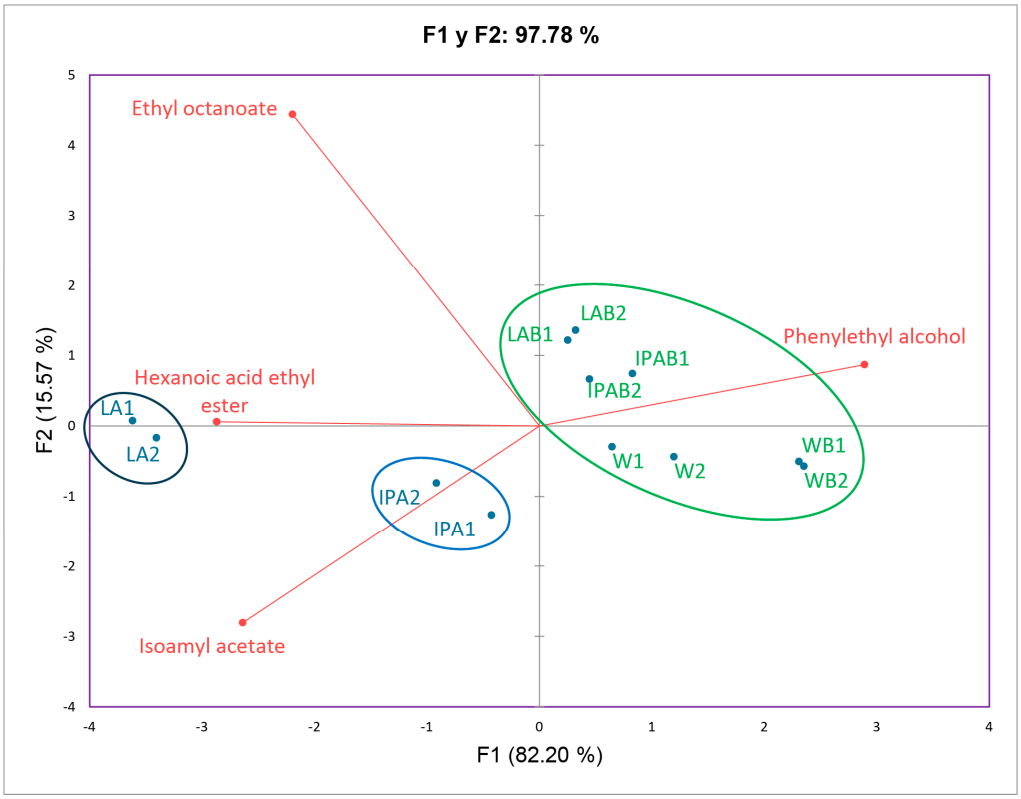

Figure 7. Principal component analysis of volatile compounds in different craft beer styles.

4. Conclusions

Our results indicate that beers brewed with whole wheat bread as a partial malt substitute showed comparable quality to control beers (100% malt) across most physicochemical parameters. Notably, all beers achieved the recommended alcoholic strength, regardless of beer style, and the lager beers showed higher color intensity, attributed to the use of bread. Furthermore, whole wheat bread was found to reduce turbidity during brewing, which was particularly evident in cloudy beer styles such as Bavarian weiss.

Despite replacing up to 50% of malt with whole wheat bread, the resulting beers maintained their antioxidant capacity and protein content and, in general, showed slightly higher values than the control beers, especially evident in weiss beers. Moreover, a significant increase in total polyphenol content was observed in bread beers of all styles.

In addition, a characteristic volatile profile including phenylethyl alcohol and ethyl octanoate was identified in all wheat beers, even when malt was partially replaced by whole wheat bread.

Overall, our findings suggested that using whole wheat bread as a partial substitute for malt added nutritional value due to the health benefits of polyphenol compounds and their antioxidant activity, which could be beneficial for moderate consumers. Furthermore, these results present an opportunity to study the shelf-life behavior of whole wheat bread beers, especially in view of the observed increases in polyphenols and antioxidant capacity compared to 100% malt beers.

Finally, our results confirm that it is feasible to replace up to 50% of malt with whole wheat bread for brewing. Whole wheat bread provided comparable physicochemical properties and bioactive benefits across all beer styles compared to the control beers.

These results represent a significant advancement for the beer industry, reducing malt costs and enabling the reuse of waste bread.

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CHAPTER 3

Whole Wheat Bread Improves the Nutritional Composition and Quality of Beer During Long-Term Storage.

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Whole Wheat Bread Improves the Nutritional Composition and Quality of Beer During Long-Term Storage

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Abstract

Beer is one of the most widely consumed alcoholic beverages worldwide, whereas surplus bread constitutes a significant environmental burden; repurposing this bread as a brewing adjunct offers a sustainable mitigation strategy. In this study, we replaced 50% of the malt grist in American Lager, India Pale Ale and Bavarian Weiss with stale whole wheat bread, brewed each beer and its malt control in duplicate, and stored them for 12 months at 15 °C. Bread addition raised turbidity and soluble protein at bottling; however, after 12 months, the bread lagers clarified to 101 NTU while the controls stayed above 600 NTU. Alcohol content, pH and titratable acidity were unaffected. All bread beers retained more total polyphenols and showed stronger DPPH radical-scavenging activity than controls, especially in lager and IPA. *Lactobacillus* (<100 CFU mL^{-1}) and *Enterobacteriaceae* (<10 CFU mL^{-1}) remained below detection limits in bread samples, whereas the malt-only Weiss displayed *Lactobacillus* spoilage. Sensory panels noted fuller body, livelier carbonation and enhanced toasted-malt aroma in bread beers, with no sensory off-flavour defects detected. Repurposing surplus bread therefore improves clarity, preserves bioactive compounds and yields distinctive, shelf-stable beers while advancing circular-economy goals.

Keywords: shelf life; craft beer; whole wheat bread; antioxidant; sensory; microbial stability

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1. Introduction

Beer is the most widely consumed alcoholic beverage in the world and, along with tea and coffee, one of the most popular [1]. Traditionally, beer is brewed from four natural ingredients: malted cereals (mainly barley), hops, yeast and water. In addition, adjuncts such as other cereals or sugar sources can be added. Beer is a dynamic food matrix that undergoes continuous chemical reactions during storage [2]. The impact of the molecules derived from these reactions will depend on both the style of beer and the storage conditions [3,4]. Changes include sensorial properties, flavour deterioration, colour increase and decrease in bitterness perception [2].

The quality and shelf life of beer are mainly determined by its appearance, flavour, aroma, and texture, and are conditioned by its flavour, microbiological, and colloidal stability [5]. To achieve and maintain beer stability, it is necessary to eliminate any form of biological contamination in the brewing process [6]. With regard to aroma, various chemical reactions can be affected during its shelf life, potentially leading to a decrease in sensory quality [5]. Generally, beer aging is related to the development of carbonyl compounds, sulphur compounds, pyridines, furans, etc. [2]. The appearance of these

typical aging flavours during beer storage has been linked to oxidation of higher alcohols, oxidative degradation of hop-derived bitter acids, Strecker degradation of amino acids, oxidation of unsaturated fatty acids and aldol condensations [7]. Various factors, both external (e.g., oxygen content, pH, raw material and brewing techniques) and internal (e.g., temperature, light and packaging), influence the rate of chemical reactions that occur during beer storage [8,9].

On the other hand, beer is considered a microbiologically stable product. Due to the high concentration of ethanol, CO_2 and relatively low pH (3.8–4.7), it is a rather hostile medium for the growth of microorganisms. This effect is enhanced by the addition of hops, as iso-α-acids have inhibitory properties on Gram-positive bacteria [10,11]. In addition, the pasteurization process, which conventional beer undergoes, also helps to maintain the microbiological stability of the final product. However, in craft breweries, this process is not usually carried out, which makes craft beer more susceptible to microbial contamination and spoilage.

Beer may contain microbial contaminants originating from several sources. Microbial contamination of beer can derive from the raw materials and the brew house vessels (primary contaminants), while the secondary contaminants occur during bottling, canning, or kegging [12]. Although beer does not develop microbiota with health effects, there are certain microorganisms that can grow under these inhospitable conditions and alter its organoleptic properties. Literature highlights that half of the microbiological troubles of both industrial and craft beers are caused by the secondary contamination [13].

Due to the increase in beer exports coupled with growing consumer concern for product quality, the brewing industry needs to offer a product capable of maintaining its stability and best organoleptic characteristics throughout its shelf life [5].

On the other hand, food waste is a complex phenomenon occurring worldwide and is gradually receiving attention from the scientific and professional communities, as well as from policy makers due to the large quantities produced worldwide and the associated environmental, social and economic impacts [14,15].

Bread is considered to be one of the most wasteful food categories. Due to its own characteristics, bread is a product highly susceptible to microbial attack, resulting in a food with a short shelf life. This, together with consumer preference for freshly baked products, is the main reason for the high levels of bread waste [16].

Despite the potential use of this bread waste for beer brewing, there are currently few studies on the matter. Almeida et al. [17] in 2018, brewed a craft beer with waste bread and concluded that the resulting beer had a 20% lower carbon footprint compared to the control craft beer. Subsequently, Brancoli et al. [18] investigated the use of leftover bread as a substitute for malted barley in brewing (25–28% by weight) and determined that the Global Warming Potential (GWP) decreased by 0.46 kg CO_2 eq. per kg of wasted bread used in brewing. In 2021, Narisetty et al. [19] showed that a maximum of 25% bread can replace barley due to the need for enzymes. Years later, McDonagh et al. [20] studied the feasibility of using waste bread to brew beer, investigating the impact on alcohol content and the environmental implications of this substitution. The results showed that beer brewed with up to 60% malted barley by weight, replaced by bread, had sufficient fermentability to produce the required volume of alcohol. In a previous study, the possible use of different types of bread (wheat bread, rye bread, whole wheat bread and corn bread) for beer production was evaluated. The results showed the possibility of replacing up to 50% of the malt with stale bread, which represents a significant saving for the brewing industry. In addition, beers brewed with whole wheat bread were found to contain a higher total polyphenol content and antioxidant capacity; therefore, the reuse of waste whole wheat

bread in beer brewing could result in obtaining a final product with healthier properties than conventional beer [21].

In this work, we continued our previous investigations into the use of whole wheat bread as a partial substitute for malt in craft beer brews. The main objective of this study was to evaluate the shelf life of bread-enriched beers stored under standard brewery conditions (15 °C) over a period of 12 months, in order to assess their nutritional, sensory, physicochemical, and microbiological stability at the end of the storage period. We evaluated the performance of the production system across different brewing styles, like American Lager, Indian Pale Ale (IPA), and Bavarian Weiss Ale. This was achieved by analyzing key physicochemical characteristics such as turbidity, pH, acidity, alcohol by volume (ABV), dry extract, colour, protein content, polyphenol content, antioxidant activity, and microbiological stability. Additionally, sensory evaluations were conducted to explore how the inclusion of bread influences the visual, aromatic, and taste profiles of these beers. The results provide important information on the potential use of whole wheat bread to improve beer quality and extend shelf life, while supporting sustainable brewing practices aligned with circular economy principles.

2. Materials and Methods

2.1. Reagents and Standards

Methanol, Folin–Ciocalteu and Gallic Acid reagents were obtained from Merck Millipore (Madrid, Spain). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). Sodium chloride (NaCl), sodium hydroxide (0.01 M), anhydrous D(+)-glucose for ACS analysis, Coomassie blue G-250 (CBBG) and Bradford reagent were sourced from Panreac (Castellar del Vallés, Spain). All solutions were prepared with analytical-grade reagents and distilled water. Finally, Agar Raka-Rai wasacquired from Scharlab (Sentmenat, Spain).

2.2. Brewing Process

Three different craft beer styles were selected based on the most widely consumed types globally: one Lager beer (American Lager) and two Ale beers (IPA and Bavarian Weiss).

Beer samples were designated according to the codification presented in Table 1.

Table 1. Nomenclature of craft beers.

Control Beers	Whole Wheat Bread Beers
Lager (LA)	Bread lager (LAB)
Indian pale ale (IPA)	Bread Indian pale ale (IPAB)
Bavarian Weiss (W)	Bread Bavarian Weiss (WB)

All trials were carried out in the pilot brewery of the University of Valladolid (Palencia, Spain). For each beer style, a reference wort was produced with 100% malted grain/flakes, using 2.381 kg for American lager (Castle Malting, Beloeil, Belgium), 2.650 kg for IPA (Weyermann, Bamberg, Germany) and 2.170 kg (Weyermann, Bamberg, Germany) for Bavarian wheat ale per 10 L of mineral water (Carbónicas Navalpotro S.A, Almazán, Spain). Experimental beers were prepared in the same way but with 50% of the malt mass replaced by an equal mass of stale whole wheat bread from La Tahona de Sahagún (Palencia, Spain). Each formulation was brewed in duplicate as independent 10 L pilot batches, and these duplicate brews were treated as the experimental units for statistical analysis.

A comprehensive description of the procedure optimized by our group and recently reported [22] is summarised in Figure 1. Briefly, malt and bread were milled separately

with a 1 mm two-roll gap immediately before mashing. Mineral water was pre-heated to 40 °C, the grist (or grist + bread) was added and stirred for 20 min, and the mash was then stepped through the temperature programme shown in Figure 1.

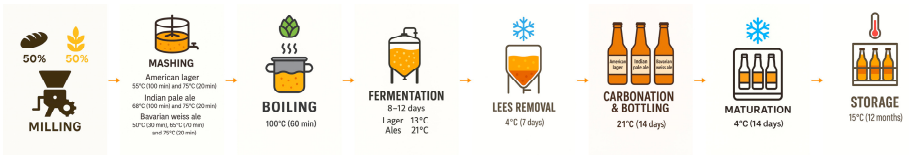


Figure 1. Brewing process methodology.

After a 24 h room-temperature rest (to maximize enzymatic conversion), the wort was lautered, and the spent grains were sparged with 80 °C water to restore the 10 L volume. Each wort was boiled for 60 min at 100 °C.

Hopping rates were 18 IBU with Saaz for Lager; 50 IBU for IPA (Cascade 35 IBU at 0 min and Citra 15 IBU at 30 min); and 15 IBU with Magnum for Weissbier. All hops were purchased from Laguilhoat (Fuenlabrada, Spain). On flame-out the trub was removed and the wort was cooled in a −25 °C chamber to the specific fermentation temperature (13 °C for lager; 21 °C for ales). Prior to pitching, oxygenation was achieved by 2 min of vigorous shaking.

Dry yeasts (SafLager S-23, SafAle US-05 and SafAle WB-06 (Fermentis, Marcq-en-Baroeul, France)) were rehydrated in sterile water (25 °C, 1 g yeast in 10 mL) for 15 min and added at 0.5 g L^{-1}. Primary fermentation proceeded for 8–12 days in 10 L stainless steel tanks fitted with over-pressure valves. When final attenuation was reached, the beers were cooled to 4 °C for 7 days to enhance sedimentation, the lees were removed and the beers were tempered back to 21 °C. Bottling was performed in 330 mL glass bottles with dextrose priming (4 g L^{-1} bar^{-1}) and SafAle F-2 to target CO_2 pressures of 2.0 bar (Lager), 2.5 bar (IPA) and 3.0 bar (Weissbier). Bottles were conditioned for 14 days at 21 °C; internal pressure was checked with a crown-cap aphrometer.

Subsequently, the beers were held at 4 °C for a further two-week maturation and later stored at 15 °C for up to 12 months. Samples were collected immediately after maturation (S0) and after 12 months of storage (S12) for analytical comparisons.

2.3. Physicochemical Analysis

For most tests, unfiltered samples were used, except for those requiring spectrophotometric analysis (Colour, Total Polyphenol Content, Antioxidant Capacity and Protein Content), which were pre-processed by centrifugation (Bunsen, model KOCH 1460, Humanes de Madrid, Spain) at 4000 rpm for 5 min, followed by filtration through a vacuum filter (model: Kitasato) and 0.45-micron Millipore filters (Merck Millipore, Darmstadt, Germany). All filtered samples were analyzed using a spectrophotometer (ThermoFisher Scientific, model Genesys 20 UV-Vis, Madrid, Spain).

- pH: pH values were measured using a calibrated pH meter (HACH-LANGE, sensiON™ + pH3 model, Hospitalet, Spain).
- Acidity: Continuous pH measurements were performed, and acidity was assessed by an acid–base titration until the sample reached a pH of 7. Results were reported as the percentage of lactic acid.
- Turbidity: Turbidity was determined using a turbidimeter (Hanna Instruments, model HI 98703, Eibar, Spain). Each beer sample was placed in a transparent glass container with a lid, which was then inserted into the turbidimeter to obtain turbidity values in NTU (Nephelometric Turbidity Units).

- Dry Extract: Dry extract content was assessed with a thermobalance (Gibertini Eurotherm, Novate Milanese, Italy). A 1 g sample of each beer was weighed, evaporated, and the remaining solid was measured to determine the dry extract percentage by difference.
- Alcohol By Volume (ABV): ABV was determined using an ebulliometer (GAB system, model 1010006, Moja, Spain), calibrated with distilled water. The boiling points of the water standard and the beer sample were compared, and the ABV was calculated to a precision of 0.1% using a ruler scale.
- Protein Content: Protein content was determined by the Bradford method [22], based on the binding of Coomassie blue G-250 dye with proteins in the beer sample. Each analysis used 3140 µL of distilled water, 200 µL of Bradford reagent, and 60 µL of the beer sample in a test tube. A calibration curve from 1 to 40 µL was prepared using serum albumin (0.1 µg/µL). Samples were then measured at 595 nm.
- Colour (EBC): A 3 mL beer sample was placed in a standard 1 cm glass cuvette. Absorbance at 430 nm was recorded, with distilled water as a blank. The absorbance value was converted to the European Brewery Convention (EBC) colour scale by multiplying by 25.
- Total Polyphenol Content (TPC): TPC was quantified using the Folin–Ciocalteu method, with absorbance measured at 760 nm [23].
- Antioxidant Activity (DPPH): Antioxidant activity was measured by the DPPH method following Abderrahim et al. [24].

2.4. Microbiological Analysis

Two microbiological methods were employed to assess potential contamination in the beer samples:

- *Lactobacillus* spp. Count: *Lactobacillus* spp. was quantified through surface plating on selective media. In this technique, a small volume of sample was spread evenly on the surface of agar plates specific to *Lactobacillus* spp., which were then incubated under conditions optimal for the growth of these bacteria. Colony-forming units (CFU) were counted after incubation, with a detection limit of <100 CFU/mL. This approach enables a reliable assessment of *Lactobacillus* spp. presence and growth, which is critical given their role in beer spoilage [25].
- Enterobacteriaceae Detection: Enterobacteriaceae presence was determined via membrane filtration. In this method, the beer samples were filtered through 0.45 micron Millipore membranes (Merck Millipore, Darmstadt, Germany). The membranes were subsequently placed on selective agar plates, designed for Enterobacteriaceae growth and incubated to facilitate colony formation. Colonies were then counted, with a detection limit of <10 CFU/mL. Membrane filtration is a widely used method in the brewing industry for detecting low levels of Enterobacteriaceae, enhancing quality control measures. This technique involves passing beer samples through a membrane filter that captures bacteria, which are then cultured on selective media to identify contaminants. Studies have demonstrated the effectiveness of membrane filtration in detecting microbial trace contaminations in beer, including Enterobacteriaceae, thereby ensuring product safety and quality [26].

2.5. Sensory Analysis

Eight professional beer tasters (five men and three women) were trained following ISO 8586:2012 standards. The training process included six two-hour tasting sessions, in which panellists learned to identify various beer descriptors using a presence–absence scale and a discontinuous three-point scale. During the first two sessions, tasters focused on

descriptor identification through a free choice profile technique paired with a control identification test. In the remaining four sessions, a standardized tasting sheet was developed, incorporating the most relevant descriptors selected through a qualitative focus group technique. Criteria for potential removal were set, with panellists scoring below 70% on the control test subject to elimination. This process resulted in the exclusion of two tasters, yielding a final panel of six highly qualified judges.

The sensory evaluation took place in a single session at 5:00 p.m. Before beginning, tasters were briefed on the project objectives and the specific beers to be evaluated. Each beer was tested in duplicate, with twelve samples presented in total. After the first six samples, a short break was given. Each beer was served at 6 °C in glasses conforming to ISO 3591:1977 standards, with a unique three-digit random code assigned to each sample, and a randomized order of presentation for each taster. The analysis was conducted in a controlled tasting room that met UNE-EN ISO 8589:2010 guidelines. Panellists spent approximately two minutes on each sample, using a descriptive approach aligned with the tasting sheet developed during training. This sheet included descriptors to facilitate the sensory profiling of the beers across three distinct categories:

- Visual: Intensity, tonality, limpidity, froth colour, and CO_2 bubbles.
- Aroma: Maltiness, cereal malt, ripe fruit malt, hoppy, exotic fruit, citric fruit, herbaceous, yeast, tropical fruit yeast, spicy yeast, bread yeast, toasted, coffee, licorice, and caramel, along with defect descriptors such as oxidized, cider, vinegar, musty, stable, and soapy.
- Taste: Acidity, CO_2, bitterness, body, and persistence.

These descriptors encompassed most of the sensory categories, including several primary terms represented in the beer sensory wheel.

2.6. Statistical Analysis

All analyses were conducted in triplicate. Statistical analyses were conducted utilizing XLSTAT v.2023.3.1 software (Addinsoft, Paris, France). For each sampling time (S0 and S12), all physicochemical variables were first assessed with a two-way MANOVA (fixed factors: Style and Bread); multivariate significance was evaluated using Wilks' lambda (Λ), which ranges from 0 to 1 ($\Lambda \approx 0$ indicates strong group differences; $\Lambda \approx 1$ indicates no multivariate effect). This statistic tests the null hypothesis that there are no significant multivariate differences among the beer types. Statistical significance was determined based on the associated p-value < 0.05, considered significant. Each variable was then analyzed with a two-way ANOVA (Style, Bread, Style × Bread) and, within the same sampling time, re-analyzed by a one-way ANOVA (12 beer batches as levels). Pair-wise differences were located with Tukey's HSD ($p < 0.05$). Additionally, the R Pearson correlation coefficients, calculated at $p < 0.05$, were used to determine relationships among the physicochemical and sensory variables examined.

3. Results and Discussion

3.1. Physicochemical Analysis

A preliminary two-way MANOVA (Style × Bread) was applied separately to S0 and S12 for turbidity, pH, colour, dry extract, acidity and % ABV. For fresh beers (S0), multivariate effects were significant for Style ($\Lambda = 0.006$, $p < 0.001$), Bread ($\Lambda = 0.32$, $p = 0.015$) and their interaction ($\Lambda = 0.08$, $p = 0.002$). After 12 months of storage (S12), the differences were even stronger: Style ($\Lambda = 0.002$, $p < 0.001$), Bread ($\Lambda = 0.27$, $p = 0.008$) and Style × Bread ($\Lambda = 0.04$, $p < 0.001$). As expected, beer style emerged as the main source of multivariate variation, yet the bread adjunct also altered the overall physicochemical fingerprint. The addition of bread did not affect all beers uniformly; its impact differed

depending on whether the base style was lager, IPA, or Weiss beer. Consequently, each variable was examined with a two-way ANOVA, and the resulting significance levels are summarized in Table 2.

Table 2. Two-way ANOVA (Style × Bread)—significance levels for physicochemical analysis at S0 and S12.

Parameter	Style	Bread	Style × Bread	Style	Bread	Style × Bread
Sampling Time	**S0**	**S0**	**S0**	**S12**	**S12**	**S12**
Turbidity	*	*	*	*	*	*
pH	*	ns	*	*	*	*
Colour	*	*	*	*	*	ns
Dry extract	*	*	*	*	*	*
Acidity	*	ns	ns	*	*	ns
% ABV	*	ns	ns	*	ns	ns

* $p < 0.05$; ns = not significant. Factors: Style (lager, IPA, Weiss) and Bread (100% malt vs. bread beers).

As shown, the factor style was significant ($p < 0.05$) for every basic physicochemical variable at both sampling times, whereas bread addition was significant for turbidity, dry extract and colour at S0, and for turbidity, dry extract and acidity at S12. The style × bread interaction was significant for turbidity, pH, colour and dry extract at S0, and for turbidity, pH and dry extract at S12, indicating that the impact of bread differs among beer styles. Because this interaction prevents a single overarching conclusion, each variable was subsequently analyzed with a one-way ANOVA within each sampling time; the resulting means and Tukey letter codes are presented in Table 3 (S0) and Table 4 (S12).

Table 3. One-way ANOVA significance for S0 values of physicochemical properties in craft beers (mean ± S.D).

Beer Sample	Turbidity (NTU)	ABV (%)	Dry Extract (%)	pH	Acidity (% Lactic Acid)	Colour (EBC)
LA1	652.67 ± 38.42^{DE}	5.43 ± 0.06^{B}	6.88 ± 0.25^{A}	3.95 ± 0.04^{B}	0.03 ± 0.001^{C}	9.77 ± 0.15^{G}
LA2	702.33 ± 11.59^{D}	5.53 ± 0.12^{B}	6.66 ± 0.26^{A}	3.9 ± 0.01^{BC}	0.03 ± 0.001^{C}	10.13 ± 0.18^{F}
LAB1	510.46 ± 25.94^{F}	5.38 ± 0.10^{B}	5.98 ± 0.15^{B}	4.12 ± 0.04^{B}	0.04 ± 0.001^{AB}	10.84 ± 0.33^{E}
LAB2	478.33 ± 14.57^{F}	5.45 ± 0.12^{B}	5.83 ± 0.21^{B}	4.12 ± 0.01^{B}	0.03 ± 0.001^{BC}	11.33 ± 0.32^{E}
IPA1	683.03 ± 19.47^{DE}	6.95 ± 0.15^{A}	5.21 ± 0.09^{C}	4.40 ± 0.17^{A}	0.04 ± 0.002^{A}	15.72 ± 0.24^{C}
IPA2	647.67 ± 28.45^{E}	6.90 ± 0.10^{A}	5.31 ± 0.09^{C}	4.28 ± 0.13^{A}	0.04 ± 0.002^{AB}	15.79 ± 0.18^{C}
IPAB1	626.67 ± 17.01^{E}	6.60 ± 0.16^{A}	5.12 ± 0.16^{C}	4.31 ± 0.03^{A}	0.04 ± 0.002^{AB}	15.96 ± 0.26^{C}
IPAB2	613.33 ± 10.07^{E}	6.59 ± 0.12^{A}	4.96 ± 0.05^{C}	4.37 ± 0.03^{AB}	0.04 ± 0.001^{AB}	17.05 ± 0.21^{B}
W1	1007.33 ± 25.38^{A}	5.43 ± 0.12^{B}	6.88 ± 0.25^{A}	3.65 ± 0.06^{C}	0.04 ± 0.001^{A}	20.92 ± 0.57^{A}
W2	988 ± 42.23^{A}	5.30 ± 0.11^{B}	6.66 ± 0.26^{A}	3.71 ± 0.09^{C}	0.05 ± 0.001^{A}	20.67 ± 0.45^{A}
WB1	789.53 ± 12.53^{B}	5.25 ± 0.05^{B}	5.98 ± 0.15^{B}	3.77 ± 0.06^{C}	0.05 ± 0.002^{A}	13.94 ± 0.65^{D}
WB2	828.21 ± 35.59^{B}	5.30 ± 0.17^{B}	5.83 ± 0.30^{B}	3.79 ± 0.13^{C}	0.05 ± 0.001^{A}	14.01 ± 0.17^{D}

$^{A–G}$ Means without any common letter within the same column are significantly different ($p < 0.05$).

No statistically significant differences in % ABV were observed between bread and control beers within any style after 12 months, although, as expected, % ABV still differed across styles (Tables 3 and 4). By contrast, pH and turbidity exhibited marked style effects: the wheat beers combined the highest turbidity with the lowest pH values (Tables 3 and 4). Consistent with Wang and Ye [27], lower pH levels favoured the formation of insoluble protein-polyphenol complexes, increasing turbidity. This relationship suggests that beer with a lower pH may present higher turbidity due to the stability of these aggregates in

solution. As shown in Table 4, Weiss-style beer made with 100% malt and matured for 12 months exhibited the highest turbidity values. So, this elevated turbidity is likely attributed to wheat malt's high levels of soluble proteins, which enhance both foam stability and turbidity in the final product. A recent study confirmed that these proteins significantly contribute to foam properties and turbidity stability in wheat beers [28].

Table 4. One-way ANOVA significance for S12 values of physicochemical properties in craft beers (mean ± S.D).

Beer Sample	Turbidity (NTU)	ABV (%)	Dry Extract (%)	pH	Acidity (% Lactic Acid)	Colour (EBC)
LA1	622.01 ± 8.19 ^{E}	5.43 ± 0.1 ^{B}	6.59 ± 0.12 ^{A}	3.98 ± 0.0 ^{B}	0.03 ± 0.001 ^{C}	6.64 ± 0.31 ^{I}
LA2	716.33 ± 1.53 ^{C}	5.50 ± 0.01 ^{B}	5.92 ± 0.09 ^{B}	4.00 ± 0.01 ^{B}	0.03 ± 0.001 ^{BC}	9.85 ± 0.09 ^{G}
LAB1	101.33 ± 10.97 ^{K}	5.33 ± 0.15 ^{B}	4.95 ± 0.08 ^{C}	4.14 ± 0.03 ^{B}	0.03 ± 0.001 ^{C}	7.01 ± 0.13 ^{HI}
LAB2	308.33 ± 4.73 ^{H}	5.47 ± 0.21 ^{B}	5.00 ± 0.11 ^{C}	4.13 ± 0.08 ^{B}	0.03 ± 0.001 ^{C}	7.21 ± 0.08 ^{H}
IPA1	125.15 ± 3.00 ^{J}	6.90 ± 0.2 ^{A}	4.01 ± 0.14 ^{DE}	4.32 ± 0.12 ^{A}	0.03 ± 0.003 ^{BC}	15.71 ± 0.28 ^{C}
IPA2	250.03 ± 6.24 ^{I}	6.97 ± 0.12 ^{A}	4.08 ± 0.07 ^{D}	4.27 ± 0.06 ^{A}	0.04 ± 0.001 ^{AB}	16.00 ± 0.04 ^{C}
IPAB1	94.07 ± 7.81 ^{K}	6.63 ± 0.19 ^{A}	3.81 ± 0.10 ^{E}	4.37 ± 0.06 ^{A}	0.04 ± 0.001 ^{AB}	14.15 ± 0.15 ^{D}
IPAB2	78.13 ± 6.66 ^{L}	6.67 ± 0.18 ^{A}	4.04 ± 0.13 ^{D}	4.42 ± 0.03 ^{A}	0.04 ± 0.001 ^{AB}	10.27 ± 0.22 ^{F}
W1	1034.00 ± 26.26 ^{A}	5.53 ± 0.24 ^{B}	6.59 ± 0.12 ^{A}	3.54 ± 0.13 ^{C}	0.04 ± 0.001 ^{A}	16.41 ± 0.08 ^{B}
W2	1016.00 ± 9.54 ^{A}	5.27 ± 0.06 ^{B}	5.92 ± 0.09 ^{B}	3.64 ± 0.07 ^{C}	0.04 ± 0.002 ^{A}	13.73 ± 0.22 ^{D}
WB1	355.33 ± 11.93 ^{G}	5.33 ± 0.14 ^{B}	4.95 ± 0.08 ^{C}	3.85 ± 0.12 ^{C}	0.04 ± 0.001 ^{AB}	14.04 ± 0.07 ^{D}
WB2	363.67 ± 8.39 ^{G}	5.45 ± 0.16 ^{B}	5.00 ± 0.11 ^{C}	3.94 ± 0.11 ^{B}	0.04 ± 0.001 ^{AB}	10.91 ± 0.22 ^{E}

$^{A–L}$ Means without any common letter within the same column are significantly different ($p < 0.05$).

Over time, turbidity decreases during long-term storage as these colloidal particles precipitate, especially in bread beers, where initial turbidity is higher due to increased protein content. Extended storage promotes the sedimentation of these complexes, resulting in improved clarity.

Furthermore, the data on dry extract content revealed significant differences among the styles. Control beers (e.g., LA1, LA2, W1, and W2) presented the highest dry extract content both immediately after brewing and after 12 months of storage. In contrast, beers produced with the addition of bread (LAB, WB) and IPA formulations exhibited significantly lower values. Additionally, a decrease in dry extract content was observed across all treatments after 12 months of storage. These results align with previous findings, which documented a decrease in dissolved solids due to the sedimentation of proteins, polyphenols, and other colloidal compounds. As these particles aggregate and precipitate over time, they stabilize the beer, resulting in improved clarity [27,29].

Beers brewed with bread exhibited even lower dry extract levels, likely due to additional proteins and fermentable compounds introduced by the bread. During long-term storage, these components either metabolize or precipitate, especially in Weiss-style beers with bread, where the high protein levels encourage colloidal sedimentation of solids [27]. These findings are in agreement with previous studies, indicating that proteins and polyphenols form haze-active complexes that tend to aggregate under lower pH conditions, eventually precipitating and leading to a gradual decrease in extract content over time [29,30].

Also, regarding the colour results, beer styles were statistically differentiated, showing significant variation among them. The decrease in beer colour intensity observed during long-term storage could be attributed to the sedimentation and degradation of polyphenols and other colour-contributing compounds. For instance, recent studies have demonstrated that polyphenols, particularly flavonoids, play a significant role in beer colour. Their gradual reduction over time, often due to precipitation, contributes to a lighter appearance in

beers. During storage, compounds like catechin and epicatechin can decrease significantly, especially in the first few weeks, which contributes to the gradual loss of colour intensity in stored beers [1,30].

According to Šibalić et al. [31], the phenolic compounds in beer derived from raw materials and the brewing process can undergo oxidation during storage, contributing to colour darkening. Based on this, we could conclude that no signs of oxidation were observed, as beer coloration did not increase after 12 months of storage.

3.2. Bioactive Compounds

A separate two-way MANOVA (Style × Bread) was applied to the three bio-active variables—protein, total polyphenols (TPC) and antioxidant activity (DPPH)—at each sampling time. For fresh beers (S0), Style produced a pronounced multivariate effect ($\Lambda = 0.073$, $p < 0.001$); Bread was also significant ($\Lambda = 0.331$, $p = 0.004$), as was the Style × Bread interaction ($\Lambda = 0.345$, $p = 0.008$). After 12 months (S12), all effects intensified: Style ($\Lambda = 0.040$, $p < 0.001$), Bread ($\Lambda = 0.230$, $p = 0.003$) and Style × Bread ($\Lambda = 0.016$ ($p < 0.001$). Thus, while beer style again accounted for most multivariate variation, the bread adjunct significantly modified the joint protein-phenol-antioxidant profile, and its influence differed by style—especially at the end of storage. Therefore, each variable was examined with a two-way ANOVA, and the resulting significance levels are summarized in Table 5.

Table 5. Two-way ANOVA (Style × Bread)—significance levels for bioactive compounds at S0 and S12.

Parameter	Style	Bread	Style × Bread	Style	Bread	Style × Bread
Sampling Time	**S0**	**S0**	**S0**	**S12**	**S12**	**S12**
Protein content	*	ns	ns	*	*	*
Total polyphenols	*	*	ns	*	*	*
Antioxidant activity (DPPH)	*	ns	*	*	*	*

* $p < 0.05$; ns = not significant. Factors: Style (lager, IPA, Weiss) and Bread (100% malt vs. bread beers).

As Table 5 indicates, Style was a significant factor for protein, total polyphenols and antioxidant activity at both sampling times. Bread addition was significant for total polyphenols at S0 and S12 and became significant for both protein and antioxidant activity only at S12. The Style × Bread interaction appeared for antioxidant activity at S0 and S12 and for protein content at S12, showing that the effect of bread varies with beer style and is more pronounced at the end of storage.

Given these style-specific patterns, the three bio-active variables are discussed separately in the following subsections.

3.2.1. Protein Content

In freshly brewed beers (S0), protein content varied considerably across styles, with average values ranging from 0.56 to 1.95 mg mL^{-1} (Figure 2). After 12 months of storage, protein content decreased in the lager and Weiss beers—especially in the bread variants. The decline in protein levels likely resulted from the precipitation of protein compounds during the 12-month storage period, an expected process observed in bottled beer over time. Other studies were consistent with our findings, particularly regarding wheat beers. Research has shown that wheat proteins tend to precipitate over time, especially in environments with lower polyphenol concentrations, resulting in reduced protein content during storage [30].

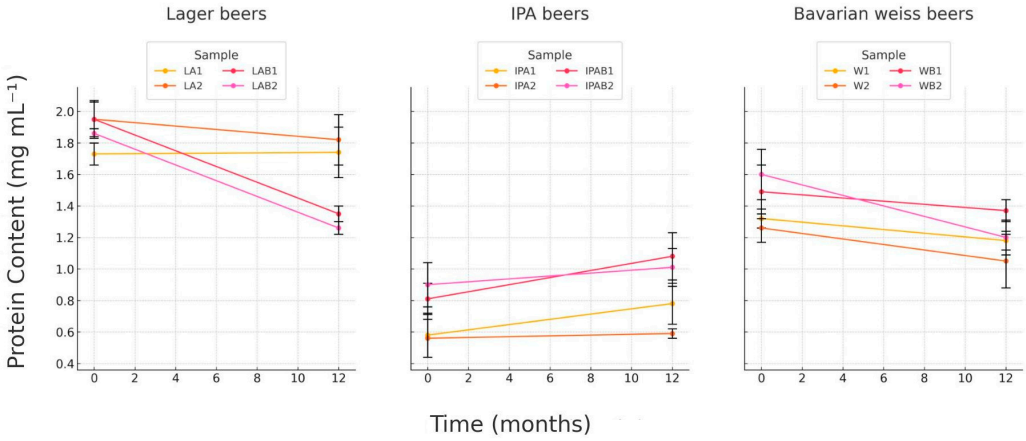


Figure 2. Protein content in the craft bread beers studied throughout the long-term storage process.

IPA beers' protein content did not display the reduction seen in other beer styles after long-term storage, suggesting a potential stabilizing effect from hop-derived compounds (Figure 2). Recent studies confirm that hop polyphenols play a crucial role in haze stability by interacting with proteins. These interactions form non-covalent protein-polyphenol complexes that resist sedimentation, thereby preserving turbidity even during extended storage. This effect is especially notable in hopped IPAs, where the high polyphenol concentration promotes the formation of stable complexes with haze-active proteins, enhancing both the durability and visual consistency of haze in these beers [27].

3.2.2. Total Polyphenol Content (TPC)

As shown in Figure 3, fresh beers showed marked differences between styles in total polyphenols, reaching their highest levels in the bread-enriched IPA and, to a lesser extent, in the bread-wheat beers. After 12 months of storage, polyphenol concentrations decreased in most samples; the steepest declines occurred in the 100% malt Lager and in the bread IPAs, while the other styles lost less than 7%.

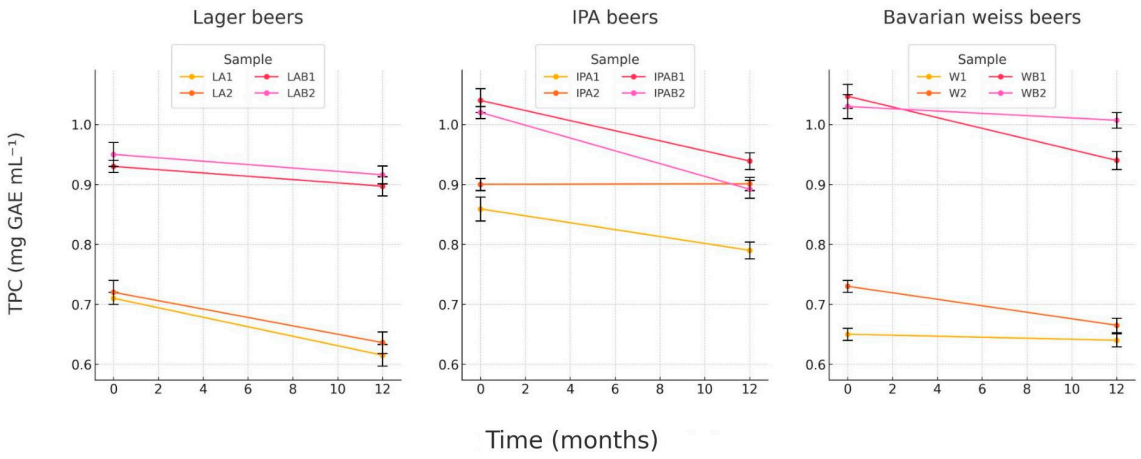


Figure 3. Total polyphenol content in the craft bread beers studied throughout the long-term storage process.

These outcomes are consistent with the findings of Jongberg et al. [32], who demonstrated that polyphenols form stable complexes with proteins such as Protein Z and LTP1 during beer storage, often leading to precipitation, particularly under oxidative conditions, which affects haze stability.

After 12 months of storage, all IPA beers maintained higher polyphenol levels compared to other styles, likely due to hop-derived protein polyphenol interactions, which contribute to haze retention [27].

In contrast, Lager and Weiss beers exhibited a more pronounced decrease in polyphenol content over time. Remarkably, this reduction was less evident in the beers brewed with bread. Bread Lagers retained higher polyphenol levels than their 100% malt counterparts, likely due to antioxidant compounds present in the bread. Similarly, bread Weiss beers demonstrated greater polyphenol retention compared to their malt-only versions, suggesting a comparable antioxidant effect [30]. These findings support the hypothesis that incorporating bread could enhance polyphenol stability during long-term storage, particularly in beer styles more susceptible to polyphenol degradation.

3.2.3. Antioxidant Activity by DPPH

Initial antioxidant activity varied by beer type, with IPAs and Weiss bread beers showing higher values than other styles. After 12 months of storage, a notable decrease in antioxidant activity was observed across most samples, especially in 100% malt Lagers and Weiss beers, suggesting antioxidant depletion over time (Figure 4).

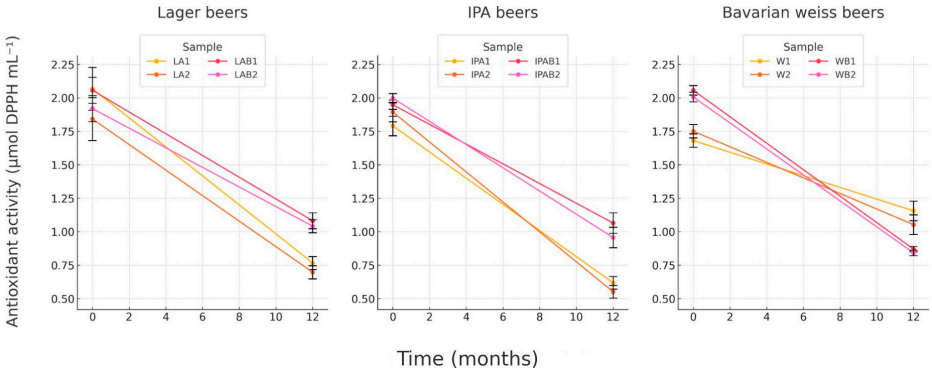


Figure 4. Antioxidant activity in the craft bread beers studied throughout the long-term storage process.

However, bread Lagers and Bread IPAs maintained higher antioxidant activity than their pure malt counterparts, implying that compounds from bread might contribute to antioxidant stability during extended storage. Similarly, at 12 months of storage, Weiss beers brewed with bread showed comparable values to those of the 100% malt Weiss beers, indicating a potential stabilizing effect from bread-derived antioxidants. This trend was consistent with another investigation by Jongberg et al. [32], showing that antioxidants in beer could degrade during storage due to oxidative reactions. Antioxidants, particularly polyphenols, interact with proteins and may oxidize, leading to reduced antioxidant activity. As indicated by total polyphenol content data, the incorporation of whole wheat bread likely introduced additional polyphenolic compounds, leading to a more stable antioxidant profile.

3.3. Microbiological Analysis

Microbiological analysis of the beer samples revealed that *Lactobacillus* spp. levels were undetectable in all beer styles, with counts below 100 CFU·mL^{-1}, except in Weiss beers 100% malt, which exhibited markedly elevated levels (8.5×10^5 CFU·mL^{-1}), indicating a potential risk for sensory alterations and reduced product stability.

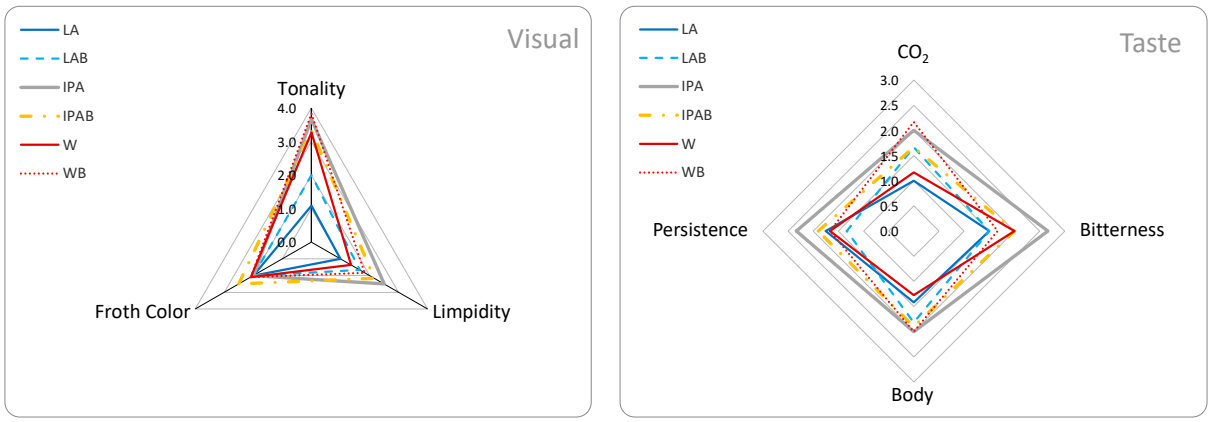
The elevated *Lactobacillus* levels observed in Weiss beer may be attributed to its higher protein and nutrient content, in combination with a lower hopping rate, which is known to create favourable conditions for lactic acid bacterial growth [25]. This aligns with previous

findings indicating that beer styles with reduced hop content, such as Weiss beers, are more susceptible to *Lactobacillus* spp. spoilage [33], which can lead to undesirable sensory attributes including sourness and turbidity. However, such elevated *Lactobacillus* spp. counts were not detected in the bread-enriched versions, suggesting that the addition of bread may have influenced microbial stability, preventing the proliferation of *Lactobacillus* spp. seen in traditional wheat formulations.

Additionally, effective sanitation and strict quality control are essential to prevent contamination and ensure beer quality. High levels of *Enterobacteriaceae* can indicate fecal contamination or poor sanitation, posing risks of off-flavours and potential health concerns. In this study, no *Enterobacteriaceae* were detected in any of the beer samples after 12 months of storage, with counts remaining below the detection limit of 10 CFU·mL^{-1}. These results suggest that the beers maintained microbiological stability with respect to *Enterobacteriaceae* throughout the long-term storage period, reflecting effective hygiene practices and a low risk of contamination [33].

3.4. Sensory Analysis

The visual sensory characteristics, collected after 12 months of storage, highlighted key differences influenced by beer styles (Figure 5a).



(**a**) (**b**)

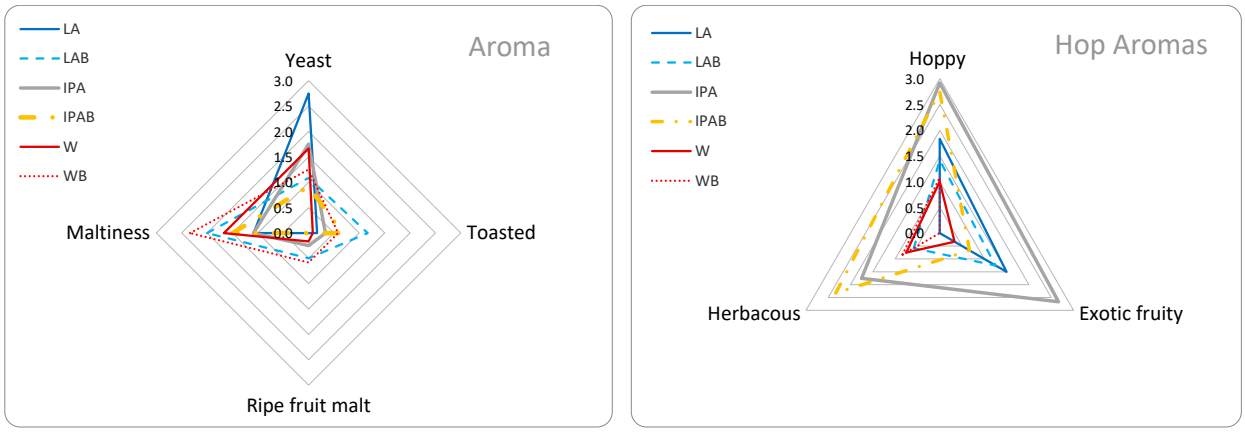
Figure 5. (**a**) Comparative visual sensory radar of beer samples stored for 12 months (Mean data); (**b**) Comparative taste profile of beer samples stored for 12 months (Mean data).

Lagers displayed higher limpidity and lighter tonality, reflecting the clearer, paler nature typical of this style. In contrast, IPAs and Weiss beers have higher tonality and lower limpidity, consistent with their known turbidity and deeper colour. The elevated turbidity in IPA and Weiss beers could result from the presence of haze-active proteins and polyphenols, which are more abundant in these beer styles due to their formulations and ingredients, such as wheat in Weiss beers and hop polyphenols in IPAs. However, the addition of bread appears to impact tonality positively in most styles, such as Lagers and Weiss, suggesting that bread contributes to a richer colour profile. This effect was less evident in IPAs, where bread-enriched IPAB showed slightly lower tonality than the standard IPA. This could indicate a different interaction between polyphenols and proteins in the presence of bread additives, possibly influenced by the high polyphenol content from hops.

In addition, Figure 5b showed how CO_2 levels, bitterness, body, and persistence varied across different beer samples in long-term storage, highlighting the impact of bread addition, increasing CO_2 taste and mouthfeel body across styles, although it slightly reduces

bitterness and persistence, especially in IPAs. All descriptors shown in Figure 5a,b were found to differ significantly ($p < 0.05$) except for froth colour.

Regarding the aroma profile of beers, all descriptors represented in Figure 6a,b exhibited statistically significant differences across the beer samples ($p < 0.05$), and no off-flavour descriptors such as oxidized, cider, vinegar, musty, stale, or soapy were detected, indicating that beers did not exhibit any sensory aromatic defects at the end of their studied shelf life. The results presented in Figure 6a showed variations in sensory attributes such as "Yeast," "Toasted," "Ripe fruit malt," and "Maltiness", differentiating the sensory profiles across the beer samples.



(**a**) (**b**)

Figure 6. (**a**) Comparative aroma sensory radar of beer samples stored for 12 months (Mean data); (**b**) Hops aroma profile of beer samples stored for 12 months (Mean data).

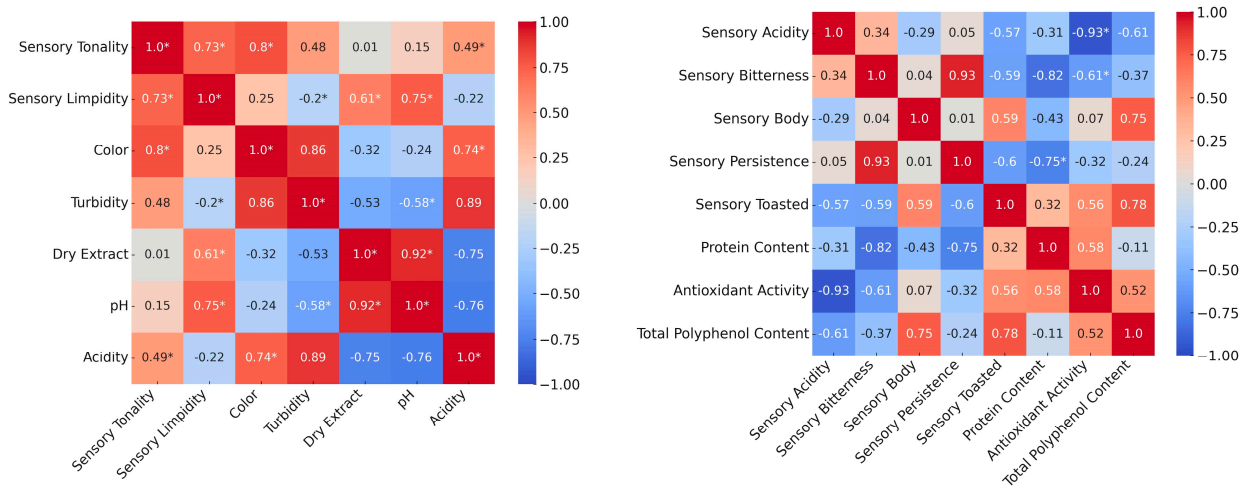
The incorporation of bread into beer formulations appears to selectively enhance specific sensory attributes, particularly toasted and malty notes, which were more pronounced in bread beers. This effect is likely attributed to the presence of Maillard reaction products and additional fermentable compounds introduced by the bread, contributing to greater aromatic complexity. In contrast, the 'Yeast' and 'Ripe fruit malt' aromas exhibited less noticeable variation. These findings are consistent with our previous research, which suggested that bread can contribute complex aroma and flavour compounds associated with roasting and Maillard-type browning reactions [21].

Finally, the hop aromas were evaluated (Figure 6b) and, as expected, were most pronounced in all IPA samples, consistent with their traditionally higher hop content. However, after 12 months of storage, the addition of bread appeared to alter specific hop-derived aromatic notes, particularly in IPAs. The observed reduction in exotic fruity aromas in bread-enriched beers may be attributed to interactions between bread components and volatile hop esters, which could become less stable in the presence of additional proteins or carbohydrates introduced by the bread [21]. On the other hand, the stronger herbaceous aroma observed in bread IPA beers suggested that bread may enhance green notes, possibly by retaining specific hop compounds or reducing the evaporation of these esters over time.

3.5. Correlations Analysis Between Sensory Attributes and Physicochemical Properties in Beer Samples

A correlation matrix heatmap between all physicochemical parameters and visual sensory attributes was analyzed. Strong positive (+1) and negative (−1) correlations are highlighted in red and blue, respectively. The variables included in Figure 7a were selected based on the most relevant pairing combinations, particularly those involving Colour,

Turbidity, Dry extract, pH, and Acidity in relation to Tonality and Limpidity, allowing for a clearer interpretation of the data.



(**a**) (**b**)

Figure 7. (**a**) Correlation matrix between key physicochemical analyses and visual sensory attributes ($p < 0.05$ indicated with *). (**b**) Correlation matrix between key physicochemical analyses and aromatic/taste sensory attributes ($p < 0.05$ indicated with *).

Turbidity showed a strong and statistically significant positive correlation with acidity, suggesting that beers with higher turbidity also tend to exhibit greater acidity. Although turbidity also displayed a high correlation coefficient with colour, this relationship was not statistically significant at $p < 0.05$.

A strong and significant correlation was observed between sensory tonality and sensory limpidity, indicating perceptual alignment in visual sensory descriptors. The strongest correlation found was between colour and sensory tonality (r = 0.80, $p < 0.05$), suggesting that the physical appearance of the beer strongly influences its perceived visual tonality.

Furthermore, beers with higher dry extract content tended to exhibit higher pH values (r = 0.92, $p < 0.05$). This relationship may be attributed to the presence of unfermented carbohydrates, dextrins, and particularly protein-derived compounds, which contribute significantly to the buffering capacity in beers [34]. These proteins and peptides, mainly from malt, help resist pH reduction during fermentation and storage, thereby stabilizing the final pH of the product.

Using the same criteria, Figure 7b presents the comparison between sensory aroma, taste attributes and physicochemical parameters, which showed the strongest correlations.

The high indirect correlation between sensory acidity and antioxidant activity in beers (r = −0.93, $p = 0.0001$) revealed a statistically significant relationship, indicating that higher antioxidant levels may lead to a perception of reduced acidity. This phenomenon could be attributed to antioxidants neutralizing acidic compounds, thereby modifying sensory perception. Previous research has indicated that polyphenolic antioxidants can interact with organic acids, potentially diminishing their sensory impact and contributing to a smoother taste profile [35]. Additionally, elevated antioxidant activity could inhibit microbial growth, preventing the production of organic acids that would otherwise increase acidity. For example, polyphenols in beer possess antimicrobial properties that reduce the proliferation of acid-producing bacteria, thus maintaining a more stable pH during storage [36].

Another inverse relationship was observed between protein content and sensory bitterness (r = −0.82, $p = 0.0015$), which was also statistically significant. This finding

supports the notion that proteins can bind with bitter substances, reducing their solubility and availability in the oral cavity and thereby diminishing bitterness perception. This phenomenon is in line with observations by Gonçalves et al. [37], who reported that proteins in beer, particularly those derived from malt, can form complexes with bitter compounds, leading to reduced bitterness intensity.

Additionally, a negative correlation was observed between protein content and sensory body ($r = -0.43$, $p = 0.14$), which, although not statistically significant, suggested a possible trend where higher protein levels might contribute to a fuller mouthfeel and balance bitter flavours. This effect could be due to proteins' ability to increase viscosity and interact with bitter compounds, reducing their perceived intensity. A recent study also reported that higher protein content enhances mouthfeel and reduces perceived bitterness in beer [38].

The correlation between total polyphenol content and sensory body ($r = 0.75$, $p = 0.052$) did not reach statistical significance, but still indicated a notable trend. This association suggests that higher polyphenol levels may contribute to a fuller mouthfeel. Polyphenols can increase viscosity and astringency, thereby enhancing the perception of body in beer. This interpretation aligns with prior studies showing that polyphenols interact with proteins and polysaccharides, leading to a more complex and rich mouthfeel [39].

Finally, a statistically significant positive correlation was observed between total polyphenol content and toasted flavour ($r = 0.78$, $p = 0.032$), indicating that beers with higher polyphenol levels tend to exhibit stronger toasted sensory characteristics. This relationship is likely influenced by the presence of polyphenols from toasted or roasted malts, as well as contributions from whole wheat components, which enhance the sensory complexity of the beer. These findings are consistent with prior research showing that Maillard reaction products from toasted malt contribute both to antioxidant capacity and to the development of toasted flavour profiles [31].

4. Conclusions

The results of this study revealed important insights into the evolution of craft beers, particularly those enriched with bread, during a 12-month storage period. Physicochemical analyses demonstrated significant reductions in turbidity and dry extract, especially in bread beer. This is likely due to the additional bioactive compounds as proteins, introduced by whole wheat bread, which contributed to increasing colloidal sedimentation, resulting in clearer beers after extended storage. The decrease in colour intensity aligned with the gradual sedimentation of polyphenols, a key factor in colour stability, which supports previous findings that link polyphenol precipitation with lighter colour profiles in extended storage beers.

Additionally, the bread beers retained higher levels of antioxidant activity and total polyphenol content compared to their 100% malt counterparts, suggesting that bread contributed positively to bioactive compound stability. This was particularly evident in IPAs and Weiss beers made with bread, where antioxidant stability was preserved despite the natural decline over time. These outcomes highlight the role of bread as a stabilizing factor for polyphenols at the end of beer's long-term storage process, which not only enhanced antioxidant activity, but also contributed to sensory properties, particularly toasted and maltiness aromas. The higher polyphenol content also correlated with a fuller body, adding complexity to the mouthfeel, a feature desirable in craft beer profiles. Moreover, the aroma data suggested that the strategic use of bread in brewing could add unique aromatic nuances, particularly in enhancing malt and toast-related attributes, potentially appealing to consumers seeking richer and more complex flavour profiles in craft beers. In addition, bread contributed to a fuller mouthfeel and increased carbonation taste, potentially altering the sensory profile and drinkability of beer.

The correlation analysis underscored the connection between physical-chemical properties and sensory attributes, revealing a strong association between polyphenol content and toasted flavours, while higher protein content was associated with reduced bitterness. Moreover, bread-enriched beers maintained microbiological stability, with no remarkable spoilage microorganisms detected, affirming that bread can enhance the sensory and bioactive profile, without compromising microbiological quality after 12 months of storage. In the context of a rising bioeconomy, using bread as a partial malt substitute ingredient not only provides a sustainable approach to resource utilization but also improves beer quality. This study supports the potential of bread as an innovative ingredient that complements the traditional brewing process, contributing to a more robust and complex sensory profile while aligning with circular economy goals by valorizing food waste.

Author Contributions: Conceptualization, C.M.-L., I.C. and C.A.B.; methodology, C.M.-L.; investigation C.M.-L.; supervision, I.C. and C.A.B.; writing—original draft, C.M.-L., I.C. and C.A.B.; writing—review and editing, C.A.B. and I.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: At the time the study was conducted (May 2023–September 2024), the University of Valladolid did not yet have a dedicated non-clinical human research board. In parallel, the Department of Agricultural and Forestry Engineering provided ethical oversight for this non-clinical sensory study and issued an Ethical-Compliance Certificate on 17 September 2024, confirming adherence to the Declaration of Helsinki and to the University's internal ethical guidelines. The Palencia Research Ethics Committee (CEIm) has now issued a favorable report (approval code: 2025/032; approval date: September 2025), acknowledging the non-clinical nature of the sensory work with adult assessors and the safeguards implemented (informed consent and data-protection compliance).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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V. CONCLUSIONES

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A partir de los resultados obtenidos en los tres trabajos que integran esta tesis doctoral, puede concluirse que la reutilización de **pan duro/pan de desecho**, y especialmente **pan integral**, constituye una alternativa tecnológica sólida para sustituir parcialmente la malta en la elaboración de cerveza, manteniendo la calidad del producto final y aportando ventajas en términos de componentes bioactivos, estabilidad y perfil sensorial. En conjunto, la investigación confirma que es posible integrar este ingrediente en condiciones controladas y reproducibles, y que su efecto depende del tipo de pan, del estilo cervecero y del almacenamiento prolongado.

En este marco, se exponen seguidamente las conclusiones particulares más relevantes:

- **Desarrollo y validación del proceso productivo**: se confirmó la viabilidad de incorporar pan duro mediante un esquema de elaboración que permite **sustituir hasta un 50% de la malta** sin comprometer el desarrollo del proceso cervecero ni el resultado final del producto, siempre que se mantenga una fracción suficiente de malta para aportar actividad enzimática.

- **Selección del pan integral como mejor opción**: el estudio comparativo inicial evidenció que el **pan integral** fue la opción tecnológicamente más equilibrada, al producir cervezas muy próximas al control en parámetros generales y, al mismo tiempo, con mejoras en variables asociadas al valor funcional. En contraste, la formulación con **pan de maíz** fue la menos favorable, al generar una cerveza más "ligera" y con peores resultados globales respecto al control.

- **Comportamiento fermentativo y parámetros de calidad a escala de estilos**: al aplicar la sustitución fija del 50% con pan integral en estilos contrastados (lager, IPA y weiss), las cervezas resultantes mantuvieron, en términos generales, un comportamiento comparable al control en los indicadores fisicoquímicos de referencia.

- **Efecto sobre la turbidez y el color**: la incorporación de pan integral provocó una disminución de **la turbidez**, con un efecto más marcado en estilos naturalmente turbios como las weiss. En los lotes comparados, la turbidez de las weiss con pan fue aproximadamente **un 15–21% menor** que la de sus controles (p. ej., de ~1024 a ~808 NTU y de ~1017 a ~868 NTU), mientras que

en lager la reducción osciló aproximadamente **entre un 13–33%** según el lote. Además, el impacto sobre el color dependió del estilo: en lager se observó un **aumento de intensidad de color** atribuible al pan integral.

- **Mejora perfil bioactivo y calidad sensorial**: en términos generales, las cervezas elaboradas con pan integral presentaron **mayor contenido fenólico total** y **actividad antioxidante** frente a sus equivalentes 100% malta. Esta ventaja se mantuvo al final del **almacenamiento prolongado**, lo que sugiere que la adición de pan contribuye a una **mayor estabilidad de los compuestos bioactivos**. Además, esta mayor presencia de polifenoles se correlacionó con atributos sensoriales deseables, especialmente **notas tostadas/maltosas** y el mayor contenido proteico con una menor percepción de amargor, además el pan integral proporcionó a las cervezas por la general, una **sensación de cuerpo más marcada**, aportando mayor complejidad en boca.

- **Perfil aromático (compuestos volátiles):** además, la sustitución parcial de malta por pan integral **no redujo la complejidad aromática**. El perfil volátil mantuvo compuestos relevantes como el **2-feniletanol** (asociado a **notas florales tipo rosa y matices dulces**) y el **octanoato de etilo** (relacionado con **aromas afrutados**). En conjunto, los resultados apoyan que el uso de pan puede aportar **matices aromáticos ligados a malta y tostado**, reforzando la riqueza sensorial sin asociarse a defectos.

- **Comportamiento durante almacenamiento prolongado: evolución física favorable y estabilidad microbiológica**: tras 12 meses a 15 °C, las cervezas con pan integral mostraron una evolución **favorable**, destacando una **reducción marcada de la turbidez**, especialmente en las cervezas con pan, en línea con fenómenos de **sedimentación coloidal**. De manera ilustrativa, en lager con pan se alcanzaron valores cercanos a **~101 NTU**, mientras que los controles se mantuvieron **por encima de ~600 NTU**. Paralelamente, el uso de pan integral **no comprometió la estabilidad microbiológica**, manteniéndose los recuentos de **Lactobacillus** por debajo del límite de detección (<100 UFC·mL^{-1}) y **Enterobacteriaceae** (<10 UFC·mL^{-1}) al final del almacenamiento.

- **Implicación tecnológica sostenible y de valorización de subproductos**: en conjunto, estos estudios demuestran que incorporar pan integral como adjunto en la elaboración de cerveza es una estrategia viable para producir

cervezas más sostenibles y de mayor calidad. Desde el punto de vista tecnológico, los elaboradores pueden reutilizar un residuo alimentario muy común (pan duro) para sustituir una parte importante de la malta, reduciendo así la huella ambiental de la producción cervecera (dado que la malta de cebada requiere muchos recursos y el pan, de otro modo, se desecharía). Este enfoque permite obtener cervezas que igualan o superan los estándares habituales de calidad, con beneficios nutricionales adicionales (más antioxidantes y polifenoles) y manteniendo su atractivo sensorial. Además, la sustitución de parte de la malta por pan de desecho, se ajusta a los principios de la economía circular al transformar residuos de panadería en un producto de alto valor añadido. Al ampliar el conocimiento sobre el rendimiento fermentativo, la estabilidad fisicoquímica y antioxidante y los resultados sensoriales asociados al uso de pan, **esta investigación doctoral contribuye tanto a la ciencia cervecera como a la sostenibilidad**. Además, abre la puerta a que los elaboradores de cerveza tanto a nivel artesanal como industrial, innoven con materias primas alternativas, convirtiendo desperdicios alimentarios en cerveza sin comprometer la calidad, y pone de relieve su potencial impacto social a través de la reducción de residuos, el ahorro de costes y la elaboración de cervezas con propiedades nutritivas beneficiosas.

V. CONCLUSIONS

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Based on the results obtained across the three studies comprising this doctoral thesis, it can be concluded that the valorisation of **stale/discarded bread**, and particularly **whole wheat bread**, represents a robust technological option to partially replace malt in beer production, while maintaining final product quality and providing advantages in terms of **bioactive composition**, **stability**, and **sensory profile**. Overall, the findings confirm that this ingredient can be integrated under controlled and reproducible conditions, and that its effects are influenced by the **type of bread**, the **beer style**, and **long-term storage**.

Within this framework, the most relevant specific conclusions are summarised as follows:

- **Development and validation of the production process:** The feasibility of incorporating stale bread through a brewing scheme enabling the replacement of **up to 50% of the malt** was confirmed, without compromising process performance or overall product outcome, provided that a sufficient fraction of malt is retained to supply the required **enzymatic activity**.

- **Selection of whole wheat bread as the most suitable option:** The initial comparative assessment showed that **whole wheat bread** was the most technologically balanced option, producing beers closely comparable to the control in general parameters while simultaneously improving variables associated with functional value. In contrast, the **corn bread** formulation performed least favourably, yielding a "lighter" beer and overall weaker results relative to the control.

- **Fermentation performance and quality parameters across beer styles:** When applying a fixed **50% substitution** with whole wheat bread in contrasting styles (lager, IPA, and weiss), the resulting beers generally displayed performance comparable to the controls in key physicochemical indicators.

- **Effect on haze and color:** The incorporation of whole wheat bread tended to reduce **haze (turbidity)**, with a more pronounced effect in naturally hazy styles such as weiss. In the batches compared, haze in bread-weiss beers was approximately **15–21% lower** than in their controls (e.g., from ~1024 to ~808 NTU and from ~1017 to ~868 NTU), while in lager the reduction ranged

approximately from **13% to 33%** depending on the batch. The impact on color was style-dependent: in lager, an increase in color intensity attributable to whole wheat bread was observed.

- **Improved bioactive profile and sensory quality:** Consistently, beers brewed with whole wheat bread exhibited higher **total phenolic content** and **antioxidant activity** than their 100% malt counterparts. This advantage persisted at the end of **long-term storage**, suggesting that bread contributes to enhanced stability of bioactive compounds. In addition, higher polyphenol levels correlated with desirable sensory attributes, particularly **toasted/malty notes**, while higher protein content was associated with a **lower perceived bitterness**. Overall, whole wheat bread tended to provide a fuller body and greater mouthfeel complexity.

- **Aroma profile (volatile compounds):** Partial replacement of malt with whole wheat bread did not reduce aromatic complexity. The volatile profile retained key compounds such as **2-phenylethanol** (associated with **rose-like floral notes** and sweet nuances) and **ethyl octanoate** (linked to **fruity aromas**). Collectively, the results support that bread can contribute aroma nuances associated with malt and toasted character, enhancing sensory richness without being linked to sensory defects.

- **Behaviour during long-term storage: favourable physical evolution and microbiological stability:** After **12 months at 15 °C**, beers brewed with whole wheat bread showed favourable evolution, notably a marked reduction in haze, consistent with **colloidal sedimentation** phenomena. Illustratively, lager brewed with bread reached values close to **~101 NTU**, whereas controls remained above **~600 NTU**. In parallel, the use of whole wheat bread did not compromise microbiological stability: **Lactobacillus** counts remained below the detection limit (**<100 CFU·mL^{-1}**) and **Enterobacteriaceae** below **<10 CFU·mL^{-1}** at the end of storage.

- **Sustainable technological implications and by-product valorisation:** Taken together, these studies demonstrate that incorporating whole wheat bread as a partial malt substitute is a viable strategy to produce more sustainable and higher-quality beers. From a technological standpoint, brewers can reuse a common food waste stream (stale bread) to replace a substantial proportion of malt, thereby lowering the environmental footprint of brewing (given the resource intensity of malted barley and the fact that bread would otherwise be discarded). This approach enables beers

that match or exceed conventional quality benchmarks, providing additional nutritional value (higher antioxidants and polyphenols) while maintaining sensory appeal. Furthermore, the partial substitution of malt with surplus bread aligns with circular economy principles by transforming bakery waste into a value-added product. By advancing knowledge on fermentation performance, physicochemical and antioxidant stability, and sensory outcomes associated with bread use, this doctoral research contributes to both brewing science and sustainability. Moreover, it supports innovation in both craft and industrial brewing by enabling the conversion of food waste into beer without compromising quality, highlighting potential societal benefits through waste reduction, cost savings, and the production of beers with nutritionally beneficial properties.

VI. REFERENCES

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CHAPTER 1: Bread as a Valuable Raw Material in Craft Ale Beer Brewing

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Para enriquecer y actualizar la redacción de la introducción de la presente tesis, se han utilizado las siguientes referencias:

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