Microbiota and associated chemical compounds in South African craft ales

by

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Submitted in fulfilment of the requirements for the Degree

Doctor of Philosophy: Environmental Health

Centre for Applied Food Sustainability and Biotechnology (CAFSaB)

Department of Life Sciences

Faculty of Health and Environmental Sciences

Central University of Technology, Free State

BLOEMFONTEIN

2025

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DECLARATION

I, Lerato Bonolo Mogotsi, Identity Number: (_____), hereby declare that this research project, submitted to the Central University of Technology, Free State for the Degree Doctor of Philosophy in Environmental Health, is my own independent work. This work complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State. This work has not been submitted before to any institution by myself or any other person for the attainment of a qualification.

……

Lerato Bonolo Mogotsi

I certify that the above statement is correct.

……

Professor O. de Smidt (Promotor)

28 October 2025

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DEDICATION

I dedicate this work first and foremost to my Lord and Saviour, Jesus Christ, whose grace, wisdom, and strength have been my constant source of guidance and endurance throughout this journey.

To my beloved husband (Biko Mogotsi), whose patience, unwavering belief in me, and steadfast support carried me through every stage of this study. Your understanding and care in managing everything while I pursued this dream made all the difference.

To my father (John Nkata), though miles away, your constant encouragement and unshakable faith in my abilities have been a source of strength and motivation.

To my children (Bogolo, Oabusa and Tumisang Mogotsi), who prayed for me, inspired me, and looked up to me even when my time was divided — you have been my greatest motivation to keep going and finish strong.

To my family and friends, your love, prayers, and encouragement sustained me through the most challenging times.

I am sincerely grateful to the National Research Foundation (NRF) for funding this study and to the Central University of Technology, Free State (CUT), for providing the resources and academic platform to make this work possible.

This thesis is dedicated to all of you who believed in me and made this achievement a reality.

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ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my promoters, Prof O. de Smidt and Dr A.O. Ojo, for their exceptional mentorship, guidance, and support throughout this research. Prof O. de Smidt provided outstanding academic leadership, combining meticulous attention to detail with extensive scientific expertise. Her insightful direction and critical feedback were instrumental in shaping the overall quality of this study, significantly enhancing both its scientific rigor and academic presentation.

I am equally grateful to Dr A.O. Ojo for her invaluable contributions, including her detailed guidance on methodology, data interpretation, and manuscript preparation. Her thoughtful feedback, dedication, and encouragement went beyond standard supervision, ensuring the development of a robust, well-articulated thesis. Both supervisors fostered a highly supportive and intellectually stimulating environment, which greatly enriched my development as an independent researcher. Their mentorship not only guided the successful completion of this study but also strengthened my professional growth, analytical skills, and academic writing.

I extend my sincere appreciation to the National Research Foundation (NRF) for funding this study and to the Central University of Technology, Free State (CUT) for providing the academic platform, facilities, and resources necessary for its completion. I am also grateful to the Central Analytical Facilities (CAF) at Stellenbosch University, SMT Laboratories (Bloemfontein), and Inqaba Biotechnical Industries (Pretoria) for their technical support during the analytical phases of this research.

Special thanks to the Centre for Applied Food Sustainability and Biotechnology (CAFSaB) and members of the Faculty of Health and Environmental Sciences (FHES) for their assistance and support. I am particularly thankful to Dr. Manduna, whose coordination of research activities, consistent reminders, and encouragement greatly facilitated my progress.

I would also like to acknowledge my Head of MSTE Department, Mr Beukes, for his unwavering support and for granting me the opportunity to take a five-month sabbatical, which provided the time and focus necessary to complete this research.

Finally, I express my heartfelt gratitude to my colleague Mr Walter Nigrini, for his invaluable assistance with the statistical analysis and data processing. His expertise and collaboration were instrumental in ensuring the accuracy and robustness of the analytical results.

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ABBREVIATIONS

ABV	Alcohol by volume
$AFLB_1$	Aflatoxin B_1
$AFLB_2$	Aflatoxin B_2
$AFLG_1$	Aflatoxin G_1
$AFLG_2$	Aflatoxin G_2
CAF	Central Analytical Facilities
D3G	Deoxynivalenol-3-glucoside
DON	Deoxynivalenol
EFSA	European Food Safety Authority
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
GC-MS	Gas chromatography–mass spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
NIV	Nivalenol
OTA	Ochratoxin A
SPE	Solid phase extraction
TDI	Tolerable dose intake
TPC	Total plate count
UPLC-ESI-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
WHO	World Health Organisation
ZEN	Zearalenone

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ABSTRACT

Craft beer is typically brewed in smaller batches compared to industrial breweries. Several factors can influence the flavour, quality, and safety throughout the craft beer brewing process, including raw materials, fermentation conditions, and the interactions between the chemical composition and microbial communities. Limited research has been undertaken on the integration of microbial and chemical profiles of craft beers. Therefore, this study aimed to profile the microbial and chemical compositions of South African craft ale beers, to evaluate their safety in relation to the detected microbial and chemical compositions, including the level of mycotoxins, and to explore correlations between the microbiota and the detected compounds.

A total of 30 beers (in triplicate) of different styles were purchased from 30 commercial craft breweries and tested for mycotoxin levels using ultra-performance liquid chromatography–electrospray ionisation–tandem mass spectrometry (UPLC-ESI-MS/MS). The most prevalent mycotoxin was deoxynivalenol (DON), which was detected in 10% of the beer samples. The highest concentration of DON (22 µg/L and 41.49 µg/L) was detected in India Pale Ale (IPA) and Stout, while fumonisin FB_1 and FB_2 (0.37 µg/L and 0.16 µg/L) were detected in IPA and Pale Ale, respectively. Nivalenol (NIV) was present in trace amounts (0.13 µg/L) in one IPA sample. The presence of these mycotoxins indicated the need for quality assurance of raw materials used in craft beer production and quality control.

Microbial profiling showed total viable microorganisms present up to 1.63×10^5 CFU/mL, yeast was detected in more than 50% of the beer samples, not unexpected since most breweries do not filter or pasteurise their final product. Moulds were present in product from 17% of breweries, and 60% of the breweries presented with lactic acid bacterial (LAB) contamination. Common foodborne pathogens *Escherichia coli* and *Salmonella spp* were not detected. Pale Ales from one brewery presented co-occurrence of LAB and *Staphylococcus aureus*, indicative of post-fermentation contamination. Next-generation sequencing results showed that *Acetobacter* and *Levilactobacillus* spp. dominated bacterial communities, while the fungal community was dominated by *Saccharomyces cerevisiae*, although other non-*Saccharomyces* genera, including *Cladosporium*, *Vishniacozyma*, and *Penicillium* spp. were also present.

Chemical profiling revealed several flavour-active compounds, including isoamyl alcohol (178.9 mg/L), 4-vinylguaiacol (10 mg/L), isoamyl acetate, and vanillic acid (76.7 mg/L), present in concentrations well above sensory thresholds in beer styles in which these compounds are not expected or preferred. The sugar and sugar alcohols varied widely in both type and concentrations. Fructose (107.4 mg/L), xylose (60.6 mg/L), and xylitol (185.4 mg/L) were among the most dominant. A strong positive correlation

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between higher alcohols, as well as a negative correlation between certain sugars (e.g., maltose, trehalose) and flavour-active compounds was also revealed.

This research provides insights into the microbial and chemical profiles of sampled South African craft ales. While these beers may be microbiologically safe and favourably complex, some of the beer samples remain susceptible to post-fermentation contamination and subtle chemical variability. The findings highlight the interconnected role of microorganisms in shaping beer flavour and stability, and they emphasise the importance of maintaining consistent quality in the craft brewery environment. This study establishes valuable baseline data to enhance product safety, sensory consistency, and meet regulatory standards of the rapidly growing craft beer industry.

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Beer is the world's most widely consumed fermented beverage and the third most popular drink after tea and coffee. Its production dates back thousands of years to the Neolithic urbanisation and civilisation period (Hayden, Canuel and Shanse, 2013). In the last two decades, the rapid emergence of flourishing independent craft breweries in countries where a beer tradition had never been established was witnessed (Garavaglia and Swinnen, 2017). Globally, craft breweries are characterised as small-scale traditional breweries that emphasise flavour and adopt a local identity (Brewers Association, 2016; Craft Beer South Africa Interim Steering Committee, 2014).

Traditionally, yeast *Saccharomyces cerevisiae* (or the related hybrid *S. pastorianus*) was the primary fermentation microorganism in beer production (Burini et al., 2021). *Saccharomyces cerevisiae* converts wort sugars into ethanol and carbon dioxide as well as a wide variety of secondary metabolites. These compounds include higher alcohols, esters, organic acids, and phenolic compounds (Paszkot, Gasiński and Kawa-Rygielska, 2023) that are key contributors to the flavour, aroma, and mouthfeel of beer (Pires and Brányik, 2015). Owing to the use of diverse raw materials and a lack of advanced quality control measures in many craft breweries, a wide range of microorganisms, including non-*Saccharomyces* yeasts, lactic acid bacteria (LAB), and opportunistic microorganisms found in the environment, are associated with craft beer brewing (Thompson and Dawson, 2023). These organisms can either contribute positively to the sensory characteristics of beer or impact it negatively, leading to spoilage and undesirable off-flavours (Suzuki, 2011). Thus, understanding the microbial diversity of craft beer is fundamental in managing product quality and safety.

Concurrently, throughout the fermentation and maturation stage of beer production, the chemical composition of beer is shaped by the raw material composition (such as malt, hops, adjuncts and water) and microbial metabolism (Humia *et al.*, 2019). The flavour profile of the beer being brewed is collectively determined by phenolic acid, volatile phenols, higher alcohols, esters, fatty acids, aldehydes, and residual sugars in beer (Coghe et al., 2004;Vanderhaegen et al., 2006). Additionally, the natural occurrence of mycotoxins, which are secondary metabolites of fungi, may persist from grain to final product, potentially causing acute health disorders when ingested at high concentrations (Berthiller *et al.*, 2011) .

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Despite the extensive global research on microbial diversity or chemical profiling, a gap remains due to limited data on the microbial and chemical characterisation of the emerging South African craft beers market. In this context, it is important to note that the variability in fermentation control and the diverse raw materials used in local craft breweries may influence microbial diversity and metabolite production in ways that may not yet be fully comprehended. Therefore, when this study was conceptualised, it was argued that an integrated analysis process of microbial diversity, chemical composition, and mycotoxin occurrence in craft beers would enhance understanding of the quality and safety implications of this type of beer. The identified knowledge gaps were systematically addressed by investigating the microbiota and associated chemical compounds in South African craft ales. A combination of culture-based and molecular methods was used to profile the selected beer styles from various breweries. The chemical profiling of the selected beer styles was done by quantifying flavour and non-flavour-active compounds, residual sugars, and mycotoxins. By integrating the microbial and chemical data, this study provides insights to support the production of safe, high-quality craft beer.

1.2 PROBLEM STATEMENT

Beer is among the most widely consumed alcoholic beverages globally. In South Africa, beer accounts for the largest share of alcohol consumption, with national consumption nearly double the average reported by the World Health Organisation (WHO). For the African region, WHO estimates that per capita alcohol consumption was approximately 6.3 litres per person per year, while South Africa's consumption is projected to reach 12.1 litres per person per year by 2025 (WHO, Global Health Observatory). A growing trend within the beer industry is the rise of craft beer, with local breweries expanding rapidly to meet increasing consumer demand (Rogerson and Collins, 2015; Pokrivčák et al., 2019). However, the limited resources and lack of advanced quality control measures in many craft breweries pose potential risks related to microbial and chemical contamination and product stability (Oldham and Held, 2023). Unlike large-scale industrial breweries that employ stringent quality control methods such as pasteurisation to eliminate microbial contaminants, craft breweries often lack these processes due to financial and infrastructural constraints (Obi, 2017; Thompson and Dawson, 2023). As a result, spoilage microorganisms such as lactic acid bacteria (LAB) can negatively impact beer quality, leading to undesirable flavours and turbidity (Suzuki, 2011). Additionally, raw materials, particularly barley, can serve as a source of contamination (Schabo et al., 2021). A key concern associated with barley is the presence of mycotoxins, which are secondary fungal metabolites such as fumonisins, deoxynivalenol, and zearalenone. These mycotoxins are prevalent in cereal crops and pose significant food safety risks in Africa (Yu and Pedroso, 2023). High concentrations of these mycotoxins can lead to adverse health

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effects (Khan, Anwar and Ghazali, 2024). Beyond concerns related to microbial contamination, volatile and aromatic compounds play a fundamental role in shaping the sensory attributes of beer. These compounds, influenced by microbial diversity and fermentation by-products, contribute to the overall flavour profile. Understanding the interactions between indicator microbiota, their metabolic activity, and beer chemistry is essential for evaluating product quality and ensuring craft beer safety.

To date, most studies in this field have either focused on microbial diversity or chemical profiling separately; hence, data on mycotoxins in locally produced craft beers are scarce. This knowledge gap is significant because integrating the analysis of microbial diversity, chemical composition, and mycotoxin occurrence will provide a comprehensive understanding of craft beers, their potential risks, and overall product quality.

1.3 AIMS AND OBJECTIVES

This study aimed to assess the microbial diversity in South African craft ale beers and to identify chemical compounds that could influence flavour development or pose risks to consumer health.

More specifically, the objectives were to:

1. Assess the occurrence of key mycotoxins, such as aflatoxin B_1 (AFB_1), deoxynivalenol (DON), fumonisins (FB_1, FB_2, FB_3), ochratoxin A (OTA), zearalenone (ZEN), and nivalenol (NIV) in various sampled styles of commercially available South African craft beers using UPLC-ESI-MS/MS.
2. Evaluate the microbial and chemical profiles of sampled South African craft ales and examine potential correlations among key variables—including volatiles, phenolic acids, off-flavours, sugars, and alcohols.
3. Evaluate microbial diversity in the selected South African craft ale beers using single-molecule real-time sequencing (SMRT) on the PacBio platform.

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1.4 CHAPTER LAYOUT

Chapter 1: Background to the study

This chapter provides a brief background to the study.

Chapter 2: Review of related literature

This chapter reviews existing research on craft beer and fermentation processes, microbial population, chemistry, safety, and potential contamination risks in South African craft beers.

Chapter 3: Mycotoxins associated with the selected South African craft ale beers

This chapter examines the occurrence and levels of different mycotoxins along with their safety implications in the sampled South African craft beers.

Chapter 4: Overview of microbial and chemical profiles of the selected South African craft ale beers

This chapter provides an integrated overview of the microbial and chemical profiles of South African craft beers, highlighting the impacts of flavour and non-flavour active compounds on beer quality and safety.

Chapter 5: Assessing microbial diversity of South African craft ale beers and the influence of flavour-active compounds

This chapter assesses the microbial composition of South African craft beers and examines how the microbial community may influence selected flavour-active compounds.

Chapter 6: General concluding remarks and future work

This chapter summarises key findings, addresses knowledge gaps, discusses limitations and provides future research and improvements in craft brewing practices

Chapter 7: List of references

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CHAPTER 2:
LITERATURE REVIEW

2.1 INTRODUCTION

Beer is one of the oldest fermented beverages in the history of mankind, and it remains the world's most widely consumed alcoholic beverage and the fourth most popular drink after water, tea, and coffee (Piacentini et al., 2015; Raihofer et al., 2022). Traditional beer brewing produced limited styles, but recently consumers have preferred varieties of beer styles with richer, more distinctive tastes and better quality (Lerro, Marotta and Nazzaro, 2020). This indicates that beer production has evolved since the emergence of microbreweries that produce craft beers with diverse flavours (Villacreces, Blanco and Caballero, 2022). The rise of craft beers, also known as the microbrewery movement, has led specific market segments to shift away from large corporate brands in favour of local alternatives. This movement gained traction in the 1970s, particularly in the United Kingdom (UK) and the United States of America (USA), with pioneers such as Fritz Maytag reviving small-scale brewing (Elzinga, Tremblay and Tremblay, 2015). The craft beer industry has grown significantly over almost five decades and is now well-known worldwide. Reports suggest an 'explosion' of this trend in the USA, New Zealand, Australia, Canada, Denmark, Sweden, Norway, and Japan (Durán-Sánchez et al., 2022). The USA identified a target of approximately 70 million young consumers aged between 21 and 30 (Aquilani et al., 2015). Even after the setbacks of the COVID-19 pandemic in 2020 to 2021, more than 9,000 craft breweries were operating in the USA, the highest total since the 1880s, and hundreds more are opening every year (Collins, Rogerson and Rogerson, 2023). In 2024, as many as 9,736 small and independent craft breweries were operating in the USA (Brewers Association, 2024). It is noteworthy that the growing number of local craft breweries in South Africa has caught up with the international craft beer revolution (Collins, Rogerson and Rogerson, 2023). Most breweries are located in the Western Cape, with high concentrations also observed in the Gauteng province and smaller numbers in other provinces (Figure 2.1: Map showing craft breweries in South Africa

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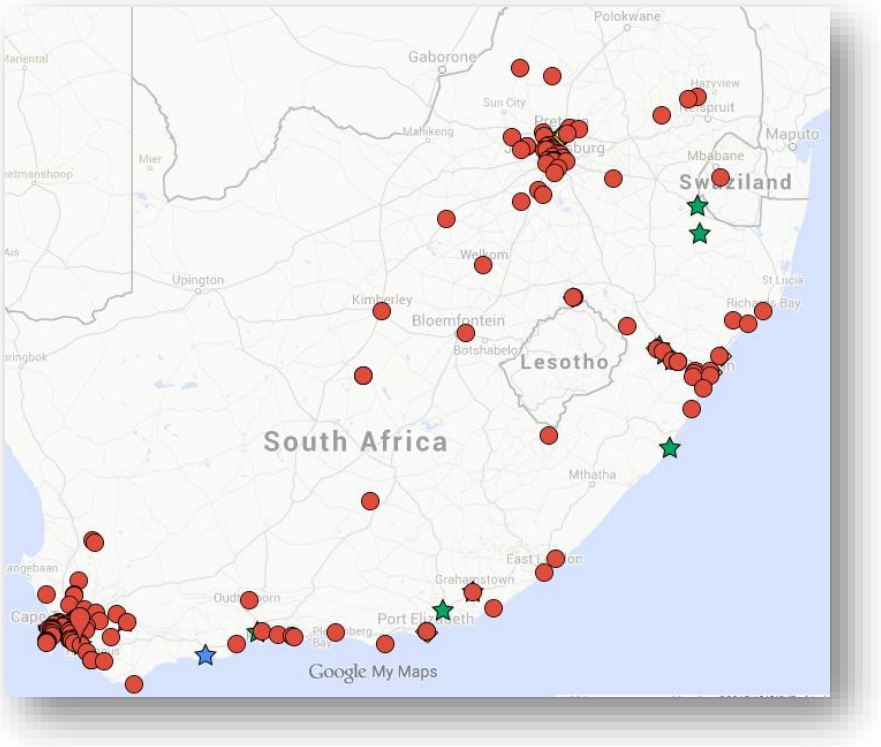


Figure 2.1: Map showing craft breweries in South Africa

Red circles (●) represent craft breweries, green stars (★) represent notable or award-winning breweries, and blue stars (★) represent historic or pioneering breweries.

Based on the belief that the craft beer niche promotes various craft beer categories, South African Breweries (SAB) has supported microbreweries by sponsoring their festivals. In addition, this large company has begun to offer flavoured traditional types of beers, such as their flavoured Flying Fish product (Mohasoa, 2016). The No 3 Fransen Street Brewery, which was previously used by SAB as a test brewery for their talented brewers to experiment with new beer styles, is now used to make ales and to embrace the opportunity to make a variety of beers. In 2024, SAB introduced three new unique brews at its No 3 Fransen Street Brewery, and it has continued to diversify its beer offerings and embrace the craft beer culture (DRINKStuff SA, 2014).

Countries such as the USA, the UK, and South Africa have diverse definitions of a craft brewery. However, globally, craft brewing is defined as a smaller-scale, traditional brewery characterised by focusing on flavour and diversifying brewing techniques (Brewers Association, 2016; Craft Beer South Africa Interim Steering Committee, 2014). In South Africa, the craft beer industry has experienced substantial growth, with microbreweries producing diverse beer styles such as Pale Ales, India Pale Ales (IPAs), Stouts, and Porters (Schabo et al., 2021). Unlike large-scale commercial breweries that source

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highly regulated malted grains, craft brewers often rely on locally sourced ingredients of varying quality. Craft beers that are in demand by consumers are assumed to have been brewed with skill and care by a suitably qualified person using high-quality, traditional or non-traditional ingredients (Miller, 2019). Many beer drinkers are particularly interested in tasting novel craft beers that exhibit different flavours and aromas from those usually associated with well-known commercial brands (Aquilani et al., 2015). A 2023 study highlights that consumers are increasingly attracted to new and novel beer styles and flavours offered by craft breweries, suggesting a shift towards more adventurous taste experiences (Pilone et al., 2023). However, craft beers are prone to contamination, so continuous quality monitoring at craft beer breweries and strict regulations must be established to protect consumers.

2.2 NATIONAL AND INTERNATIONAL CRAFT BEER REGULATIONS

Craft brewers use wild yeast, different strains of bacteria, experimental hop varieties, spices, and fruits to produce a variety of craft beers (Miller et al., 2011). However, craft beers are more prone to microbial contamination than industrial beers because some craft breweries lack pasteurisation and microfiltration equipment (Oldham and Held, 2023). Today, many craft breweries do not pasteurise their beers as pasteurisation equipment is expensive. The proliferation of Gram-negative bacteria, which are anaerobic members of *Pectinatus* species and *Megasphaera* species, along with some wild yeasts such as the *Brettanomyces* species, are frequent beer spoilage microorganisms. Nonetheless, most spoilage incidents are caused by lactic acid bacteria (LAB) (Rodríguez-Saavedra, González de Llano and Moreno-Arribas, 2020). Depending on the species and strain, LAB can cause acidification, sedimentation, turbidity, and off-flavours associated with highly active volatile phenols such as 4-vinylguaiacol (4-VG) and 4-ethylguaiacol (4-EG) (Coghe et al., 2004; Lentz, 2018). Microbial contamination can adversely affect beer quality and can cause detrimental financial consequences for the brewing industry (Vaughan, O'Sullivan and Van Sinderen, 2005; Oldham and Held, 2023; Thompson and Dawson, 2023). It is for this reason that quality assurance is pivotal, and this requirement ranges from raw materials and individual process stages to the finished beer (Piacentini et al., 2015).

In South Africa, the quality of craft beer is governed by national legislation. For instance, the Foodstuffs, Cosmetics and Disinfectants Act No. 54 of 1972 states that quality beer must be produced from malted barley, hops, water, and yeast (Republic of South Africa, 1972). Although malted barley is a primary ingredient, unmalted maize, rice, or wheat are frequently added to beer to produce different flavours at lower manufacturing costs (Ruiz-Medina and Fernández-de Córdova, 2015; Yorke, Cook and Ford, 2021) Hence, the use of grain products such as barley, wheat, and maize is common in big industrial breweries

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(Shewry, 2010; Dabija et al., 2021). These grains are associated with the natural occurrence of mycotoxins, which are secondary metabolites of fungi that can cause disease and even death in humans and animals (Berthiller et al., 2013; Pascari et al., 2022). In fact, mycotoxins can cause acute health disorders when ingested in high concentrations (Berthiller et al., 2013; Piacentini et al., 2015; Nagda and Meena, 2024). Generally, mycotoxins are thermally and chemically stable compounds that can survive the cereal production process and contaminate finished processed foods (Köppen et al., 2010; Schaarschmidt and Fauhl-Hassek, 2021). The survival of mycotoxins has compelled most countries to establish and enforce regulations that guide the maximum levels of mycotoxins in food.

Fungi of the genera *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium* are predominant producers of mycotoxins (Berthiller et al., 2013; Adelusi et al., 2023). Fumonisins, deoxynivalenol (DON) and its derivatives are among the most important mycotoxins in terms of economic impact and toxicity (Deng et al., 2023).These mycotoxins belong to the major class of *Fusarium* toxins that are frequently produced by various *Fusarium* species (Shale et al., 2012;Bryła et al., 2018; Ekwomadu, Akinola and Mwanza, 2021). Their prevalence means that mycotoxins must be frequently tested using specific analysis protocols. Studies have shown that in a commodity contaminated with mycotoxins, derivatives such as conjugated or masked mycotoxins, formed through plant metabolism, may display unique chemical behaviours that can easily escape routine analysis (Ekwomadu et al., 2020). For instance, microorganisms used in fermentation can transform mycotoxins into products that cannot be detected by analytical or conventional methods (Galaverna et al., 2009; Berthiller et al., 2011; Abraham et al., 2022). These microorganisms can enzymatically transform mycotoxins through hydrolysis, de-epoxidation, reduction, conjugation or complete degradation (Berthiller et al., 2013). These forms could be hydrolysed to their precursors in the digestive tracts of animals and humans, resulting in adverse toxic effects (Galaverna et al., 2009; Daud et al., 2020).

Many countries have devised regulations to protect their citizens and livestock against the potential harm caused by mycotoxins. In fact, the maximum allowable level of specific mycotoxins in food and feed is regulated in more than 70 countries worldwide. The most regulated mycotoxins globally include deoxynivalenol (DON), zearalenone (ZEN), fumonisins (B_1, B_2), aflatoxins, ochratoxin (OTA), and patulin (Codex Alimentarius Commission, 2023). The European Union (EU) (2023) has imposed some of the strictest regulations to curb their prevalence. For instance, in 2024, it established strict new threshold limits for ochratoxin. On the other hand, the USA follows regulations from the FDA with specific levels for DON, fumonisins, and aflatoxin M_1 (Cai, McLaughlin and Zhang, 2020). In South Africa, the levels of only two mycotoxins in food and milk, namely aflatoxin and patulin, are regulated by the South African National

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Regulations (News24, 2014). The regulations established new limits for aflatoxin B_1 by setting it at 5 µg/kg. Total aflatoxins were restricted to 10 µg/kg in food, and limits for aflatoxin M_1 in milk were also established (Republic of South Africa, 2024). However, no legislation governs the maximum allowable levels for *Fusarium*-related mycotoxins in food and feed (Matumba et al., 2015). The problem is that, even though some major mycotoxins are being regulated internationally, monitoring and regulation remain inconsistent. This gap in regulation impacts the craft brewing sector, where small-scale producers often use locally sourced grains that are usually susceptible to mycotoxins (Gonzalez Pereyra et al., 2011). Based on the increasing trend in craft breweries both internationally and in South Africa, this study sought to understand how variability in raw ingredients might influence the risk of mycotoxin contamination in such beer in order to contribute to guidelines that will ensure the overall safety of craft beer consumers.

2.3 TRENDS IN CRAFT BEER BREWING INTERNATIONALLY AND IN SOUTH AFRICA

The tradition of brewing beer began 13,000 years ago during the Neolithic period of urbanisation and civilisation (Meussdoerffer, 2009; Hayden, Canuel and Shanse, 2013). Since those early times, beer has played a significant role in society across different cultures and lifestyles (Miller, 2019). In this era, the Egyptians used beer as a form of currency, and even slaves were paid with beer rations. Beer was considered both food and drink during celebrations, with women primarily responsible for brewing beer (Frankel, Benjamin and Stephens, 2023).

Today, the brewing industry is a global enterprise run by a few multinational companies. However, the emergence of flourishing independent craft breweries has recently been observed even in countries where the beer brewing tradition had never existed (Garavaglia and Swinnen, 2017). It is especially in Italy where craft beer brewing has recently emerged and where consumers have become interested in tasting new craft beers that elicit different aromas, flavours, and bitterness levels than those associated with well-known commercial brands (Lerro, Marotta and Nazzaro, 2020).This growing interest is reflected in a considerable increase in production from 290,000 hectolitres per year in 2008 to approximately 17.27 million hectolitres in 2020 in this European country (Cimini and Moresi, 2021). Moreover, Italian microbreweries increased from 175 in 2007 to 653 in 2013 (Esposti, Fastigi and Viganò, 2017). As a traditional wine-producing country, this trend shows that the rise of the craft brewing sector in Italy might have occurred as a consequence of significant interest in the beer market (Garavaglia and Mussini, 2020).

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In 2010, craft brewers in the USA accounted for 4.9% of overall beer production and 7.6% of retail expenditure across beer categories (Brewers Association, 2011). The legalisation of homebrewing and brewpubs in 1979 led to reduced excise taxes for brewers selling specialised beers of less than 2 million barrels (Carroll and Swaminathan, 2011). Even during the difficult 2020/2021 period, it is estimated that 400,000 jobs were created in the US through the growth of the craft brewing industry (Brewers Association, 2020). Statistics show that Colorado hosts over 400 established craft breweries that make a significant contribution to the state's economy (Moore, Reid and McLaughlin, 2016). The rise of craft breweries in this state exemplifies a thriving industry within local, state, and national economic settings.

South Africa has more than 200 microbreweries (Collins, Rogerson and Rogerson, 2023), with the vast majority located in the Western Cape and Gauteng and smaller numbers in other provinces. However, compared to its international counterparts, South Africa's craft beer industry remains relatively underexploited (Hadisi and Snowball, 2022). Based on its market share volume, South Africa is probably 10 to 15 years behind the USA in terms of craft beer penetration (IOL, 2024). Hence, limited literature is available on the craft beer brewing industry in South Africa, where it is a relatively fragmented and recent trend (Rogerson and Collins, 2019). Nonetheless, due to the increasing consumption of craft beer varieties, the growth of the craft beer market is likely to continue in South Africa in the coming years. However, due to the COVID-19 pandemic, 27 breweries were compelled to shut down, with 31 more on the brink of closure due to four periods of alcohol banning imposed on this industry by the national government in 2020 (Pillay, 2021).

Recently, the demand for craft beer in SA has increased significantly. According to Josef-Schmid, co-founder and managing director of MadMead Brewing, South Africa's first majority black-owned microbrewery, the strong consumer demand for the product indicates that the craft beer industry in this country, which is still in its infancy, is still expanding (Collins, Rogerson and Rogerson, 2023). For instance, the Darling Brewery in the Western Cape could not meet consumers' demand, prompting it to explore opportunities to expand its brewing capacity (The Beer Connoisseur, 2015).

The definition of the microbrewery movement varies depending on the preferences or regulations of different countries. For example, the US Brewers Association and the Society of Independent Brewers in the UK support craft breweries that meet their criteria for being a microbrewery, such as being independent, producing on a small scale, and maintaining authenticity (Waehning, Karampela and Pesonen, 2018). In South Africa, there is no single, formally established interim steering committee for

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the entire craft beer industry. However, the Beer Association of South Africa (BASA) works to support the sector, including craft breweries, and collaborates with stakeholders to address challenges such as high excise taxes. Key industry issues, including the increased excise tax on alcohol, are often discussed by industry bodies like BASA and the Craft Beer Association of South Africa (CBASA). However, a recently established Craft Beer Interim Steering Committee (2014) willingly manages and administers the activities of craft brewers. To be considered a craft brewer under CBASA in South Africa, the following conditions generally apply:

- The brewery's annual volume capacity must be under 5 million litres (BusinessTech, 2018)
- No more than 25% of the craft brewery can be controlled or owned by any alcoholic beverage industry shareholder who is not a craft brewer or craft owner itself (Green, 2015)
- Concerted attention must be paid to tradition. The craft brewer or craft brand owner must use either traditional or innovative brewing ingredients and adhere to traditional fermentation processes (Brewers Association of South Africa (BASA).

Craft breweries in South Africa are regulated by the National Liquor Authority (NLA) under the Department of Trade and Industry (DTIC), but each of the country's nine provinces also has its own Liquor Regulatory Authority (LRA) that oversees licensing and regulations within its borders. Industry bodies such as the Beer Association of South Africa (BASA) and the Craft Brewers Association of South Africa (CBASA) also play significant roles in advocating for the industry and promoting responsible practices, but they are not regulatory bodies themselves.

2.4 CRAFT BEER INGREDIENTS AND THE BREWING PROCESS

Typical ingredients in the craft beer brewing process include a starch source (usually malted barley), water, hops, and yeast (Ruiz-Medina and Fernández-de Córdova, 2015). Water makes up roughly 90% of the beer volume, and its mineral content influences flavour (Bettenhausen et al., 2018). The malted barley supplies sugar for the yeast and gives body to the beer, while hops provide bitterness and aroma (Anderson et al., 2019).

2.4.1 Malted barley

Malt is derived from germinated cereals such as barley, maize, rye, oats, rice, sorghum and millet that have undergone the malting process (Yang and Gao, 2022). Barley grain (or any suitable grain) and malt are inherently linked, as one cannot exist without the other (Schabo et al., 2021). Barley (*Hordeum*

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vulgare) is a flowering plant belonging to the family Poaceae or Gramineae (herbs), and it is seasonally cultivated across temperate regions. The outermost layer of the barley grain, known as the husk or hull, protects the inner kernel and aids in gaseous exchange during grain development. Barley is the most dominant grain used in beer brewing, although other grains, such as wheat, are also used to produce different styles of beer (Dabija et al., 2021; Adelusi et al., 2023). Barley comprises starch (70%), proteins (11.5-14.2%), minerals (1.8-2.4%), vitamins, husks, lipids, and polyphenols (Kaur et al., 2024). The structure of the barleycorn includes the hull, bran, endosperm, and germ (Figure 2.2). To promote gluten-free beers, some international and local craft breweries are now using sorghum and rice (Taylor, Dlamini and Kruger, 2013; Cela et al., 2023). Grain's spectrum also includes a variety of adjuncts, such as malted and non-malted wheat, rye, millet, and maize, which serve as main sources of starch (Yeo and Liu, 2014). Due to customers' increased demand for new flavours, spices, fruits, and flavouring ingredients are added during production to create or enhance the unique flavours and aromas of special beers (Yeo and Liu, 2014).

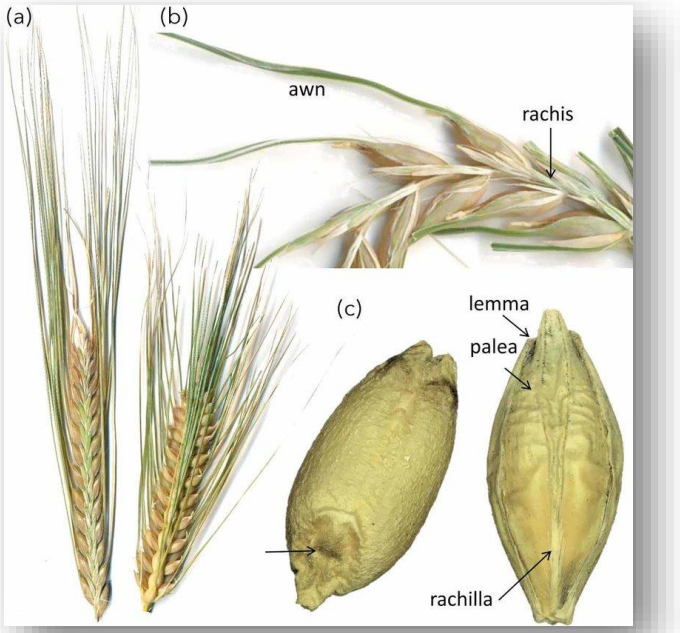


Figure 2.2: Barley spike with rows (left) and a cross-section of barley grain (right).

Source: (Borisjuk, Rolletschek and Radchuk, 2020).

The quality of grains and adjuncts directly impacts the safety and quality of the final product; therefore, they should undergo scrupulous quality control before use (Fox and Bettenhausen, 2023). Microbiological

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contamination of grain can result in fast beer spoilage caused by uncontrollable growth of bacteria, yeasts, and moulds (Luarasi, Troja and Pinguli, 2016). Moreover, cereal grains and fruits used in beer production can be contaminated with mycotoxin-producing fungi in the field, or during storage or malting (Rodhouse and Carbonero, 2019; Roselli et al., 2024).

A traditional process used in 90% of global beer production is fermentation with malted barley (Kok et al., 2019; De Simone et al., 2021). The malting process of barley decreases the starch content in a kernel by 18% and causes a two-fold increase in the amount of polyphenols and an 80% increase in glucan content (Vingrys et al., 2022). Properly malted barley should be characterised by suitable activities of enzymes such as α-amylase, β-amylase, proteases, and β-glucanase that convert starches, proteins, and glucans in the grain into fermentable sugars, amino acids, and polysaccharides. Most industrial breweries use alternative starch sources as adjuncts to the malted barley, a technique implemented to reduce the final product cost and attain the targeted beer's style and characteristics (Willaert, 2012; Cadenas et al., 2021).

2.4.2 Water

Beer primarily consists of water, accounting for about 92-95%, with 15-18% being alcohol and flavour compounds. Therefore, water is vital in craft beer brewing (Punčochářová et al., 2019). Water can be classified as soft or hard depending on the concentration of trace minerals in it, such as calcium and magnesium (Ingin, Mahringer and El-Athman, 2024). Soft water contains 0-60 mg/L of calcium, while hard water exceeds 120 mg/L of calcium. Water with 61-120 mg/L of calcium is considered moderately hard (Punčochářová et al., 2019). The mineral composition of the water influences the style of beer produced (Xu et al., 2018). For example, the hard water of Burton-on-Trent yields bitter pale-ale styles, whereas the soft water of Pilsen produces smooth lagers with rounded palates (Palmer, 2006). Consequently, hard water is typically used for brewing dark beers, such as stout and strong bitter, while soft water is often preferred for brewing light-bodied beers, such as pale lagers (Archana et al., 2024). The water quality used for craft beer production is crucial as it impacts beer flavour and enzyme activities. Water thus plays a vital role in determining the flavour and quality of the final product. The water used for brewing beer must meet key quality parameters, including pH, water hardness, mineral content, and chemical and microbiological standards (Punčochářová et al., 2019). The pH and ionic strength of water affect enzyme activity during the mashing and yeast fermentation stages. Deviating from the optimal pH range between 5.2 and 5.6 can result in undesirable flavour development and incomplete fermentation (Silva et al., 2022).

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Hard water contains calcium and other alkaline materials with a pH level of about 8, while soft water has a pH of around 6. As a result, introducing hard or soft water into the brewing process can alter how enzymes react and influence the efficiency of starch conversion as well as the flavour profile of the product (Tóth et al., 2013). To determine the mineral composition of water, brewers can consult with local water companies for a report or send samples to laboratories. Water quality checks are crucial in brewing beer to produce a safe and high-quality product.

Conversely, because craft breweries often lack filtration and heat treatment, craft beers may be susceptible to microbial contamination, which can lead to the deterioration of their physicochemical and sensory properties (Tirado-Kulieva et al., 2023). Mycotoxins from fungi that pose health and quality concerns can also contaminate the product via water and raw materials, emphasising the importance of quality control (Azam et al., 2021 ; Pallarés et al., 2022 ; Zhang, Tang, et al., 2023). Brewing water should be free from contaminants and possess a mineral composition suited to a particular beer profile. When local water does not meet these standards, craft breweries can apply sterilisation methods such as boiling, ultraviolet treatment, or dissolving chlorine dioxide in the water to reduce or eliminate microbes (Zaborowski, Poćwiardowski and Długosz, 2024). If the mineral composition of the water is incompatible with a specific beer profile, the mineral content can be adjusted with brewing salts such as magnesium or calcium sulphate.

Water is also used in the brewing process for cleaning, rinsing, and as a coolant. Therefore, it must meet the microbiological standards of drinking water and be free from microbial contamination before and after boiling (Willaert, 2012; Moretti, 2013). Monitoring the water supply for craft beer production is crucial to ensure safe water for brewing and to maintain a consistently high-quality beer. A routine microbiological analysis of the water supply should be conducted to detect the presence of pathogenic microorganisms (Luarasi, Troja and Pinguli, 2016). In breweries, water is typically pre-treated with reverse osmosis, carbon filtration, or another type of filtration system, depending on the location of the brewery and the quality of incoming water.

Regarding water usage in beer production, craft breweries are no longer just a small part of the industry, as their numbers and size have increased significantly. Like industrial breweries, craft breweries generate large quantities of wastewater and solid waste mainly from the brewing, rinsing, and cooling processes, making water management a critical concern. As such, wastewater must be treated according to a strict environmental guideline (Aremanda et al., 2022). However, the cost of wastewater treatment for craft

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breweries is very high. As a result, craft breweries are exploring strategies to reduce wastewater by installing affordable treatment systems that allow for safe disposal and reuse (Olajire, 2020).

2.4.3 Hops

Hops[1] (*Humulus Lupulus*) are the pinecone-like flowers of a female climbing plant belonging to the cannabis family (Kavalier et al., 2011). The main reason for using hops is that they act as a bittering, flavouring, and preservative agent in the brewing industry (Steenackers, De Cooman and De Vos, 2015). Before hops were introduced in the fifteenth century, ales were flavoured with herbs and spices (Verberg and Abstract, 2020). Hops primarily consist of resins, polyphenols, proteins, essential oils, lipids, and cellulose, and it is the resins and essential oils they contain that impart bitterness and flavour to beer (Sun et al., 2022).The alpha acids (α-acids), known as humulones, are the key component of resins that are the main bittering agent in hops. These α-acids undergo thermally induced isomerisation during boiling within 60 minutes. The longer the boiling process, the greater the degree of isomerisation and the more bitter the beer becomes (Schönberger and Kostelecky, 2011). When α-acids are isomerised during boiling, they become iso-α-acids. Hops can be categorised into two types: bitter and aroma hops. Bitter hops are high in alpha acids that occur commonly in a range of 2-18%, and aroma hops are low in alpha acids that are commonly in a range of 2.5-6% (Korpelainen and Pietiläinen, 2021). Several varieties of hops (ranging from 6-10%) serve a dual-purpose (Schönberger and Kostelecky, 2011). The hop plant has several aromatic oils, with myrcene, humulene, and caryophyllene being the most dominant.

- Myrcene is the most significant hop oil, making up 30-60% of the oil in all hop varieties.
- Humulene, a naturally occurring monocyclic sesquiterpene, is the second most abundant oil in hops by percentage and accounts for 12-50% of the total hops oil.
- Caryophyllene accounts for 6-15% of total hops oil.

Theoretically, hops might pose a risk of microbiological contamination, but they have not been identified as a source of contaminants in beer due to the bacteriostatic properties displayed by their α-acid (Krofta et al., 2021). However, certain lactic acid bacteria (LAB), such as *Lactobacillus* and *Pediococcus* species,

[1] One uses 'hops' to refer to the plural flowers of the hop plant that are added to beer to impart bitterness, flavour, and aroma, while 'hop' (or the verb 'to hop') is used to describe the action of adding these hops to the beer, as in 'to hop the beer'. For example, "The brewer added the hops during the boil" refers to the ingredient, whereas "The brewer decided to hop the beer with Mosaic hops" describes the action of adding them. Hop is also the name of the plant from which the hops (flowers) are derived.

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have developed mechanisms to resist these hop compounds. These bacteria possess hop resistance genes, notably *horA* and *horC*, which enable them to survive and potentially spoil beer despite the presence of iso-α-acids (Sun et al., 2022).

2.4.4 Yeast

Yeast and single-celled microorganisms have historically played a crucial role in fermentation during brewing. The strains used in beer production worldwide are mainly classified into two categories: ale and lager yeasts (Burini et al., 2021). Yeasts belong to the species *Saccharomyces cerevisiae* and are highly diverse, having been isolated from numerous locations around the world. Ale yeast, often called top-fermenting yeast, tends to rise to the surface of the beer foam during fermentation, with an optimal temperature range of 18-22°C. Lager yeast is *Saccharomyces pastorianus*, formerly known as *Saccharomyces carlsbergensis*. Its fermentation temperature ranges from 7-13°C, which is significantly lower than that of *S. cerevisiae*, which ferments between 6-15°C (Lawrence, 2002). It flocculates at the bottom of the fermenting vessel and is typically harvested for re-pitching. Lager yeast was discovered relatively recently by Louis Pasteur and is a more complex organism than ale yeast, as it is a hybrid of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (Mertens et al., 2015).

The genome of *S. cerevisiae* has been sequenced fully (O'Donnell et al., 2023). Strains of S288C used for sequencing are haploid, while brewing strains are polyploid or aneuploid, with three or more copies of each chromosome (Mozzachiodi et al., 2022). The main objective of yeast strain development in beer brewing is to improve the fermentation process in terms of velocity and efficiency while preserving or improving the quality of the final product (Saerens et al., 2010).

In the lifecycle of yeast during fermentation, different yeast strains produce different amounts of specific metabolites and volatile compounds. These volatile compounds (VCs) are responsible for the complex flavours and aromas found in beer (Paszkot, Gasiński and Kawa-Rygielska, 2023). The main representations of this group of VCs are higher alcohols, esters, ketones, aldehydes, branched alcohols, and methanol. While some of these compounds are required to achieve the stylistic accuracy of the beer, others are negatively impacting its quality, and their formation is therefore suppressed (Kucharczyk, Zyła and Tuszyński, 2020; Manzano et al., 2011). Hence, the strains' genotype and the environmental factors during fermentation determine the presence of volatile compounds in the final product. (Paszkot, Gasiński and Kawa-Rygielska, 2023). Ale beers are marked by a broad array of higher alcohols and esters, while lager beers are generally characterised by a limited number of flavours and aromas (Murmann et al., 2024).

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S. cerevisiae can take up and metabolise a broad range of sugars, namely glucose, mannose, fructose, galactose, sucrose, maltose, maltotriose, and partly raffinose. *S. cerevisiae* can also utilise dextrin and partially hydrolysed starch (Walker and Stewart, 2016; Donzella, Sousa and Morrissey, 2023). Conversely, *S. pastorianus* can utilise melibiose (galactose-glucose) in addition to the range of sugars utilised by *S. cerevisiae* (Walker and Stewart, 2016; Timouma et al., 2024). In malted wort, maltose is present at the highest concentration, followed by maltotriose and glucose (Skendi and Papageorgiou, 2018). The sequence of sugar uptake by the yeast occurs in a distinct order, with monosaccharides, such as glucose and fructose, being utilised first. Maltose and maltotriose, compared to glucose, require energy (active transport) to be taken up by the yeast cell. The concentration of each sugar and its proportion influence the flavour profile of the beer (Methner et al., 2022).

2.4.5 The brewing process

Overall, the beer production process has not changed fundamentally over the years. The process includes various stages, such as malting, mashing, wort filtration, wort boiling, fermentation, maturation, filtration, and pasteurisation (Pires et al., 2014; Archana et al., 2024). In some craft breweries, certain stages such as filtration and pasteurisation are omitted or minimised to preserve the beer's flavour. Some breweries maintain the tradition of malting floors, where barley is germinated manually, although this is no longer common. The conventional brewing process is outlined in Figure 2.3. The flow diagram depicts the overall beer production process for ales and lagers.

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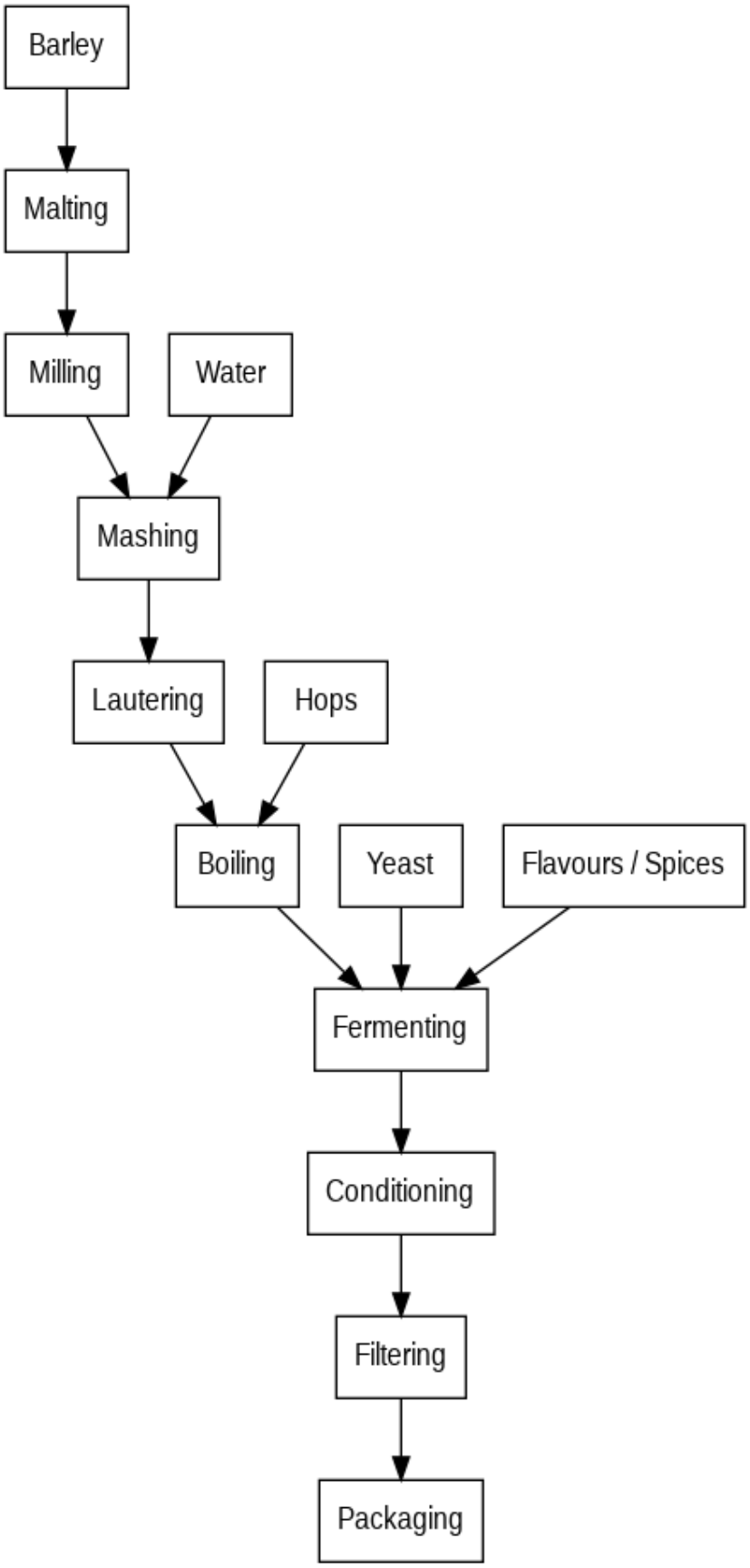


Figure 2.3: Simplified diagram illustrating the brewing process.

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The first step in the brewing process is malting, which involves soaking and draining barley to initiate the germination of the plant seed (Palmer, 2006; Belcar et al., 2021). It consists of three stages: steeping in warm water, germination and kilning (Gupta, Abu-Ghannam and Gallaghar, 2010).

- The purpose of steeping in warm water is to increase the moisture of the grain from 12% to at least 40%.
- Germination modifies the structure of the barley endosperm by breaking down the cell walls and the protein matrix through enzyme synthesis.
- Kilning is used to halt the sprouting process, prevent loss of extract-yielding starch, and ensure product stability.

After the seed sprout has reached the required length, usually two-thirds of the kernel length, the grains are dried in a kiln to stop the enzyme activity (Hertrich, 2013). The grains are termed malt after kilning, and they are ready for dispatch. The malt is allowed to cool to room temperature, cleaned of rootlets and any loose light materials, and then stored in a cool, dry place (Hertrich, 2013; Nylon et al., 2020).

After malting, the next step is milling the malt. Milling helps release the sugars inside the grains, which are later used in brewing (Géczi et al., 2014).The brewing process varies depending on the type of beer being produced (Puligundla, Smogrovicova and Mok, 2021). For example, in ale brewing, the initial mash-in temperature of the liquor to which malt is added is 65°C, the temperature at which starch gelatinises. In contrast, mashing begins at lower temperatures around 45°C in lager brewing, allowing proteases and glucanases to break down the materials before the temperature rises. These enzymes degrade proteins and β-glucans, which are more relevant in lager malts. Research indicates that the time taken to convert starch into sugar is typically one hour.

In ale brewing, the conversion of starch into sugar takes place in a mash-tun, where the sugar extract is separated from solids through filtration (Palmer, 2006). In lager brewing, the separation of the mash occurs in a vessel called a lauter-tun. During the mashing process, flavour and colour compounds are extracted from malt grist, resulting in malt extract known as wort. Other cereals, such as maize, wheat, sorghum, and brewing syrups, can also be added as adjuncts, leading to different beer styles (Cadenas et al., 2021). In addition to enzyme activity, flavour and colour, compounds are extracted from the malt grist during mashing to form the wort (i.e., the malt extract) (Palmer, 2006; Castro, Affonso and Lehman, 2021). The sugar components of the wort mainly include fructose, glucose, sucrose, maltose, and maltotriose, along with dextrin material (Zarnkow et al., 2010). Table 2.1 summarises the sugars present in the wort.

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Table 2.1: Typical sugar spectrum concentration in beer wort

Sugar	Composition (%)
Glucose	10-15
Fructose	1-2
Sucrose	1-2
Maltose	50-60
Maltotriose	15-20
Dextrins	20-30

Source: Walker and Stewart, 2016. Values shown are based on wort produced from 100% malted barley.

The sweet wort is pumped into a kettle where it undergoes boiling with hops and sometimes other ingredients, such as herbs or sugars that are added later. Although boiling consumes a lot of energy, it is a vital stage because it is used to:

- Extract bitterness from the hops. Adding hops at the start of boiling mainly contributes to the bitterness of the beer, while adding hops at the end of boiling releases more volatile aromatic compounds, resulting in a beer with a stronger hop aroma. Not much bitterness is extracted at this stage due to the short boiling time of the hops (Klimczak and Cioch-Skoneczny, 2023).
- Sterilise the wort and reduce undesirable aromatic compounds.
- Remove undesired substances.
- Precipitate unwanted proteins and lipids (Böhm et al., 2023).

After cooling, the yeast is pitched to start the fermentation process. The amount of yeast added varies depending on the beer style required. An ale beer can use approximately 207 billion cells. Conversely, brewing lager requires twice as much yeast for a batch of comparable size (Saerens et al., 2010). Fermentation can take up to one week. During this period, the yeast metabolises the sugars in the wort through a series of biochemical processes, resulting in the production of carbon dioxide, ethanol, higher alcohols, sulphur compounds, organic acids, aldehydes, ketones, and esters as a result of cellular metabolism, ensuring the beer meets quality and stability standards (Maicas, 2020).

In contrast to industrial beer brewing, craft beer breweries often re-pitch yeast, meaning yeast from completed fermentation is reused. To prevent yeast quality decline, re-pitching is usually limited to less than ten times (Vidgren and Londesborough, 2011; Kordialik-Bogacka and Diowksz, 2013). The fermentation process can take up to two weeks, and once complete, the brewer may condition the beer in a bright beer tank for one week to several months to balance flavours and allow unwanted flavours to

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dissipate (Archana et al., 2024). After conditioning, the beer is carbonated either artificially or naturally with CO_2 and then packaged into bottles, cans, or kegs. Depending on the brewer's style and preferences, most industrial and some craft beers are filtered for clarity and stability prior to packaging (De Francesco, Sileoni and Marconi, 2025).

In industrial beer production, pasteurisation is used to sterilise the beer, while some craft breweries omit it, as unpasteurised beer offers a more appealing, fresher taste for modern consumers. However, the absence of pasteurisation could result in microbial growth, leading to beer spoilage over time (Bose et al., 2021; Eizaguirre et al., 2024).

2.5 MICROBIAL CONTAMINATION ASSOCIATED WITH BEER

Beer is generally considered a safe food product because it is widely accepted that pathogens cannot grow in beer due to its low pH values (3.8-4.7) (Ciont et al., 2022).Other factors, such as the presence of ethanol (0.5-10% w/w), the prevalence of hops-derived bittering compounds (typically 17-55 ppm of iso-α-acids), the high carbon dioxide content (approx. 0.5% w/v), and low-temperature storage, can also reduce contamination (Manzano et al., 2011; Oldham and Held, 2023). However, it remains plausible that, under favourable conditions, certain microorganisms can cause spoilage during brewing due to the nutrient-rich wort environment (Roselli et al., 2024). Additionally, the long production times of up to several weeks allow unwanted microorganisms sufficient time to develop (Bokulich and Bamforth, 2013; Suzuki, 2020). Beer favours the growth of microaerophilic and anaerobic microorganisms due to its high CO_2 and low O_2 levels (Oldham and Held, 2023). Furthermore, the anaerobic environment of beer can adversely affect its quality, resulting in off-flavours or turbidity. These quality issues can therefore cause significant financial losses for the craft beer industry (Manzano et al., 2011; Oldham and Held, 2023).

Spoilage microorganisms that can grow under certain beer brewing parameters include Gram-positive bacteria, with the main representatives being lactic acid bacteria (LAB) species that are highly prevalent in nature and human environments (Bokulich and Bamforth, 2013). Beer contamination can occur either through primary contamination (e.g. from raw materials during beer production) or secondary contamination in the packaging hall (Ciont et al., 2022). LAB can be responsible for up to 90% of microbial beer spoilage incidents (Oldham and Held, 2023). Most LAB are prevented from growing in beer due to hops-derived antimicrobial compounds such as flavonoids, polyphenols, prenylflavonoids, and glucosides (Bokulich and Bamforth, 2013). Regarding LAB, the strains *Lactobacillus brevis* and *Pediococcus damnosus* are considered major causes of beer spoilage because they are widely found in

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the food industry and natural environments (Suzuki, 2011; Rodríguez-Saavedra, González de Llano and Moreno-Arribas, 2020). *Lactobacillus brevis* is a Gram-positive, heterofermentative bacterium with an optimal growth temperature of 30°C and a pH of 4-6. Other *Lactobacillus* species that can cause beer spoilage include *L. plantarum*, *L. casei*, *L. buchneri*, and *L. coryniformis*. However, even if these species are less frequent as beer spoilers compared to *L. brevis*, they can still cause turbidity or precipitation in the beer and produce off-flavours through the generation of lactic acid, acetic acid, and diacetyl (Munford, Chaves and Sant'Ana, 2020).

On the other hand within the subset of strictly anaerobic spoilage bacteria, Gram-negative genera of *Pectinatus* and *Megasphaera* are responsible for approximately 90% of reported cases attributed to this group (Rodríguez-Saavedra et al., 2021). *Pectinatus* and *Megasphaera* are anaerobic contaminants of unpasteurised beer (Rodríguez-Saavedra et al., 2021). In the 1990s, the presence of *Pectinatus* and *Megasphaera* increased due to a significant reduction of oxygen content in the packaged beer of modern breweries (Paradh, Mitchell and Hill, 2011; Vriesekoop et al., 2012). *Pectinatus* species produce notable amounts of propionic acid and hydrogen sulphide (H_2S) in beer, accompanied by extensive turbidity and a bad aroma similar to rotten eggs (Paradh, Mitchell and Hill, 2011). Similarly, spoilage caused by *Megasphaera* species results in turbidity and an unpleasant odour due to H_2S production, as well as the presence of butyric, valeric, and caproic acids (Suzuki, 2011).

Pediococcus damnosus is a Gram-positive, homofermentative, coccoid-shaped bacterium that grows in pairs or tetrads and is found in wort and finished beers (Shimokawa and Suzuki, 2021; Vermote et al., 2023). *P. damnosus* can grow at pH levels above 4.2 in beer and environments with low concentrations of hops and 0,5% ethanol (Vermote et al., 2023). *P. damnosus* is characterised by its metabolite diacetyl, which at concentrations above the taste threshold of 0.12 mg/L produces a buttery off-flavour (Xu et al., 2020). *Pediococci* can be resistant to commonly used sanitisers, making it difficult to eradicate from contaminated manufacturing equipment (Haakensen et al., 2009; Ahn et al., 2017). This can result in bacteria surviving the fermentation process and being present in packaged beer.

2.6 THE PREVALENCE AND EFFECTS OF MYCOTOXINS IN BEER

Mycotoxin is derived from the Greek word 'mykes', meaning fungus, while the Latin word 'toxicum' means poison (Mertens et al., 2015; Agriopoulou, Stamatelopoulou and Varzakas, 2020). Mycotoxins are toxic, low molecular weight secondary metabolites produced by fungi and are associated with disease (Shekhar, Raghavendra and Rachitha, 2025). They are classified into polycetoaids, cyclopeptides,

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terpenes, and nitrogenous metabolites based on their structures and biological origins (Bhat, Rai, and Karim, 2010). Mycotoxins can harm vertebrates through ingestion, inhalation, or dermal contact (Cimbalo et al., 2020). They can cause a condition called mycotoxicosis, which occurs without direct involvement of an infectious fungus (Khan, Anwar and Ghazali, 2024). Important mycotoxins linked to mycotoxigenic fungi include aflatoxins, fumonisins, ochratoxins, deoxynivalenol/nivalenol, zearalenone, patulin, and citrinin (Zahra et al., 2019;.Khan, Anwar and Ghazali, 2024).

To date, approximately 500 different mycotoxins have been identified, most of which are produced by moulds belonging to the genera *Fusarium*, *Aspergillus*, *Trichothecium*, and *Penicillium*. The major mycotoxins of concern and their fungal sources are summarised in Table 2:2. A small subset of these is linked to cases of acute or chronic intoxications, including the induction of cancers and immune deficiency (Dass et al., 2023). Several factors influence fungal colonisation and mycotoxin production, but no specific optimal conditions for the production of various fungi have been established (Daou et al., 2021). Generally, fungal growth is mainly affected by temperature and water activity (aw) in grains before and during harvesting, handling, shipment, and storage (Tola and Kebede, 2016). The ideal temperature for grain development ranges between 20-30°C, and the optimal water activity (aw) is approximately 0.980-0.995, with a minimal range of 0,70 to 0,91 (Erazo et al., 2023). The water activity influences spoilage and mycotoxin production in grains. Maize is particularly vulnerable to fungal infestation and subsequent mycotoxin contamination, while rice is the least susceptible (Nji and Mwanza, 2024).

Due to the growing consumer culture supporting microenterprises, craft beer production and consumption are at their highest levels ever (Peters et al., 2017; Gobbi et al., 2024). Mycotoxin contamination of beer is a public health concern, especially as many people now frequently consume beer and because barley, one of its main ingredients, is prone to mycotoxin contamination (Piacentini, Rocha, et al., 2019). Moreover, mycotoxins are thermostable and may survive the malt production process, thereby contaminating the final product (Mastanjević et al., 2019; Piacentini, Běláková, 2019). It was thus deemed vital to explore the use of craft beer ingredients in the context of food safety and quality.

The prevalence of mycotoxins in beer has been extensively studied using immunoassay-based methods (Peters et al., 2017). According to a report by the Food and Agriculture Organisation (FAO, 2003), at least 99 countries had by that time implemented regulations on mycotoxin levels in food and animal feed, marking a substantial increase from previous years (FAO, 2003). This trend reflects growing global concern over the health risks posed by mycotoxins and their impact on international trade. While numerous mycotoxins have been identified, this study focused on deoxynivalenol (DON), nivalenol (NIV),

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fumonisin (FB_1, FB_2, FB_3), Zearalenone (ZEA), Ochratoxin A (OTA), and Aflatoxin because of their prevalence in cereal crops and co-occurrence in food and feed products (Lee and Ryu, 201; Peters et al., 2017; Palumbo et al., 2020).

2.6.1 Deoxynivalenol

Deoxynivalenol (Figure 2.4) is known colloquially as 'vomitoxin' because of its emetic effects in pigs. Deoxynivalenol (DON) is routinely found in cereal grains worldwide, especially in barley (Iwase et al., 2023). Structurally, DON is a polar organic compound which belongs to the type B trichothecenes. Its chemical name is 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on (Brera et al., 2013). The structure determines the toxicity associated with 3 free hydroxy groups -OH (Brera et al., 2013; Zhai et al., 2019). DON contamination in cereal grains remains a global problem and is predominantly caused by *Fusarium graminearum* and *Fusarium culmorum* (Gab-Allah, Choi and Kim, 2023; Nji and Mwanza, 2024). Acute toxicity of DON can cause diarrhoea, vomiting, haemorrhaging, circulatory shock, and ultimately death (Deng et al., 2023).

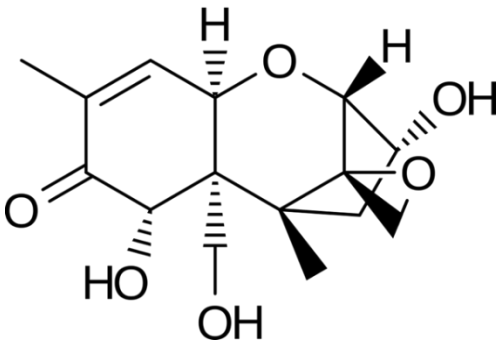


Figure 2.4: Chemical structure of Deoxynivalenol

Source: Sobrova et al., 2010.

Deoxynivalenol (DON) is one of the most important trichothecenes B produced by *Fusarium* species (Pierzgalski et al., 2021), as its presence in food and feed has been detected in more than 90% of samples (Sobrova et al., 2010). *Fusaria* also produce two prominent acetylated derivatives of DON, namely 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON) (Gilbert et al., 2010). DON-3-G is formed during the germination of cereal grains when host UDP-glucosyltransferases conjugates a portion of the DON with glucose (Schiwek et al., 2022; Berthiller et al., 2013). However, DON-3-G is also frequently referred to as a masked mycotoxin because many routine analytical methods do not detect it. The human colonic microbiota can convert DON-3-G back to DON, and the inclusion of

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DON-3-G in food safety assessments has been increasingly under debate by the scientific and regulatory communities (Gratz et al., 2018; Daud et al., 2020).

In the last few years, DON has repeatedly been linked to causing gushing in beer, which is excessive foaming and overflowing when opening a bottle or can. Gushing damages the reputation of relevant breweries and indicates the presence of DON, which might pose a health risk (Schabo et al., 2021). Due to its toxicity and widespread occurrence, DON has received considerable attention in studies in recent years (Deng et al., 2023).

2.6.2 Nivalenol

Nivalenol (NIV) (Figure 2.5) is a trichothecene mycotoxin mainly produced by *Fusarium* species such as *F. cerealis*, *F. poae*, *F. graminearum*, and *F. culmorum* (Schiwek et al., 2022). NIV's chemical name is 12,13-epoxy-3,4,7,15-tetrahydroxy-(3α,4β,7α)-trichothec-9-en-8-one (Kumar et al., 2022). Nivalenol (NIV) and DON mycotoxins frequently co-occur in various cereal crops such as maize, barley, oats, rye, and wheat. They also occur in processed grains and grain products such as malt, beer, and bread (Kumar et al., 2022). However, NIV is less frequently found in food than DON, but studies indicate that its toxicity may be higher in animal-based than in human-based studies (Pierzgalski et al., 2021). A test done on mice revealed that the lethal doses that killed half of the mice (LD_{50}) were 78 mg/kg and 39 mg/kg of DON and NIV, respectively (Gratz et al., 2018; Jiang et al., 2024). Similar to other trichothecenes, NIV was found to have a limited effect on primary eukaryotic cell metabolism, including DNA, RNA, and protein synthesis. Early experimental evidence states that exposure to dietary NIV could be associated with an increased incidence of gastric carcinomas and oesophageal cancer in some areas in China (Hsia et al., 2004). However, more recent evaluations reveal that human epidemiological evidence remains insufficient to substantiate this association (Zingales, Fernández-Franzón and Ruiz, 2021). Moreover, available NIV toxicological data are limited, as consumers do not consider NIV a risk because the daily intake remains below the tolerable daily intake level compared to DON. However, evidence has shown that the synergistic effect of combined NIV and DON can have negative implications for consumers (Alassane-Kpembi et al., 2017). Therefore, while NIV is less prevalent in food compared to DON, its potential health impacts should not be underestimated. Ongoing research and monitoring are essential to ensure consumer safety regarding these mycotoxins.

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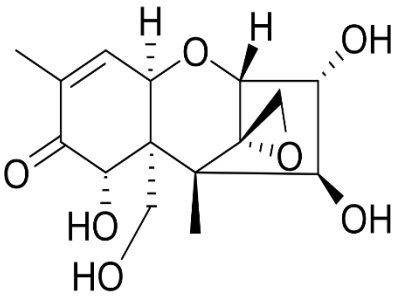


Figure 2.5: Chemical structure of nivalenol (NIV).

Source: Kumar et al., 2022.

2.6.3 Fumonisins

Fumonisins are polyketide mycotoxins that can be divided into groups of toxins, namely fumonisin B_1 (FB_1), B_2 (FB_2), B_3 (FB_3), and analogues, which are similar in structure to sphingosine (Anumudu et al., 2025).

FB_1 has the structure 2S−amino−12S,16R−dimethyl−3S,5R,10R,14S,15R−penta−hydroxy−eicosane, with C14 and C15 hydroxyl groups esterified by a terminal carboxyl group of propane−1,2,3−tricarboxylic acid. Fumonisins (FBs), mycotoxins B_1, B_2, and B_3, are closely related compounds produced by *Fusarium verticillioides* (formerly known as *F. moniliforme*) and *Fusarium proliferatum* (Al-Rashdi et al., 2024)Al-Rashdi et al., 2024). *Fusarium verticillioides* was first isolated in 1970 in South Africa from a batch of mouldy corn. It was believed to be the main cause of a field outbreak of leukoencephalomalacia, a hole-in-the-head syndrome in horses (Wilson et al., 1990; Echenique et al., 2019). Fumonisins were first described and characterised in 1988 and synthesised by condensing the amino acid alanine into an acetate-derived precursor (Bezuidenhout et al., 1988). Fumonisin B_1 (FB_1) (Figure 2.6) is the most prevalent toxic fumonisin associated with the high level of human oesophageal cancer in South Africa and China (Yu et al., 2021).

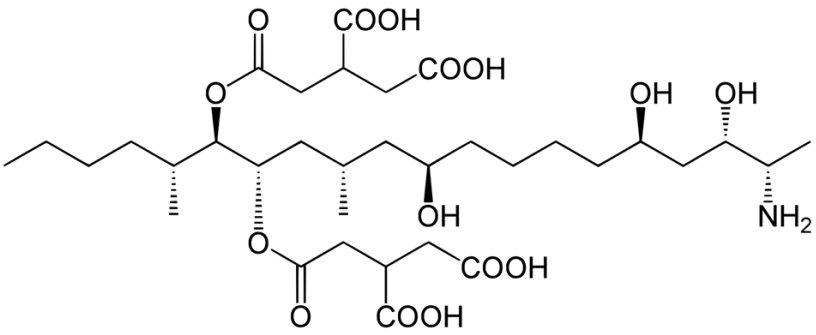


Figure 2.6 : Chemical structure of fumonisin B_1.

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Source: ApSimon, 2001

FBs are mainly associated with maize and maize-based products as they cause maize ear and stalk rot, leading to significant yield losses and deterioration of grain quality (Singh et al., 2025). FBs can also be found in beer, sorghum, rice, cowpea seeds, soybeans, and asparagus (Chen et al., 2021; Bouelet Ntsama et al., 2023). Studies have shown that, in thermally treated or extruded products such as cornflakes, FB_1 may be bound to macromolecular components such as carbohydrates and proteins (Anumudu et al., 2025). Bound mycotoxins often lead to underestimating FB's level by analytical methods (Berthiller et al., 2013). According to the WHO Food Additives Series 47, it is estimated that the mean daily intake of FB_1 may range between 12 and 140 µg per person, depending on geographical factors. However, doses as high as 2500 µg/day are consumed by some individuals (World Health Organisation, 2001).

2.6.4 Zearalenone

Zearalenone (Figure 2.7), a macrocyclic β-resorcyclic acid lactone, is a non-esteroidal estrogenic mycotoxin produced by various *Fusarium* fungi in temperate and warm countries (Perczak et al., 2016). The *Fusarium* species include *F. culmorum*, *F. roseum*, *F. graminearum*, *F. crookwellense*, *F. equiseti*, and *F. semitectum* (Manshor et al., 2012). *Fusarium culmorum* and *F. graminearum* are the primary fungi responsible for Zearalenone (ZEA) production; however, *Fusarium* species can also produce the toxin in varying amounts (Al-Rashdi et al., 2024). ZEA appears regularly with trichothecenes like DON (Mertens, 2016; Al-Rashdi et al., 2024). Grain and grain products that are the main sources of ZEA contaminants are found in wheat, corn, barley, sorghum, rice, and bread (Yu et al., 2022). In Nigeria, it was observed that about 50% of ZEA was transferred from naturally contaminated maize into beer (Kpodo and Bankole, 2008). However, more recent research on this specific transfer rate is limited. Most toxin production occurs in high moisture feeds when moulds develop under humid and cold weather conditions (Shabangu, 2009).

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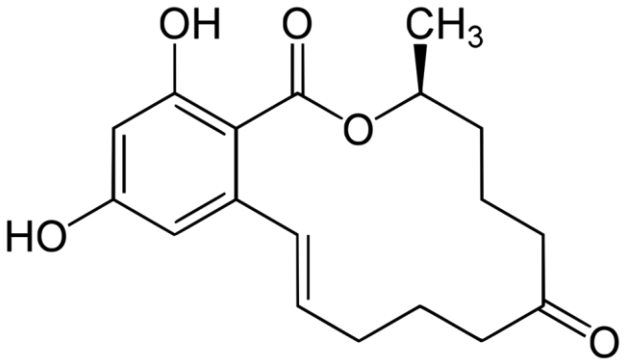


Figure 2.7: Chemical structure of zearalenone.

Source: Harčárová et al., 2020

ZEA remains stable during milling, storage, and food processing/cooking, particularly because it does not degrade at high temperatures. It may co-occur with DON in wheat, barley, oats, and corn and co-occurs with fumonisins in maize (Al-Rashdi et al., 2024). Zearalenone derivatives include α-Zearalenol (α-ZEA), β-Zearalanol (β-ZAL), and α-Zearalanol (β-ZAL) found in maize stem isolates of *Fusarium* species (Pierzgalski et al., 2021). Zearalenone and its derivatives produce estrogenic effects in various animal species, such as vulval oedema, infertility, vaginal prolapse, and mammary hypertrophy in females and the feminisation of males (Faisal et al., 2018) due to atrophy of testes and enlargement of mammary glands (Wang et al., 2022). Zearalenone is metabolised in the gastrointestinal tract and subsequently in the liver to α- and β-zearalenone that can exhibit higher estrogenic activity than zearalenone. These compounds may be further reduced to α- and β-zearalanone. Zearalenone involvement in human toxicoses has not been confirmed, but it is classified as an endocrine disrupter and considered potentially hazardous to humans (Bennett and Klich, 2003).

2.6.5 Ochratoxin A

Ochratoxin A (OTA) (Figure 2.8) is an innately fluorescent compound produced by fungi of the genera *Aspergillus* and *Penicillium* (Heussner and Bingle, 2015). OTA is also the most abundant and toxic metabolite in the ochratoxin group (Heussner and Bingle, 2015). Chemically, OTA is a phenylalanyl derivative of a substituted isocoumarin(R)-N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-y1)-carbonyl]-L-phenylalanine (Marin et al., 2013).

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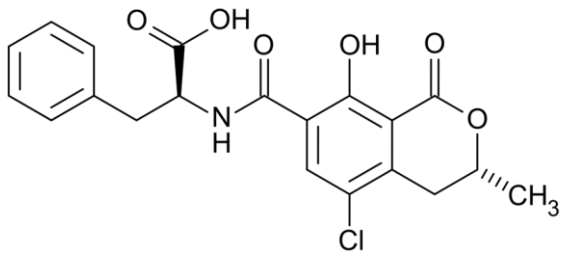


Figure 2.8: Chemical structure of ochratoxin

Source: Kőszegi and Poór, 2016.

The major *Aspergillus* species involved in OTA production include *Aspergillus auricomus*, *Aspergillus melleus*, *Aspergillus carbonarius*, *Aspergillus alliaceus*, *Aspergillus glaucus*, and *Aspergillus niger* (Bennett and Klich, 2003; Nazareth et al., 2024). OTA frequently contaminates natural products such as coffee, cocoa, spices, cassava, flour, cereals, fish, dried fruits, wine, peanuts, poultry eggs, and milk (Bhat, Rai and Karim, 2010). Mould that is visible in major species that produce ochratoxin varies from blue-green with *Penicillium* species to yellowish-tan with *A. ochraceus* and black with *A. niger* (Richard, 2007; Cabañes, Bragulat and Castellá, 2010). However, in some instances, mould may not be visible for ochratoxin that occurs in grain and other commodities, but a musty odour on the grain can be a sign of mycotoxin and ochratoxin presence (Richard, 2007; Rayhana et al., 2025).

OTA is associated with improper storage conditions of cereals, such as high temperatures, humid conditions, and inappropriate practices during crop storage (Akkaya et al., 2025). OTA is nephrotoxic, and very high concentrations can affect the liver (Wang et al., 2019). In addition, it has immunosuppressive, carcinogenic, and teratogenic effects, and immunosuppression can be induced with low concentrations of OTA.

2.6.6 Aflatoxins

Aflatoxins (Figure 2.9) are highly toxic secondary metabolites derived from polyketides produced by several *Aspergillus* species, mainly *A. flavus* and *A. parasiticus*, that grow in warm conditions in tropical and sub-tropical regions of the world (Schamann et al., 2024). There are over 20 known aflatoxins, but the four major aflatoxins most significant for human and animal health are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G1 (AFG_1), and aflatoxin G2 (AFG_2). This categorisation is based on their

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fluorescence under UV light (blue or green) and relative chromatographic mobility in thin-layer chromatography (Bennett and Klich, 2003). Aflatoxin B_1 (AFB_1) is the most toxic and prevalent aflatoxin, with strong evidence that it is a liver carcinogen in humans and a genotoxic carcinogen in laboratory animals. The detrimental effects associated with aflatoxins, in general, include mutagenic, teratogenic, and immunosuppressive effects in humans and animals (Okechukwu et al., 2024).

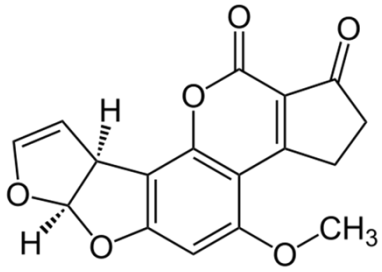


Figure 2.9: Chemical structure of aflatoxin.

Source: Asao et al., 1965.

Aflatoxins are among the most widely reported mycotoxins in major dietary and export-targeted crops in Southern Africa. The foods most susceptible to aflatoxin B_1 contamination are beer and raw materials such as barley and corn (Ruiz-Medina and Fernández-de Córdova, 2015; Okechukwu et al., 2024). Contamination can occur before or after harvest and/or during transport and storage (Gachara et al., 2022). Chemically, aflatoxins (AFTs) are difuranocoumarin derivatives in which a bifuran group is attached at one side of the coumarin nucleus, while a pentanone ring is attached to the other side in the case of the AFTs and AFTs-B series, or a six-membered lactone ring may be attached in the AFTs-G series (Schincaglia et al., 2023). AFB_1 is heat-resistant at various temperatures, including those reached during commercial processing (Sabina, Uzoamaka and Chinedu, 2023). The maximum limits of total aflatoxins and AFB_1 in all foods in South Africa are 10 µg/kg and 5 µg/kg, respectively (Chilaka et al., 2012). Fungal sources of mycotoxins and their maximum allowed limits are presented in Table 2.2.

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Table 2.2: Mycotoxin maximum limits and their fungal sources.

Mycotoxin	Commodity	Fungal Sources	US FDA (µg/kg)	EU (EC 2006) (µg/kg)
Deoxynivalenol/nivalenol	Wheat, maize, barley	*Fusarium graminearum* *Fusarium crookwellense* *Fusarium culmorum*	1000	200–50
Zearalenone	Maize, wheat	*F. graminearum* *F. culmorum* *F. crookwellense*	Not set	20–100
Ochratoxin A	Barley, wheat, and many other commodities	*Aspergillus* ochraceus *Penicillium verrucosum*	Not set	2-10
Fumonisin B_1	Maize	*Fusarium moniliforme* plus several less common species	2000–4000	200–1000
Aflatoxin B_1, B_2	Maize, peanuts, and many other commodities	*Aspergillus flavus*	20 for total	2-21 for B_1 4-15 for total
Aflatoxin B_1, B_2, G_1, G2	Maize, peanuts	*Aspergillus parasiticus*	20 for total	2-21 for 4-15 for total

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CHAPTER 3:
MYCOTOXINS ASSOCIATED WITH THE SOUTH AFRICAN CRAFT ALE BEERS

ABSTRACT

Mycotoxin contamination in craft beer poses a potential public health risk. Although data on mycotoxin occurrence in South African beers are limited, several studies have reported mycotoxin contamination in beers worldwide, including African-brewed beers. Mycotoxins such as deoxynivalenol (DON), fumonisins (FB_1 and FB_2), and nivalenol NIV) are produced by fungal species commonly present in grains used in beer production. This study aimed to assess the prevalence of these mycotoxins in South African craft beers. Thirty craft beer samples, representing various styles, namely Pale Ales, India Pale Ales (IPAs), Stouts, Blond Ales, Irish Red Ales, Amber Ales, Best Bitter Ales, Altbier Ales, Porter Ales, and Imperial Ales, were collected from microbreweries and stores across South Africa. Mycotoxin analyses were performed using ultra-performance liquid chromatography–electrospray ionisation–tandem mass spectrometry (UPLC-ESI-MS/MS) to precisely quantify DON, OTA, aflatoxins, fumonisins, and zearalenone. The alcohol by volume (ABV) levels of the craft beers were also determined.

The results indicated that DON was the most prevalent mycotoxin, detected in 10% of the samples, with Stout and IPA exhibiting the highest concentrations of 41.49 µg/L and 22 µg/L, respectively. FB_1 and FB_2 were detected in IPA and Pale Ale samples with concentrations of 0.37 µg/L and 0.16 µg/L, respectively. NIV was found in a trace concentration of 0.13 µg/L in one IPA sample. The ABV of the tested craft beers ranged from 4.95% to 7.34%. IPAs and stouts did not consistently show increased mycotoxin levels,. These findings are consistent with large scale screening which have reported that mycotoxin level in beer was not consistently found in higher levels in certain styles with higher malt content (Peters et al., 2017). International bodies such as FAO (2023) set maximum limits in cereals at 1750 µg/kg for DON, 1000 µg/kg for fumonisins (FB_1 + FB_2), and 4 µg/kg for ochratoxin A, but not explicitly for beer.

This study emphasises the significance of continuous mycotoxin monitoring in craft beer, particularly as the South African craft beer industry continues to grow. Improving quality control measures throughout the brewing process, including better grain sourcing, storage, and brewing practices, is essential to minimise potential health risks.

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3.1 INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by fungal species, primarily from the genera *Aspergillus*, *Penicillium*, and *Fusarium*, which can contaminate cereal grains used in food and beverage production (Schabo et al., 2021). The primary grains utilised in beer production, particularly barley (Hordeum vulgare), maize (Zea mays), and sorghum (Sorghum bicolour), are highly susceptible to fungal contamination during cultivation, storage, and processing (Martiník et al., 2023). Ingestion of mycotoxins at high concentrations has been associated with severe health disorders, including hepatotoxicity, nephrotoxicity, immunosuppression, and carcinogenic effects (Smith et al., 2016). DON and fumonisins are partially excreted from the body, whereas OTA can accumulate in the kidneys due to its long half-life (Pestka, 2010). Even low-level chronic exposure can result in accumulation, thus suggesting that removing them from beer is critical for public health. These findings could further inform recommendations on alcohol consumption limits in the Food-Based Dietary Guidelines (Vorster, Badham and Venter, 2013). As beer is one of the most widely consumed alcoholic beverages worldwide, the potential presence of mycotoxins in raw brewing materials must be closely monitored as they pose significant public health concerns (Tolosa et al., 2021).

In South Africa, mycotoxins are regulated in staple grains such as maize and wheat under the Foodstuffs, Cosmetics and Disinfectants Act No. 54 of 1972 (Republic of South Africa, 1972). However, no maximum permissible levels of mycotoxins for beer or other cereal-based beverages have been established, despite evidence that mycotoxin contamination may persist from the field through to harvesting, storage, malting, and brewing (Benešová et al., 2022). Conversely, in the European Union (EU), legislation extends beyond raw grain to include finished beer (Bryła et al., 2022). In the EU, maximum permissible levels of these mycotoxins in grains are as follows: 1,750 µg/kg deoxynivalenol (DON), 1,000 µg/kg total fumonisins B_1 and B_2, 4 µg/kg total aflatoxins, 4 µg/kg ochratoxin A, 3 µg/kg aflatoxin B_1, 4 µg/kg total aflatoxins, and 400 µg/kg of zearalenone (Cheli et al., 2014). This variation emphasises a regulatory gap in South Africa, where limits mainly cover cereal-based products while other products, such as various porridges, flour and maize meal, are still unregulated (Tola and Kebede, 2016). Permissible levels of mycotoxin are commonly expressed in micrograms per kilogram (µg/kg) of grain, whereas beer is consumed as a liquid (µg/L), complicating the direct comparison of safety levels (US FDA, 2007). Considering the ever-increasing number of craft breweries, addressing this gap is crucial because the risk of mycotoxin contamination from using large quantities of grain may negatively impact the final product. (Andronaco et al., 2025). Several studies have reported the presence of deoxynivalenol (DON), nivalenol (NIV), fumonisins (FB_1, FB_2, FB_3), zearalenone (ZEN), ochratoxin A, and aflatoxins (AFs) in

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commercially available beers (Pack, Meyerhoff and Schmale, 2022). Beers from African countries, including South Africa, Malawi and Cameroon, showed the highest levels of DON derivatives, OTA, and fumonisins in a global beer review study conducted by Schabo et al. (2021), with the highest cases exceeding 100 µg/L. The same study shows that OTA exposure through beer poses notable health risks in countries like Brazil and Belgium.

The *Fusarium* species, which thrive in humid environments, are known producers of DON, NIV, FB_1, FB_2, FB_3, and ZEN in malted barley, while *Aspergillus* species predominantly produce aflatoxins in maize and sorghum (Kumar et al., 2022; Pascari, Ortiz-Solá, et al., 2018). The extent of fungal contamination in grains is influenced by various factors, including pre-harvest environmental conditions such as temperature, humidity, and rainfall, as well as post-harvest storage and handling practices (Neme and Mohammed, 2017; Dövényi-Nagy et al., 2020). Of the most commonly detected mycotoxins in malted barley, DON cause excessive beer foaming, known as 'gushing' (Smith et al., 2016), but to date, it is unknown what specific amount of DON causes gushing. However, it has been determined that the prevalence of hydrophobins, surface-active proteins produced by fungal contamination, is the primary cause of gushing (Sarlin et al., 2005). Additionally, DON exposure has been linked to acute and chronic toxic effects, including teratogenicity, embryotoxicity, and immunosuppression. It is important to mention that mycotoxins can survive the brewing process (especially boiling) and end up in the final product because the molecules are heat-stable.

Numerous studies worldwide have documented the occurrence of mycotoxins in beers. Schabo et al. (2021) conducted a comprehensive meta-analysis of beers from around the world and revealed that 31% of the beers tested positive for mycotoxins. The most common were DON and its derivatives (53%), followed by ochratoxin A (52%), fumonisins (47%), and aflatoxins (12%). Xu et al (2023) detected eight mycotoxins in 158 beer samples purchased in Shanghai. Their findings revealed that 48.1% of beer samples were contaminated with Deoxynivalenol-3-glucoside (D3G) and zearalenone (ZEN). DON, 3A-DON, 15A-DON, NIV, FB_1, and OTA were reported to be below the limit of detection (LOD). D3G was the mycotoxin most frequently detected across the tested beer samples at concentrations ranging from 5-495 µg/L.

In European markets, mycotoxin contamination in beer is subject to rigorous monitoring and control. This means that the EU actively regulates mycotoxin contamination at the grain stage and indirectly ensures safer beer through routine checks and surveillance of the final product (FAO, 2023). However, the persistence of mycotoxins throughout the brewing process highlights the need for stringent monitoring protocols and improved brewing practices to mitigate potential health risks (Schabo et al., 2021). Previous

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studies have indicated that traditional African beers, particularly those brewed with sorghum and maize, often contain aflatoxins and fumonisins due to suboptimal grain storage and processing methods (Shephard et al., 2005). Reports from South Africa, Zimbabwe, Kenya, Tanzania, Zambia, and Burundi have highlighted the presence of these mycotoxins, especially in sorghum and maize-based brews. However, limited data exist on mycotoxin contamination in South African beers, especially in the craft space, highlighting the need for comprehensive investigations through rigorous analyses.

It was in this context that the current study aimed to assess the occurrence of key mycotoxins, particularly aflatoxin B_1 (AFB_1), deoxynivalenol (DON), fumonisins (FB_1, FB_2, FB_3), ochratoxin A (OTA), zearalenone (ZEN), and nivalenol (NIV) in various styles of commercially available South African craft beers.

3.2 MATERIALS AND METHODS

3.2.1 Beer samples

A total of 270 beers of different styles were purchased from 30 commercial craft breweries across South Africa (Table 3.1). At the time of sample collection, approximately 200 craft beer breweries existed in South Africa, and the study collected samples from 15% of these. The ale beers tested were randomly collected based on availability and accessibility. These samples were used for various analytical procedures described in subsequent chapters. For mycotoxin analysis, one beer sample per brewery (a total of 30 beers) was analysed in triplicate, yielding 90 samples. The primary goal was to obtain samples of as many different craft beers as possible to conduct a detailed quality assessment and to detect potential safety concerns. At the beginning of the study, the aim was to sample beers of the same style (Indian Pale Ale). However, it was difficult to obtain the same style of beer brewed by the various breweries, hence various styles were tested. The craft beers were mainly collected from restaurants, supermarkets, and craft breweries between 2019 and 2021. The samples were labelled and stored at 4°C until the analysis processes commenced.

The beers were degassed using a magnetic stirrer at 50 rpm for 5 min. After stirring, the beer samples were transferred into 50 mL centrifuge tubes and centrifuged at 5,000 rpm for 5 min. The supernatant was then stored at -20°C until analysis. The centrifugation step was the pre-analytical clarification of the beer samples, intended to remove yeast cells and debris prior to storage and shipment. Mycotoxins are low molecular weight, soluble, and are not expected to pellet under these conditions. The targeted beer styles were grouped into 11 categories (Table 3.2).

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Table 3.1.: Full brewery style names with abbreviations of 30 commercial breweries sampled across South Africa.

Full name	Brewery	Abbreviation
Altbier	20	Altbier
American Amber Ale	14	Amber-ale
Best Bitter	1	Best-bitter1
Best Bitter	23	Best-bitter2
Blonde Ale	9	Blonde-ale1
Blonde Ale	2	Blonde-ale2
Blonde Ale	10	Blonde-ale3
Indian Pale Ale (Belgium)	5	IPA-belg
Indian Pale Ale (English1)	6	IPA-Eng1
Indian Pale Ale (English2)	7	IPA-Eng2
Indian Pale Ale (English3)	24	IPA-Eng3
Indian Pale Ale	4	IPA01
Indian Pale Ale	12	IPA02
Indian Pale Ale	13	IPA03
Indian Pale Ale	17	IPA04
Indian Pale Ale	18	IPA05
Indian Pale Ale	22	IPA06
Indian Pale Ale	25	IPA07
Indian Pale Ale	28	IPA08
Indian Pale Ale	29	IPA09
Indian Pale Ale	30	IPA10
Irish Red Ale	19	Irish-red-ale
Pale Ale	11	Pale-ale1
Pale Ale	16	Pale-ale2
Pale Ale	21	Pale-ale3
Pale Ale	27	Pale-ale4
Pale Ale	26	Pale-ale5
Imperial Porter	15	Porter-imp
Stout	3	Stout1
Stout	8	Stout2

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Table 3.2: Samples of South African beer styles analysed for mycotoxins.

Beer styles	Number analysed	Brewery
Altbier	3	20
Amber Ale	3	14
Best-bitter 1-2	6	1
Blonde Ale 1-3	9	9, 2, 10
IPA-Belg	3	5
IPA-Eng 1-3	9	6, 7, 24
IPA 1-10	30	4, 12, 13, 17, 18, 22, 25, 28, 29, 30
Irish-red-ale	3	19
Pale Ale 1-5	15	11, 16, 21, 27
Porter-Imp	3	15
Stout 1-2	6	3, 8

3.2.2 Multi-mycotoxin analysis

To ensure accuracy and reliability, multi-mycotoxin analysis was performed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-ESI-MS/MS) at the Central Analytical Facility at Stellenbosch University.

3.2.2.1 Chemicals and reagents

Liquid Chromatography-Mass Spectrometry (LC-MS) grade chemicals such as methanol (HPLC grade, purity ≥99.9%), acetonitrile (HPLC grade, purity ≥99.9%), formic Acid (purity ≥98%), acetic acid (99.8%), anhydrous, magnesium sulphate (analytical grade), and sodium chloride (analytical grade) were purchased from Merck, South Africa. Acetonitrile (HPLC grade, purity ≥99.9%) was used as the primary solvent for mycotoxin extraction due to its effectiveness in dissolving various mycotoxins and as the mobile phase for chromatographic analysis. Water (Milli-Q grade or equivalent) was used as a solvent component in both the extraction phase and as part of the UPLC mobile phase, ensuring minimal contamination and high analytical quality. Formic acid (purity ≥98%) was added to the extraction solvent

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(acetonitrile-water mixture) at a concentration of 1% (v/v) to improve the extraction efficiency of acidic mycotoxins by enhancing their solubility and recovery. Magnesium sulphate ($MgSO_4$) (analytical grade) was used during the salting-out phase to assist in phase separation by reducing the solubility of mycotoxins in the aqueous phase, thus promoting partitioning into the acetonitrile-rich organic layer. In the salting-out process, sodium chloride (NaCl) (analytical grade) was employed alongside magnesium sulphate to achieve a clean separation between the organic and aqueous layers during extraction. Methanol (HPLC grade, purity ≥99.9%) was used to enhance the overall performance of the chromatographic separation. This resulted in sharper peaks and better resolution during the analysis process, as proposed by Yang et al. (2019).

3.2.2.2 Standard solutions

The following mycotoxins and metabolites (standards) purchased from Sigma-Aldrich (USA) were used: deoxynivalenol (DON), zearalenone (ZEA), fumonisins (FB_1, FB_2, and FB_3), nivalenol (NIV), ochratoxin A (OTA), and aflatoxin B_1 (AFB_1). The working standards were prepared as follows: a fumonisin solution containing FB_1, FB_2, and FB_3, each at 5 µg/mL was diluted in acetonitrile/water (50/50 v/v) and stored at 4ºC. Aflatoxin B_1 at 1 µg/mL and the other mycotoxin solutions containing nivalenol, deoxynivalenol, zearalenone, and ochratoxin, each at 10 µg/mL were diluted in acetonitrile/water (50/50 v/v) and stored at −20ºC. These working standards were diluted differently and used to prepare calibration solutions. The calibration solutions were analysed with the ACQUITY UPLC/MS system under optimised chromatographic conditions.

3.2.2.3 Sample preparation

Samples were extracted using the mycotoxin extraction method described by (Sulyok, Krska and Schuhmacher (2007) with some modifications. Briefly, 2 mL of filtered beer was extracted in 50 mL Falcon tubes using an organic extraction solution (acetonitrile:water: acetic acid, 59:40:1) and vortexed for 30 seconds. This was followed by adding 4 g of $MgSO_4$ and 1 g of NaCl to the solution followed by vigorous mixing using a vortex. Each upper organic layer (5 mL) was transferred into a 15 mL glass tube and dried through evaporation under nitrogen gas for 1 h. A solution of 75% water:25% methanol solvent was prepared, and 200 µL of this solution was transferred into the 15 mL glass tubes containing the extracted samples and vortexed. The solution was then transferred into respective 2 mL Eppendorf tubes and centrifuged for 5 min at 13 000 rpm. Each diluted sample was filtered through a 0.22 µm PTFE filter, and 1 mL of each filtered sample was aliquoted into a vial for UPLC-ESI-MS/MS analysis.

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3.2.2.4 Instrumentation and chromatographic conditions

Chromatographic separation was performed using a Waters ACQUITY UPLC system coupled with a Quattro Premier XE tandem mass spectrometer. This system included a binary pump, degasser, column oven, and autosampler. A 2.1 mm × 100 mm ACQUITY UPLC BEH C18 column with a particle size of 1.7 µm was used to achieve optimal separation, with the column temperature maintained at 50°C throughout the analysis.

The mobile phase consisted of two solvents: Phase A was water with 0.1% formic acid, and Phase B was acetonitrile with 0.1% formic acid. A gradient elution program effectively separated multiple mycotoxins in the beer matrix. The mobile phase composition was initially set to 90% Phase A and maintained at this level for the first 3 minutes. Over the next 7 minutes, Phase A was gradually reduced to 30%. From 10.1 to 12 minutes, Phase A was further decreased to 10% to facilitate the elution of more hydrophobic compounds. Finally, the gradient was returned to 90% Phase A from 12.1 to 14 minutes to re-equilibrate the column. The flow rate was set to 0.4 mL/min, with an injection volume of 20 µL per sample.

3.2.2.5 Mass spectrometric conditions

The Quattro Premier XE tandem quadrupole mass spectrometer was operated in positive electrospray ionisation (ESI+) mode, optimal for ionising most mycotoxins. Detection was conducted in multiple reaction monitoring (MRM) mode, providing high selectivity and sensitivity for each targeted mycotoxin. The cone voltage ranged from 10-50 volts for optimal performance, while collision energy varied between 7 and 42 electron volts, depending on the target compound. The source temperature was maintained at 140°C, and the desolvation temperature was set at 400°C to efficiently remove solvent from the ions. The desolvation gas flow was 800 L/h, and the cone gas flow was 50 L/h to ensure a stable spray and consistent ion generation.

3.2.2.6 Determination of alcohol by volume (ABV)

The alcohol content of each beer sample was measured using the Anton Paar Alex 500 instrument. Prior to measurement, the samples were degassed with a magnetic stirrer at room temperature for 5 min to remove dissolved CO_2. The degassed beer samples were then filtered through folded Whatman Grade 1 paper (185 mm) purchased from Lasec, South Africa, to remove suspended solids. After filtration, the clear samples were used directly for the measurement on the Alex 500.

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3.3 RESULTS AND DISCUSSION

3.3.1 Mycotoxin contamination in craft ale beers

The triplicate measurements of 30 beer samples representing 11 distinct styles were evaluated for mycotoxins. Figure 3.1 illustrates the distribution of different mycotoxins across the various beer styles in a column chart. A secondary axis was also added to enhance visibility, as some samples had low mycotoxin concentrations. The standard deviation of the samples, ranging from 0-1.23 µg/L, indicated that some values were consistent across samples, while others exhibited greater variability among the replicates. This could be due to sampling errors because the distribution of mycotoxins is often highly heterogeneous (Milićević, Škrinjar and Baltić, 2010). The heterogeneity of mycotoxins can be minimised by sample homogenisation, such as freeze drying (Andronaco et al., 2025). Table 3.3 aids in the interpretation of these results.

The results indicated low levels of mycotoxin contamination across the samples, with contamination found in 3 of the 11 beer styles, accounting for approximately 27%. As shown in Table 3.3, the contaminated styles were Indian Pale Ale (IPA01, IPA09, IPA-Eng), Stout, and American Amber Ale (Amb-ale). The detected mycotoxins were DON, FB_1, FB_2, and NIV (Figure 3.1). Aflatoxin B_1 (AFB_1), fumonisin B_3 (FB_3), zearalenone (ZEN), and ochratoxin A (OTA) were not detected.

DON was the most frequently detected mycotoxin, occurring in 9 of the 90 analysed samples (10%), of the analysed beers, with levels ranging from 7.13 ± 1.24 µg/L to 41.49 ± 0.37 µg/L (Table 3.3). Stout (brewery 3) had the highest DON concentration of 41.49 ± 0.37 µg/L, followed by IPA from brewery 4 (22.72 ± 0.00 µg/L). In a study conducted by Grumi et al. (2020), a comprehensive analysis of Italian beers was performed on 72 craft and industrial beer samples. DON was detected in all the samples, with the concentrations between 6.1 and 111.2 µg/L. Similarly, DON was detected in 96 Polish beer samples, with concentrations ranging from 6 to 70.2 µg/L (Kuzdraliński, Solarska and Muszyńska, 2013).

The prevalence of DON in beer samples is consistent with the dominance of DON-producing *Fusarium* species associated with cereal grains used in brewing (Pestka and Smolinski, 2005; Iwase et al., 2023). Previous research indicates that DON and its derivatives, such as Deoxynivalenol-3-Glucoside (D3G), which is a masked plant-derived conjugated form of DON, are not inherently highly toxic; however, D3G becomes toxic when it is in the human and animal gut, where it is hydrolysed back into DON (Berthiller et al., 2011; Peters et al., 2017). As established by the European Food Safety Authority (EFSA), DON is considered toxic at levels exceeding the tolerable dose intake (TDI) of 1 µg/kg body weight (European

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Food Safety Authority, 2013). Based on the EFSA group TDI for DON of 1 µg/kg bw/day, a 70 kg adult will reach the tolerable intake by consuming ~14 L/day if the beer contains 50 µg/L DON. Thus, exceedance of the TDI will require sustained high daily consumption of alcohol, whereas higher outlier concentrations substantially reduce the margin of safety (Pestka, 2010; Knutsen et al., 2017). While the TDI provides a threshold for health risk assessment, it is crucial to note that international regulatory limits for mycotoxins are set for raw cereals and cereal-based products, not directly for beer Codex Alimentarius; FAO, 2023). Typical DON concentrations in commercial and craft beers range from 5 to 500 µg/L, with higher levels occasionally reported in dark or high-alcohol beers (Peters et al., 2017; Ksieniewicz-Woźniak et al., 2019). Similar considerations apply to fumonisins, NIV, and OTA, which are primarily regulated in grains but may appear in beer depending on the contamination of the raw materials used. This approach allows measured beer concentrations to be assessed against health-based guidance, showing that while average beer consumption is unlikely to exceed the TDI, outlier beers with elevated mycotoxin levels could pose a more immediate risk.

Stout is a high-gravity beer with an alcohol by volume (ABV) above 6%. Typically, the alcohol concentration in beer is directly related to the amount of grain used in the mash. The finding of elevated DON aligns with a study by Ksieniewicz-Woźniak et al. (2019), who reported elevated DON concentrations in dark malt beers compared to pale beers. However, not all dark beers consistently showed high DON levels during testing, indicating that variability in raw materials and brewing processes plays a significant role. Given that DON is heat-stable, it is likely that brewing conditions do not significantly reduce its presence. This aligns with the finding that typical brewing processes result in only partial degradation of DON (Pascari, Ortiz-Solá, et al., 2018).

India Pale Ale (IPA) had the highest number of contaminated samples across the participating breweries, followed by Stout. This variation in mycotoxin levels across beer styles can be attributed to several factors, including the brewing process (e.g., malting, mashing, fermentation), regional fungal contamination of cereal grains prior to brewing and the raw materials used, such as barley, malt, hops, or adjuncts (Pascari et al., 2018). Additionally, origin and quality of the ingredients can influence, batch-to-batch variation in mycotoxin levels in beer resulting from seasonal climate conditions and differences in suppliers, storage, and handling of raw materials (Adekoya et al., 2018; Pascari et al., 2018). In a comprehensive study by Peters et al. (2017), samples from 1,000 beers across 47 countries were collected, with 60% of the samples being craft beers. Strong beers, such as IPAs and imperial stouts, were affected by the concentrations of DON ranging from 10 µg/L to 474 µg/L.

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In this study, fumonisins FB_2 and FB_1 were detected in beers from breweries 11 and 12. The FB_1 concentration was recorded in IPA02 (brewery 12) at a level of 0.37 µg/L ± 0.02 µg/L, followed by FB_2 in Pale-ale1 (brewery 11) at 0.16 µg/L ± 0 µg/L (Table 3.3).

These results aligned with a review by Buczynski et al. (2023), who documented a strong correlation between fumonisin occurrence and the use of maize-based adjuncts in brewing. The presence of fumonisins in beer can be attributed to the fungal contamination of raw grains, particularly maize, as reported by Shephard et al. (2005). Moreover, FB_2 was detected in trace amounts in the IPA samples, supporting the observation by Piacentini et al. (2015) that FB_2 is generally found at lower levels compared to FB_1 in beer matrices. The observed variations in fumonisin levels could be due to differences in brewing techniques, raw material sources (suggesting the use of adjuncts such as maize), and fungal contamination during grain storage (Wu and Munkvold, 2008). The concentrations of FBs (FB_1, FB_2) in the studied samples were considerably lower than the estimated mean daily intake range of 12-140 µg/L per person of FB_1 mycotoxin (Ariño et al., 2007).

NIV was detected in IPA09 (brewery 25) at the lowest concentration of 0.13 ± 0.01 µg/L. This result aligns with findings by Peters et al. (2017) and supports literature findings that NIV is rarely detected in beer, with most values below 5 µg/L and only in isolated cases, such as in Dutch Pale Ale (21 µg/L) and some African traditional beers. This confirms that NIV occurs sporadically compared to DON. Krska et al. (2005) argued that the occurrence of NIV in beverages is highly variable and depends on the extent of fungal contamination in the grain before processing. Although the NIV levels detected in the current study were relatively low, Ahad et al. (2017) emphasise that combined exposure to multiple trichothecenes, including DON and NIV, could lead to synergistic toxic effects, necessitating further investigation. Reports have shown that NIV toxicological data are limited because the tolerable daily intake compared to DON remains very low; thus, consumers do not consider its consumption a risk.

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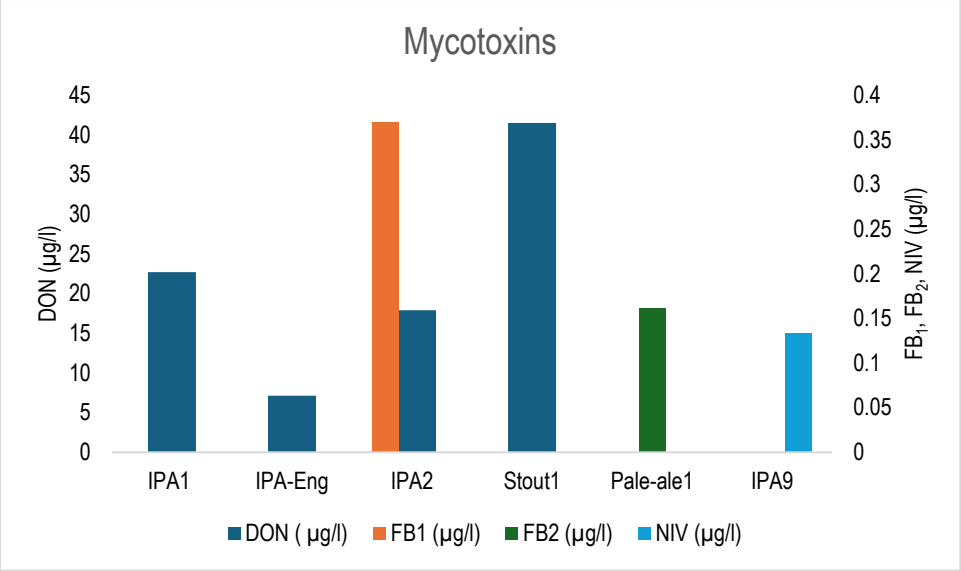


Figure 3.1: Clustered column chart representing the distribution of mycotoxin concentrations in positive craft beer samples. Each plot represents a specific mycotoxin in triplicate. IPA01 (brewery 4), IPA-Eng (brewery 6), IPA02 (brewery 12), Stout1 (brewery 3), Pale-ale1 (brewery 11), and IPA09 (brewery 25).

Table 3.3: Beer samples with mycotoxin contamination and alcohol content.

Mycotoxin	Beer style	Brewery	Mycotoxin concentration (µg/L)	ABV (%)	Province
DON	IPA01	4	22.72 ± 0.00	5.80 ± 0.02	Western Cape
	IPA-Eng	6	7.13 ± 1.24	7.34 ± 0.02	Western Cape
	IPA02	12	17.92 ± 1.21	5.86 ± 0.07	Western Cape
	Stout1	3	41.49 ± 0.37	6.73 ±. 0.03	Gauteng
FB_1	IPA02	12	0.37 ± 0.02	5.86 ± 0.02	Western Cape
FB_2	Pale-ale1	11	0.16 ± 0.37	2.63 ± 0.04	Free State
NIV	IPA09	25	0.13 ± 0.02	4.95 ± 0.02	Gauteng

Each value is the mean of triplicate ±SD of triplicate.

3.3.2 Influence of alcohol by volume (ABV) on mycotoxin levels

The ABV of the tested craft beers ranged from 2.63 to 7.34% with beers having a higher alcohol content (IPA-Eng; Stout) (Table 3.3) However, these did not consistently exhibit increased mycotoxin levels. This was in contrast with a study by Bertuzzi et al. (2018), who found that beers with higher malt content and

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extended fermentation times were more prone to mycotoxin retention. This discrepancy may be attributed to differences in raw material quality, brewing conditions, and yeast adsorption effects. In the current study, Stout exhibited elevated DON levels, reinforcing findings from studies that attributed this to dark malt composition and prolonged brewing conditions, such as that by Ksieniewicz-Woźniak et al. (2019).

3.4 CONCLUSIONS

This chapter reported on the presence of chemical contaminants, such as mycotoxins, in South African craft ales. Mycotoxins are known to have detrimental effects on human health, and their presence in food and beverages warrants attention. The primary objective was to highlight the occurrence of *Fusarium*-related mycotoxins, including deoxynivalenol (DON), fumonisins (FB_1 and FB_2), and nivalenol (NIV), in commercially available South African craft beers.

The key findings indicated that six of the 30 craft beer samples analysed contained mycotoxins DON, FB_1, FB_2, and NIV. Of these detected mycotoxins, DON was prevalent in two beer styles (IPA and stout), with stout1 and IPA01 having the highest DON concentrations of 41.49 µg/L and 22 µg/L, respectively. The high DON contamination levels in these two beer styles may have been due to their malt-rich composition. Stout1 and IPA-Eng that contained mycotoxins also had higher alcohol contents than the other beers tested. However, there was no significant connection between ABV and mycotoxin levels. Following the international mycotoxin safety thresholds of 500 µg/L for DON and tolerable levels of fumonisins and NIV set by EFSA and WHO, it was concluded that the levels of mycotoxins detected in this study did not pose immediate concern. Yet, the implications of the findings are profound as the detection of DON, FB_1, FB_2, and NIV in the samples of six craft beers reinforces the global trend of mycotoxins in craft beers.

Furthermore, in light of the growing expansion of craft breweries in South Africa, the presence of multiple mycotoxins in the beer samples underscores the importance of implementing rigorous quality control measures throughout the brewing process in all craft breweries. Additionally, the occurrence of mycotoxins across different beer styles highlights the importance of establishing effective guidelines for quality control measures through adequate continuous monitoring of the quality of raw materials, such as the grains used in craft beer production, rather than focusing solely on specific beer styles.

Since only 30 South African craft beers were sampled and tested in this study, this scope may have limited a more detailed assessment of these craft beers' quality in terms of safety. Future research should therefore increase its sample sizes and collect multiple beer styles from the same brewery. Ingredient

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sources and storage conditions should also be investigated. In conclusion, only a small number of craft beers (six samples) contained mycotoxins at low concentrations, which indicated that exposure to consumers was generally minimal. However, the detection of mycotoxins in craft beer highlights the importance of ongoing monitoring and safety guidelines to further reduce potential contamination and ensure consumer safety.

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CHAPTER 4:
OVERVIEW OF MICROBIAL AND CHEMICAL PROFILES OF SOUTH AFRICAN CRAFT ALE BEERS

ABSTRACT

The growing South African craft beer sector is distinguished by its high-quality craftsmanship; however, variability in fermentation control and microbial management can impact both safety and sensory outcomes. This chapter presents an integrated overview of the microbial and chemical profiles of sampled South African craft ales, with emphasis on the interplay between microorganisms, flavour-active compounds, residual sugars, and fermentation dynamics.

The aim of the study was to evaluate the microbial composition, chemical profile, and flavour potential of sampled craft ale beers, and to identify correlations between microbial activity, sugar metabolism, and key flavour-active compounds. Specifically, this chapter examined microbial plate counts (yeasts, moulds, lactic acid bacteria, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* species), quantified flavour and non-flavour-active compounds, phenolic acids, higher alcohols, esters, and residual sugars/sugar alcohols, and explored their interrelationships using Spearman's correlation analysis.

Chemical and microbial analyses were performed on multiple craft ale styles, including IPA, stout, pale ale, blond ale, best bitter, and porter. Microbial counts were determined using culture-based methods, while sugars and phenolic acids were analysed by Gas Chromatography–Flame Ionisation Detector (GC–FID), volatile compounds by Gas Chromatography–Mass Spectrometry (GC–MS), and mycotoxins by High-Performance Liquid Chromatography–Mass Spectrometry (HPLC–MS). Flavour thresholds from literature were used to evaluate sensory thresholds, and Spearman's correlation was applied to investigate relationships among microbial counts, sugars, and flavour metabolites.

The results demonstrated marked variability across breweries. Total plate counts ranged up to 1.63×10^5 CFU/mL, with yeast detected in just over half of the samples and mould contamination occurring in 17% of breweries. LAB were present in 60% of samples, with high counts in certain beers, while *S. aureus* co-occurred with LAB in Pale-ale2, suggesting post-fermentation contamination. Chemically, several compounds exceeded sensory thresholds, including isoamyl alcohol (up to 178.9 mg/L), 4-vinylguaiacol (0.14–10 mg/L), isoamyl acetate (selected beers), and vanillic acid (up to 76.7 mg/L), confirming strong

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contributions to flavour. Residual sugars and sugar alcohols varied widely, with xylose (60.6 mg/L), xylitol (185.4 mg/L), and fructose (107.4 mg/L) reflecting incomplete attenuation in some styles .Xylose and fructose detection is common in beers, while xylitol although less frequently reported is not rare in craft beers, it is known as a by-product of yeast xylose metabolism, which can accumulate under metabolic stress. Correlation analysis revealed strong positive associations between ferulic acid and higher alcohols (isobutanol, isoamyl alcohol, phenylethyl alcohol) and negative correlations between sugars (maltose, trehalose, glucose) and flavour-active metabolites, highlighting the metabolic interplay between sugar utilisation and secondary metabolite formation.

Overall, the findings highlight the chemical richness, microbial variability, and fermentation challenges in the sampled South African craft ales. While threshold-exceeding compounds enhance sensory complexity, the presence of residual sugars and microbial contaminants reflects inconsistent brewing control, with implications for quality, balance, and product stability in the emerging craft beer industry.

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4.1 INTRODUCTION

Over the past two decades, the global beer industry has undergone considerable changes, driven by the expansion of craft brewing (Faganel and Rižnar, 2023). Traditionally led by industrial lagers, the beer sector now accommodates an accelerating diversity of small-scale breweries that emphasize artisanal practices, experimentation, and unique sensory impressions (Garavaglia and Swinnen, 2017; Faganel and Rižnar, 2023). South Africa reflects this global trend, with the number of craft breweries significantly growing since the early 2000s (Collins, Rogerson and Rogerson, 2023). The use of indigenous raw materials, such as South African hop cultivars and regionally sourced malted barley, has further contributed to a distinct beer identity (De Simone et al., 2021). The craft beer sector remains relatively small compared to mainstream beer markets in Europe or North America. However, it is experiencing dynamic growth, with over 200 microbreweries, a significant increase in recent years (Rogerson and Collins, 2015).

Craft beers are celebrated for their complexity, frequently exhibiting marked hop character, speciality malts, unique fermentation profiles, and application of diverse ingredients (Garavaglia and Swinnen, 2017). This diversification has reshaped consumer preferences across many regions, driving increased demand for beers that offer authenticity and variety. However, the rapid rise of craft brewing and consumption has also brought challenges. Contrary to Industrial-scale breweries, which are equipped with quality control systems, small craft producers often operate with limited resources (Mainardis, Hickey and Dereli, 2024). This limitation can increase susceptibility to microbial contamination and inconsistencies in chemical compositions (Collins, Rogerson and Rogerson, 2023). While variability in chemical composition in craft beer creates unique flavours and authenticity, it also raises concerns about product stability, consumption safety, and reproducibility. Fermentations without stringent control and poor microbial oversight may result in off-flavours, spoilage, or the presence of hazardous metabolites (Ciont et al., 2022).

Craft beers contain chemical and microbial constituents, and their composition is substantially shaped by the raw materials and microbial communities used in their production (Collins, Rogerson and Rogerson, 2023). Consequently, craft beers can be contaminated by complex chemical systems that extend beyond microbial contamination. Yeast, primarily *Saccharomyces cerevisiae*, is vital for fermentation, as it produces ethanol and carbon dioxide along with a variety of secondary metabolites such as esters, higher alcohols, fatty acids, and phenolic compounds that influence aroma, flavour, and mouthfeel (Willaert, 2012). However, beyond the controlled action of brewing yeast, the presence of wild yeasts, lactic acid

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bacteria (LAB), and filamentous fungi is a growing concern in craft breweries. These microorganisms can introduce variability and, in some cases, produce mycotoxins in the beer (Garofalo et al., 2015;.Sohlberg, Sarlin and Juvonen, 2022).

Several studies have been conducted on microbial contamination and mycotoxin production. Penha et al., (2025), conducted a study on 60 commercial beers from different cereal grains (barley malt, malt with corn, and malt with rice) in São Paulo, Brazil, results revealed that DON, fumonisins (FB_1, FB_2), ZEN, OTA, ochratoxin A (OTA); T-2 toxin; fand aflatoxins mycotoxins were detected in the beer samples. This finding indicated the presence of fungal contamination in the raw materials, necessitating rigorous quality control monitoring of the raw materials. In a study by Xu et al. (2023), 158 beer samples bought in Shanghai, China, were analysed for 8 mycotoxins that included DON, ZEN, FB_1, FB_2, NIV, T-2 toxin, OTA, and aflatoxins. Nearly half of the samples were contaminated with *Fusarium* mycotoxins, while 34.81% and 16.46% of the total samples were contaminated with Deoxynivalenol-3-glucoside (D3G) and zearalenone (ZEN), respectively. This study showed the persistence of fungal metabolites surviving the brewing process. Andronaco et al. (2025) looked at nine mycotoxins (DON, FB_1, FB_2, OTA, AFB_1, AFB_2, AFG_1, AFG_2, and HT-2) using freeze-dried samples and HPLC/ESI-MS/MS in craft beers from Italy.

In this study certain mycotoxins, in particular deoxynivalenol (DON) and Fumonisins (FB1, FB2), were consistently detected, indicating their prevalence in accordance with other studies. These studies collectively indicate that mycotoxin contamination, rather than microbial activity during fermentation, originates from fungal-infected raw materials.

Lactic acid bacteria (LAB) are essential for sour beer production but can be problematic contaminants in non-sour styles, as they can produce lactic acid and diacetyl at undesirable levels (Bokulich and Bamforth, 2013). LAB display two functions in brewing; they are essentially used in beer souring, but can also be the main spoilage organisms in conventional beer. *Lactobacillus* and *Pediococcus* genera can produce diacetyl and lactic acid; some strains are hop-resistant, complicating detection in their nonculturable states (Bokulich and Bamforth, 2013; Xu et al., 2020; Oldham and Held, 2023).

Fungi, particularly moulds associated with malted barley or adjunct grains, pose an additional risk because they can produce mycotoxins such as deoxynivalenol (DON), fumonisins, and nivalenol, which are toxic secondary metabolites that pose a significant threat to food and feed safety (Al-Rashdi et al., 2024). Fermentation by-products, such as esters (e.g., ethyl acetate, isoamyl acetate), higher alcohols (e.g. isobutanol, phenylethyl alcohol), and medium-chain fatty acids (e.g., hexanoic, octanoic, and decanoic acids), significantly shape the sensory quality of beer. These compounds provide fruity, floral,

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creamy, or fatty notes, many of which are considered desirable when present within specific thresholds but can become overpowering or unpleasant at higher concentrations (Roberts et al., 2023). Conversely, aldehydes such as furfural or acetaldehyde, often associated with stressed yeast metabolism, may impart harsh or stale flavours. They can also be naturally present in some dark beers, including strong ales or stouts (Vanderhaegen et al., 2004). The balance of these compounds reflects the delicate interplay between microbial physiology, raw material availability, and brewing practice. In small-scale breweries, where variability in fermentation conditions is often more pronounced, these chemical outcomes can be especially unpredictable.

The integrated microbial and chemical profiling of South African craft ales have not received much attention. While global studies have documented the influence of yeast, bacteria, and fungi on beer quality and safety, the unique raw materials, brewing practices, and local conditions of South African breweries necessitate a more targeted investigation.

This chapter aims to present an overview of the microbial and chemical profiles of South African craft ales. It examines the role of microorganisms in shaping fermentation outcomes, their interaction with the chemical complexity of beer, and potential correlations among key variables (including volatiles, phenolic acids, off-flavours, sugars, and alcohols). This integrative approach seeks to generate deeper insights into fermentation dynamics, links among microbial diversity, flavour development, and contamination risks within 30 South African breweries.

4.2 MATERIALS AND METHODS

4.2.1 Microbial content analysis using plate counts

4.2.1.1 Culture media

Microbial analysis using conventional cultivation methods (enriched and selective media) was used to differentiate between several groups of microorganisms. The culture media used included sterile buffered peptone water (BPW), Plate Count Agar (Oxoid), De Man, Rogosa and Sharpe agar (Oxoid) (MRS), violet, red bile agar, Baird-Parker medium, Rappaport Vassiliades medium with soya broth (RVS) and Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn), Xylose Lysine Deoxycholate agar (XLD) and Brilliant green agar. The microbiological analysis, involving the enumeration of total aerobic bacterial counts (total viable count), detection of lactic acid bacteria (LAB), coliforms, *Staphylococci, Salmonella*, moulds, and yeasts, was conducted in triplicate at SMT LABS according to (International Standard

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Organisation, 2005)(International Standard Organisation, 2005)(International Standard Organisation, 2005)(International Standard Organisation, 2005) Clause 5.4.

Briefly, serial dilution was done where each beer sample (1 mL) was transferred into a test tube containing 9 mL of sterile buffered peptone water (BPW) of the following composition (g/L): peptone (10), disodium phosphate (5), monopotassium phosphate (1.5) and sodium chloride (5). This process was performed in triplicate and kept on ice. The 10−2 dilution (1ml) of each beer suspension was then used for the downstream application.

4.2.1.2 Total viable count

Total viable count was done by transferring 1ml of the diluted beer sample (10−2 dilution) to two sterile labelled petri dishes and adding 15ml of molten Plate Count Agar (Oxoid) of the following composition (g/L): enzymatic digest (5), yeast extract (2.5), glucose (1 and 15) on each petri dish containing each diluted beer sample. These were allowed to solidify and incubated aerobically at 30°C ± 2°5, C for 72 ± 2 hours (South African Bureau of Standards, 2007).

Colony-forming units (CFU) were enumerated on plates containing 30–300 colonies, and the results were calculated using the equation described in (ISO, 2003) for the preparation and dilution of test samples. Results were expressed as CFU/mL and rounded to two significant figures.

4.2.1.3 Detection of lactic acid bacteria

Mesophilic lactic acid bacteria were enumerated using the horizontal colony count technique as described in ISO 15214:1998 (International Organisation of Standardisation, 1998). Each beer sample (1 mL) was transferred into labelled sterile petri dishes. Approximately 15 mL of molten MRS agar De Man, Rogosa and Sharpe agar (Oxoid) of the following composition g/L: peptone 10, meat extract 5, yeast extract 5, D(+) glucose 20, dipotassium hydrogen phosphate 2, triammonium citrate 2, sodium acetate 5, Magnesium sulphate (equivalent to 0.2 g/L heptahydrate) 0.1, Manganous sulphate monohydrate 0.01, and agar 12 was poured into the petri dish. The samples were mixed into the agar by gently swirling the plates and allowed to solidify on the benchtop. The plates were incubated at 30°C ± 1°C for 72 ± 3 hours, followed by bacterial enumeration. Colonies with typical white growth morphology were recorded as presumptive LAB and subjected to catalase confirmation by applying 3% hydrogen peroxide. Only catalase-negative isolates were considered confirmed LAB. Counts were performed on plates containing 1–300 colonies and expressed as CFU/mL. Positive controls (growth control) and sterility controls

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(negative control) were included for quality assurance according to SABS ISO 7218:1996 (SABS ISO, 1996).

4.2.1.4 Detection of coliforms and *Escherichia coli*

Coliforms and Escherichia coli were enumerated using the colony count technique in accordance with SANS 4832:2007 (SANS, 2007) and SANS 9308-1:2004 (SANS, 2004).

Enumeration of coliforms and *Escherichia coli* was performed on violet and red bile agar media. The same procedure used above was followed using violet, red bile agar containing the following constituents (g/L): yeast extract (3), enzymatic digest of gelatin (7), bile salts mixture (1.5), lactose (10), NaCl (5), neutral red (0.03), crystal violet (0.002), 4-Methylumbelliferyl-β-D-Glucuronide (0.1) and agar (15) as a growth medium. The media (41.6 g) was suspended in 1 L of distilled water. With frequent agitation, the mixture was heated and boiled for 1 minute to dissolve and then cooled to 45ºC. Plates were allowed to solidify on a bench. Each diluted beer sample (1mL) was plated out, and the plates were allowed to dry. This was followed by incubation at 37°C ± 1°C for 18 to 24 hours. Coliforms and *Escherichia coli* colonies were enumerated under the UV light of wavelength 366 nm, and the purple-red colonies surrounded by a reddish zone of precipitated bile and red colonies surrounded by a zone of precipitated bile were counted.

Samples with >150 colonies were reported as ">150 × dilution factor". Positive and negative controls were included in all runs, and sterility checks were performed in accordance with SANS 5221:2011 (SANS, 2011).

4.2.1.5 Detection of *Staphylococcus aureus*

Staphylococcus spp. was enumerated according to the horizontal colony count method described in SANS 6888-1:1999 (SANS, 1999).

Screening of *Staphylococcus aureus* was done on BPA (Baird-Parker medium). As previously indicated, 1 mL of each beer sample was prepared in triplicate, transferred into a test tube containing 9 mL of sterile buffered peptone water, and thoroughly mixed by vortexing. A volume of 0.5 mL of each beer sample was transferred into Baird-Parker medium (BPA) containing (g/L) pancreatic digestion of casein (10), meat extract (5), yeast extract (1), sodium pyruvate (10), L-glycine (12), lithium chloride (5), agar (20). Using the spreader, the inoculum was spread evenly over the plates. The plates were then placed in a

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laminar flow hood for 15 min to allow the suspension to dry and were subsequently incubated at 37°C ± 1°C for 48 ± 2 h.

Colonies were identified by their characteristic black or grey, shiny convex appearance with a surrounding clear zone. The Staphaurex Latex kit was used to confirm the presence of *S. aureus*, following the manufacturer's instructions (coagulase test). Counts were performed on plates containing 1–150 colonies, and results were expressed as CFU/mL. Positive, negative, and sterility controls were included for quality assurance (SABS ISO, 1996; ISO/IEC, 2005).

4.2.1.6 Detection of *Salmonella* spp.

Salmonella spp. were detected using a horizontal method based on SANS 6579:2003 (SANS, 2003) and SANS 19250:2011 (SANS, 2011). Beer samples (100 mL) were initially transferred to 10 ± 0.5 mL of Rappaport Vassiliades medium with soya broth (RVS) and 10 ± 0.5 mL of Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn), respectively. Incubation of RVS and MKTTn was done at 41.5°C ± 1°C for 24 ± 3 hours and 37°C ± 1°C for 24 ± 3 hours. The cultures from RVS and MKTTn broth were transferred to Xylose lysine deoxycholate agar (XLD) and Brilliant green agar with a loop by streaking out for single colonies. The plates were incubated at 37°C ± 1°C for 24 h ± 3 h. Typical colonies were identified as pink or black (H_2S-producing) on XLD and pink on brilliant green agar. Presumptive colonies were confirmed using a commercial *Salmonella* confirmation kit following the manufacturer's instructions. Results were expressed as CFU/100 mL. Plates with no growth were recorded as Not Detected (ND), and overgrown plates were reported as "> dilution factor × number of colonies". Positive, negative, and sterility controls were included for quality assurance (ISO/IEC, 2005; SABS ISO, 1996).

4.2.1.7 Detection of yeast and mould

Yeasts and moulds were enumerated according to the colony count method described in SANS 7954:1987 (SANS, 1987). Enumeration of yeasts and moulds was done on Rose Bengal Chloramphenicol Agar (RBA, g/L mycological peptone 5, glucose 10, potassium dihydrogen phosphate 1, magnesium sulphate 0.5, rose bengal 0.05, chloramphenicol 0.1, agar 15.5) after preparing a 1:10 dilution of the beer samples in Buffered Peptone Water (BPW). Quality assurance included daily positive controls using *Candida albicans* and *Aspergillus brasiliensis*, negative controls with *Escherichia coli*, and sterility controls of BPW (SABS ISO, 1996). All analyses were performed in compliance with ISO/IEC 17025:2005 (ISO/IEC, 2005). Decimal dilutions were prepared as described in SANS 6887-1:2017 (SANS, 2017). Plates were swirled to mix and incubated aerobically, inverted, at 25 ± 1°C for 72 ± 4 h

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(SANS, 1987). Yeast colonies were recognised as large, cream to dull-white colonies, while moulds were identified as white/pale colonies that darkened to green or black with conidia formation. Yeast morphology was confirmed by wet-mount microscopic observation of oval-shaped budding cells. Counts were recorded for plates with 1–300 colonies. Quality assurance included daily positive controls using *Candida albicans* and *Aspergillus brasiliensis*, negative controls with *Escherichia coli*, and sterility controls of BPW (SABS ISO, 1996). All analyses were performed in compliance with ISO/IEC 17025:2005 (ISO/IEC, 2005).

Multi-mycotoxin analysis in craft beer is fully detailed in Chapter 3; details of sample preparation, extraction, and quantification are not repeated in this Chapter.

4.2.2 Chemical analyses of the craft beers

4.2.2.1 Sample preparation

To ensure precision and accuracy, 50 mL of degassed and frozen craft beer was collected from each sample and shipped under cold conditions to the Central Analytical Facilities (CAF), Stellenbosch University. Samples were handled and transported according to established protocols required for advanced chromatographic and spectrometric instrumentation. For preparation, 10 mL of each beer sample was transferred into 15 mL Falcon tubes. Afterwards, 100 µL of a 100 ppb Brett (Brettanomyces-derived compounds) and smoke internal standard solution and 100 µL of a 1 ppm naphthol internal standard solution were added and vortexed thoroughly prior to analysis. All the analyses were conducted in triplicate (three different bottles of beer).

4.2.2.2 Quantification of sugars, phenolic acids, volatile phenols, and major volatiles

The chemical profiling targeted two broad classes of metabolites:

- Non-volatile compounds (sugars and phenolic acids) were analysed by solid phase extraction (SPE) and gas chromatography with flame ionisation detection (GC–FID) after derivatisation.
- Volatile compounds (volatile phenols, esters, higher alcohols, aldehydes) were analysed by solid phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS).

This dual approach enabled comprehensive coverage of both flavour-active compounds and non-volatile fermentation products.

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4.2.2.3 Solid phase extraction (SPE) for volatile phenols

SPE was used to isolate volatile phenols (e.g., 4-vinylguaiacol, 4-ethylguaiacol, vanillin, eugenol) using SDBL (100 µm styrene-divinylbenzene) cartridges, which provide a hydrophobic surface suitable for retaining phenolic volatiles (Pizarro, Pérez-del-Notario and González-Sáiz, 2010).

Calibration: Calibration curves were prepared using internal standards (100 µL of 100 ppb Brett and smoke solution, 100 µL of 1 ppm naphthol, and 100 µL of 10 ppm methanol in 6% ethanol buffer, pH 3.5).

Cartridge preparation and extraction was conducted as follows: Cartridges were conditioned with 12 mL methanol and 10 mL deionised water. A 10 mL aliquot of degassed, filtered beer spiked with internal standards was loaded, dried under pressure (15 min), and eluted with 2 mL ethyl acetate into Pyrex tubes containing anhydrous sodium sulphate. Eluates were vortexed, concentrated, and transferred to GC vials for analysis.

GC–MS analysis: Separation was performed using an Agilent 6890N GC coupled to a 5975B MSD. Samples were injected via a CTC PAL autosampler onto a STABILWAX column (60 m × 0.25 mm ID, 0.25 µm). The oven program began at 35°C (5 min), ramped to 50°C (3°C/min, held 3 min), then to 120°C (3°C/min, held 5 min), and finally to 240°C (8°C/min, held 5 min). Helium was used as a carrier gas at 1 mL/min. The MSD was operated in EI mode at 70 eV, scanning 30–500 m/z.

4.2.2.4 Quantification of sugars and phenolic acids (GC–FID)

Given the non-volatile nature of sugars and phenolic acids, samples were extracted with 70% methanol and derivatised prior to analysis. In the next step, 15 mg of each beer sample was weighed into 1.5 mL Eppendorf tubes, vortexed with 70% methanol, and incubated at 60°C for 3 h. Extracts were dried under vacuum overnight.

Derivatisation was conducted as follows: Dried samples were treated with 100 µL of 2% methoxyamine in pyridine (40°C, 2 h), followed by 50 µL BSTFA + 1% TMCS (60°C, 30 min). Dried samples were derivatised with 100 µL acetonitrile + 50 µL BSTFA + 1% TMCS (80°C, 60 min).

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GC–FID analysis: These analyses were performed on an Agilent 6890N GC equipped with an RXi-5MS column (30 m × 0.25 mm ID, 0.25 µm). Helium was the carrier gas at 1 mL/min, with the injector temperature set to 250°C and the detector at 300°C. 1 µL of each sample was injected (5:1 split).

Oven program: 80°C (1 min) ramped to 300°C (7°C/min, 2 min held).

4.2.2.5 Quantification of Major Volatiles (SPME–GC–MS)

Chemicals and reagents: Analytical-grade standards of esters (e.g., ethyl acetate, isoamyl acetate) and higher alcohols (e.g., isoamyl alcohol, 1-propanol) were obtained from Sigma-Aldrich. Internal standards (3-octanol, 4-methyl-2-pentanol) were prepared in 6% ethanol (50 ppm).

For sample preparation, 5 mL of beer was mixed with 5 mL of 20% NaCl solution in headspace vials to increase ionic strength. The vials were spiked with 50 µL of the internal standard solution, vortexed, and analysed. Internal-standard corrected calibration curves were prepared as above to ensure accuracy.

GC–MS analysis: Conditions were identical to those described in Section 4.2.2.3, using SPME fibre extraction and the same GC–MS system with STABILWAX column and oven program.

4.2.2.6 Data analysis

Spearman's rank correlation was employed to assess monotonic relationships between variables. Data were examined for monotonicity and outliers prior to analysis. The correlation coefficient (ρ) was calculated using ranked data, and significance was determined at $p < 0.05$. Correlation strengths were interpreted as strong, moderate, or weak based on the magnitude of ρ (Schober and Schwarte, 2018). All analyses were conducted using [RStudio in Microsoft Excell], with results presented in Tables and Figures.

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4.3 RESULTS AND DISCUSSION

4.3.1 Microbial and chemical analysis strategy

The complex interaction of a microbial population can influence the safety and quality characteristics of craft beer. Comprehensive profiling of microbial and chemical parameters is therefore essential for analysing flavour development and determining the potential contamination risk in finished beers.

A total of 30 beers (3 bottles of the same beers) of different styles were purchased from 30 commercial craft breweries. Some beers were purchased directly from the producing microbreweries in provinces like Gauteng and the Eastern Cape (Port Elizabeth), while other beers were obtained from retail breweries to ensure an accurate representation of breweries from various provinces. The beer styles that were analysed were: Blonde Ale, Pale Ale, Indian Pale Ale (IPA), Best Bitter Ale, American Amber Ale, Irish Red Ale, Altbier Ale, Porter, Stout, and Imperial Ales.

Microbial analyses were conducted to determine total plate counts (TPC), yeasts, filamentous fungi, lactic acid bacteria, and foodborne pathogens such as *Staphylococcus aureus, Escherichia coli* and *Salmonella* spp. These tests are crucial for determining the overall microbial load, as excessive microbial growth can lead to spoilage and off-flavour development (Rodríguez-Saavedra et al., 2020). Filamentous fungi may contribute to the production of mycotoxins and are therefore associated with safety concerns (Azam et al., 2021). The presence of pathogenic bacteria, such as *Staphylococcus aureus, Escherichia coli*, and *Salmonella* spp., is a sign of inadequate sanitation and poses a threat to consumer safety (Bintsis, 2017).

The chemical analyses focused on specific groups of compounds that are known to contribute to flavour, microbial spoilage, safety, and fermentation dynamics. The investigation included the detection of fermentable sugars, sugar alcohols, esters, fatty acids, higher alcohols, aldehydes, and phenolic compounds.

The discussion that follows covers the occurrence, sensory implications, and relevance of the measured parameters, and this background supports the results and discussion that follow later.

4.3.2 Microbial profile of craft beer

To better assist in the interpretation of the data, microbial counts were grouped into three main groups: total plate counts (TPC), yeast and filamentous fungi, and spoilage and pathogenic bacteria. Spoilage

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bacteria were represented by LAB and *Staphylococcus aureus*, which was the only pathogen detected in one beer sample. Other common foodborne pathogens, such as *Escherichia coli* and *Salmonella* spp., were not detected in any beer style. This could be attributed to the harsh intrinsic condition of the beers, which included alcohol content, hops, and a low pH. Some of the triplicate beer samples showed inconsistent microbial distribution in one or two samples in some breweries, suggesting an uneven microbial dispersion due to manual bottling practices. This observation was across all microbial counts measured in this study and was indicative of variability due to the non-homogenous distribution of microbes, which is a relevant factor in microbial data interpretation from packaged craft beers.

4.3.3 Total plate counts (TPC)

The total plate count (TPC) test was applied to assess the beer quality and safety in terms of contamination and spoilage. This was done because specific bacteria and yeasts can thrive under certain conditions and spoil the beverage, despite the unfavourable conditions in beer for most microorganisms. The TPCs differed substantially among the targeted South African craft beer styles (Figure 4.1). Depending on the beer style, counts ranged from non-detectable to 1.63×10^5 CFU/mL. Stout2, Best Bitter1, and IPA had the highest counts at 1.63×10^5 CFU/mL, 1.53×10^5 CFU/mL, and 1.55×10^4 CFU/mL, respectively. Standard acceptable ranges for food and beverages are often below a few hundred, with acceptable values often cited as 2.50×101 to 2.50×10^2 CFU/mL (Sutton, 2011). Of the beers purchased from the 30 breweries, 17 breweries had microbial loads above 2.50×10^2 CFU/mL.

This high microbial presence could be attributed to yeast and/or spoilage bacteria. However, such high levels are not entirely unexpected in craft beers, especially in unfiltered beer styles. Similar findings were reported by Jeon et al. (2015), who found the highest microbial counts in microbrewed beer at 3.16×10^3 CFU/mL, which was significantly higher than the other beer samples tested in that study. In another study, Queirós et al (2024) found that applying high-pressure processing to IPA showed that untreated craft beers contained high microbial loads of up to 1.00×10^5 CFU/mL. The highest levels found in IPA, Best Bitter, Stout, and other beer styles in this study could be attributed to limited filtration, unfiltered styles, or certain spoilage bacteria that can survive in craft beer, such as lactic acid bacteria triggered by factors like low alcohol content, high residual sugars, and reduced hops bitterness (Bokulich and Bamforth, 2013; Bose et al., 2021).

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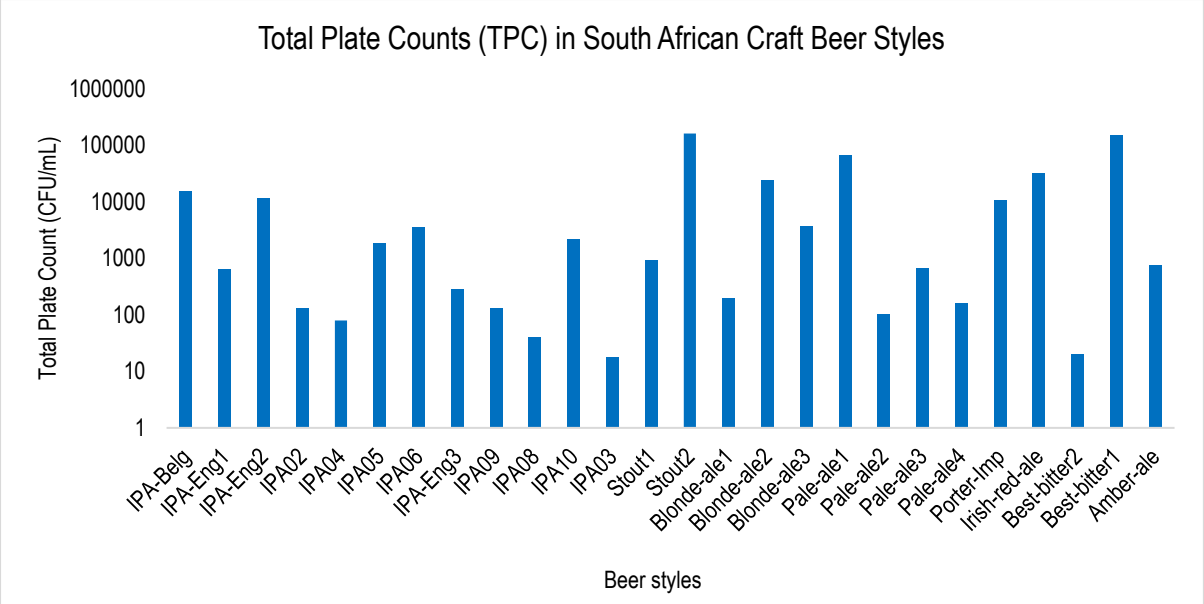


Figure 4.1: Total plate counts (TPC) levels across selected South African craft beer styles.

4.3.4 Yeast and mould counts

The presence of viable brewing yeast in yeast counts is crucial in beer quality measurement, as yeast is critical for flavour development, fermentation, and microbial stability (Maicas, 2020). Depending on the beer style, acceptable yeast levels in beer should be in the range of 0.52 million cells/mL (≈ 3.50×10^5 CFU/mL) (Ciont et al., 2022). In filtered beers, high yeast cells may indicate contamination.

In the current study, yeast counts were detected in only 16 of the 30 breweries in the different beer styles that were tested (Figure 4.2). The highest counts were Best-bitter1 (1.166×10^5 CFU/mL), Pale-ale1 (8.45×10^4 CFU/mL), and Blonde-ale2 (1.03×10^4 CFU/mL). The detected values were within the threshold expected for yeast counts in beer, especially unfiltered beers (Ciont et al., 2022). In a comparative study by Manzano et al. (2011), microbial communities were investigated before and after sanitation in craft breweries. The study revealed that filtered beers showed yeast counts below 1.00×10^1 CFU/mL after cleaning, which was close to undetectable levels. This finding supports the current study, which determined that the targeted breweries produced craft beer with no detectable yeast counts. This could be attributed to the filtration process in these breweries, which is known to significantly reduce or eliminate viable yeast populations in the final product (Manzano et al., 2011). Yeast viability and ageing related to alcohol toxicity or storage conditions could be another reason for these non-detectable counts, even initially (Bokulich and Bamforth, 2013).

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It is crucial to determine mould (filamentous fungi) counts in beer, as such data provide information regarding the fermentation outcomes, product quality, and rates of mycotoxin production (Ciont et al., 2022). In the current study, beer from 5 of the 30 breweries (17%) contained mould (Figure 2.1). These included IPA-Eng2 (1.00×10^1 CFU/mL), IPA10 (1.35×10^2 CFU/mL), Blonde-ale1 (1.00×10^1 CFU/mL), Blonde-ale2 (1.50×10^1 CFU/mL), and Stout 2 (2.80×10^1 CFU/mL). No mould was detected in the remaining breweries, possibly due to controlled grain storage environments, quality raw materials, and/or effective filtration steps (Obi, 2017). In the affected beer styles, the mould counts ranged from 1.00×10^1 CFU/mL to 1.35×10^2 CFU/mL. These values are considered low, but could pose consumer safety impacts. IPA10 with a value of 1.35×10^2 CFU/mL exceeded the expected threshold. If such levels occur, they could potentially result in early spoilage depending on the mould species, leading to undesirable flavour, aroma, or even health concerns (Ciont et al., 2022).

Contrary to the results obtained in the current study, Kim et al. (2015) detected large populations of fungi, as ≈ 4.6 log CFU/mL (~4.00×10^4 CFU/mL) were reported in a microbrewed beer, indicating that contamination must have occurred after the boiling stage. In another comparative study by Adekoya et al. (2018), the mean fungal contamination in traditional African beer was 3.66×10^5 CFU/mL, which indicated poor stabilisation. In the latter study, no filtration was linked to extreme fungal loads.

In the current study, IPA-Eng2 showed the highest yeast count at 4.80×10^3 CFU/mL, which could likely have contributed to the suppression of mould growth (1.00×10^1 CFU/mL). The literature proposes that active yeast metabolism, fuelled by nutrition consumption, produces ethanol and organic acids that can limit fungal proliferation (Maicas, 2020; He, Degraeve and Oulahal, 2024). Conversely, in IPA10 and Blonde-ale1 and Blonde-ale2, there were lower yeast levels (2.30×10^2 CFU/mL, 1.00×10^1 CFU/mL, and 1.50×10^1 CFU/mL, respectively). These counts corresponded with mould counts of 1.00×10^1 CFU/mL, 1.35×10^2 CFU/mL, and 2.80×10^1 CFU/mL, with the counts in Blonde-ale1 and Blonde-ale2. These higher counts could be attributed to insufficient yeast activity to control mould growth (Ädel Druvefors and Schnürer, 2005). The presence of LAB in the beer samples, namely IPA-Eng2 (2.97×10^3 CFU/mL) and Blonde-ale2 (1.70×10^2 CFU/mL), also indicated possible microbial interaction between LAB and yeast. LAB could have outcompeted the yeast for nutrients or produced organic acids that might have affected fungal and yeast growth, as proposed by Bokulich and Bamforth (2013).

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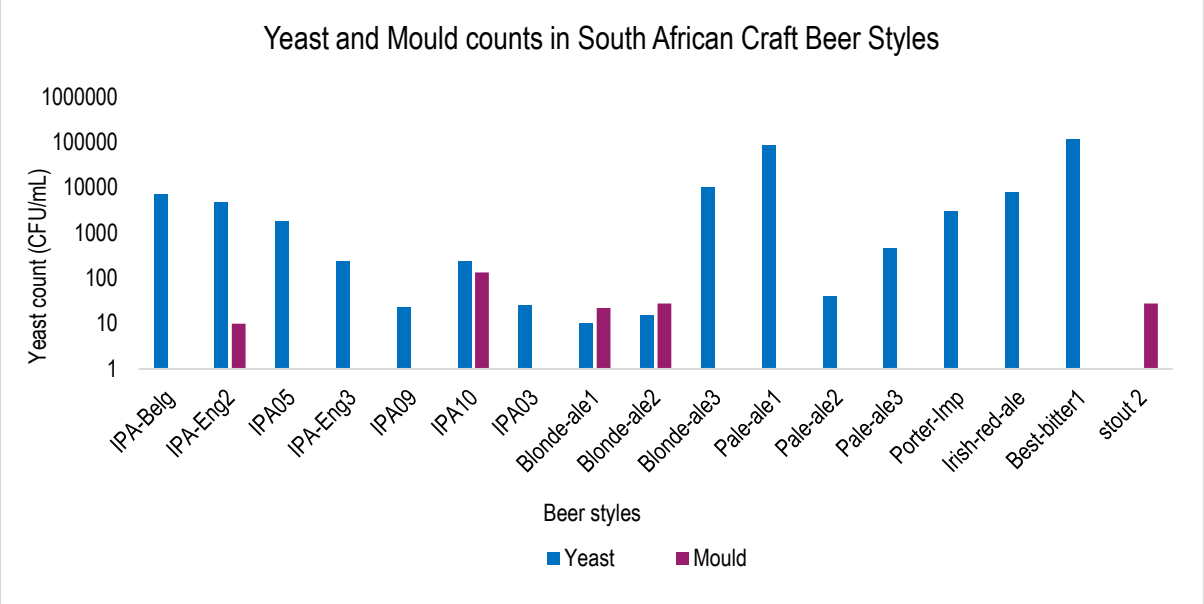


Figure 4.2: Yeast and mould count levels across the selected South African craft beer styles.

4.3.5 LAB and *Staphylococcus* count

Lactic acid bacteria were detected in 18 (60%) of the 30 targeted breweries. This indicates that cleaning and sanitation in half of the breweries required attention, as all such breweries are required to ensure healthy fermentation and safe beer throughout the production and packaging stages (Habschied, Krstanović and Mastanjević, 2024). LAB counts on MRS agar ranged from non-detectable to 1.25×10^5 CFU/mL (Figure 4.3). Best-bitter1 had the highest recorded LAB level at 1.25×10^5 CFU/mL, followed by Irish-red-ale at 6.1 × 103 and IPA-Eng 2 at 2.97×10^3. These levels, especially in Best-bitter 1, exceeded the commonly accepted spoilage level of 1.00×10^3 CFU/mL (López et al., 2020). In the same sample, a TPC of 1.53×10^5 and a yeast count of 1.17×10^5 were also observed. These counts also raised concern as elevated levels of LAB indicate potential sanitation and handling issues.

The combined detection of LAB and *Staphylococcus aureus* at 9.50×10^2 CFU/mL and 1.10×10^2 CFU/mL, respectively, in the Pale-ale2 beer style was an indication of post-fermentation contamination that possibly occurred during the bottling stage (Hofmann and Fischer, 2015). Although the detected levels of *S. aureus* were low, their presence in the beer raised hygiene and potential health risk concerns (Menz et al., 2011). In the same Pale-ale2 sample, TPC of 2.44×10^4 CFU/mL and a relatively low yeast count of 4.00×10^2 CFU/mL were recorded, highlighting microbial imbalance that could point to the presence of unwanted microbes, particularly as *S. aureus* and LAB were detected in this sample.

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In contrast to the findings of a study conducted by Kim et al. (2015), the TPC of the beer was 7.90 × 10^2 CFU/mL and LAB count was 7.90 × 10^0 CFU/mL, which were lower than the levels observed in Best-bitter1 and Pale-ale2. These findings suggest that contamination might have occurred during bottling when spoilage and pathogenic microbes were introduced.

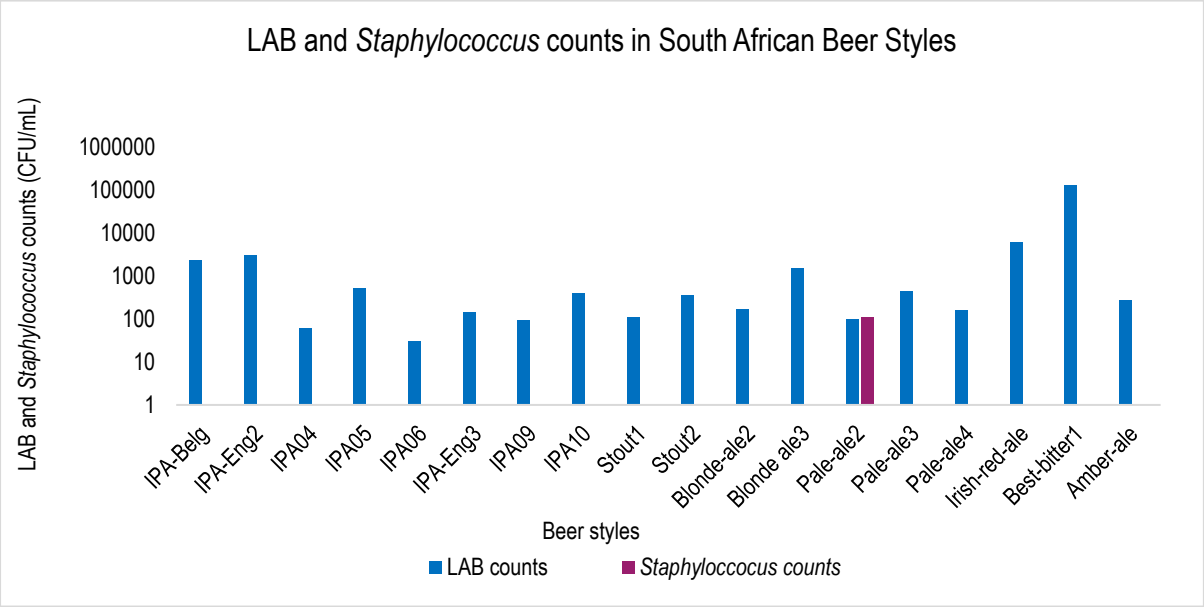


Figure 4.3: LAB and *Staphylococcus aureus* levels across the selected South African craft beer styles.

4.3.6 Chemical profile of craft beer

Given the complexity of craft beer composition, this study distinguished between compounds that were flavour-active and those that were not. Flavour-active compounds are those present at or above their sensory thresholds where they can contribute directly to aroma, taste, and/or mouthfeel. Threshold values, as reported in the literature, were used as the primary filtering criterion to classify the compounds (Lentz, 2018). Threshold indicates the concentration at which a compound is detectable by 50% of tasters (ISO 13301, 2002). Both detected and undetectable compounds were evaluated across the various beer styles to provide a comprehensive chemical profile. In particular, the selected flavour-active compounds in this study included higher alcohols, esters, and phenolic volatiles, as they are widely recognised for their strong sensory impact at relatively low concentrations, their role in defining beer style characteristics, and their extensive documentation in brewing science (Pires et al., 2014; Wannenmacher, Gastl and Becker, 2018; Humia et al., 2019; Ucharczyk, Zyła and Tuszyński, 2020; Gribkova et al., 2023). Microbes such as spoilage bacteria and wild yeast that contaminate beer can produce flavour-active compounds at low concentrations, altering the aroma and flavour of the beer. Even though both brewing yeast and contaminating microbes produce flavour-active compounds, metabolites from wild yeast or spoilage

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bacteria can be distinguished by their specific chemical signatures, such as volatile phenols, and their low concentrations, which are detectable using SPME-GC-MS. This allows identification of compounds that alter aroma and flavour due to contamination rather than standard fermentation. These flavour-active compounds may interact with those produced intentionally by the brewing yeast (Roselli et al., 2024). Other compounds, such as phenolic acids and sugars, are abundant and relevant to beer stability and quality, but they were considered non-flavour-active in this context, as they are regarded as background or matrix contributors rather than primary drivers of flavour diversity. Importantly, only the flavour-active compounds that were detected above their reported sensory thresholds were constructed into bar graphs to visualise their sensory relevance, while non-flavour-active compounds were discussed separately in terms of occurrence and brewing significance.

4.3.6.1 Flavour-active compounds

The flavour-active compounds in this study included isobutanol, isoamyl alcohol, phenylethyl alcohol, ethyl acetate, 2-phenylethyl acetate, ethyl caproate, ethyl caprylate, 4-vinylguaiacol, 4-ethylphenol, 4-ethylguaiacol, vanillin, guaiacol, and eugenol.

The beers were not formally tasted in this study. The classification of beers in this study was guided by the Beer Judge Certification Program (BJCP) Beer Style Guidelines (2021). These guidelines provide standardised descriptions of recognised beer styles, including typical ranges for alcohol by volume (ABV), bitterness (IBU's) colour (SRM) and expected sensory characteristics such as aroma, flavour and mouthfeel. The BJCP framework categories beers into major families (ales, larger, speciality beers) and defines stylistic expectations. The following section presents detailed information on the presence of each of the identified flavour-active compounds. For each compound, the concentrations detected across the beer styles are presented relative to reported sensory thresholds, along with their known sensory descriptors, origins, and relevance to beer style characteristics, as described in the literature and the Beer Style Guidelines (BJCP, 2021).

Isobutanol was detected in all the beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, mg/L in Porter-Imp. Isobutanol is a by-product of yeast fermentation and is a fusel alcohol produced through the Ehrlich pathway, or through the de novo synthesis of branched-chain amino acids (Branduardi et al., 2013). It can contribute to the overall alcohol content in beer, but high amounts can

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infuse the beer with undesirable flavours (Lachenmeier, Haupt and Schulz, 2008). Despite the high concentration detected in Porter-Imp, all the levels were still below the sensory threshold of 200 mg/L. They did not exceed the BJCP guidelines' stylistic expectations for warming, especially in higher-strength beers (Blanco, Andrés-Iglesias and Montero, 2016).

Isoamyl alcohol was also detected in all the beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3 IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale) (Figure 4.4A). Isoamyl alcohol is the most quantitatively important compound for flavour in the higher-alcohol group (Olaniran et al., 2017). It is produced during amino acid metabolism through the Ehrlich pathway by *Saccharomyces cerevisiae*, which produces a fruity, sometimes banana- or pear-like flavour in beer (Hazelwood (Hazelwood et al., 2008). Isoamyl alcohol concentrations ranged from 16.38 mg/L (Amber-ale) to 178.99 mg/L (IPA01). These concentrations exceeded the reported flavour threshold of 70 mg/L. The effect was more pronounced in IPA01 (178.99 mg/L), indicating that isoamyl alcohol likely contributed to a detectable fusel flavour in this beer sample. In American Amber Ale (Amb-ale), the isoamyl alcohol level (16.38 mg/L) remained below the threshold and was unlikely to contribute noticeably to the fusel character. Moderate fusel alcohols are acceptable in many ale styles as they add complexity, while elevated levels may result in a harsh quality beer, especially in lighter or hops-forward beers (BJCP, 2021).

All samples contained isoamyl alcohol, with concentrations varying due to yeast strain, wort composition, and fermentation management (Olaniran et al., 2017). Pitching rate and inoculum size have been identified as key factors in fusel alcohol production. For example, higher inoculum sizes have been attributed to deleterious effects in fusel alcohol production, while correct pitching rates and good aeration result in moderate production (Wang et al., 2019). The content of amino acids, as well as carbohydrates (glucose, fructose, sucrose, maltose, maltotriose, and non-fermentable dextrin), vitamins, minerals, and other constituents too numerous to mention in the wort, affects higher alcohol formation (Ferreira and Guido, 2018). Temperature is also another factor that influences the production of isoamyl alcohol concentrations. High or fluctuating fermentation temperatures can accelerate amino acid uptake, raising isoamyl alcohol concentrations (Godillot et al., 2023).

Phenylethyl alcohol was also detected across all the beer styles (IPA-Belg, IPA-Eng1, IPA02, IPA04, IPA05, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2,

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and Amber-ale) at varying concentrations. The concentrations ranged from 3.43 mg/L in Stout1 to 1.06 mg/L in Best-bitter2. Phenylethyl alcohol is an aromatic fusel alcohol that is yielded from yeast fermentation as a by-product of amino acid metabolism, specifically from phenylalanine. It commonly contributes to a rose-like aroma in beer. The concentrations in this study were significantly lower than the beer flavour threshold of 125 mg/L reported in the literature (Blanco, Andrés-Iglesias and Montero, 2016). The low concentrations detected indicated that phenylethyl alcohol contributed a dominant rose-like or floral aroma to the beer. Floral notes are acceptable for certain beer styles, such as IPA-Belg, Blonde Ale, and some Pale Ales. However, they are more subdued in malt-forward styles like Porter, Stout, and Altbier. The detection of phenylethyl alcohol in all styles indicated yeast metabolism via the Ehrlich pathway from phenylalanine, and this was active during fermentation conditions.

Phenyl ethyl acetate was not detected in the following beer styles: IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale. Phenyl ethyl acetate, also known as 2-phenylethyl acetate, is produced through esterification of ethanol and phenylacetic acid during yeast fermentation via the Ehrlich pathway (Adler et al., 2011 ;Bernardino et al., 2024). According to the BJCP (2021) guidelines, its absence in the targeted beer styles resulted in cleaner, style-appropriate flavours. Moreover, floral and honey-like esters are acceptable in some ales but should be properly maintained in hop-forward or malt-forward beers.

However, phenyl ethyl acetate was detected in the following beer styles: IPA01, IPA-Belg, IPA-Eng1, IPA-Eng2, IPA02, IPA-Eng3, IPA08, IPA09, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, and Porter-Imp. (Figure 4.4B). The phenyl ethyl acetate concentrations ranged 0.007 mg/L to 11.49 mg/L, a level that contributes to a noticeable floral and honey-like flavour to beer. Blonde-ale2 had the highest concentration, which exceeded the 3.80 mg/L threshold and thus contributed to a perceptible floral flavour in this beer style. The BJCP guidelines (2021) suggest moderate floral esters in certain ales, but moderate expression in hops-forward or malt-focused beers.

Ethyl caproate was not detected in some beer styles (IPA02, Blonde-ale2, Blonde-ale3, Pale-ale1, Altbier, and Amber-ale). Ethyl caproate (also known as ethyl hexanoate) is a by-product of fatty acid and alcohol metabolism during yeast fermentation. It contributes to fruity and tropical notes in beer, and these flavours are often described as pineapple- or apple-like (Olaniran et al., 2017; Fan et al., 2021). The absence of ethyl caproate in these beer styles aligns with the BJCP (2021) guidelines that specify that tropical fruit

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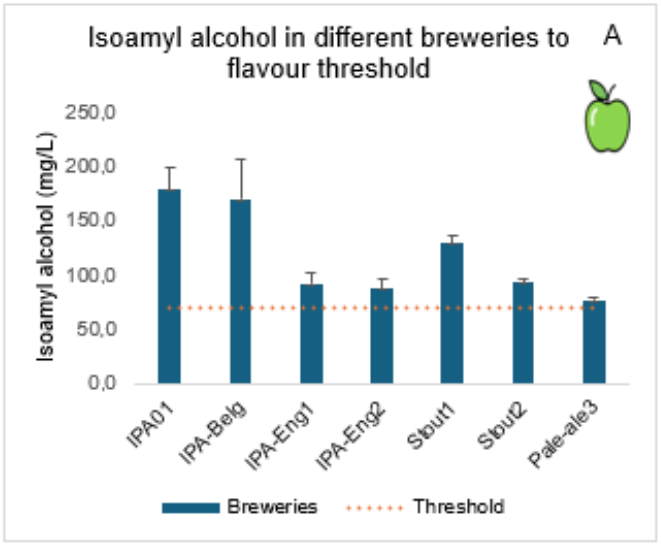
esters are not required in malt-forward or hops-forward beers, thereby preventing unwanted fruitiness and maintaining the desired flavour profile.

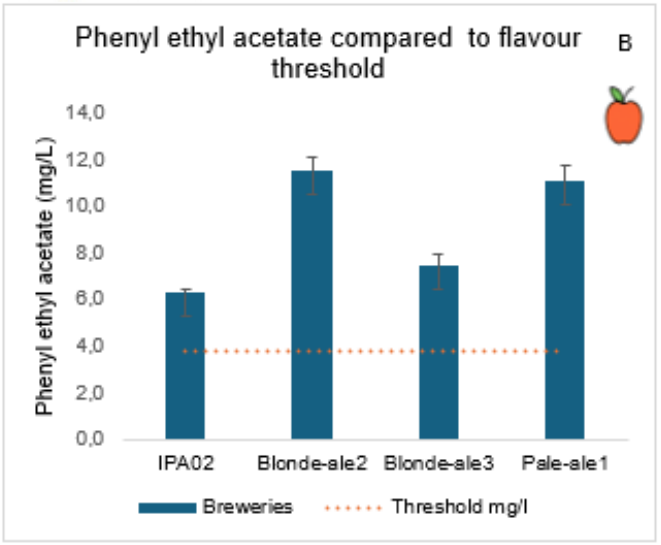
However, ethyl caproate was detected in the majority of beer styles, namely IPA01, IPA-Belg, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale (Figure 4.4C). Ethyl caproate concentrations ranged from 0.007 mg/L to 469.277 mg/L. The ethyl caproate concentrations detected in most beers in this study exceeded the flavour threshold of 0.21 mg/L indicated in the literature, indicating an appreciable fruity aroma. High levels, such as that detected in Stout2 (469.277 mg/L), may produce an excessive tropical fruit character and intensify complexity in malt-forward beer styles, but may modify the flavour balance in hops-forward beers (Olaniran et al., 2017; .McKay and Buica, 2020). According to the BJCP guidelines (2021), moderate fruity esters are desirable in some ales (e.g., Belgian Ale and Pale Ale) but should be controlled in hops-forward styles. Differences in yeast metabolism, fermentation, and wort composition could be the factors involved in variations in concentrations, indicating the sensory and stylistic relevance of this ester.

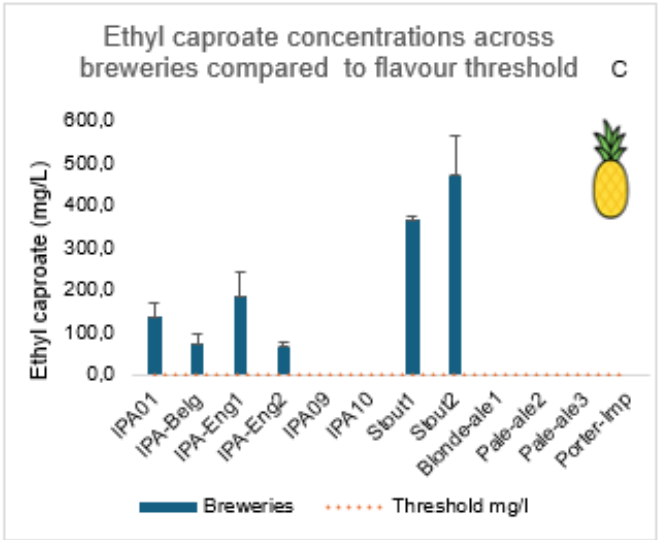
Ethyl caprylate was not detected in some beer styles (IPA02, Pale-ale1, Pale-ale2, Pale-ale3, and Porter-Imp). Ethyl caprylate, also known as ethyl octanoate, is formed during yeast fermentation through the interaction of caprylic acid and ethanol (Saerens et al., 2010). Ethyl caprylate is associated with fruity and apple- or pear-like flavours in beer (Zhang et al., 2025). The absence of ethyl caprylate in some beer styles could be attributed to yeast, precursor, or fermentation conditions (Olaniran et al., 2017). Fruity esters are desirable in some ales but should be moderated in hops-forward or malt-focused beers. Its absence supports a cleaner, desirable flavour profile (BJCP, 2021).

Ethyl caprylate was detected in many of the beer styles (IPA01, IPA-Belg, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale4, Pale-ale5, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale) (Figure 4.4D). Ethyl caprylate concentrations ranged from 0.004 mg/L to 1.024 mg/L, and IPA06 had the highest concentration. Ethyl caprylate exceeded its flavour threshold of 0.90 mg/L only in IPA06, contributing to a detectable apple- or pear-like aroma, while the other beers remained below the threshold with negligible flavour impact. Moderate fruity esters enhance certain ale styles but should be limited in hops-forward or malt-focused beers. The variations detected in ethyl caprylate across the beer samples reflected differences in yeast strain, fermentation, and wort, which impacted the beer flavours while maintaining their style-specific profiles.

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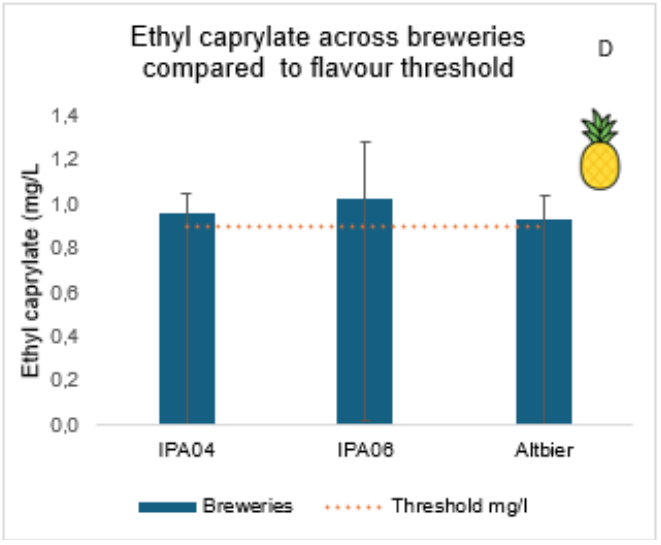


Figure 4.4: Concentrations of selected flavour compounds across the selected South African breweries compared to sensory flavour thresholds, A=Isoamyl alcohol, B=Phenyl ethyl acetate, C=Ethyl caproate and D=Ethyl caprylate. Each value is the mean of triplicate ± SD of triplicate.

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4-Vinylphenol (4-VP) was not detected in some beer styles, namely IPA04, IPA05, IPA07, IPA10, Pale-ale4, Altbier, Irish-red-ale, Best-bitter1, and Best-bitter2. 4-VP is a phenolic compound produced during fermentation by yeast decarboxylation of p-coumaric acid, a hydroxycinnamic acid present in malt (Vanbeneden, Gils, et al., 2008). Phenylacrylic acid decarboxylase enzymes found in yeast strains, particularly phenolic-off-flavour (POF) positive yeast strains, are responsible for this transformation 4-VP is attributed with a strong medicinal, clove-like aroma when present in excessive concentrations (Vanbeneden et al., 2008; Cui et al., 2015). Its absence in these beer styles aligned with BJCP (2021) style expectations, which favour clean fermentation profiles in these categories, probably due to POF-negative yeast and low p-coumaric acid in the raw materials (Coghe et al., 2004).

4-Vinylphenol (4-VP) was detected in several other beer styles (IPA01, IPA-Belg, IPA02, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA09, IPA08, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale5, Porter-Imp, and Amber-ale) (Figure 4.5E). The concentration of 4-VP ranged from 0.004 mg/L to 3.36 mg/L with IPA-Belg having the highest concentration. The literature flavour threshold for 4-VP is 0.2 mg/L, and it is argued that samples above this level are likely to display noticeable phenolic, medicinal, or smoky flavours (Cui et al., 2015). 4-VP exceeded the 0.2 mg/L threshold in IPA-Belg, producing a detectable phenolic character appropriate for Belgian-style beers, while other ales with lower levels preserved a clean malt and hops balance that was consistent with the BJCP (2021) guidelines.

4-Vinylguaiacol (4-VG) was detected in all the beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale) (Figure 4.5F). The concentrations of 4-VG ranged from 0.14 mg/L in Blonde-ale2 to 10 mg/L in IPA-Belg. The flavour threshold of 4-VG is accepted at 0.3 mg/L (Cui et al., 2015). Most samples exceeded this threshold and were therefore likely to be infused with perceptible phenolic flavours. Clove-like phenolic aromas are preferred in certain Belgian style and wheat beers, aligning with the high level observed in IPA-Belg (10 mg/L) (BJCP, 2021). However, in most hops-forward or malt-balanced ales, such as Pale Ale or Blonde Ale, excessive 4-VG may be regarded as an off flavour that can interfere with the beer's intended malt or hops balance. The lowest concentration (0.14 mg/L) was observed in Blonde-ale2, and this was below the threshold, indicating minimal sensory contribution in that beer sample.

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4-Ethylguaiacol (4-EG) was not detected in several of the beer styles (IPA-Belg, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale2, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). 4-EG is a phenolic compound produced during fermentation by yeast when it converts ferulic acid into 4-EG. It imparts smoky, spicy, and clove-like aromas and flavours to beverages like beer (Sunao et al., 2016). Its absence in these beer styles demonstrated that ferulic acid conversion was either absent or minimal. It is averred that this conversion is influenced by the type of yeast used and the presence of ferulic acid in the malt (Coghe et al., 2004).

However, 4-Ethylguaiacol (4-EG) was detected in various other beer styles (IPA02, IPA-Eng1, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale3, Porter-Imp, Stout1, and Stout2). 4-EG concentrations ranged 0.002 mg/L to 0.38 mg/L in Blonde-ale2 (Figure 4.5G). The flavour threshold of 4-EG is reportedly 0.13 mg/L (Miller, 2019). Blonde-ale2 exceeded this threshold by nearly double, resulting in perceptible phenolic notes present in this beer that arguably resulted in a sensory deviation from the expected style. In Stout1 and Stout2, exceedances were more stylistically compatible, as richer malt-forward profiles introduced phenolic complexity without appearing as off-flavours. Most beer samples remained below the threshold, indicating minimal sensory contribution. Phenolic and smoky notes are generally undesirable in hops-forward or Pale Ales, while they might be acceptable in malt-forward or speciality beers (BJCP, 2021).

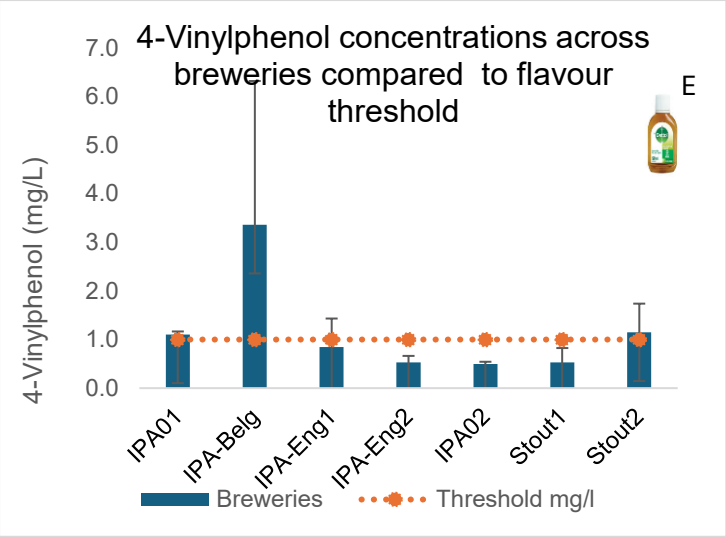
Vanillin was detected in all the targeted beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). Vanillin concentrations ranged from 0.002 mg/L in IPA06 to 0.64 mg/L in IPA-Eng1. Vanillin is a phenolic aldehyde derived mainly from malt and, in some cases, from oak maturation due to the breakdown of lignin, contributing to a characteristic vanilla aroma (Sterckx et al., 2011). Additionally, phenolic compounds such as 4-vinylguaiacol (4-VG) can degrade during beer storage to produce vanillin, especially in the absence of oxygen (Sterckx et al., 2011). Contaminant yeast can also contribute to vanilla flavours in beer. The flavour threshold of vanillin reported by the Siebel Institute of Technology (2019) is 0.004 mg/L. Most concentrations in the current study surpassed this threshold, indicating that vanillin likely contributed to the perceptible flavour in several samples, especially in IPA-Eng1 (0.64 mg/L). Vanilla flavours can be favourable in malt-forward styles such as stouts and certain speciality ales, while being less compatible in hops-forward or clean ales where a neutral fermentation profile is desired (BJCP, 2021).

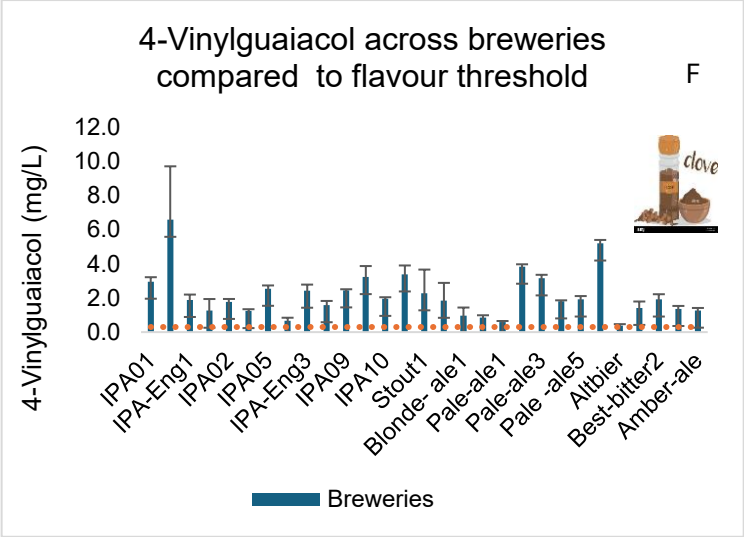
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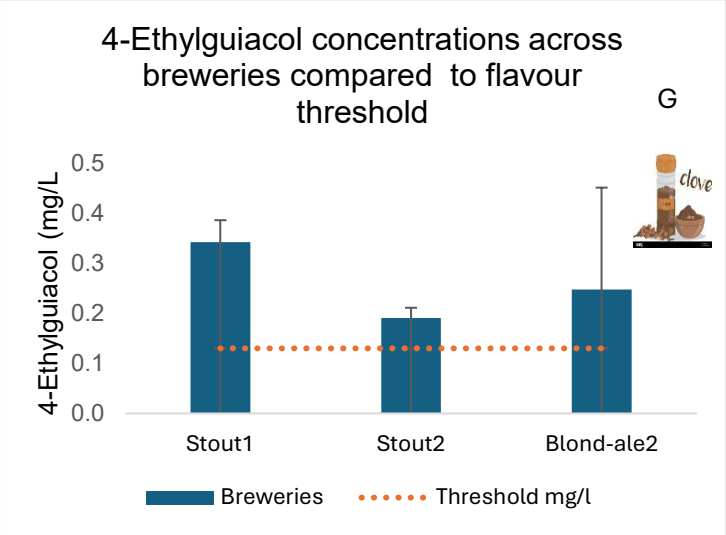
Guaiacol was not detected in some beer styles (IPA04, IPA06, IPA-Eng3, IPA07, IPA09, Pale-ale4, Pale-ale5, Best-bitter1, Best-bitter2, and Amber-ale). Guaiacol is a volatile phenolic compound formed in beer through thermal lignin degradation in smoked or heavily kilned malts. It offers minor contributions from microbial metabolism, and it contributes to a smoky or medicinal flavour in beer (Álvarez-Rodríguez et al., 2003; Vanbeneden, Gils, et al., 2008). The BJCP (2021) guidelines support the absence of this compound, indicating that smoky or phenolic aromas are unlikely to be present in hops-forward or malt-balanced ales.

However, guaiacol was detected in several other beer styles (IPA01, IPA-Belg, IPA02, IPA05, IPA-Eng1, IPA-Eng2, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Porter-Imp, Altbier, and Irish-red-ale) (Figure 4.5H). Guaiacol concentrations ranged from 0.01 mg/L to 0.48 mg/L in Stout2. In the literature, the flavour threshold of guaiacol is 0.04 mg/L. (Osafune et al., 2020). Several samples, such as IPA01, IPA-Eng1, Stout1, Stout2, and Port-Imp, exceeded this threshold, demonstrating that guaiacol could contribute to smoky, medicinal, and phenolic flavours. Stout2 showed the highest guaiacol concentration of 0.48 mg/L. However, the BJCP (2021) guidelines reveal that low phenolic notes are acceptable and even desirable in malt-forward, darker style beers like stout due to their roasted chocolate and caramel malt flavours. This is contrary to hops-forward beers like IPA, in which a similar concentration is deemed undesirable.

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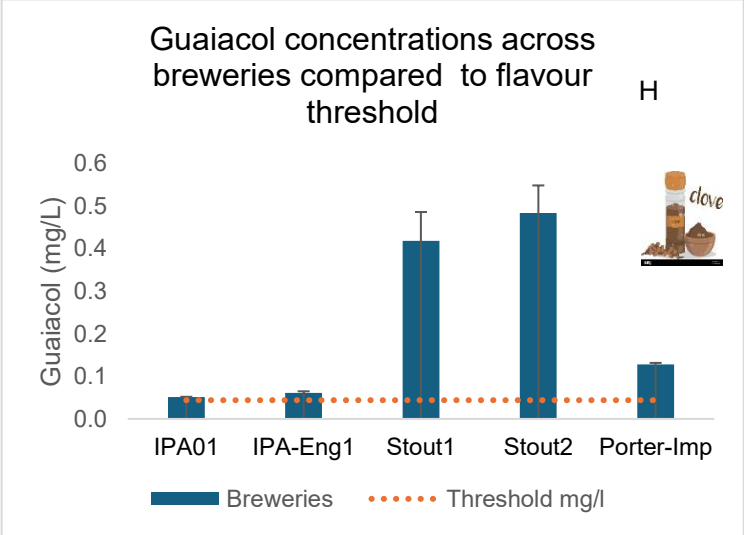


Figure 4.5: Concentrations of selected flavour compounds across the selected South African breweries compared to sensory flavour thresholds, E=4-Vinylphenol, F=4-Vinylguaiacol, G=4-Ethylguiacol and H=Guaiacol. Each value is the mean of triplicate ± SD of triplicate.

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Eugenol was undetected in several beer styles (IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Blonde-ale2, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). Eugenol is a phenolic compound that can be produced during fermentation by yeast, producing a spicy, clove-like flavour profile (Bendre and Rajput, 2016). Its absence in these styles reflected controlled use of POF-negative yeast strains, which preserved a clean malt and hops balance in line with BJCP (2021) guidelines. This prevented unwanted clove-like or spicy characteristics.

However, eugenol was detected in other beer styles (IPA01, IPA-Belg, IPA-Eng2, Stout1, Stout2, Blonde-ale3, and Pale-ale1). Eugenol concentrations ranged from 0.01 mg/L to 0.03 mg/L in IPA01. The flavour threshold of eugenol in beer is reportedly 0.04 mg/L (Siebel Institute of Technology, 2019). The eugenol concentrations of these beer styles remained below the flavour threshold, likely contributing to mild malt complexity without influencing the style-specific flavour aligned to the BJCP (2021) guidelines of clean, appropriate beer styles.

Isoamyl acetate was not detected in IPA02, Blonde-ale2, Blonde-ale3, and Pale-ale2 beer styles. Isoamyl acetate is a common ester that is produced when isoamyl alcohol and acetyl-CoA react during yeast fermentation. Isoamyl acetate is responsible for the fruity, banana-like aroma in beer (Yoshimoto and Bogaki, 2023). The absence of isoamyl acetate in these beer styles was consistent with the BJCP (2021) guidelines that describe these styles as clean-fermenting with hops- or malt-driven profiles and minimal ester expression.

However, isoamyl acetate was detected in several beer styles (IPA01, IPA-Belg, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Pale-ale1, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale) (Figure 4.6J). Isoamyl acetate ranged from 0.005 mg/L to 5,50 mg/L in Porter-Imp. In the literature, the flavour threshold of isoamyl acetate is 1.40 mg/L (Blanco, Andrés-Iglesias and Montero, 2016) but most of the beer styles were below this threshold, indicating minimal sensory contribution. However, several beer styles, specifically Porter-Imperial (5.503 mg/L), exceeded the threshold, making a detectable fruity aroma likely in those beers. The BJCP guidelines (2021) describe the acceptable ester levels for each style; hence, the high isoamyl acetate concentration in the Porter-Imperial beer probably reflected fermentation conditions such as high original gravity, yeast strain characteristics, and warmer temperatures (Yilmaztekin, Cabaroglu and Erten, 2013).

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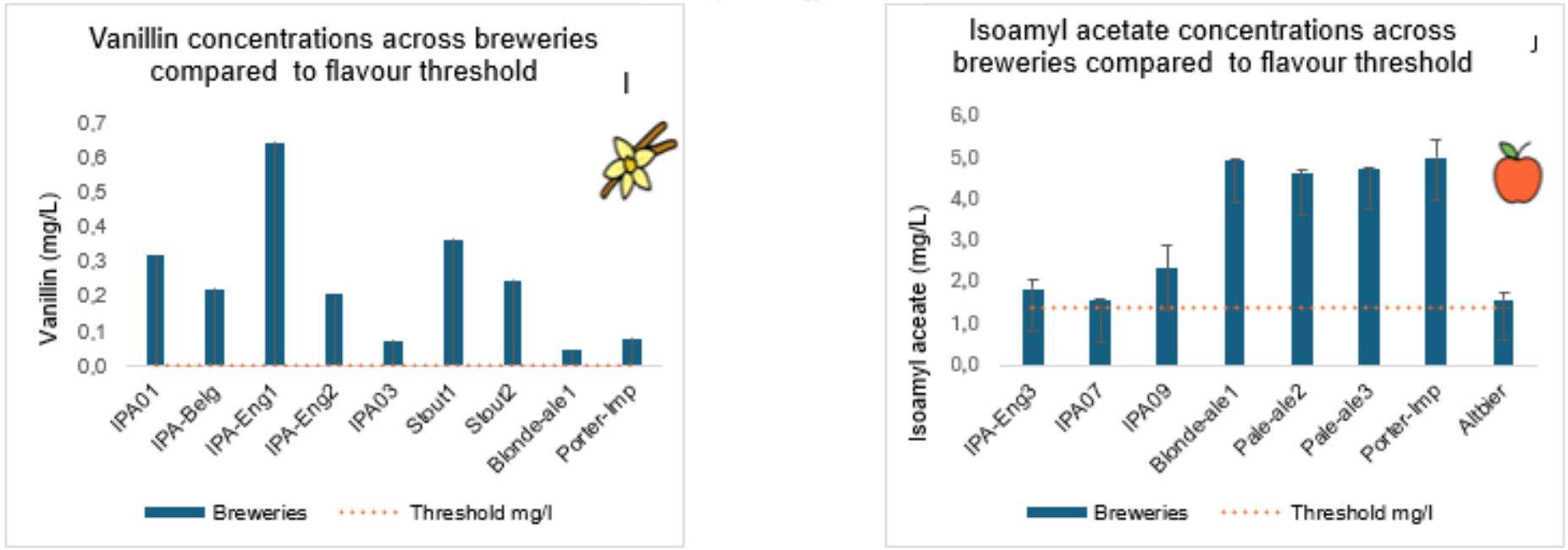


Figure 4.6: Concentrations of selected flavour compounds across the sampled South African breweries compared to sensory flavour thresholds, I=Vanillin and J=Isoamyl acetate. Each value is the mean of triplicate ± SD of triplicate.

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4.3.6.2 Non-flavour-active compounds

The remaining compounds were classified as non-flavour-active because they are generally not considered style-defining. Most were detected at levels below their reported sensory thresholds, although some exceeded these thresholds. These compounds do not significantly contribute to characteristic beer aromas but are important for beer stability, balance, mouthfeel, and overall composition.

2,6-Dimethylphenol, which was not detected in two beer styles (IPA02 and IPA06), can be produced from malt and hops during yeast fermentation. It contributes to a medicinal, smoky, ashy, musty, or phenolic aroma in beer (Wang and Chambers, 2018). Its absence in IPA02 and IPA06 indicated a clean fermentation profile not associated with unwanted medicinal or smoky phenolic profiles, which is consistent with the BJCP (2021) beer style guidelines for hops-forward beers.

However, 2,6-dimethylphenol was detected in the rest of the beer styles (IPA01, IPA-Belg, IPA04, IPA05, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). 2,6-dimethylphenol concentrations ranged from 0.008 mg/L to 0.04 mg/L. The flavour threshold of 2,6-dimethylphenol in beer is not definitively established in the available literature. However, the low levels detected in these beer styles were unlikely to produce medicinal or smoky phenolic characteristics, supporting a clean fermentation profile and stylistic expectations in line with the BJCP (2021) guidelines (Yoshimoto and Bogaki, 2023; Blanco, Andrés-Iglesias and Montero, 2016; Yilmaztekin, Cabaroglu and Erten, 2013).

Protocatechuic acid was detected in all the beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA08, IPA09, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale) with concentrations ranging from 0.002 mg/L in IPA06 to 2.51 mg/L in Blonde-ale2. Protocatechuic acid originates from malt and hops and contributes to antioxidant properties in beer. It contributes to mild astringency, antioxidant properties, and a beer ‘mouthful’ sensation (Callemien and Collin, 2010; Huang and Xu, 2021; Nardini, 2023). The flavour threshold of protocatechuic acid in beer is not clearly stated in the literature, as it can vary depending on the specific beer style. This is attributed to variable grain phenolic content and malting conditions (Piazzon, Forte and Nardini, 2010). In the current study, the concentration range aligned with existing literature on protocatechuic acid content in beer. For instance, Dvořáková et al., (2007) reported

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protocatechuic acid levels from 0.7 mg/L to 5.1 mg/L in the beer samples they tested. In the current study, elevated protocatechuic acid levels in Blonde-ale2 could have subtly influenced its sensory character, though it most likely remained below the level of stylistic concern without supporting sensory evidence.

Syringic acid was also detected in all the beer styles with concentrations ranging from 0.01 mg/L in Pale-ale6 to 21.96 mg/L in Blonde-ale3. Syringic acid originates from malt and hops and contributes to mild astringency or bitterness if present in higher than usual concentrations (Callemien and Collin, 2010; Huang and Xu, 2021). Compared to existing literature, some concentrations in the beer styles in the current study were relatively high. Nardini and Foddai (2020) reported a syringic acid concentration range up to 0.07 mg/L in the beer samples they tested Nardini (2023) reported the highest value in the literature to date of 2.46 mg/L in grape beer. Blonde-ale2 had the highest level of all the analysed beer styles, nearly nine times higher than previously reported. The high levels in Blonde-ale2 could be attributed to malt composition or to prolonged mashing (Wyler et al., 2015; Gribkova et al., 2023). According to the BJCP (2021) guidelines, Blonde Ale is expected to have a clean fermentation character with low to moderate maltiness and minimal phenolic notes. Even though syringic acid is not associated with off-flavour descriptors, such high concentrations can lead to flavour-active derivatives under certain conditions, such as microbial metabolism (Lentz, 2018).

Gallic acid was detected in all the samples, with concentrations ranging from 0.002 mg/L to 0.15 mg/L in IPA01. Gallic acid is naturally found in barley and hops used in brewing and contributes to beer aroma and flavour profiles while enhancing bitterness and mouthfeel (Abadio et al., 2015; Niu et al., 2024). The literature suggests that its sensory threshold in beer is not well defined but is generally considered to be much higher than the concentrations observed in the current study (Humia et al., 2019). Its low presence in the beer styles may be associated with clean fermentation or extraction during the brewing process, or prolonged mashing. Gallic acid remains an important marker of the antioxidant and polyphenolic content of beer, even at low concentrations (Nardini, 2023).

Caffeic acid was undetected in altbier sample, but it was detected in all other beer samples (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale) at the highest concentration of 0.50 mg/L in IPA01. Caffeic acid originates from malted barley and hops, which are the main ingredients in beer. It is a precursor to volatile phenols that impart clove-like, medicinal, and spicy notes and can also influence bitterness and astringency in beer (Oladokun et al., 2016; Carvalho and Guido, 2022). The

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concentration range detected in this study was at the lower end compared to the range reported by Nardini (2023) in an article titled “An overview of bioactive phenolic molecules and antioxidant properties of beer: Emerging trends”. Caffeic acid data across multiple beer types and studies were summarised, showing concentrations varying from 0.002 mg/L to 2.53 mg/L. The latter concentration range aligns with the BJCP (2021) guidelines that state that a phenolic character is not a dominant feature in IPA, and beers are expected to have a clean, hops-forward profile.

Trans-cinnamic acid was detected in all the beer styles at concentrations ranging from 0.002 mg/L to 0.24 mg/L, with the highest concentration in Stout2. Trans-cinnamic acid is a phenolic compound derived from malt and hops. Trans-cinnamic acid contributes a distinctive, spicy, clove-like flavour, often described as phenolic when produced in high amounts (Richard et al., 2015; Baigts-Allende et al., 2021). Its levels in beer vary depending on the beer type and brewing process; the levels in the study are lower than the threshold for cinnamic acid reported in the literature, ranging from 67 mg/L to 139 mg/L (Habschied et al., 2021). Stouts are expected to have a robust malt character, which can include roasted and slightly phenolic notes, according to the BJCP 2021 guidelines. Although below the flavour threshold, trans-cinnamic acid can contribute indirectly to beer stability through its antioxidant properties, particularly in malt-forward beer styles such as stout (Guido and Ferreira, 2023).

Vanillic acid was detected in all the beer styles in a range of 0.0019 mg/L in Amber-ale to 76.75 mg/L in Porter-Imp (Figure 4.6l). This compound is derived from ferulic acid during the brewing process and contributes to vanilla-like, sweet, and slightly wood-like flavour notes in beer (Wyler et al., 2015). The vanillic acid impact in beer is subtle, yet its exact sensory threshold in beer has not been widely reported In this current study, the upper value level of 76.75 mg/L in Porter-Imp was higher than the range reported in the literature by Nardini (2023), which, according to various sources, notes 0–3.6 mg/L free vanillic acid in different beer styles. Lower concentrations have often been reported in the literature in standard ales compared to other phenolic acids, like ferulic acid (Piazzon, Forte and Nardini, 2010; Nedyalkov et al., 2025).The BJCP (2021) guidelines for this Porter-Imp style highlight the use of highly kilned and roasted malts that may increase phenolic acid content and thermal degradation of lignin and ferulic acid (Wyler et al., 2015).

p-coumaric acid was detected in all the beer styles in the range of 0.003 mg/L in Irish red-ale to 0.81mg/L in IPA01. p-Coumaric acid is a type of hydroxycinnamic acid that is primarily sourced from malt and hops (Carvalho and Guido, 2022). This acid is a precursor to 4-vinylphenol (4VP) and 4-vinyl guaiacol (4VG) and contributes a clove-like, smoky, or spicy flavour to beer. A specific yeast converts p-coumaric to 4VG

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depending on the yeast type, style of the beer, and fermentation temperature (Holt et al., 2018). The flavour threshold of p-coumaric in beer is 520 mg/L; however, it can be decarboxylated into 4VP and 4VG, which are known to be low at 0.2 mg/L and 0.3 mg/L, respectively (Cui et al., 2015). The highest concentration of this compound was detected in IPA01 at a level that was consistent with the BJCP (2021) guidelines. It emerged with a bold hop aroma and flavour with minimal malt dominance.

m-coumaric acid was detected in the following beer styles: IPA01, IPA-Belg, IPA-Eng1, IPA-Eng2, IPA02, IPA08, IPA10, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Porter-Imp, and Altbier, at trace concentrations between 0,001 mg/L and 0.01 mg/L (IPA-Belg exhibited the highest concentration). m-Coumaric acid is a hydroxycinnamic acid and an isomer of p-coumaric acid. It originates from barley malt, with hops making a small contribution (Lentz, 2018). m-Coumaric is less studied than p-coumaric, but it also possesses antioxidant properties and contributes indirectly to flavour development through microbial transformations (Montanari et al., 1999; Rodríguez et al., 2009). No data could be traced that explicitly state the threshold of m-coumaric acid, but the literature indicates that p-coumaric acid is closely related to m-coumaric acid at a threshold of 520 mg/L (Vanbeneden, Delvaux and Delvaux, 2006). According to the BJCP (2021) guidelines, the slightly elevated m-coumaric acid in IPA-Belg. style may support the formation of volatile phenols, contributing to its distinctive clove- or spice-like notes. While m-coumaric acid levels were below threshold levels in the current study, its role as a biochemical precursor means it still has relevance in the development of phenolic aromas.

Ferulic acid was detected across all the beer samples in concentrations ranging from 0.005 mg/L in Pale-ale2 to 2.27 mg/L in IPA-Eng1. Ferulic acid originates from barley malt and wheat malt during the brewing process (Coghe et al., 2004). Ferulic acid is a precursor to flavourful compounds such as 4-vinyl guaiacol (4VG), which is produced by decarboxylation by certain yeast strains. It produces a spicy, clove-like flavour that is often associated with that of wheat beers (Coghe et al., 2004). Ferulic acid has a high threshold in beer, usually at 600 ppm, and doesn't contribute to the flavour of beers at these typical concentrations. However, when converted to its precursors (such as 4VG) that have a lower threshold, it contributes phenolic clove-like or spicy off-flavours (Vanbeneden, Van Roey, et al., 2008). Compared to the reported threshold values in the literature, the data in the current study showed that all the analysed beer samples were below the detection limit of ferulic acid. In accordance with the BJCP (2021) guidelines, the IPA-Eng1 value (highest in the study) allowed for a rich malt backbone with minor complexity. Its higher value compared to other styles could suggest a high malt contribution that could have occurred when the ferulic acid was converted to 4-VG biochemically, while the beer was sitting in the fridge and slowly aging. It was questionable whether its presence meant anything for flavour stability.

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Therefore, the question was posed whether the phenolic off-flavour would still develop if there was ferulic acid in the finished beer.

Furfural was not detected in any of the following beer styles: IPA03, IPA06, Pale-ale5, and Best-bitter2. It originates from Maillard reactions during wort boiling and malt production (Li et al., 2009). According to the BJCP (2021) guidelines, these styles are expected to be fresh with a clean malt character and without oxidative notes. The presence of furfural imparts almond-like, stale flavours that are inconsistent with these beer styles (Vanderhaegen et al., 2004). The absence of furfural in these beers signified appropriate malt selection, limited thermal stress, and oxygen exposure during production and storage (Vanderhaegen et al., 2004; Mertens et al., 2022).

Furfural was detected in beer styles IPA-Belg, IPA-Eng1, IPA02, IPA04, IPA05, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, and Amber-ale. Its presence or increase in beer indicates oxidation, which can occur at any stage of brewing, particularly during the packaging process. Poor oxygen control during filling and capping can lead to furfural formation (Vanderhaegen et al., 2006; Wietstock, Kunz and Methner, 2016). The detection of furfural in beer samples suggests that many craft breweries face challenges in managing oxygen during packaging. Furfural concentrations ranged from 0.005 mg/L to 0.54 mg/L in Stout1. The threshold flavour of this compound, according to the literature, is 25 mg/L, which is higher than the threshold obtained in this study (Ferreira and Guido, 2018). This showed that the level of concentration found in Stout1 was well below the threshold required to produce a stale, almond-like flavour in this beer style. Low levels of furfural in the beer samples indicated clean malt profiles without oxidative defects and effective brewing and storage practices, which aligned with BJCP guidelines.

Ethyl butyrate was also not detected in the following beer styles: IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale4, Pale-ale5, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale. Ethyl butyrate is an ethyl ester that is formed when ethanol reacts with butyric acid during yeast fermentation. It contributes to a fruity aroma like pineapple or mango, and excessive levels result in cheesy off flavours (Ma et al., 2020). The absence of ethyl butyrate in the beer samples aligned with the BJCP (2021) guidelines that prioritise hops bitterness, a roasted character, and malt complexity over fruity esters. Additionally, its absence demonstrated that fruity ester notes were absent or minimal in these beer styles (Xu et al., 2017).

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Ethyl butyrate was detected in the following beer styles: IPA01, IPA-Belg, IPA-Eng2, Stout1, Stout2, Blonde-ale1, Pale-ale2, Pale-ale3, and Porter-Imp. Ethyl butyrate concentrations ranged from 0.02 mg/L 3.28 mg/L in Porter-Imp. The ethyl butyrate threshold level in the beer was 0.4 mg/L and in accordance with the literature (Xu et al., 2017). In the current study, 3.28 mg/L in Porter-Imp was significantly higher than earlier detected values, indicating that the ethyl butyrate likely imparted an elevated fruity aroma in the tested beer, as the detected concentration was approximately 8x higher than the reported threshold value. Such high ester levels may conflict with the malt-forward character of Porter beer, which is a finding that deviates from the BJCP (2021) guidelines.

However, 4-methyl-1-pentanol was not detected in the following beer styles: IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA02, IPA04, IPA05, IPA06, IPA07, IPA09, IPA08, IPA10, IPA03, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale. 4-methyl-1-pentanol is a higher alcohol produced by yeast in beer during fermentation, and it contributes to a fruity, alcoholic, and sometimes flower-like flavours in beer (Pires et al., 2014; Holt et al., 2019). Elevated levels of this compound in beer are often associated with high fermentation temperatures or excessive yeast growth (Willaert, 2012). The absence of 4-methyl-1-pentanol across these beer styles aligned with the BJCP (2021) guidelines of stylistic integrity and the quality of fermentation.

4-methyl-1-pentanol was detected in the following beer styles: IPA01, IPA-Belg, Stout1, and Stout2. The detected 4-methyl-1-pentanol concentrations ranged from 48.53 mg/L to 1220.946 mg/L in Stout2. This represented a significant variation across styles. The most desirable threshold level of 4-methyl-1-pentanol in beer is not yet indicated in the literature. In the current study, 1220.95 mg/L in Stout2 was excessively higher than the concentrations generally reported in the literature for higher alcohols, which range from 100-300 mg/L (Blanco, Andrés-Iglesias and Montero, 2016). The high concentrations most certainly resulted in elevated levels of a solvent-like, harsh fusel character that exceeded the Stout beer style's stylistic acceptability in line with the BJCP (2021) guidelines.

Ethyl caprate was not detected in the following beer styles: IPA02, Blonde-ale1, Blonde-ale2, Blonde-ale3, and Pale-ale1. Ethyl caprate is a fatty acid ester that is produced in beer during yeast fermentation. This compound contributes to a fruity, apple-like aroma in beer (Zhang et al., 2025). According to the BJCP guidelines, this medium-chain ester is not essential to these beer styles, as it favours hops-forward or clean profiles with restrained ester expression.

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Ethyl caprate was detected in the following beer styles: IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA03, IPA04, IPA05, IPA06, IPA07, IPA08, IPA09, IPA10, Stout2, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale. Ethyl caprate concentrations ranged from 0.006 mg/L to 3.28 mg/L in Porter-Imp. A threshold concentration of ethyl caprate in beer is not mentioned in the literature.

Phenylethyl acetate was not detected in the following beer styles: IPA04, IPA05, IPA06, IPA07, IPA08, IPA10, IPA-Eng3, Pale-ale4, Pale-ale5, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale. Phenylethyl acetate is formed from the enzymatic esterification of phenyl ethanol and acetic acid during yeast fermentation in beer, and it contributes to rose, honey, fruit, and floral aromas (Xu et al., 2017). The beer styles mentioned above generally have hop-derived aromas, malt complexity, and low esters over intense floral sweetness. The absence of phenylethyl acetate in these beers was consistent with the BJCP guidelines.

However, phenylethyl acetate was detected in multiple beer styles, namely IPA01, IPA-Belg, IPA-Eng1, IPA-Eng2, IPA09, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale5, and Porter-Imp. Phenylethyl acetate concentrations ranged from 0.007 mg/L to 11.60 mg/L, while the flavour threshold concentration of 2-phenylethyl acetate in beer is 3.8 mg/L (Methner et al., 2022). The concentration detected in Blonde-ale2 of 11.60 mg/L was nearly 3x above the sensory threshold, leading to an overly perfumed character. Elevated levels of compounds such as 2-phenylethyl acetate can result from elevated temperatures, an increase in ester synthesis, and yeast type (Olaniran et al., 2017; Mitri et al., 2022). Certain styles aligned with the BJCP (2021) guidelines, such as IPA-Belg, the Blonde Ales, and some Stouts, in terms of moderate ester notes due to the 2-phenylethyl acetate compound.

Hexanoic acid was detected in all the tested beer styles (IPA-Belg, IPA-Eng1, IPA02, IPA04, IPA05, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Stout2, Blond-ale1, Blond-ale2, Blond-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, Amber-ale). The concentrations ranged from 0.49 mg/L in Stout2 to 7.78mg/L in Pale-ale1. Hexanoic acid, also known as caproic acid, is a medium-chain fatty acid, primarily produced by yeast during fermentation with apple-like fruity flavours (Cheng et al., 2025). If concentration levels are present above the threshold, it will result in fatty, cheesy, and undesirable off-flavours (House and Acree, 2002). The threshold concentration of hexanoic acid reported in beer is 8 mg/L (Blanco, Andrés-Iglesias and Montero, 2016). All the detected

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concentrations were below this threshold, which aligned with BJCP guidelines (2021) regarding the clean fermentation character of the styles analysed.

Octanoic acid was not detected in the following beer styles: Blonde-ale1, Pale-ale2, Pale-ale3, and Porter-Imp. Octanoic acid, also known as caprylic acid, is a medium-chain fatty acid produced by yeast fermentation. It produces fatty, cheesy flavours when present at high concentrations (Duan et al., 2015). The absence of this compound in these beer styles could be attributed to yeast metabolism that limited fatty acid formation, aligning with the BJCP (2021) guidelines that support clean and balanced flavour profiles without extreme off-flavours.

Octanoic acid was detected in several beer styles, namely IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale4, Pale-ale5, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale. Octanoic acid concentrations ranged from 0.43 mg/L to 5.01 mg/L in IPA01 Its flavour threshold in beer is 14 mg/L (Blanco, Andrés-Iglesias and Montero, 2016). The concentrations of octanoic acid in the detected styles were lower, indicating a negligible direct flavour contribution that resulted in a clean fermentation profile as expected for these beer styles.

Decanoic acid was not detected in the following beer styles: IPA02, IPA04, IPA05, IPA06, IPA08, IPA10, IPA03, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Irish-red-ale, Best-bitter1, and Best-bitter2. Decanoic acid is a medium-chain fatty acid produced by yeast during fermentation. It can contribute soapy, fatty, waxy, or rancid off-flavours when present in excess (Bravi et al., 2017).The absence of this compound aligns with a stylistic expectation of a clean flavour profile, as its presence above the desired threshold can introduce undesirable flavours.

Decanoic acid was detected in several beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, Stout1, Stout2, Altbier, and Amber-ale). Decanoic concentrations ranged from 0.09 mg/L to 0.51 mg/L in IPA01. The decanoic acid threshold in beer is 10 mg/L (Blanco, Andrés-Iglesias and Montero, 2016). The concentrations detected in the study were substantially lower than the reported threshold. These levels were unlikely to impart noticeable flavours, aligning with BJCP expectations for clean fermentation profiles in these styles.

Coniferaldehyde was detected in all the beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2 Blonde-ale3, Pale-

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ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). Coniferaldehyde concentrations ranged from 0.01 mg/L in IPA-Belg to 76.94 mg/L in Best-bitter1. Coniferaldehyde is a cinnamic aldehyde that originates from the thermal degradation of lignin in malt husks during kilning/roasting, and some coniferaldehyde-induced flavours can be derived from oak wood in the barrels during beer maturation. It contributes to a spicy, vanilla-like flavour in beer (Wyler et al., 2015). However, there is no published sensory threshold for coniferaldehyde in beer. Its occurrence across Stout, IPA, Amber Ale, and other styles reflected the wide use of specialty malts in craft ale brewing, regardless of style-specific malt composition. Although the threshold of coniferaldehyde in beers is not documented, the observed concentration range in this study highlights the potential sensory relevance of coniferaldehyde in some craft ales, especially those with elevated levels of this compound.

Syringaldehyde was detected in all the beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). Syringaldehyde concentrations ranged from 0.001 mg/L (IPA04, IPA06, Pale-ale2, Pale-ale3, Porter-Imp, Altbier, and Amber-ale) to 0.05 mg/L in Blond-ale2. Syringaldehyde originates from the degradation of lignin during the malting and brewing processes and contributes smoky, woody, and sweet vanilla-like flavours to beer (Jung et al., 2023). The published flavour threshold for syringaldehyde is 0.02 mg/L (Sterckx et al., 2011). However, earlier studies reported higher thresholds (15 mg/L-50 mg/L), meaning the values obtained in this study were unlikely to contribute a strong, dominant flavour. The syringaldehyde in the beers could have originated from lignin degradation in the speciality malts during kilning or roasting. According to the BJCP (2021) guidelines, syringaldehyde can minimally enhance malt complexity when well balanced, but at high levels (if present), it is undesirable in hops-forward styles.

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4.3.6.3 Residual sugars and sugar alcohols

Residual sugars and sugar alcohols are non-flavour-active compounds that can influence the body and mouthfeel of beer. They do not directly contribute to beer flavour unless present at high concentrations. Their presence or absence in a particular beer style reflects the type of raw materials, yeast, and brewing techniques used (Palmer, 2006; BJCP, 2021). The levels of the following residual sugars and sugar alcohols were measured in this study: D-xylose, L-(+)-arabinose, D-ribose, xylitol, D-fucose + rhamnose, arabitol, D-fructose, D-galactose, D-mannose, D-glucose, mannitol, sorbitol, myo-inositol, sucrose, alpha-lactose, and D-maltose + trehalose.

Xylose was not detected in some beer styles (Irish-red-ale, IPA-Eng2, and Altbier). These styles of beer have dry, well-attenuated finishes in line with the BJCP guidelines; hence, moderate to low residual sugars were expected. Additionally, xylose is a pentose sugar that is not typically fermented by brewing yeast (Lagos et al., 2023). It can contribute to residual sweetness in the final product or indicate the use of adjuncts in brewing (Moysés et al., 2016). The relevance of quantifying this and various other sugars is due to their impact on flavour, body, and microbial stability (Bamforth, 2006). The persistence of non-fermentable sugars influences sweetness and stability, especially in beers where a dry finish is desired.

Xylose was detected in the following beer styles: IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA01, IPA-Eng1, IPA-Eng2, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Best-bitter1, Best-bitter2, Irish-red-ale, Altbier, and Amber-ale. The concentrations ranged from 0.40 mg/L to 60.61 mg/L. Its presence in the finished beer could be attributed to incomplete enzymatic breakdown during the mashing stage or the use of unmalted adjuncts (Laureys et al., 2023). In Best-bitter1, the xylose concentration was the highest compared to the other beer styles; however, it aligned with the BJCP (2021) guidelines for malt-rich beers. These malts are known to contain elevated levels of arabinoxylans, which are a primary source of xylose upon enzymatic hydrolysis during mashing.

L-(+)-arabinose was undetected in some beer styles (Irish-red-ale, Altbier, IPA-Eng2, and IPA03). L-(+)-Arabinose in beer originates from wheat and barley used in brewing (Li and Du, 2019). However, arabinose, just like xylose, is not fermentable by standard yeast ales, so it remains in the final beer as residual sugar. High arabinoxylan content contributes to increased beer viscosity, and its absence in the above beer styles reflected minimal contribution from the raw materials (Moremi, Jansen Van Rensburg and La Grange, 2020).

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However, L-(+)-arabinose was detected in several beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng1, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale5, Porter-Imp, Best-bitter1, Best-bitter2, and Amber-ale). L-(+)-Arabinose concentrations ranged from 1.24 mg/L to 57.94 mg/L, while Best-bitter1 had the highest concentration. Thus, styles like Best-bitter1 often use speciality or husk-rich malts with more arabinoxylan, increasing the potential release of arabinose.

Best-bitter1 showed high levels of xylose and arabinose; these pentoses are not fermentable by *Saccharomyces cerevisiae* and could originate from hemicellulose breakdown in the malt husk or spent grain during mashing or milling, especially when speciality malts are used (Subtil and Boles, 2011; Nijland and Driessen, 2020). Their persistence in the beer may indicate that the brewer's mashing regime may favour high residual sugar, contributing to body and sweetness (Briggs et al., 2004). However, a high level of residual sugars can increase the beer's susceptibility to microbial spoilage, as spoilage microbes can metabolise these substrates.

D-ribose was not detected in two beer styles (Irish-red-ale and Altbier). D-Ribose is not a naturally occurring component in beer that is produced through the brewing process; thus, it is sometimes added to the beer as a dietary supplement (Fico et al., 2008). The absence of ribose, xylose, and arabinose post-fermentation implied negligible carryover of these sugars from raw materials, since these styles are typically described as crisp and clean and are not major contributors to beer flavour. D-ribose was detected in (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA010, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale 1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). The concentrations ranged from 1.47 mg/L to 48.01 mg/L in Best-bitter1.

Xylitol was not detected in the following beer styles: Irish-red-ale, Best-bitter2, IPA01, IPA03, IPA-Eng2, Stout1, Altbier, and Pale-ale4. Xylitol is a sugar alcohol used as a low-calorie sweetener in many products, but it is not a traditional component of beer (Rahayu, Hidayah and Adiandri, 2022). Xylitol is not usually produced by ale yeast in standard fermentation, and its absence supports microbial stability of hops-forward and malt-balanced styles, as per the BJCP guidelines. However, xylitol was detected in several beer styles, namely IPA-Belg, IPA02, IPA05, IPA06, IPA-Eng1, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale5, Porter-Imp, Best-bitter1, and Amber-ale. Xylitol concentrations ranged from 1.19 mg/L to 185.40 mg/L, with Best-bitter1 yielding the highest concentration. This sugar alcohol is not fermentable by ale

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yeast strains(Lambrechts and Pretorius, 2019). Its detection in the latter beer styles could be linked to speciality or husk-rich malts that are often used in these beer styles (Pérez-Flores et al., 2019). The relatively high concentration in Best-bitter1 could be attributed to endogenous or microbial xylanases during mashing, or microbial metabolism during fermentation, resulting in the conversion of xylan-rich material (arabinoxylan) into xylitol. Its presence in certain beers may also suggest intentional addition as a sweetener. This might be attributed to back-sweetening finished beer that is too bitter without non-fermentable sugars to balance it (Toivari et al., 2004; Ha et al., 2011).

D-fucose and rhamnose were not detected in all beer styles except in Amber-ale and Pale-ale1. They are rare in beer, and their presence could be indicative of complex usage of adjuncts or microbial activity (Terebieniec et al., 2025). D-fucose and rhamnose concentrations ranged from 20.92 mg/L 21.23 mg/L in Best-bitter-1. These are 6-deoxyhexose sugars that are not fermentable by the yeast strain and hence persist in the beer (Tao et al., 2021). Rhamnose possibly originates from the plant cell wall of cereal grains, while D-fucose may be derived from glycoproteins associated with malt or fermentation. Although the detected concentrations of these sugars were low, their occurrence could be linked to malt composition and potential microbial activity during brewing.

Arabitol is a type of sugar alcohol produced by certain osmophilic yeast and bacteria, and it was not detected in several beer styles (IPA01, IPA-Belg, IPA-Eng1, IPA-Eng2, IPA02, IPA03, Stout1, Stout2, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale4, Altbier, Irish-red-ale, and Best-bitter2). As it was absent in most beers, it confirmed hygienic practices, as it can be produced by certain undesirable yeasts and fungi (Farias et al., 2022).

However, arabitol was detected in some beer styles (IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Blonde-ale1, Pale-ale2, Pale-ale3, Pale-ale5, Porter-Imp, Best-bitter1, and Amber-ale). Arabitol concentrations ranged 0.52 mg/L to 23.16 mg/L, while Best-bitter1 exhibited the highest concentration. At these observed concentrations, it was unlikely to contribute to flavour or sweetness and was arguably derived from non-*Saccharomyces* yeasts or lactic acid bacteria, or possibly from arabinoxylan-rich malts (Ravikumar et al., 2022).

D-fructose is normally present in malt and adjunct ingredients. Fructose is fermentable by yeast; however, it is consumed at a slower pace than glucose. Fructose was not detected in a few beers (IPA01, Altbier, Irish-red-ale, and IPA-Eng2). Its absence in these beers demonstrated complete fermentation that was

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indicative of their dry nature (BJCP, 2021). Glucose and fructose are simple sugars utilised first by yeast in beer (Maicas, 2020).

D-fructose was detected in most beer styles (IPA-Belg, IPA-Eng1, IPA02, IPA04, IPA05, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Porter-Imp, Best-bitter1, and Amber-ale). Fructose concentrations ranged from 1.58 mg/L to 107.36 mg/L, with Pale-ale3 having the highest concentration. The presence of lactic acid bacteria and non-*Saccharomyces* yeast might have contributed to residual fructose, as they could have impacted the yeast metabolism by competing for simple sugars. Pale Ales, according to the BJCP guidelines, are clean and well attenuated with little residual sugars. At the detected concentrations, they would have no impact on the beer flavour (Cason, Reid and Gatner, 1987; Berthels et al., 2008).

Galactose can be found in beer as it is derived from the breakdown of beta-glucans and arabinoxylans found in barley, through enzymatic hydrolysis during mashing at temperatures around 35–50°C (Li et al., 2020). Galactose was not detected in the following beer styles: IPA-Eng2, IPA03, Blonde-ale2, Altbier, and Stout2. Galactose is less fermentable by standard yeasts. Its absence in the above styles indicated clean fermentations that aligned with the BJCP guidelines for moderate to dry finishes in these beer styles (Quarterman et al., 2016).

Galactose was detected in most beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Blonde-ale1, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, IPA-Eng1, IPA-Eng3, Porter-Imp, Irish-red-ale, Best-bitter1, Best-bitter2, and Amber-ale). Galactose concentrations ranged from 1.31 mg/L to 29 mg/L. Pale-ale3 had the highest concentration. Though at low concentrations, most *Saccharomyces cerevisiae* strains do not ferment galactose efficiently. Its presence could be associated with the metabolism of the brewing yeast; at this concentration, it could influence mouthfeel and potentially serve as a substrate for spoilage bacteria (Huuskonen et al., 2010; Moktaduzzaman et al., 2015).

Mannose is a hexose sugar derived from cereal grains due to mannan-rich hemicelluloses in yeast cell wall degradation during mashing and malting (Faustino et al., 2022). Mannose was not detected in some beer styles (IPA-Eng2, IPA03, Stout1, Blonde-ale2, Pale-ale2, Pale-ale3, Porter-Imp, Altbier, Irish-red-ale, and Best-bitter2). It is partially fermentable by *Saccharomyces cerevisiae* and remains in trace amounts after fermentation to the major wort sugars, with most remaining bound in mannoproteins or other polysaccharides (Walker and Stewart, 2016); Yoon, Mukerjea and Robyt, 2003). The detection of

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small amounts reflected complete fermentation and a low mannose-rich contribution. This finding supported the crisp finish of these beer styles, which was consistent with the BJCP (2021) guidelines.

Mannose was detected in the following beer styles: IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Blonde-ale1, Blonde-ale3, Pale-ale1, Pale-ale4, Pale-ale5, IPA-Eng1, IPA-Eng3, Best-bitter1, and Amber-ale). Mannose concentrations ranged from 1.75 mg/L to 19.01 mg/L in Blonde-ale1. The BJCP (2021) guidelines for Blonde Ales require a malt-balanced and fully attenuated character. However, the low residual sugar in this style is craft-beer-specific and may include limited filtration.

Glucose originates primarily from the starch in grains, especially barley. It is essential for fermentation, providing the fuel for yeast to produce alcohol and carbon dioxide. Glucose was not detected in IPA-Eng2 and Irish-red-ale beer styles. Its absence in these styles indicated full attenuation of the wort, resulting in readily fermentable sugar (Johnson et al., 2022). Glucose was detected in most beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Blonde-ale1, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, IPA-Eng1, IPA-Eng3, Porter-Imp, Best-bitter1, Best-bitter2, and Amber-ale). Glucose levels ranged from 1.55 mg/L to 66.63 mg/L, with Pale-ale3 displaying the highest concentration. Glucose is primarily fermentable by *S. cerevisiae*. The residual sugar in the samples could be linked to incomplete fermentation, malt composition differences, or even microbial interactions (Roselli et al., 2024). Any Pale Ale should be clean, moderately malty, and well attenuated. The trace levels found in this study could be attributed to fermentation variability in craft brewing.

Mannitol was not detected in the following beer styles: Irish-red-ale, Altbier, and IPA03. It is a sugar alcohol that does not originate from the fermentation process. Mannitol is a sugar alcohol that is not typically produced by *S. cerevisiae* but is commonly associated with non-*Saccharomyces* yeasts and food spoilage bacteria such as lactic acid (Wisselink et al., 2004). Thus, its absence suggested microbial control in brewing, aligning with style expectations.

Mannitol was detected in various beer styles (IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Blonde-ale1, Blonde-ale3, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, IPA-Eng1, IPA-Eng3, Porter-Imp, Best-bitter1, Best-bitter2, and Amber-ale). Mannitol concentrations ranged from 1.43 mg/L to 68.26 mg/L, and Pale-ale2 exhibited the highest concentration.

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Thus, the presence of mannitol at this concentration could be attributed to microbial diversity or fermentation dynamics in the craft breweries.

Sorbitol is a sugar alcohol that is not produced by *S. cerevisiae*, but it can be produced by certain lactic acid bacteria and non-*Saccharomyces* yeasts. Sorbitol was not detected in IPA-Eng2, IPA03, Altbier, Irish-red-ale, and Best-bitter2 beer styles. This finding supported the traditional clean fermentation profiles of these styles, as aligned to the BJCP (2021) guidelines. Hence, its presence could indicate unwanted bacterial activity (Zhou et al., 2023).

In fact, sorbitol was detected in some beer styles, including IPA01, IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, Stout1, Blonde-ale1, Blonde-ale3, Pale-ale1, Pale-ale1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, IPA-Eng1, IPA-Eng3, Porter-Imp, Best-bitter1, and Amber-ale. Sorbitol concentrations ranged from 0.79 mg/L to 28.05 mg/L, with Pale-ale2 having the highest concentration. Its detection in Pale-ale2 could be associated with wild yeasts or LAB that are linked to unfiltered craft beers. According to literature, sorbitol in beer may be associated with wild yeasts or LAB, linked to unfiltered craft beers (Park et al., 2016). However, LAB concentration in Pale-ale2 sample was 9.5×10^1 CFU/mL, which was low and not the highest among other samples, even too low to contribute to the production of sorbitol in this sample. Therefore, yeast metabolism with contribution from wort composition and fermentation processes could likely be the primary source of sorbitol (Xu et al., 2018). According to the BJCP (2021) guidelines, clean, well-attenuated Pale Ale styles should not display polyol sweetness.

Sucrose is a sugar that is intentionally added to beer, while some very minor amounts of sucrose might naturally be derived from certain grains; yet, it is not a significant component of beer's original ingredients (Methner et al., 2022). Sucrose was not detected in beer styles Stout1, Stout2, Altbier, Irish-red-ale, the Pale Ales, Best Bitters, Amber Ale, and multiple IPAs. It is fully fermentable, and its absence thus supported complete fermentation (Lopes, Oliveira and Guido, 2025). It can influence body and fermentation characteristics that are appropriate for most ale styles.

Sucrose concentrations ranged from 2.33 mg/L to 6.78 mg/L, while IPA03 had the highest concentration. Sucrose is a disaccharide composed of glucose and fructose. It is rapidly fermented by *S. cerevisiae* through a yeast enzyme, invertase, and is either absent or present in trace amounts in beer. The highest concentration (detected in IPA03) suggested minimal but detectable levels. IPAs should be dry to

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medium-dry with high attenuation, according to the BJCP (2021) guidelines. The trace levels in sucrose concentrations could indicate production variability that is common in IPAs.

Myo-inositol concentrations ranged from 2.61 mg/L to 44 mg/L, with the lowest concentration in Stout1 and the highest in Pale-ale3. Myo-inositol, a cyclic sugar alcohol, is naturally present in barley malt, especially in speciality or husk-rich malts. It is also not fermentable by *S. cerevisiae* and is often found in the final beer, especially in unfiltered beers or beers produced from complex malt grist. The differences in concentrations in Stout1 and Pale-ale3 were associated with differences in malt, microbial activity, and mash conditions. Myo-instil is non-flavour active and its presence doesn't contradict the styles but rather highlights craft-specific practices, such as those associated with unfiltered beers.

Alpha-lactose was not detected in beer styles IPA01, IPA04, IPA05, Stout1, Stout2, Blonde-ale3, Pale-ale2, Pale-ale3, Porter-Imp, Altbier, Irish-red-ale, Best-bitter1, and Amber Ale. The origin of alpha-lactose in beer is by its addition as milk sugar during the brewing process to impart sweetness and a fuller body, particularly in styles like milk stouts and sweet stouts. Its absence in the above beer styles confirmed compliance with the BJCP guidelines. Alpha lactose was detected in (IPA-Belg, IPA02, IPA04, IPA05, IPA06, IPA-Eng3, IPA07, IPA09, IPA08, IPA10, IPA03, Stout1, Stout2, Blonde-ale1, Blonde-ale2, Blonde-ale3, Pale-ale1, Pale-ale4, Pale-ale5, Best-bitter2). ranged from 2.25 mg/L to 15.06 mg/L, while Pale-ale4 had the highest concentration. Lactose is non-fermentable by *S. cerevisiae* and is useful in increasing residual sweetness and body, especially in milk stouts. The presence of alpha lactose at the concentration of 15.06 mg/L in the Pale-ale4 and other ale styles despite its absence from the declared ingredient list, was unexpected. This could be attributed to the addition of adjuncts such as lactose-containing ingredients or cross-contamination from the equipment used in brewing lactose-based flavourings.

D-maltose and trehalose were not detected in some beer styles (IPA01, IPA04, IPA05, Irish-red-ale, Altbier, IPA-Belg, Stout1, Stout2 and Blond-ale3, Pale-ale2, Pale-ale3 Best-bitter1). Maltose is a disaccharide composed of two glucose units. It makes up 60% of total fermentable sugars and is efficiently metabolised by *S. cerevisiae* during beer fermentation (Rautio and Londesborough, 2003). Its absence indicated complete sugar conversion and fermentation efficiency, which are typical of these beer styles and aligned with BJCP (2021) expectations for dry or moderate residual sweetness. D- maltose and trehalose concentrations was detected in several beer samples (IPA-Belg, IPA-Eng1, IPA02, IPA03, IPA06, IPA07, IPA09, IPA08, IPA1, IPA-Eng1, IPA-Eng2, Blonde-ale1, Blonde-ale2, Pale-ale1, Pale-ale4, Pale-ale5, Best-bitter2) with concentrations range from 1.31 mg/L to 1.10 mg/L and Pale-ale4 had

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the highest concentration. Maltose is a disaccharide composed of two glucose units fermentable by *S. cerevisiae*. The persistence of maltose in the beer at this level could be attributed to incomplete fermentation, high original gravity (or sugar load), or inefficiency of maltose uptake by yeast strains. According to the BJCP guidelines (2021), this style should be well attenuated and dry; however, in modern or unfiltered craft beers, it can remain within acceptable stylistic levels.

4.3.7 Spearman correlation analysis

The previous section focused on individual flavour-active and non-flavour-active compounds. It was subsequently important to determine the relationships among all the measured parameters across the beer styles. Hence, a multivariate Spearman correlation analysis was performed to identify correlations among microbial content, sugar levels, alcohol content, phenolic compounds, and mycotoxins. Spearman's rank correlation is suitable for chemical and microbiological datasets, as it identifies monotonic relationships without assuming normal distribution. The strength of the correlation (R-values) was interpreted according to the ranges applied in this study: 0.40–0.69 = moderate, 0.70–0.89 = strong, 0.90–1.0 = very strong (Schober and Schwarte, 2018). Only correlations within these ranges are discussed (Figure 4.7)

A strong positive correlation was observed between yeast and LAB, which was consistent with their synergistic interactions during fermentation. Yeast and LAB often co-exist in natural fermentation systems and can interact in both mutually supportive and inhibitory interactions, depending on environmental conditions (Carbonetto et al., 2020). Additionally, vitamins and amino acids produced by the yeast can support the growth and proliferation of LAB in beer (Chen et al., 2022). This may lead to an increase in LAB populations that can result in off-flavours and taste such as bitterness, sourness, and a cheesy taste (Kordialik-Bogacka, 2022). Moderate correlations were observed between TPC and LAB counts, indicating that while an increase in microbial population might have been accompanied by an increase in LAB, other factors (pH, temperature, storage conditions, etc.) most likely influenced the LAB population and the total beer fermentation quality (Preetha and Narayanan, 2020). Similarly, moderate correlations between mould counts and TPC were observed, indicating that moulds contributed partially to the total microbial population. This means that other microorganisms, like bacteria and yeast, also played a role in the total determination of TPC.

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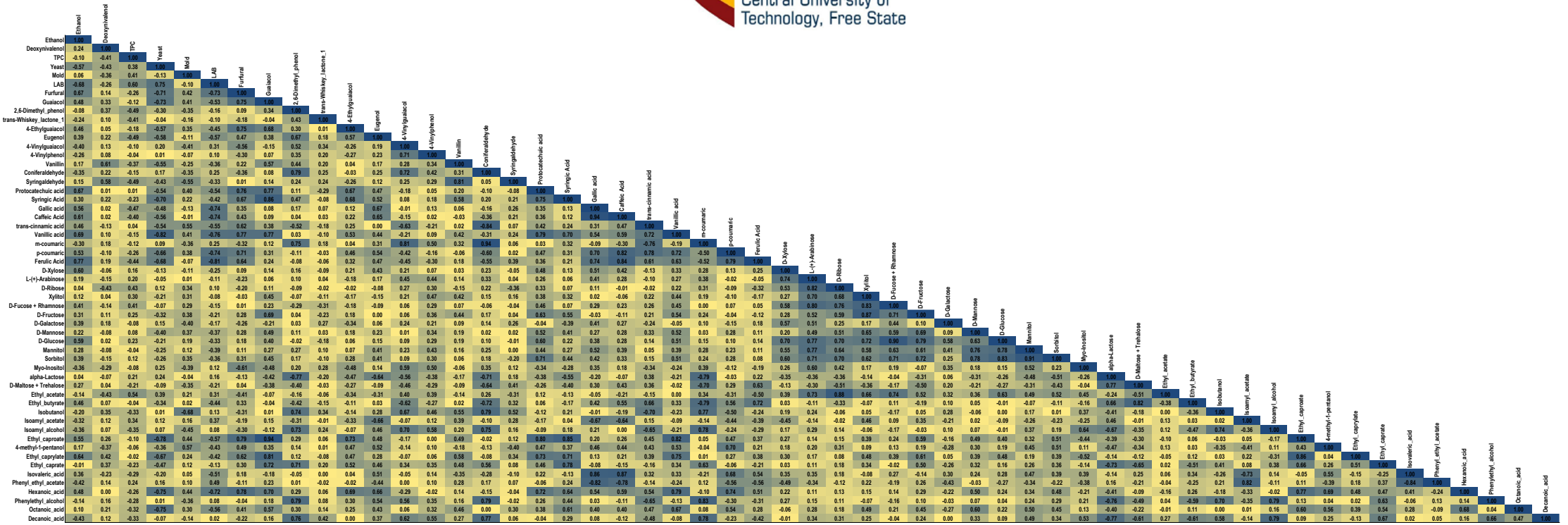


Figure 4.7: Correlation matrix of measured viable microbes and chemical compounds. The strength of the correlation (R-values): 0.40–0.69 = moderate (yellow), 0.70–0.89 = strong, 0.90–1.0 = very strong (blue) Click or tap here to enter text.

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Ethanol demonstrated moderate negative correlations with yeast, LAB, and decanoic acid, which was consistent with its antimicrobial properties and suppressive effects on some microbial communities (Viegas et al., 1989; Sivaranjani, Liu and White, 2021). Contrarily, ethanol correlated moderately positively with furfural, hexanoic acid, guaiacol, 4-ethylguaiacol, protocatechuic acid, gallic acid, caffeic acid, trans-cinnamic acid, vanillic acid, p-coumaric acid, D-xylose, D-fucose + rhamnose, D-glucose, ethyl butyrate, ethyl caproate, and ethyl caprylate, and strongly positively with ferulic acid. These relationships revealed that alcohol reflected fermentative activity of the yeast, leading to phenolic release, sugar metabolism, and ester formation that, collectively, contributed to aroma and flavour development (Besançon et al., 2024). Negative moderate correlations with 4-vinylguaiacol and phenylethyl acetate highlighted pathway competition, where ethanol accumulation could have coincided with reduced conversion of certain phenolic precursors and acetate ester synthesis.

Deoxynivalenol (DON) demonstrated a moderate negative correlation with TPC and yeast, reflecting its potential inhibitory or toxic effects on microbial populations. This was consistent with the known toxic properties of DON on biological systems (Jeong et al., 2025). DON showed moderate positive correlations with vanillin, ethyl caprylate, and syringaldehyde; for this reason, a question arises whether elevated levels of these compounds may be linked to mycotoxin presence. This potential association could have been mediated by yeast stress responses where mycotoxins acted as mild stressors, prompting yeast to alter metabolism and produce higher levels of phenolic compounds or esters (Bauer and Pretorius, 2019). Negative moderate correlations with ribose and ethyl acetate suggested that DON contamination could be associated with altered sugar metabolism and reduced formation of certain aroma compounds. These findings indicated that mycotoxin accumulation did not occur in isolation but was integrated into broader microbial and chemical networks that influenced both safety and sensory outcomes, as posited by Berthiller et al. (2013).

Microbial activity strongly regulated phenolic and volatile compound dynamics. Yeast exhibited strong negative correlations with furfural, syringic acid, guaiacol, vanillic acid, ethyl caproate, hexanoic acid, and octanoic acid, demonstrating yeast-driven depletion or transformation of these compounds, as suggested by Bauer and Pretorius (2019). Moderate negative correlations with broader phenolics, including 4-ethylguaiacol, vanillin, caffeic acid, ferulic acid, and p-coumaric acid, highlighted yeast phenolic acid decarboxylase activity, converting hydroxycinnamic acids into vinyl and ethyl phenols (Swiegers et al., 2005). LAB exhibited strong negative correlations with p-coumaric, gallic, caffeic, ferulic, vanillic acids, as well as furfural and hexanoic acid, reflecting LAB-mediated metabolism of phenolic precursors and aldehydes (Filannino et al., 2014; Olaniran et al., 2017). Conversely, LAB correlated positively with

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octanoic acid and phenyl ethyl acetate, suggesting that LAB metabolism could have promoted medium-chain fatty acid and ester accumulation either directly or indirectly through yeast-mediated metabolic changes (Swiegers et al., 2005). Mould showed moderate positive correlations with furfural, guaiacol, trans-cinnamic acid, and medium-chain fatty acids, including hexanoic acid and ethyl caproate. This was consistent with fungal metabolism of lignocellulosic-derived compounds and lipoxygenase-driven fatty acid degradation (Andlar et al., 2018). Negative correlations with sugars, such as D-galactose, and higher alcohols, including isobutanol and isoamyl alcohol, highlighted its competitive role in carbon utilisation.

P-coumaric acid displayed strong positive correlations with ferulic acid and hexanoic acid, while moderate positive correlations occurred with ethyl butyrate, ethyl caproate, 4-methyl-1-pentanol, isovaleric acid, and octanoic acid, which indicated that higher p-coumaric levels coincided with enhanced esterification and higher alcohol synthesis (Coghe et al., 2004; Hazelwood et al., 2008). Moderate negative correlations with isobutanol, isoamyl acetate, and phenyl ethyl acetate indicated pathway competition, where extensive hydroxycinnamic acid metabolism could have diverted precursors away from branched-chain amino acid catabolism and acetate ester formation (Verstrepen et al., 2003; Swiegers et al., 2005; Hazelwood et al., 2008). In contrast, m-coumaric acid correlated negatively with p-coumaric and ferulic acids, α-lactose, D-maltose plus trehalose, and ethyl butyrate, while exhibiting strong positive correlations with isobutanol, isoamyl alcohol, phenyl ethyl alcohol, and decanoic acid, highlighting its association with fusel alcohol and long-chain fatty acid synthesis (Swiegers et al., 2005). Gallic acid showed strong positive correlations with the caffeic, ferulic, p-coumaric, and isovaleric acids, linking phenolic metabolism with fatty acid degradation products. Trans-cinnamic acid correlated positively with the p-coumaric and vanillic acids, indicating its role as a central branching point in phenolic catabolism.

Sugars and sugar alcohols formed highly interconnected networks. D-xylose correlated strongly positively with L-(+)-arabinose, moderately with D-ribose, D-fucose + rhamnose, D-galactose, D-glucose, mannitol, and sorbitol, and negatively with isoamyl acetate and phenyl ethyl acetate. L-(+)-arabinose strongly correlated with D-ribose, xylitol, D-fucose + rhamnose, D-glucose, mannitol, sorbitol, and ethyl acetate, and moderately with D-fructose, D-galactose, and D-mannose, while myo-inositol, D-ribose, xylitol, D-fucose + rhamnose, D-fructose, D-galactose, D-mannose, D-glucose, mannitol, sorbitol, and myo-inositol all showed a web of moderate to strong positive correlations among one another, with aroma-active esters, and with fatty acids (ethyl acetate, isoamyl acetate, ethyl caproate, ethyl caprylate, and octanoic acid), reflecting coordinated sugar utilisation and metabolite synthesis (Piddocke et al., 2011; Olaniran et al., 2017). Conversely, α-lactose and D-maltose + trehalose displayed negative correlations with fusel (higher) alcohols and medium-chain fatty acids, indicating that residual complex sugars were inversely

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associated with higher alcohol and fatty acid formation, as proposed by Postigo et al. (2022). Mannitol and sorbitol are strongly intercorrelated and positively associated with 4-methyl-1-pentanol, hexanoic acid, and octanoic acid. Myo-inositol was moderately positively correlated with ethyl acetate, isoamyl alcohol, and decanoic acid, and negatively with ethyl caproate and ethyl caprylate.

Volatile compounds demonstrated interdependent behaviour, which was expected as it reflects yeast metabolic connectivity. Isobutanol correlated strongly with isoamyl alcohol and phenyl ethyl alcohol, and moderately with decanoic acid. Isoamyl alcohol showed strong positive correlations with phenyl ethyl alcohol and decanoic acid, whereas isoamyl acetate displayed moderate negative correlations with 4-methyl-1-pentanol, strong negative correlations with isovaleric acid, and strong positive correlations with phenyl ethyl acetate. Ethyl caproate correlated moderately with 4-methyl-1-pentanol, octanoic acid, and ethyl caprate, and strongly with ethyl caprylate and hexanoic acid. 4-Methyl-1-pentanol showed moderate positive correlations with isovaleric acid, hexanoic acid, and octanoic acid. Ethyl caprate displayed moderate positive correlations with phenyl ethyl alcohol, octanoic acid, and decanoic acid. Isovaleric acid exhibited a strong negative correlation with phenyl ethyl acetate. Hexanoic acid was moderately positively correlated with octanoic acid, octanoic acid was moderately positively correlated with decanoic acid, and phenyl ethyl alcohol correlated positively with decanoic acid. Positive correlations among higher alcohols originate from shared amino acid precursors via the Ehrlich pathway, while correlations between alcohol and esters reflect acetyl-CoA availability and alcohol acetyltransferase activity. Medium-chain fatty acids and ethyl esters are linked through fatty acid biosynthesis. Negative correlations could likely arise from percussor competition (Swiegers et al., 2005; Hazelwood et al., 2008; Saerens et al., 2010).

Overall, these results demonstrated that beer composition, aroma, and safety emerged from highly interconnected microbial and chemical networks, where yeast and LAB dominated fermentation dynamics, mould contributed to aldehyde and fatty acid accumulation, phenolic acids acted as metabolic hubs, sugar and sugar alcohol metabolism regulated ester and higher alcohol formation, ethanol mediated both microbial suppression and metabolite accumulation, and mycotoxins such as DON integrated with microbial and chemical processes to influence both the quality and safety of the craft beer products.

4.4 CONCLUSIONS

This chapter provided an integrated assessment of the microbial and chemical profiles of the selected South African craft ales, with a focus on microbial plate counts, chemical thresholds of flavour-active

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compounds, residual sugars and sugar alcohols, and correlations among key variables. The results highlighted substantial variability across breweries and beer styles, reflecting both the speciality production styles and potential contamination risks associated with the various beer breweries.

Microbial analyses revealed that total plate counts (TPC) varied widely, ranging from non-detectable to 1.630×10^5 CFU/mL, with the highest levels recorded for Stout2, Best-bitter1, and IPA, where the highest counts were 1.630×10^5 CFU/mL, 1.530×10^5 CFU/mL and 1.550×10^4 CFU/mL, respectively. Yeast counts were detectable in just over half of the samples, with particularly high populations in Best-bitter1 (1.165×10^5 CFU/mL) and Pale-ale1 (8.45×10^4 CFU/mL). In contrast, mould contamination was relatively rare but present in 17% of the beer sampled from the breweries. Notably, it was found in Blonde Ales and one IPA, where low yeast levels coincided with higher mould counts, suggesting weaker competitive suppression. LAB were detected in 60% of the breweries' beer, reaching as high as 1.25×10^5 CFU/mL in Best-bitter1. The co-occurrence of LAB and *Staphylococcus aureus* in Pale-ale2 indicated possible post-fermentation contamination, which most likely occurred during bottling, and this finding highlighted gaps in hygiene management in some microbreweries.

Chemical profiling demonstrated the complexity of flavour-active compounds and their potential sensory impact. Several compounds consistently exceeded flavour thresholds, suggesting direct contributions to aroma and taste. Isoamyl alcohol, detected in all beer styles at concentrations of up to 178.9 mg/L, was far above its threshold, confirming its major role in fusel character. Likewise, 4-vinylguaiacol (0.137–10 mg/L) was present in all beer styles, frequently above its clove-like sensory threshold. Isoamyl acetate exceeded its fruity threshold in the selected beers, such as in Imperial Porter, while ethyl caprylate surpassed its threshold only in one IPA sample. Syringic and vanillic acids were detected in all beers, with vanillic acid reaching an exceptionally high level (76.7 mg/L) in Porter-Imp, exceeding reported literature ranges. Similarly, coniferaldehyde was 76.9 mg/L in Best-bitter1, indicating a significant phenolic contribution. Esters such as phenylethyl acetate and ethyl butyrate, though variable across beers, also surpassed thresholds in specific styles, enhancing fruity and floral complexity. These findings suggest that while many compounds remained below thresholds, a subset—particularly fusel alcohols, phenolic acids, and esters—likely shaped the dominant sensory attributes of the sampled South African craft ales in this study.

Residual sugars and sugar alcohols were also widely detected, providing insight into the degree of fermentation completeness and their potential role in shaping body and mouthfeel. Xylose reached up to 60.6 mg/L in Best-bitter1, while arabinose concentrations peaked at 57.9 mg/L in the same beer style.

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Xylitol was found at a comparatively high level (185.4 mg/L), suggesting its persistence as an unfermented polyol. Other sugars such as fructose (up to 107.4 mg/L in Pale-ale3) and glucose (66.6 mg/L in Pale-ale3) reflected incomplete attenuation in some styles, while mannose and sorbitol, though lower in concentration, were also present in selected Blonde and Pale Ales. Myo-inositol (2.6–44.0 mg/L) and mannitol (up to 68.3 mg/L) were consistently detected, further indicating variable sugar metabolism during fermentation. The broad range of residual sugars and sugar alcohols across styles highlighted differences in yeast performance and raw material contributions, with potential implications for mouthfeel, sweetness perception, and overall balance of the beers. Although not directly flavour-active at the concentrations observed, these compounds may have contributed to fuller body and sweetness in beers where concentrations were elevated.

Correlation analyses further revealed very strong, strong, and moderate associations among compounds, emphasising the interconnected nature of fermentation biochemistry. Positive correlations between higher alcohols (e.g., isobutanol, isoamyl alcohol) and esters (e.g., phenylethyl acetate, ethyl caprate) reflected the shared precursor pathways of amino acid and sugar metabolism, linking sugar availability to ester synthesis and fruity-floral aroma potential. Similarly, strong correlations between phenolic acids (such as ferulic and p-coumaric acid) and their volatile derivatives highlighted the influence of raw material composition and yeast decarboxylation activity. Negative correlations between certain residual sugars (e.g., maltose, trehalose) and flavour-active compounds pointed to differences in attenuation efficiency, where incomplete fermentation resulted in higher sugar levels but fewer secondary metabolites. The co-occurrence of sugars with alcohols such as mannitol and xylitol also suggested differential yeast utilisation patterns, particularly in beers with weaker fermentation control. These correlations provided mechanistic insight into how yeast metabolism, raw material contributions, and microbial interactions collectively shaped both the chemical and sensory profiles of the beers.

Overall, the results demonstrated that sampled South African craft ales may be characterised by microbial diversity, chemical richness, and complex metabolic linkages. The detection of threshold-exceeding flavour-active compounds confirmed their strong sensory potential, while the presence of measurable residual sugars and sugar alcohols added another layer of complexity related to fermentation performance and beer texture. Correlation patterns further emphasised the interconnected nature of sugar metabolism, higher alcohol and ester production, and phenolic transformation, highlighting both the strengths and weaknesses of the craft brewing processes under study. However, in some cases, concentrations of flavour compounds and sugars reached levels that risked sensory imbalance, pointing to variable control over fermentation outcomes. Moreover, microbial counts revealed that a considerable

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proportion of the breweries operated near the margin of acceptable quality, with yeast–mould dynamics and LAB contamination pointing to inconsistent hygiene practices. The integration of microbial counts, flavour thresholds, residual sugar profiles, and correlation analysis offered valuable insight into the interplay between fermentation quality, sensory attributes, and contamination risks in the emerging South African craft beer sector.

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Chapter 5:
ASSESSING MICROBIAL DIVERSITY IN SOUTH AFRICAN CRAFT ALE BEERS AND THE INFLUENCE OF FLAVOUR-ACTIVE COMPOUNDS

ABSTRACT

In South Africa, the craft brewing industry is experiencing a surge, with an increasing number of beer enthusiasts seeking to revive and refine local beer recipes. There is an increasing number of studies that show the role of microbial diversity in the production of flavour-active compounds, yet little is known about the microbial diversity of South African craft ales and the possible influence of flavour-active compounds on the microbiota. In this study, different craft beer styles from thirty microbreweries were analysed to evaluate their microbial compositions and the possible influence of flavour-active compounds on the microbial composition and structure.

Microbial diversity was assessed using Single Molecule Real-time sequencing (PacBio platform), a high-throughput DNA sequencing technique targeting the 16S rRNA gene for bacteria and the ITS region for fungi. The results showed that at the phylum level, the most abundant bacterial taxa were Proteobacteria (relative abundance, RA: 62%), followed by Firmicutes (RA: 25%), while Ascomycota (RA: 99%) was the dominant fungal taxon. At the genus level, *Acetobacter* (RA: 100%) and *Levilactobacillus* (RA: 92.26%) were the most predominant bacterial genera, while *Saccharomyces* (85.14%) dominated the fungal genera across craft beer samples. At the species level, *Acetobacter orientalis* (RA: 100%) and *Kluyvera ascorbate* (RA: 100%) were the most abundant bacterial taxa, and *Saccharomyces cerevisiae* (RA: 88.45%) dominated the fungal taxa. Furthermore, *Enterobacter cloacae*, *Cutibacterium acnes, Cladosporium herbarum* and *Raoultella ornithinolytica*, as well as spoilage and a mycotoxin-producing organism such as *Penicillium brevicompactum*, were also present in a few craft beer samples.
The alpha diversity analysis of bacterial and fungal species showed no statistically significant differences in bacterial or fungal richness and evenness across the craft beers ($p = 0.4561$). Likewise, permutational analysis of variance (PERMANOVA) revealed that there are no statistically significant differences in the microbiota among the beer styles ($p = 0.223$ (bacteria) and $p = 0.085$ (fungi)). A distance-based Redundancy Analysis (db-RDA), used to investigate the influence of key flavour-active compounds on the microbial community compositions across the South African craft beer samples suggested limited

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predictive power of the environmental variables (key flavour-active compounds) in explaining microbial community composition because each overall model was not statistically significant ($p = 0.43$ (bacteria) and $p = 0.60$ (fungi).

These findings revealed the microbial composition and structure of the examined South African craft beers and suggested that the selected flavour-active compounds do not influence the microbial community. Furthermore, the presence of microbial contaminants, such as *Enterobacter cloacae*, *Cladosporium herbarum, Cutibacterium acnes* and *Raoultella ornithinolytica*, as well as a spoilage mycotoxin-producing organism, such as *Penicillium brevicompactum* in some of the craft beers highlights the need for critical quality control strategies to prevent beer contamination and preserve the product's stability.

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5.1 INTRODUCTION

Beer is one of the most widely consumed alcoholic beverages globally, with craft brewing gaining significant popularity due to the unique flavours, ingredients, and techniques (Medina et al., 2023). South Africa is no exception to this trend, as it boasts a growing craft beer industry that emphasises quality and innovation (Collins, Rogerson and Rogerson, 2023). Craft beers often showcase a diverse range of flavours and aromas that are influenced by both the choice of raw materials and microbial activity during fermentation and storage. Unlike mass-produced larger beers, the fermentation process of ale beers allows a broad range of microorganisms to contribute to their flavour complexity. Microbial diversity plays a significant role in the chemical composition of craft beers because microorganisms produce flavour-active compounds (Olaniran et al., 2017).

Beer is a complex chemical matrix resulting from chemical and metabolic reactions (Gastl and Henle, 2023). The quality attributes of these beers are crucial for consumers who desire specific characteristics associated with their visual appearance, such as brightness, sparkle, and a stable head form (Carvalho et al., 2017). The second most important impression is the flavour and aroma of the beer; hence, brewers strive to balance characteristics appropriately while avoiding off-flavours (Lentz, 2018). There are approximately 1,000 to 2,000 chemical compounds in beer that may react and interact at all stages of the brewing process (Olaniran et al., 2017; Lentz, 2018). The proportional amount is composed of over 800 different volatile compounds. Still, only a small proportion of these can be flavour-active, meaning they produce a flavour sensation when the product is consumed (Olaniran et al., 2017). The mouthful attributes and the array of flavours and aromas in beer styles worldwide result from varying degrees of many inorganic and organic compounds (Lentz, 2018). These compounds belong to various chemical groups, including volatile phenols, higher alcohols, esters, fatty acids, sulphur compounds, carbonyl compounds, furanic compounds, C13-norisoprenoids, and monoterpenols.

Although there are many different beer styles, they are commonly classified into two categories: ale produced by ‘top’ fermentation, and larger beers produced by bottom fermentation (Salanță et al., 2020). Most ale beers are fermented by *Saccharomyces cerevisiae*, while the fermentation of lagers is usually attributed to *Saccharomyces pastorianus* (Monerawela and Bond, 2017). However, non-Saccharomyces yeasts, lactic acid bacteria (*Lactobacillus* and *Pediococcus*), acetic acid bacteria, and other environmental microbes can contribute to unique sensory flavours in beer by producing flavour-active compounds such as phenolic compounds, higher alcohols, and esters (Holt et al., 2018). While

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Saccharomyces yeasts carry out the main fermentation, wild yeasts such as *Brettanomyces* can produce phenolic and ester compounds in beer, contributing to spicy and fruity aromas (Bossaert et al., 2019).

Owing to its intrinsic nature, such as low pH (3.8 – 4.7), hop bitter compounds (17 – 55 ppm of iso-ά-acids), high carbon dioxide (0.5% w/w) and a professed lack of nutritive substance, beer is protected from unfavourable microorganisms (Olaniran et al., 2017). However, despite these attributes, some microorganisms can survive in beer, leading to spoilage or unpleasant sensory changes in beer flavour. Lactic acid bacteria, for example, are known to produce lactic acid and diacetyl, which are appreciated in certain beers, whereas in others, they are considered off-flavours in high concentrations (Bokulich and Bamforth, 2013). Additionally, many strains of brewer's yeast (*Saccharomyces cerevisiae*), including *Lactobacillus* and *Pediococcus*, encode a phenolic acid decarboxylase enzyme (PAD) (Vanbeneden, Van Roey, et al., 2008). This enzyme converts hydroxycinnamic acids, which are compounds with high flavour thresholds naturally present in beer and wort, into aroma-active volatile phenols such as 4-vinylguaiacol (4VG) and 4-vinylphenol (4VP). These have been appreciated in certain beers, including Belgian Wit, German Weissen, and Rauchbeers, where they contribute to the characteristic spice, clove-like, and phenolic aromas and flavours. However, in other beer styles, they cause off-flavours at high concentrations (Lentz, 2018).

Microbial diversity studies in South African craft ales are of interest due to the potential influence of microorganisms on raw material selection, local brewing environments and the final quality of the craft beer. In a study conducted by Rodhouse and Carbonero (2019) on several American craft beers, Illumina MiSeq sequencing indicated that bacterial populations were substantially shaped by both brewing processes and equipment, with similar taxa detected in beer and equipment swabs. Piraine, Leite, and Bochman (2021) applied deep sequencing to sour beers produced in European craft breweries, revealing diverse bacterial and fungal communities between spontaneous and inoculated fermentation methods and demonstrating the influence of production techniques on microbial diversity.

In a South African beer study, Hlangwani et al. (2023) used full-length 16S rRNA and fungal sequencing to investigate traditional sorghum beer (umqombothi), documenting the dominance of lactic acid bacterial and fungal diversity shaped by local raw materials and small-scale production practices. Similar observations were recorded for other fermented beverages, such as Chinese liquors and Daqu starters, where complex microbial communities closely associated with flavour compounds were revealed by High-Throughput Sequencing (HTS) (Wei et al., 2018; Wang et al., 2021). These findings have provided valuable parallels for understanding microbial diversity and flavour development in craft beers.

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Collectively, the cited studies indicate that, while some knowledge exists on microbial diversity in craft and traditional beers, gaps remain in the context of South African craft ales. Therefore, investigating microbial diversity in South African craft ales is highly significant, as microorganisms influence not only the process of fermentation, but also the use of raw materials, the selection of brewing environments and, overall, the quality and safety of the final product. The previous chapter revealed microbial contamination—particularly lactic acid bacteria—in 60% of the selected South African craft breweries, which could be associated with small-scale production practices, minimal use of pasteurisation and filtration, and reliance on basic brewing equipment (Hlangwani et al., 2020). However, those findings were based on culture-dependent methods, which, although useful, have inherent limitations in detecting the full microbial community. The present chapter reports on the application of genetic screening approaches to gain a more comprehensive understanding of the problem under investigation and to offer a broader and more accurate view of the microbial diversity in the selected South African craft ales.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection

Section 4.2 provided a detailed description of the craft beer sampling procedures. Briefly, beer samples were purchased from different breweries and stores to ensure a diverse representation of microbial and chemical profiles. They were transported to the laboratory and refrigerated until further analysis.

5.2.2 DNA extraction, 16S rRNA, and ITS gene high-throughput sequencing

Genomic DNA was extracted from each craft beer sample using the DNeasy PowerSoil Pro kit (QIAGEN, USA). The genomic DNA extracts were sent to Inqaba Biotech™, Pretoria, South Africa, for high-throughput sequencing. Briefly, each DNA extract was quantified using the Qubit™ fluorometer with a Qubit dsDNA BR assay kit (ThermoFisher Scientific, South Africa). The universal primer sets of 27F (AGRGTTYGATYMTGGCTCAG) and 1492R (RGYTACCTTGTTACGACTT) for bacteria, and ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) for fungi were used to amplify the full-length 16S rRNA and ITS genes from the genomic DNA. The forward and reverse 16S rRNA and ITS primers were tailed with sample-specific PacBio barcode sequences (PacBio M13 barcodes) for multiplexed sequencing. Negative controls were included.

The KAPA HiFi Hot Start DNA Polymerase (KAPA Biosystems) was used to perform PCR amplification, with initial denaturing at 95°C for 3 min followed by 25 amplification cycles consisting of denaturing at

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98°C for 20 s, annealing at 57°C for 15 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 60 s. Post amplification, quality control was performed. The DNA amplified from each sample was pooled in equimolar concentration before library preparation, and the SMRTbell clean-up bead-based (PacBio, USA) purification step was then performed. Following the manufacturer's procedure, the amplified DNA pool was processed using the SMRTbell Prep kit 3.0 (PacBio, USA).

The fluorescence concentration and average size of the library pool were measured using the Qubit 1X dsDNA HS assay kit (Qubit™ Fluorometer, Invitrogen, USA) and the 5400 Fragment Analyzer (Agilent Technologies, USA). Sequencing primer annealing and polymerase binding steps were carried out using the Sequel II Binding kit 2.1 and the Sequel II DNA Internal Control Complex 1.0 (PacBio, USA) before loading the pool into the sequencer. Sequencing was performed using the Sequel II Sequencing Kit 2.0 (PacBio, USA) on the Sequel II PacBio system at Inqaba Biotec, Pretoria, South Africa.

5.2.3 Data processing and statistical evaluation

The PacBio data were processed using the DADA2 R Statistics package (v1.20.0) (Callahan et al., 2016; R Core Team, 2012). The primer sequences were first removed, and the reads were trimmed by length. Reads exceeding 5 expected errors were removed, sequencing errors were dereplicated, and the error rate was estimated using loessErrfun. True sequence variants were inferred, and the forward and reverse reads were merged to obtain the full denoised sequences with a minimum overlapping region of 15 bp. Amplicon Sequence Variants (ASVs) identified as chimeric were removed, and the non-chimeric reads were annotated to SILVA v.138.1 and UNITE v.8.2 databases (Kõljalg et al., 2013; Quast et al., 2013; Yilmaz et al., 2014; Bengtsson-Palme et al., 2015).

The data were demultiplexed and the quality check was done following the DADA2 R Statistics package (v1.20.0) (Dessau and Pipper, 2008; Callahan et al., 2016) that briefly, reorients the sequences, removes forward and reverse primers, filters and trims the sequences by length and average quality, dereplicates and estimates sequencing errors using PacBio error's model, and infers the compositions of the samples. ASVs identified as chimeric were removed, and the remaining ones were annotated (Callahan et al., 2016; Quast et al., 2013; Yilmaz et al., 2014).

The alpha rarefaction curve was constructed with 1,000 iterations, to assess whether the diversity present in all samples was adequately captured. Alpha diversity indices (Observed, Chao 1 and Shannon index) were calculated using the vegan R package (Oksanen et al., 2020). Principal coordinate analysis (PCoA)

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based on the Bray-Curtis distance index was used to map the bacteria and fungi in the craft beer samples. Subsequently, the permutational multivariate analysis of variance (PERMANOVA) was used to test the significance of differences in the bacterial and fungal communities of the craft beer samples. Distance-based redundancy analysis (db-RDA) was conducted to assess the relationship between the microbial community (response group) and the chemical parameters (explanatory group) in the selected South African craft beers. Statistical analyses and visualisation were performed using R4.1.0 (Dessau and Pipper, 2008).

5.3 RESULTS AND DISCUSSION

5.3.1 Microbial diversity in the selected South African craft ale beers

5.3.1.1 Composition of bacterial communities

High-quality sequencing reads were classified at the phylum, genus, and species levels. At the phylum level, the most abundant bacterial taxa were Proteobacteria (62%), followed by Firmicutes (25%), Cyanobacteria (9.79%), and Actinobacteriota (2.5%) (Figure 5.1). Other phyla, including Bacteroidota (0.23%) and Planctomycetota (0.05%), exhibited low relative abundances that were below the defined threshold (<1%) (Nie et al., 2024).

The dominance of Proteobacteria across all samples aligned with global observations in fermented grain beverages, confirming their prevalence in the sampled South African craft ale beers. Proteobacteria, especially the family Acetobacteraceae, are known for their role in oxidative fermentation, which results in the accumulation of acetic acid, which, in turn, contributes to increased acidity and the formation of vinegar-like off-flavours (Suzuki, 2011). Their abundance indicated possible exposure to oxygen during fermentation and could accelerate spoilage. On the other hand, species of Proteobacteria may be involved in ester production, thereby increasing the complexity of the fruity aroma of craft ale beer (De Roos and De Vuyst, 2019). The presence of Firmicutes in beer was an indication of either intended mixed fermentation, raw material or brewery equipment contamination(De Roos, Vandamme and De Vuyst, 2018; Rodríguez-Saavedra, González de Llano and Moreno-Arribas, 2020).

Cyanobacteria (9.79%) and Actinobacteriota (2.5%) were also found. Cyanobacteria, which are generally found in freshwater, can impart unwanted medicinal flavours if the treatment of brewing water is not regulated (Peces-Pérez et al., 2022). Its presence at high levels raises questions about its potential impact on beer quality and flavour, although sensory effects were not directly evaluated in this study. Smaller breweries may be more prone to cyanobacteria contamination, but water quality and hygiene are

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likely more important than production scale. Actinobacteriota are commonly found in malted barley and are known to intensify earthy aromas through secondary metabolic products. However, their effect on beer flavour remains unknown (Cordovez et al., 2015; Schulz-Bohm, Martín-Sánchez and Garbeva, 2017).

At the genus level, bacterial taxa were assigned to all samples except for Stout1 and Stout2 (Figure 5.2), possibly because there were no reference sequences available in the database. The dominant bacterial genera identified in the remaining samples include *Acetobacter*, *Levilactobacillus*, and *Raoultella*. *Acetobacter* emerged as the predominant genus in several beer samples, particularly Stout2 with 100% relative abundance (RA), IPA-Belg at 96.46%, IPA04 at 94.67%, IPA05 at 73.24%, Blonde-ale1 at 36.14%, Best-bitter1 at 25.93%, and Blonde-ale2 at 23.99%. *Acetobacter* species, particularly *A. pasteurianus*, are known for their role in ethanol oxidation, which leads to excessive acetic acid formation and imparts a sharp, vinegar-like taste to beer (De Roos and De Vuyst, 2019). In controlled environments, *Acetobacter* can contribute to the formation of complex esters and higher alcohols, thereby enhancing beer aroma. However, excessive levels are detrimental to flavour balance.

Levilactobacillus is another dominant genus that plays a significant role in beer fermentation because of its lactic acid production, which influences sensory characteristics and pH (Snauwaert et al., 2016). *Levilactobacillus*, a LAB genus, is generally known to contribute to beer souring by producing lactic acid and diacetyl, which affect the beer's buttery flavour (Snauwaert et al., 2016). Although LAB are preferred in sour beers, uncontrolled LAB result in undesirable flavour compounds. *Levilactobacillus* is commonly introduced through raw ingredients such as malt or through biofilm formation on brewing equipment. Samples, including Red Ale, IPA, and Stout, exhibited the highest relative abundance of *Levilactobacillus* at 96.26%, 95.93%, and 82.96%, respectively. The presence of this genus at such high levels raised important questions regarding its influence on beer quality and flavour. In the previous chapter, LAB counts on MRS agar (de Man, Rogosa) ranged from 0 to 1.25×10^5 CFU/mL, with Best-bitter1 exhibiting the highest count. However, in this chapter, microbial diversity analysis indicates that Iris Red Ale (Iris-red-ale), IPA, and Stout had the highest relative abundance of *Levilactobacillus* at 96.26%, 95.93%, and 82.96%, respectively. This discrepancy highlights the limitations of culture-dependent methods, which can underestimate microbial diversity by failing to detect viable but non-culturable (VBNC) cells. Studies have demonstrated that LAB can become viable but non-culturable under stressful conditions, such as low temperatures or suboptimal growth environments. In contrast, culture-independent methods, such as 16S rRNA gene sequencing, can identify VBNC populations, providing a more comprehensive understanding of the microbial communities in beer.

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While lactic acid bacteria are often associated with desirable souring effects in specific beer styles, such as Berliner Weisse or Lambics, their dominance in non-sour ale styles, including Irish Red Ale (Irish-red-ale), IPA, and Stout, suggested potential contamination rather than intentional fermentation. Recent studies have highlighted that excessive lactic acid production can interfere with the expected flavour profile of such beer styles, introducing tartness where it is not usually required (De Roos and De Vuyst, 2019).

In addition, the metabolic activity of *Levilactobacillus* is linked to diacetyl formation, a buttery off-flavour compound that may be undesirable in IPAs and Stouts, but may be acceptable at low concentrations in malt-centric styles such as Irish-red-ale (Yeo and Liu, 2014). The high relative abundance of *Levilactobacillus* in Irish-red-ale, IPA, and Stout suggested that contamination occurred post-boil, likely due to biofilms in the fermentation tanks, racking hoses, or bottling equipment. LAB populations can survive sanitation processes and proliferate, causing cross-contamination between batches (Storgårds et al., 2006). *Raoultella*, closely related to Klebsiella, was present in IPA-Eng1 (RA: 98.95%) from brewery 6, Pale-ale1 (RA: 67.87%), Pale-ale4 (RA: 64.41%) and Best-bitter2 (RA: 33.92%) collected from breweries 11, 27, and 23. *Raoultella* has been detected in numerous fermented beverages and plays a role in nitrogen metabolism, which may impact amino acid availability during yeast fermentation (Kim et al., 2016). The source of *Raoultella* species in beer could be from barley malt, which can carry these bacteria from the field. While its growth in finished beer is usually minimal, its presence indicates poor hygiene and can lead to the production of undesired substances (Matoulková et al., 2018).

At the species level, the most prevalent taxa found among the sampled craft ale beers were *Acetobacter orientalis* (RA: 100%), *Kluyvera ascorbate* (RA: 100%), and *Acetobacter orleanensis* (RA: 100%), followed by *Cutibacterium acnes* (RA: 95.88%) (Figure 5.3). Of the 30 breweries sampled, 26 had heterogeneous microbial profiles, with species richness varying from 2 to 29 species per sample, and 2 samples had homogeneous microbial profiles. However, no taxa were assigned to two samples (Stout1 and Stout2), possibly due to the absence of reference sequences in the SILVA database. Species diversity variation may result from differences in raw material processing, brewing processes, and hygiene practices (Bokulich, Bamforth and Mills, 2012; Spitaels et al., 2014) Within India Pale Ales (IPAs), sample IPA07 had the highest species diversity, with *Bradyrhizobium elkanii* being dominant (RA: 38.63%). *Bradyrhizobium* in beer environments is rare and may be caused by water sources or raw materials such as malt and hops. The brewing process, including techniques like dry hopping, can also influence microbial populations in the final product (Vaughan, O'Sullivan and Van Sinderen, 2005). Dry

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hopping involves the addition of hops to the beer after boiling, which can affect the growth of various microorganisms due to their antimicrobial properties (Gribkova et al., 2022).

In terms of Pale Ales, the Pale-ale3 sample contained 12 species, with *Enterobacter cloacae* being dominant (RA: 72.24%). The main sources of *Enterobacter* species are poor hygiene, contaminated raw materials, and wort exposure. They can occasionally be detected in pitching yeast if the slurry has been mishandled or stored under unhygienic conditions (De Roos and De Vuyst, 2019; Bokulich and Bamforth, 2013). Blonde Ales had high species richness; Blonde-ale1 had 20 species, with *Acetobacter lovaniensis* being the most abundant, representing 12 species. *Acetobacter lovaniensis* can contribute to undesirable souring effects when present in high quantities (Snauwaert et al., 2016). Porter-Imp (Porter Imperial) and Amber-ales (America Amber Ale) had low microbial diversity, with only two species identified. For instance, Porter-Imp was dominated by *Enterobacter hormaechei* (RA: 98.65%), while *Cutibacterium acnes* (RA: 95.88%) dominated in the Amber Ale. The presence of *Cutibacterium acnes*, a commonly occurring constituent of the human skin microbiota, was detected in Porter-Imp and suggested possible contamination by brewery personnel (Bokulich, Bamforth, and Mills, 2012).

Seven species were detected in Best-bitter1, namely *Citrobacter braakii*, *Citrobacter murliniae*, *Citrobacter freundii*, *Enterobacter kobei*, *Acetobacter malorum*, *Acetobacter cerevisiae*, and *Acetobacter orleanensis*. *Citrobacter freundii* was the most abundant (RA: 45.48%), while Best-bitter2 contained eight species, with *Raoultella ornithinolytica* (RA: 42.73%) being the most dominant. *Citrobacter* species have been implicated in brewery contamination and can produce off-flavours by metabolising sugars and forming volatile compounds (Matoulková et al., 2018). The presence of *Raoultella ornithinolytica* in Best-bitter2 suggested contamination, as it is pathogenic (Chen et al., 2021). Moreover, while beer's low pH, ethanol, and hop iso-α-acids inhibit many microorganisms, they do not automatically prevent the growth of all Gram-negative bacteria, including *R. ornithinolytica*. This bacterium, which can produce excessive dry hopping, may have introduced these yeasts from the hops, as they can survive post-boil and influence fermentation outcomes. Although hops possess antimicrobial properties, they do not entirely prevent yeast introduction, emphasising the need for careful monitoring of raw materials and fermentation processes (Bruner, Marcus and Fox, 2021). Histamine, which can cause food poisoning symptoms like scombroid poisoning (Hwang et al., 2020). Therefore, while the presence of *R. ornithinolytica* DNA is noteworthy, it does not necessarily imply a health risk to consumers (Hajjar et al., 2020). *Raoultella* species have occasionally been found in fermented beverages and water systems, emphasising the importance of sanitation and good manufacturing practices to prevent potential contamination. *Acetobacter* species in the sample indicated potential exposure to oxygen during packaging or

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fermentation, as the bacteria thrive under aerobic conditions and produce acetic acid, resulting in vinegar-like off-flavours (Bouchez and De Vuyst, 2022). Similarly, the presence of *Enterobacter* and *Citrobacter* species suggested possible post-fermentation contamination, as these are not typical fermentative bacteria but may transfer sulphurous and vegetal off-flavours when they metabolise specific wort components (Roselli et al., 2024). Additionally, *Cutibacterium acnes* in the Amber Ale samples indicated possible human-associated contamination, as it occurs naturally on the skin and can be introduced through improper handling or inadequate sanitation protocols (Bokulich, Bamforth and Mills, 2012).

Overall, the variations noted among the beer styles suggest that environmental, ingredient, and brewing conditions significantly influenced the observed microbial diversity. The high microbial diversity in IPAs and Blonde Ales may be attributed to the complexity of the raw materials used in their brewing, including dry hopping and higher sugar levels. On the other hand, the lower microbial diversity in Porter and Amber Ales suggests that these styles may have been exposed to limiting conditions, such as reduced pH or elevated alcohol content, which inhibited microbial survival (Spitaels et al., 2014).

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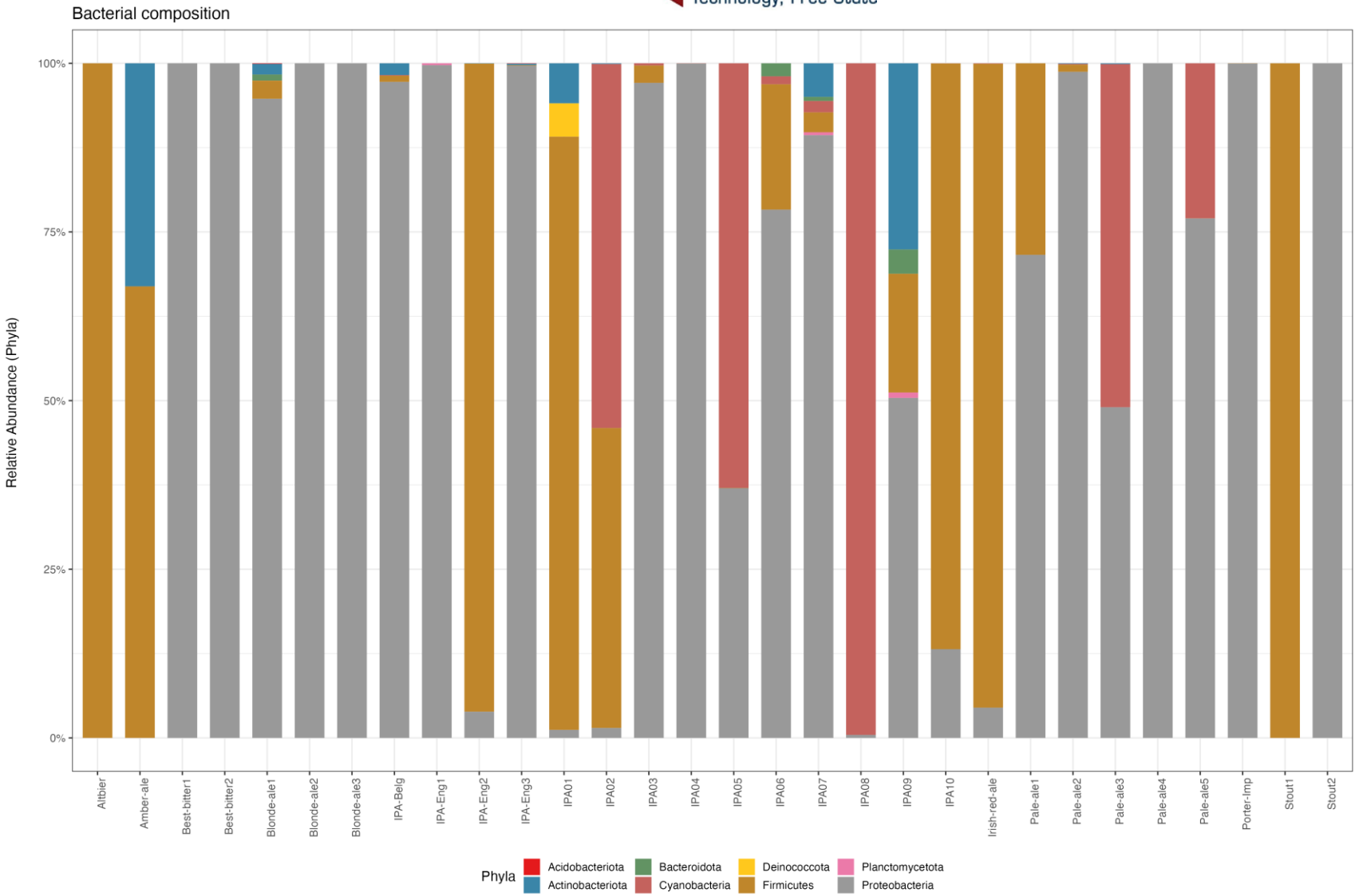


Figure 5.1: The relative abundance of bacterial composition in the sampled South African craft ale beer styles at the phylum level.

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Figure 5.2: The relative abundance of bacterial composition at the genus level in the selected South African craft ale beer styles.

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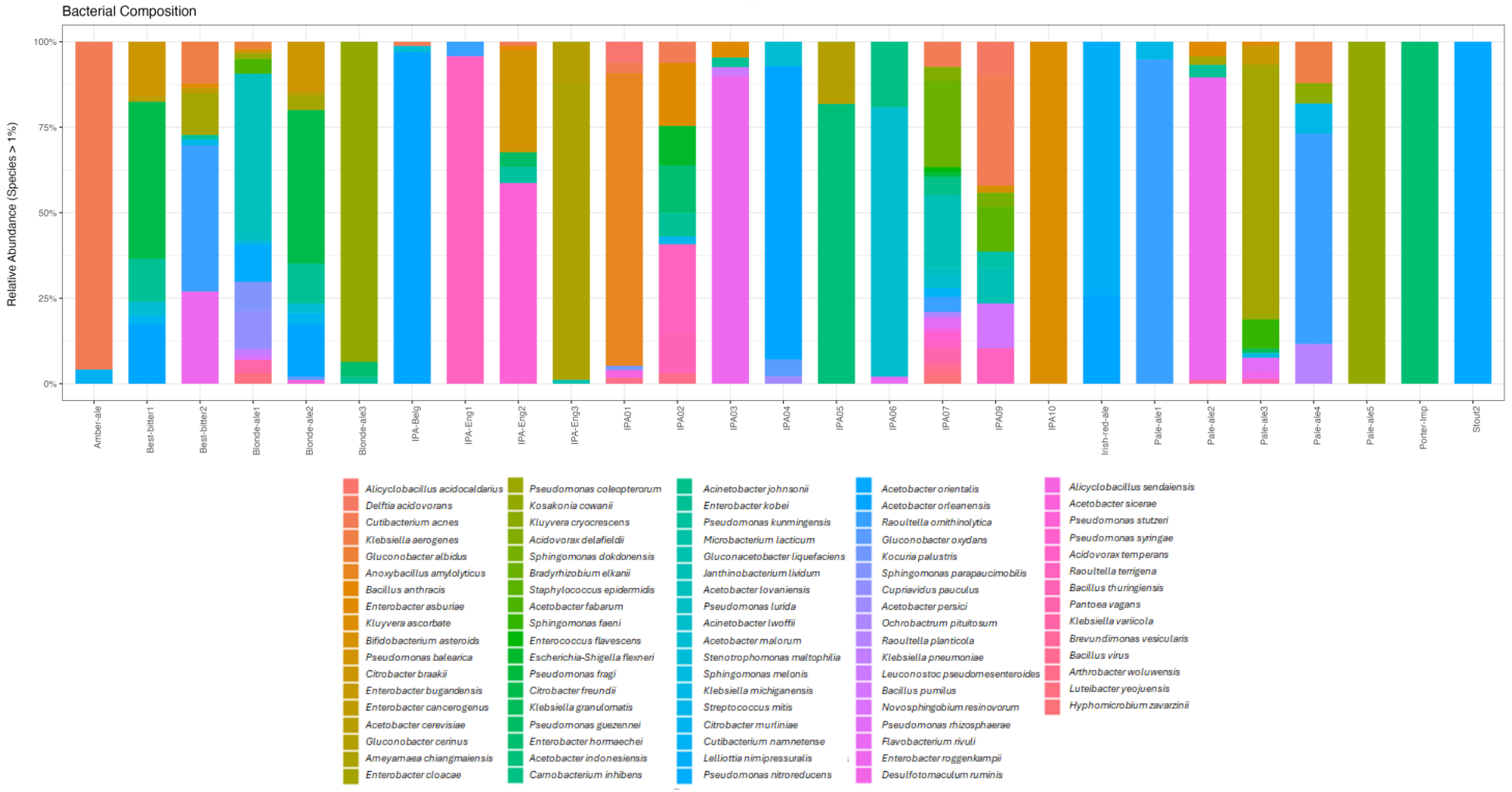


Figure 5.3: The relative abundance of bacterial composition at the species level in the selected South African craft ale style.

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5.3.1.2 Composition of fungal communities

Community profiling of the fungal population in the selected beer styles revealed the overall dominance of the phylum Ascomycota, with a relative abundance of 99%. In contrast, Basidiomycota was present at very low levels in IPA09 and IPA10 (Figure 5.4). This finding aligns with earlier studies that reported Ascomycota as the dominant fungal phylum in brewery environments. This dominance has been attributed to its ability to acclimate to fermentation conditions (Cason et al., 2020). Within Ascomycota, the most abundant class was Saccharomycetes, which has been widely documented as useful in beer fermentation and brewing (Spitaels et al., 2014).

At the genus level, no fungal taxa were assigned to IPA01, probably due to a lack of reference sequences available in the UNITE database. However, the most dominant genus across most samples had a relative abundance of 85.14%, represented by *Saccharomyces* (Figure 5.5). The prevalence of *Saccharomyces* was expected because its members are the main yeasts involved in the alcoholic fermentation process during beer production (Steensels and Verstrepen, 2014). *Saccharomyces* can efficiently metabolise sugars, produce ethanol, and outcompete other microbial genera to dominate in brewing environments. However, in the Amber-ale sample, *Wickerhamomyces* accounted for a higher relative abundance (100%). *Wickerhamomyces anomalus* possesses the ability to contribute complexity to beer flavour through the development of esters and phenolic compounds, which can contribute to mouthfeel and pleasant sensory characteristics (Zhang, Tang, et al., 2023). Interestingly, sample IPA10 contained a more diverse and complex fungal community compared to the other beer samples, featuring *Cladosporium* (RA: 2.07%), *Vishniacozyma* (RA: 0.93%), and *Penicillium* (RA: 0.34%). *Cladosporium*, being the most frequent non-Saccharomyces genus, is not traditionally associated with fermentation. However, it has been routinely isolated from brewery settings and is likely introduced through raw materials or airborne contamination (Pereyra et al., 2010). Similarly, *Penicillium* has been detected as a rare contaminant in mature beers, and some species have been implicated in the production of off-flavours and potential mycotoxins (Odhav and Naicker, 2002). The presence of the fungi validates the importance of having strict hygiene protocols in craft beer breweries to ensure that there are no impurities that compromise beer quality.

At the species level (Figure 5.6), *Saccharomyces cerevisiae* dominated in the majority of the beer samples (RA: 88.45%). Being one of the primary yeast species utilised in brewing beer, *S. cerevisiae* accounts for flavour-forming compounds and ethanol yield. It thus gives ale beer a distinct flavour profile (Steensels and Verstrepen, 2014). In Amber Ale, the dominant species with a relative abundance of

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7.32% was *W. anomalus*, which has gained attention in brewing science due to its potential to enhance beer aroma and flavour complexity by increasing ester and higher alcohol production (Li et al., 2025). Its presence in the Amber Ale samples could have contributed to creating distinctive sensory characteristics in this beer, making its investigation in craft beer brewing highly significant. Conversely, at the species level, IPA10 had a diverse microbial species population, as *Vishniacozyma psychrotolerans* (RA: 0.66%), *Debaryomyces prosopidis* (RA: 0.22%), *Cladosporium herbarum* (RA: 1.95%), *Penicillium brevicompactum* (RA: 0.23%), and *Vishniacozyma follicola* (RA: 0.35%) were identified.

The occurrence of more than a single *Saccharomyces* and non-Saccharomyces species in a single beer sample demonstrated either an uncontrollable fermentation process or environmental contamination. For example, *Cladosporium herbarum* is a filamentous fungus that was previously highly present in brewing industries. Although this organism will not directly affect fermentation, it is likely to impact the stability and maturation of beers (Hutzler, 2022). The presence of *Vishniacozyma psychrotolerans* and *Debaryomyces prosopidis* in IPA10 indicated potential variations in fermentation control or microbial interactions that could have affected beer quality. Excessive dry hopping may have introduced these yeasts from the hops, as they can survive post-boil and influence fermentation outcomes. Although hops possess antimicrobial properties, they do not entirely prevent yeast introduction, emphasising the need for careful monitoring of raw materials and fermentation processes (Bruner, Marcus and Fox, 2021). The presence of *Penicillium* and *Cladosporium* species in some samples raised concerns regarding potential spoilage. Some species of *Penicillium* and *Cladosporium* can produce mycotoxins and secondary metabolites that are undesirable as they negatively affect beer safety and quality (Ciont et al., 2022; Ogorek et al., 2012). Their presence, even at low levels of abundance, highlights the need for effective quality control measures during craft beer production to prevent fungal contamination.

The microbiome of the craft ales assessed in this study showed the presence of *Saccharomyces cerevisiae* in conjunction with non-Saccharomyces yeasts and filamentous fungi. While *Saccharomyces cerevisiae* was the dominant strain, the presence of non-Saccharomyces yeasts and filamentous fungi could also have influenced the flavour and quality of the beer. *Wickerhamomyces anomalus* is frequently found in malt, cereal grains, and other raw materials, and its presence in beer may indicate contamination (Padilla, Gil, and Manzanares, 2018).

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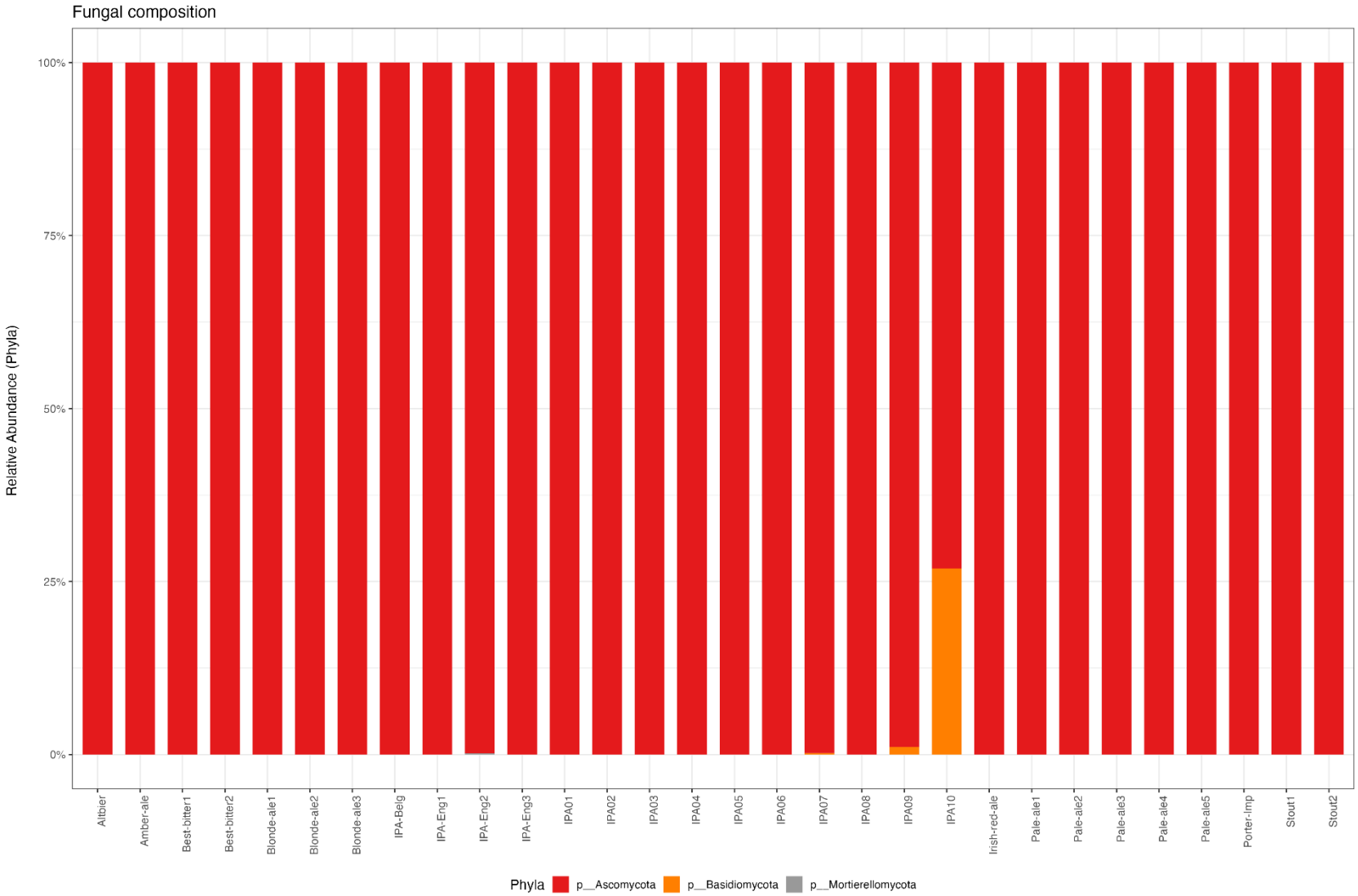


Figure 5.4: The relative abundance of fungal composition in the selected South African craft ale beer styles at the phylum level.

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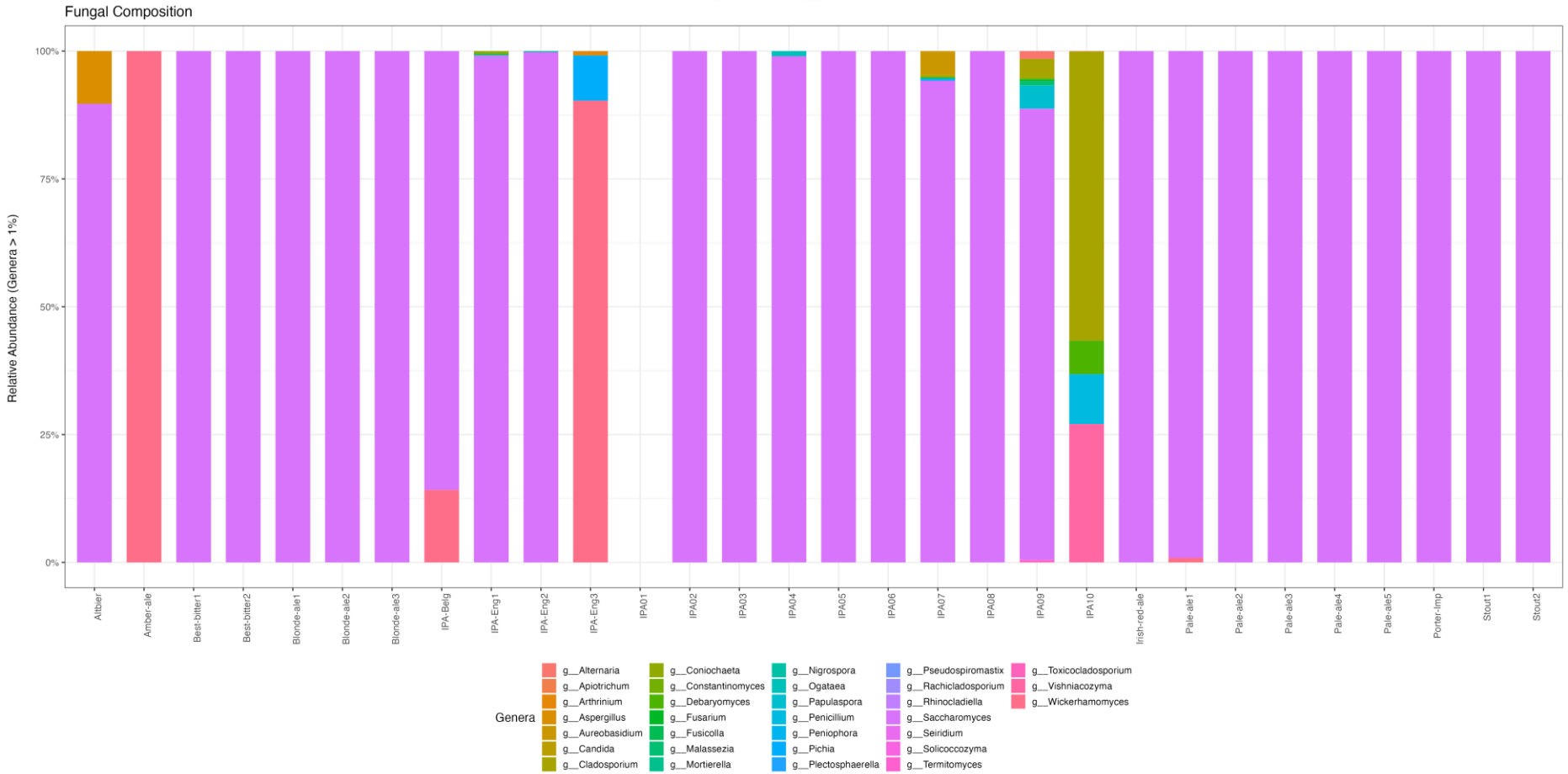


Figure 5.5: The relative abundance of fungal composition at the genus level in the sampled South African craft ale beer styles.

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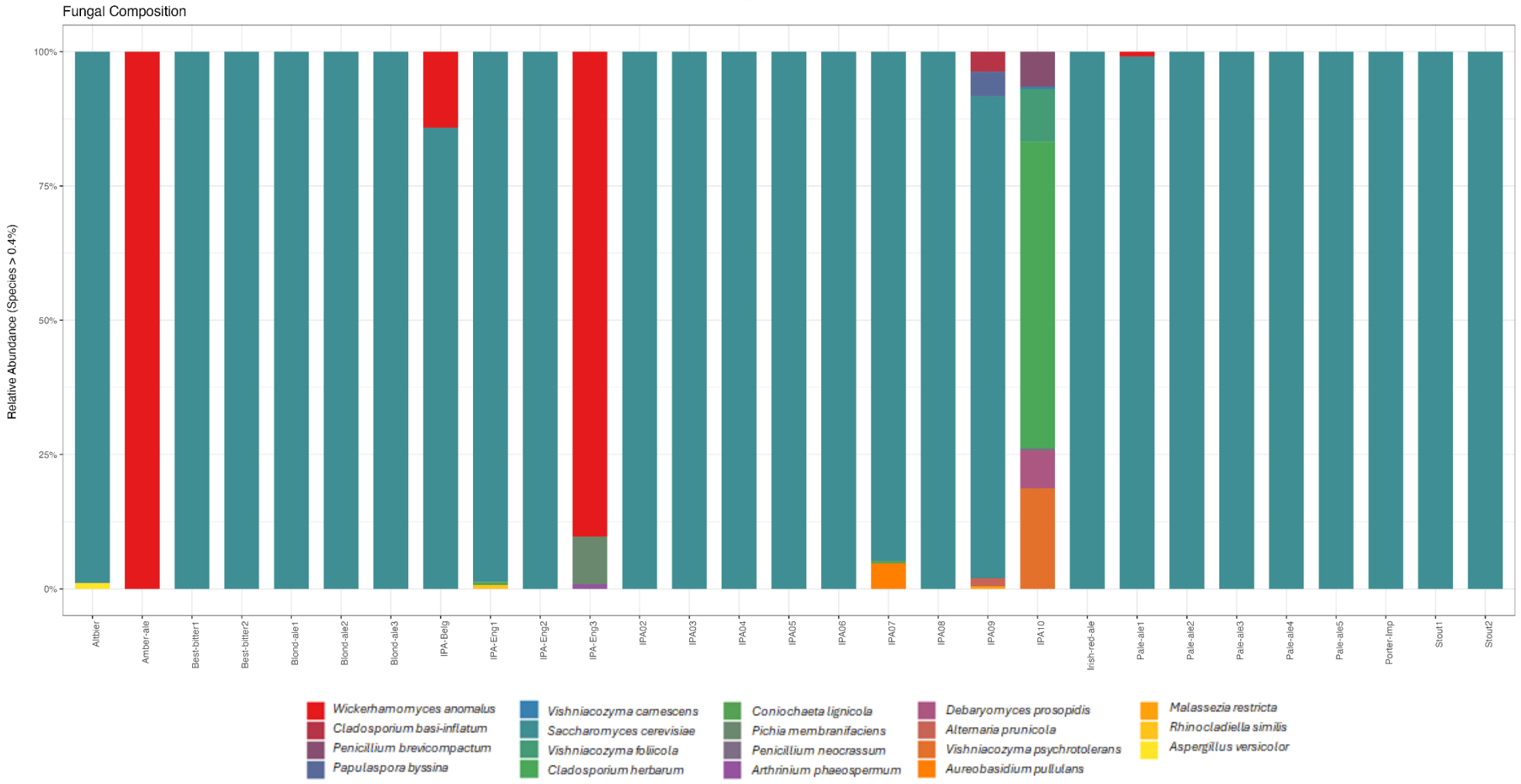


Figure 5.6: The relative abundance of fungal composition at the species level in the sampled South African craft ale beer styles.

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5.3.1.3 Measures of diversity

The diversity of bacterial and fungal species in each beer sample was assessed using Chao1, Observed, and Shannon alpha diversity indices (Figure 5.7). Chao1 index estimated a sample's total richness, including the observed and non-observed ASVs. Observed considered only information on the observed ASVs, and the Shannon index provided richness and evenness information.

Bacterial alpha diversity indices (Figure 5.7) differed among the beers. Best-bitter2 showed the highest species richness and diversity (H >4) compared to the rest of the samples. Best-bitter1, Blonde-ale2, Blonde-ale3, IPA06, IPA07, IPA10, Pale-ale1, Pale-ale4, Pale-ale5, and Stout1 exhibited very high diversity (H = 3.00 – 3.99), and moderate diversity was observed in Altbier, Blonde-ale1, IPA-Eng2, IPA01, IPA03, IPA04, IPA09, and Pale-ale2 (H = 2.5 – 2.99). The rest of the samples had low diversity (H = 2 – 2.49), except for IPA-Belg, IPA-Eng1, IPA-Eng3, IPA08, Irish-red-ale, and Stout2, which were very low (H = 1.99 – below).

High fungal species richness was observed in IPA01, IPA07, and IPA10 (Chao1 and Observed), with very low diversity (H >1.05) (Figure 5.8). However, compared to the other samples, these samples had the highest species richness and diversity. Altbier, Amber-ale, IPA-Belg, IPA08, and IPA09 also exhibited very low species richness and diversity (H >0.60). Notably, the H index below 0.60 to 0.00 was observed in the remaining 22 samples.

A Kruskal-Wallis test conducted to determine whether fungal and bacterial alpha diversity measures were significantly different across the beer styles. However, the results showed no statistically significant differences (p = 0.4561), indicating that there were no differences in bacterial or fungal richness and evenness across the craft beers. This suggests that beer style, generally defined by ingredients, brewing methods, and storage conditions, did not substantially influence microbial diversity in the selected craft beers.

The relatively low Shannon diversity observed in some beer samples suggested selective pressures caused by fermentation conditions, alcohol content, and hops-associated antimicrobial activity (Hlangwani et al., 2023). Also, interactions between yeast and lactic acid bacteria could have been accountable for the patterns, as metabolic interaction and competitive exclusion can control microbial community structures in beer (Liu, 2015; Hlangwani et al., 2023). The detection of fungi and bacteria related to spoilage, such as *Saccharomyces cerevisiae* variants, non-Saccharomyces yeasts, and

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Lactobacillus spp., could also have been responsible for variations in richness, as was previously documented in spontaneous fermentation and barrel-aged beer studies (Xu et al., 2020;Suiker and Wösten, 2022).

For beta diversity, the principal component analysis (PCoA) revealed the taxonomic structures of the microbial communities of the beer styles, as the beer styles could be clustered into five and four distinct bacterial and fungal groups (Figure 5.9 and Figure 5.10). No clear grouping was observed based on the geographical location of the breweries, suggesting that microbial community composition was more strongly influenced by beer style and fermentation characteristics than by the breweries' locations. The ability to cluster the beers into groups suggests similarities in the beer style microbiota. The clustered bacterial groups were: Group 1: Fourteen beer styles namely Altbier, Blonde-ale1 Blonde-ale3, Best-bitter2, IPA01, IPA06, IPA07, IPA09, IPA10, IPA-Eng1, IPA-Eng3, Pale-ale1, Pale-ale4, and Porter-Imp. Group 2: Amber-ale was close to the first group, with three beer styles, namely Irish-red-ale, IPA-Eng2, and Stout1. Group 3: Four beer styles, namely Best-bitter1, Blonde-ale2, IPA04, and IPA-Belg. Group 4: Two beer styles, namely IPA03 and Pale-ale2. Group 5: Four beer styles, namely IPA2, IPA05, IPA08, and Pale-ale3. Pale-ale5 was an outlier as it did not fit into any of the five groups.

For fungi, the outliers were IPA07 and IPA01 (2 beer styles), while groups were: Altbier and Amber-ale (2 beer styles); Blonde-ale1 and IPA-Eng2 (two beer styles); IPA09, IPA10, IPA-Belg, IPA-Eng3, Irish-red-ale, Pale-ale1, Porter-Imp and Stout1 (8 beer styles); and Best-bitter1, Best-bitter2, Blonde-ale2, Blonde-ale3, IPA02, IPA03, IPA04, IPA05, IPA06, IPA08, IPA-Eng1, Pale-ale2, Pale-ale3, Pale-ale4, Pale-ale5, and Stout1 (16 beer styles). However, permutational analysis of variance showed that there were no statistically significant differences among the beer styles as per PERMANOVA: $F = 1.0405$, $R^2 = 10.72\%$, $p = 0.223$ (bacteria), $F = 1.5013$, $R^2 = 14.76\%$, and $p = 0.085$ (fungi). The quantification of dissimilarity in microbial community compositions among the beer styles using the Bray-Curtis dissimilarity distance matrix showed a bacterial variation of 18.6% between the x (10.8%) and y (7.8%) axes (Figure 5.9), and a fungal variation of 50.4% between the x (38.2%) and y (12.2%) axes (Figure 5.10).

5.3.1.4 The microbial community structure in response to flavour-active compounds in craft beers

A distance-based Redundancy Analysis (db-RDA) was conducted to investigate the influence of key flavour-active compounds on the microbial community compositions across the sampled South African craft beer samples (Figure 5.11). The constrained db-RDA1 and db-RDA2 axes accounted for a

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proportion of the explained variation (10.08% for bacteria and 15.31% for fungi), but each overall model was not statistically significant, i.e., p = 0.43 (bacteria) and p = 0.60 (fungi), suggesting limited predictive power of the environmental variables in explaining microbial community composition. Nonetheless, individual variables showed differing degrees of association with community patterns. Additional markers such as diacetyl, organic acids, and volatile fatty acids may better indicate microbial influence or contamination in beer.

For bacteria, several Pale Ale styles could be clustered together (Figure 5.11 A). Some of the beer samples, particularly Blonde Ale and Irish-red-ale (dark ales), appeared to be slightly influenced by phenyl ethyl acetate (an ester compound) and ethylguaiacol (a phenolic compound). In contrast, the rest of the pale and dark ale styles were loosely clustered. They appeared to be influenced by vanillin, ethyl caproate, ethyl acetate, vinyl phenol, isoamyl alcohol, and ethyl caprylate. Furthermore, the ordination plot indicates that only Porter Imperial (a dark ale) was aligned along the direction of the vinylguaiacol and isoamyl-acetate vectors, suggesting a possible association. Despite the lack of overall significance, only vanillin showed a marginally significant relationship with bacterial community composition (p = 0.104), suggesting a potential influence. Vanillin, a lignin-derived phenolic aldehyde responsible for a characteristic vanilla-like aroma, was not statistically significant in terms of the bacterial community. This demonstrated that bacterial taxa possibly weakly influenced vanillin levels, potentially through the bioconversion of phenolic precursors. Vanillin can originate from malt-derived lignin, barrel-aging, or oxidative processes (Bokulich and Bamforth, 2013), and is considered desirable in dark ales and barrel-aged beers where it contributes to flavour complexity. Its alignment with the Porter Imperial sample was therefore in alignment with its style profile and could indicate either malt-derived or aging-related contributions.

For fungi, the individual variables showed differing degrees of association with fungal community patterns (Figure 5.11 B). For instance, the ordination shows most Pale and Blonde Ales tightly clustered together and aligned closely to phenyl ethyl acetate, while Stout2 (dark ale) aligned along the direction of the ethyl caprylate vector. The clustering of Pale and Blonde Ale near phenyl ethyl acetate was expected, as this ester contributes rose-like and honey-like aromas that are considered desirable according to the BJCP (2021) guidelines. A Pale Ale (IPA07) aligned closely with isoamyl acetate, while the remaining Pale, Dark, and Amber Ales formed a looser cluster associated with vinylguaiacol, isoamyl alcohol, ethyl acetate, and vinyl phenol.

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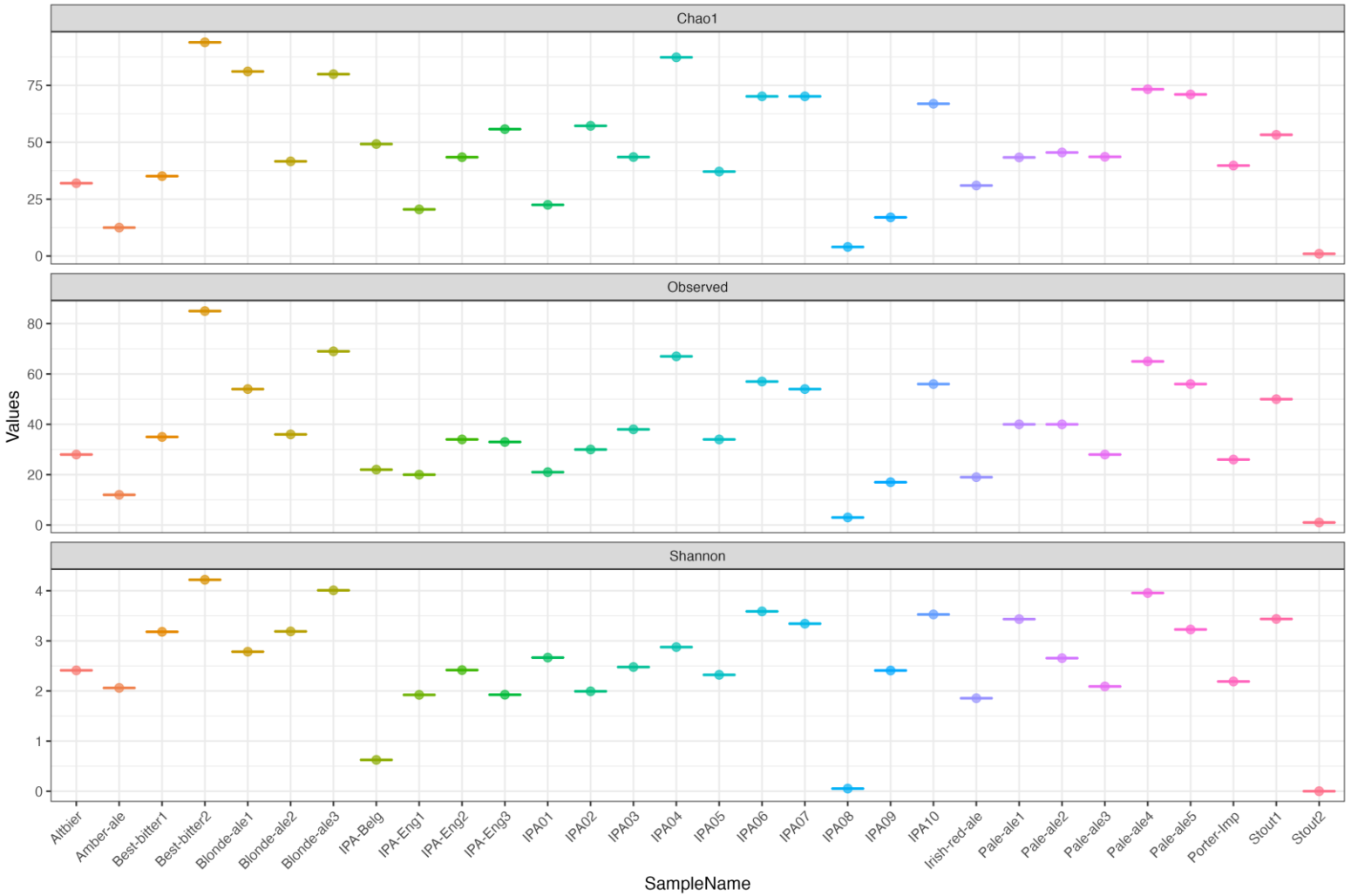


Figure 5.7: The α-diversity indices of bacteria in the sampled South African craft ale beer styles.

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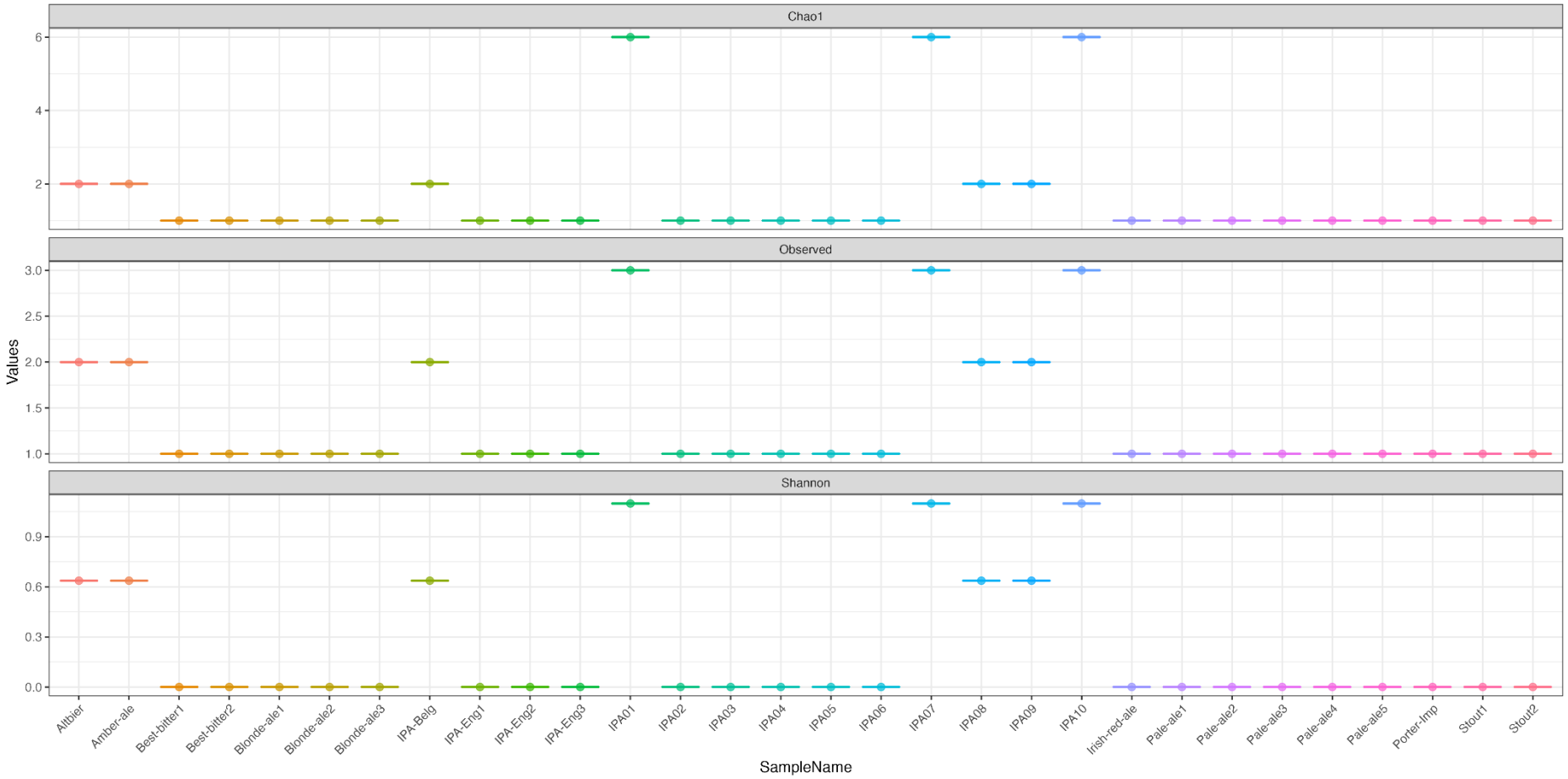


Figure 5.8: The α-diversity indices of fungi in the sampled South African craft ale beer styles.

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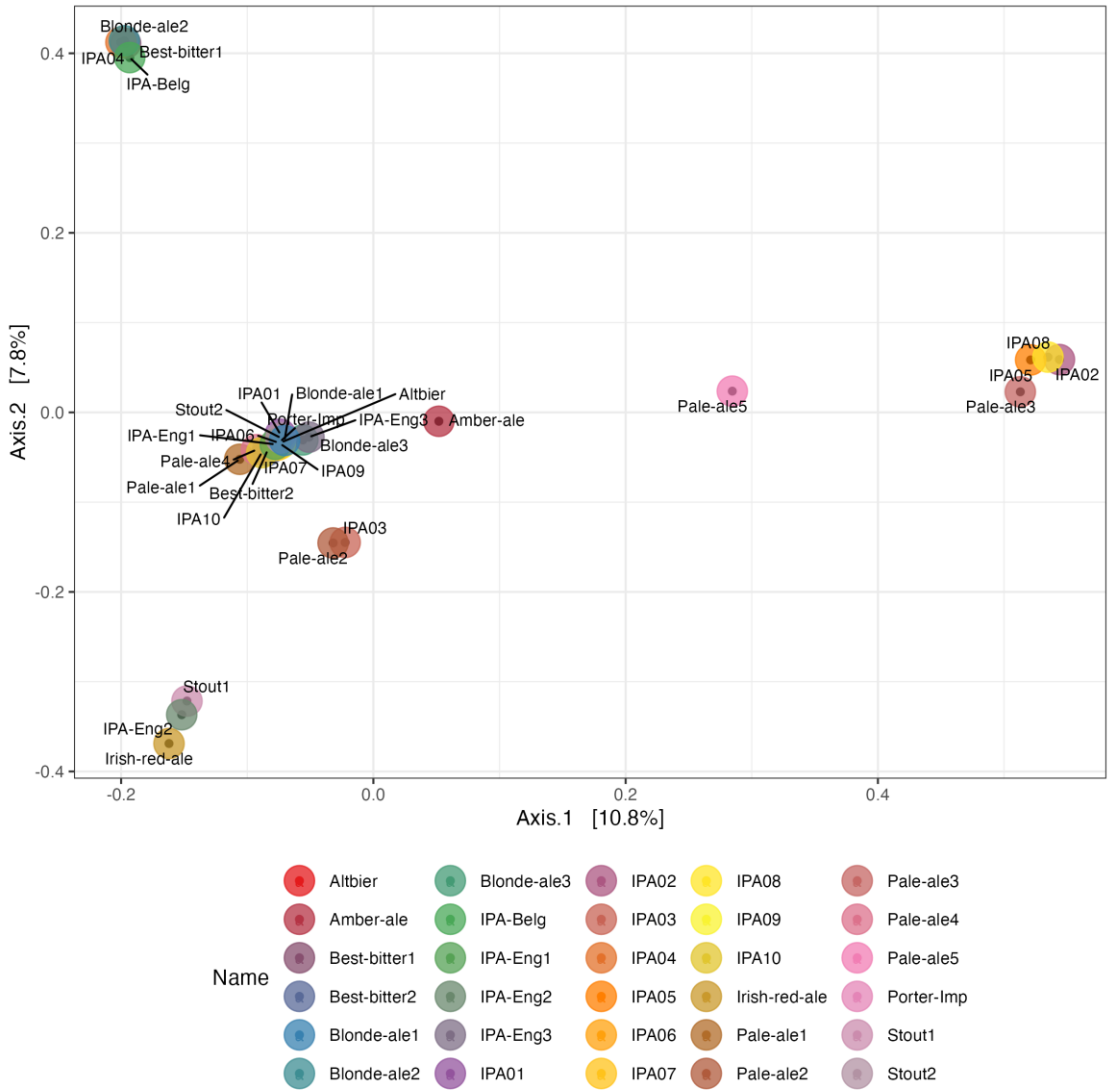


Figure 5.9: PCoA of bacterial composition across the sampled South African craft ale beer styles.

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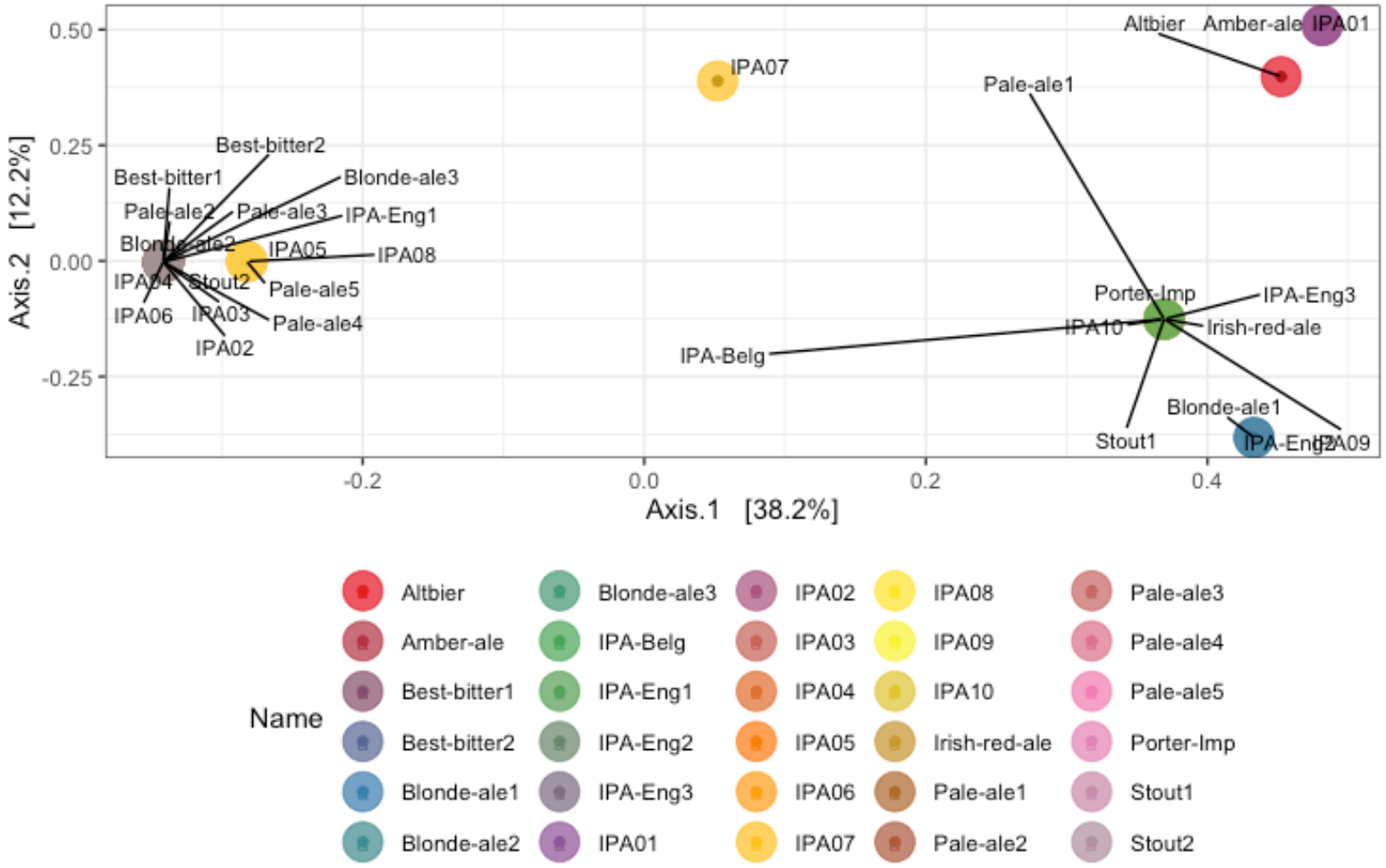


Figure 5.10: PCoA of fungal composition across the sampled South African craft ale beer styles.

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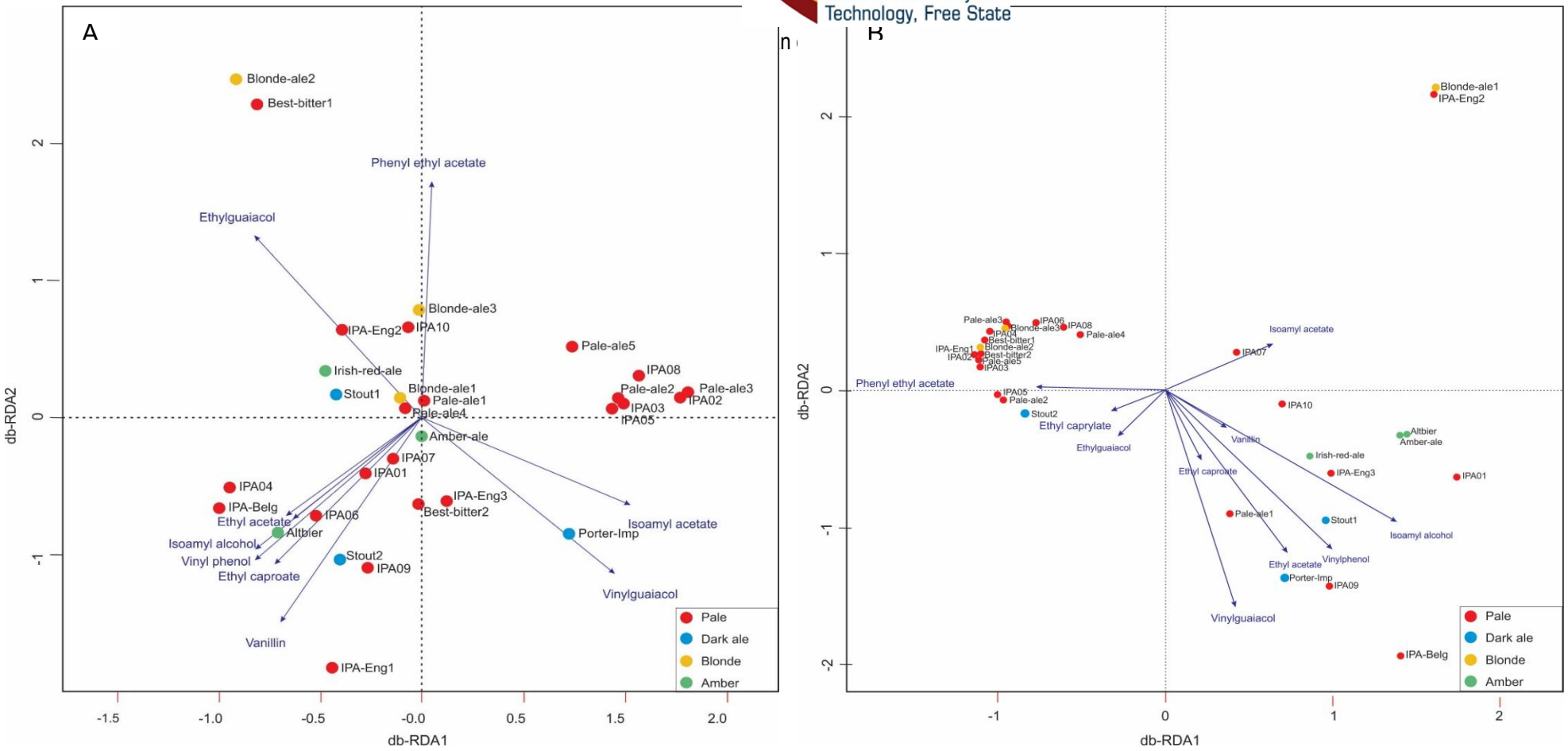


Figure 5.11: Distance-based redundancy analysis (db-RDA) of the sampled South African craft beers.

(A). Bacterial communities and (B). Fungal communities. Red, blue, amber and green circles represent bacterial and fungal communities, and the arrows represent flavour-active compounds. The first two constrained axes accounted for 5.93% and 4.15% (for bacteria) and 9.53% and 5.78% (for fungi) of the explained variance, respectively. The length of each arrow is proportional to the flavour active compound's influence on the overall variation of the bacterial and fungal communities.

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5.4 CONCLUSIONS

This study assessed the microbial diversity among selected South African craft beer styles. It focused on the bacterial and fungal composition and structure, as well as the possible influence of flavour-active compounds on the microbiota.

The key findings showed that the selected South African craft beer styles contained various bacteria and fungi in different proportions. For instance, the bacterial community contained Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteriota, Bacteroidota, and Planctomycetota, but Proteobacteria was dominant, followed by Firmicutes. Ascomycota dominated the fungal community, but only two samples contained Basidiomycota. At the species level, *Acetobacter orientalis*, *Kluyvera ascorbate*, and *Acetobacter orleanensis* were the prevalent bacterial taxa, while *Saccharomyces cerevisiae* dominated the fungal community.

Enterobacter cloacae was detected in one Blonde Ale (Blonde-Ale-2: 4.26%), two Pale Ales (Pale-Ale-2: 2.46%, Pale-Ale-3: 71.63%, and Best-bitter: 12.17%). This suggests post-fermentation contamination, possibly due to inadequate cleaning procedures. Spoilage and mycotoxin-producing organisms, such as *Cladosporium herbarum* and *Penicillium brevicompactum*, were also detected. These findings highlight the need for improved brewery hygiene and monitoring of fungal contaminants to prevent potential spoilage and mycotoxin production.

Microbial community composition and structure assessments using alpha and beta diversity measures showed no statistically significant differences in bacterial or fungal richness and evenness across the craft beers, nor in the taxonomic structures of bacteria or fungi among the beer styles. The assessment of the influence of key flavour-active compounds on microbial community compositions across the sampled South African craft beer samples using db-RDA showed that key flavour-active compounds had limited predictive power in explaining microbial community composition. Furthermore, these individual flavour-active compounds showed differing degrees of association with microbial community patterns, but they did not influence the microbial community present in the beer.

Although individual flavour-active compounds are produced by the metabolic activity of yeast and bacteria during fermentation, this finding suggests that flavour-active compounds may not influence the microbial community of the selected craft ales. Furthermore, the presence of pathogens, spoilage and mycotoxin-producing organisms highlights the need for critical quality control strategies to prevent beer

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contamination and to preserve products' stability. It is important to note that these data were generated from DNA rather than RNA, and that the results only confirmed that the microbes were present at some stage in the product, regardless of whether they were alive or inactive.

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CHAPTER 6:
CONCLUDING REMARKS AND FUTURE WORK

Craft beer quality, flavour, and safety can be influenced by several factors, including raw materials such as grain, yeast, water, and hops, as well as brewing and fermentation conditions and microbial and chemical compositions. Data on mycotoxins in locally produced craft beers are scares. Moreover, most studies have concentrated either on microbial diversity or chemical profiling, resulting in limited research on integrating microbial and chemical data from craft beers, which would enhance understanding of product quality and safety. To understand the overall quality of craft beer, this study assessed the chemical compositions and microbiota of selected South African craft beer styles, focusing on bacterial and fungal communities, their relationships with chemical constituents, and the influence of flavour-active compounds on the microbial community.

Mycotoxins Deoxynivalenol (DON), Fumonisin B1 ($FB_{1)}$, Fumonisin B2 (FB_2), and Nivalenol (NIV) were detected in six breweries, with DON being the most prevalent. The detected mycotoxin concentrations in this study do not pose an immediate concern because they were well below established regulatory limits for safe consumption. However, the occurrence of the mycotoxins in some of the beer styles emphasises the need for routine monitoring of the quality of raw materials, such as grains used in craft beer production, for quality control and safety.

Microbial loads revealed the presence of spoilage and opportunistic pathogenic microbiota as well as yeast and mould contamination. Total plate counts of microorganisms varied across beer styles, ranging from 0 to 1.63×10^5 CFU/mL. Yeast counts were present in over half of the samples. This finding is in the context of craft brewing, where some craft beers are unfiltered, in which residual yeast is expected and forms part of the beer's character. However, uncontrolled yeast activity can be a challenge, as it may trigger re-fermentation through the consumption of residual sugars, which can alter the intended flavour profile and negatively impact the beer's stability and shelf life. Although mould was present in 17% of the samples, its presence in finished beer is unexpected and concerning. Its presence raises concerns about the possible introduction of mycotoxins, as they pose potential risks to consumers even at low concentrations. Lactic acid bacteria (LAB) were detected in 18 beers samples, confirming that post-fermentation contamination was widespread among different breweries. LAB are not pathogenic; however, they are the most common spoilage organisms in brewing, producing acidity, turbidity and off-flavour compounds, which reduce the shelf life and compromise the sensory quality of the beer. Contrarily, *Staphylococcus aureus* was detected in one pale ale sample, highlighting that opportunistic

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microbes are not widespread but can arise from breaches in process hygiene. Across the 18 beer samples contaminated with LAB, a single Pale Ale stood out, as it was the only case where LAB co-occurred with *Staphylococcus aureus*, indicating post-fermentation contamination. The detection of both LAB and *Staphylococcus aureus* in a Pale Ale sample indicates post-fermentation contamination.

Flavour-active compounds, such as isoamyl acetate, phenyl ethyl acetate, ethyl caproate, ethyl caprylate, 4-vinylphenol, 4-vinylguaiacol, 4-ethylguaiacol, guaiacol, and vanillin, were detected above sensory thresholds. Although many flavour-active metabolites contribute positively to specific style characteristics, in high concentrations, they likely contribute to pronounced fruity, floral, spicy, clove-like, smoky, and vanilla-like sensory attributes characteristic of some ale styles, thereby compromising overall flavour balance and reflecting variability in fermentation management. The interpretation values are crucial to understanding their contribution to the flavour of the beer. There were several compounds described as 'non-flavour' active (e.g., syringic acid, ethyl butyrate, 4-methyl-1-pentanol, phenylethyl acetate, and coniferaldehyde) present at concentrations above their sensory thresholds, implying that they may have contributed perceptibly to the aroma and flavour of the beer. Likewise, high concentrations of alcohols such as isoamyl alcohol and 4-methyl-1-pentanol were detected above their sensory thresholds in some samples, which could result in a risk of harsh or solvent-like notes. Furthermore, residual sugars, including both fermentable and non-fermentable sugars detected in some beer samples, indicated variability in yeast performance and potential post-fermentation adjustments by brewers. Together, these findings suggest that even the compounds in beer that are not usually the main contributors to flavour can still affect the taste, aroma and overall perception of the beer, especially when present at levels detectable by the senses.

Spearman's correlation revealed a strong positive correlation between yeast and lactic acid bacteria, as well as negative correlations between residual sugars (maltose, trehalose, glucose) and flavour-active compounds. Additionally, there were strong associations between ferulic acid and higher alcohols (isobutanol, isoamyl alcohol, phenylethyl alcohol). The correlation shows how microbial and chemical interactions shape the quality of the beer. The activity of the yeast was positively correlated with LAB growth, indicating a risk of spoilage under favourable conditions. The higher levels of flavour-active compounds were associated with efficient sugar utilisation, while ferulic acid correlated with higher alcohols, demonstrating active yeast metabolism. For brewers, this highlights the need for stringent process control, while for consumers, these interactions determine whether the beer exhibits balanced complexity or develops off-flavours.

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Evaluation of microbiota at the genetic level revealed *Acetobacter orleanensis* as the prevalent bacterial taxon, while *Saccharomyces cerevisiae* dominated the fungal community. The latter was not unexpected since the beers included in this study were all bottom-fermented ales, and the majority were not filtered or pasteurised. Furthermore, the detection of *Enterobacter cloacae* in three beer styles suggests a possible inadequate cleaning regime, while *Penicillium brevicompactum* and *Cladosporium herbarum,* which were detected, are environmental fungi. *Penicillium brevicompactum* can produce mycophenolic acid, a mycotoxin that was not detected in this study. Thus, the presence of these microbial contaminants necessitates rigorous microbial monitoring and adherence to good manufacturing processes. However, the genetic analysis provides a snapshot of the microbial diversity in the beer samples, but was performed on the DNA level and therefore does not necessarily reflect viability. For a true reflection of viable microbial diversity in the final products, analysis at the RNA level should be considered for future investigations. The db-RDA analysis showed that the microbial community compositions across the sampled craft beer samples did not depend mainly on the flavour-active compounds.

Overall, these findings shed light on the microbial and chemical compositions of the sampled craft beers, providing insight into the content of these beers. The study highlights the importance of routine microbial monitoring, beer spoilage bacteria, in particular LAB, which was detected in over 50% of the beer samples. Yeast and mould monitoring is also recommended, especially in unfiltered beers, to track the development of off-flavours and potential mycotoxin production. Opportunistic pathogens, such as *Staphylococcus aureus*, though rare, should also be tested to ensure production hygiene. In terms of chemical composition, both flavour and ‘non-flavour’ compounds can influence beer sensory perception if present above threshold. Thus, routine chemical analysis could focus on monitoring LAB metabolites, such as lactic acid, during fermentation and after packaging to detect microbial activity. Key flavour active compounds, such as esters and higher alcohols, can be monitored during and after fermentation to ensure flavour targets are met. Phenolic compounds, including 4-vinylguaiacol, 4-vinylphenol, guaiacol, vanillin, and coniferaldehyde, should be monitored at the bottling or packaging stages, as their concentration can increase or change after fermentation by yeast or contaminant bacteria, oxidation, or changes in storage conditions. If present above sensory thresholds, they can contribute to off-flavours. By systematically monitoring these microbial and chemical profiles in craft beers, craft breweries can demonstrate compliance with food safety and quality regulations. This includes compliance with microbial safety limits, hygiene standards and mycotoxin regulation as required by South African food and beverage legislation and relevant industry standards.

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While this study provides valuable insights, it is important to acknowledge several limitations when interpreting the findings. These include the restricted geographic scope of the samples, a relatively small sample size and metagenomics performed on the DNA level. For future research, the geographic scope should be extended to at least include beer samples from craft breweries in every province. Although including beer samples from every craft brewery in South Africa may never be feasible, a sample size representative of at least 50% should be considered. To expand our understanding of the metagenomics of active or transient microbes in shelf-ready fermented beverages, analysis should focus on metatranscriptomic analysis of RNA.

In conclusion, the concentrations of mycotoxins detected in this study do not pose an immediate concern, and flavour-active compounds in some craft beers exceeded their desired sensory thresholds, which could negatively affect the desirable beer flavour. Furthermore, microbial contamination—including LAB, *Staphylococcus aureus*, *Enterobacter cloacae*, *Cladosporium herbarum,* and *Penicillium brevicompactum*, detected in some of the samples—highlights the need for adequate hygiene during and after craft beer production, as well as controlled storage of craft beer raw materials.

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CHAPTER 7: REFERENCES

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