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Exploring temporal changes in the malting barley seed microbiome with meta-omics to understand nitrogen content effects.

by

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Thesis

Submitted in fulfilment of the requirements for the degree

Philosophiae Doctor

in

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Dr. Angela Botes

Co- Supervisor: Prof. Pieter De Maayer

October 2024

Declaration

I, Kalonji Abondance Tshisekedi (1423566), am a student registered for the degree of Philosophiae Doctor by Thesis in the academic year 2021-2024.

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NRF Declaration

The financial assistance of the National Research Foundation (NRF) and Anheuser-Busch InBev SA/NV (AB InBev) towards this research is hereby acknowledged.

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be contributed to the NRF and/or AB InBev.



DEDICATION

This thesis is dedicated to my parents.

Chers Papa et Maman, les mots ne seront jamais suffisants pour dire merci. Votre amour et soutien ont sculpté ma personne. Chaque jour, je m'efforce d'être le reflet de votre bonté.

ACKNOWLEDGEMENTS

I am deeply grateful for the paths that have led me to this thesis. This journey would not have been possible without the grace of God, who has blessed me with countless opportunities.

I would like to extend my heartfelt thanks to Professor Rob Veale, who took a chance on me. This decision has left an indelible mark, and I am forever grateful.

To my supervisor, Dr. Angela Botes, and my co-supervisor, Prof. Pieter De Maayer, I could not have asked for better mentors. Thank you for your trust and confidence in me, for challenging me to step out of my comfort zone, and for steering me away from the danger zone whenever I veered off course. I hope to continue learning from you.

I am immensely grateful to Anheuser-Busch InBev and the National Research Foundation (NRF) for their generous funding and support, which were instrumental in the completion of this research.

To the past and present members of the Botes Lab, thank you for your assistance, encouragement, and for always checking up on me.

To Nondumiso, thank you for believing in me, supporting me through your prayers, and most importantly, for becoming my home.

To Jouissance, my little sister, all these years I thought I was taking care of you, but it turns out you were the one taking care of me. I wouldn't be here without your support.

To my friend, Uncle Graham Hewett. Your consistent check-ins over the last decade have meant the world to me.

To the Weru family, thank you for being my home away from home. I think of Clare and David often, and may their souls continue to rest in peace.

To all my friends, thank you for always being there for me.

To my siblings, thank you for your unwavering support.

Lastly, to my parents, thank you for never giving up on me and for being the best parents I could have ever asked for.

Research outputs

Journal Article

Tshisekedi, K.A., De Maayer, P. & Botes, A. Metagenomic sequencing and reconstruction of 82 microbial genomes from barley seed communities. Sci Data 11, 484 (2024). https://doi.org/10.1038/s41597-024-03332-x

Plenary talks

Tshisekedi KA, De Maayer P, Botes A (2023). Elucidating Nitrogen Cycling and Microbial Adaptation in Malting Barley Grain: A Meta-Omics Approach. South African Society for Microbiology (SASM) conference. Stellenbosch, September 2023. **Award:** Best Oral Presentation 2nd place.

Conference outputs – Oral presentations

Tshisekedi, K.A., De Maayer P., and Botes A (2023). Unveiling the role of nitrogen in shaping microbial communities and malting quality in barley grain through meta-omics analysis. International Symposium on Microbial Ecology (ISME) #19. Cape Town. 18th Aug 2024 – 23rd Aug 2024 **Award:** African Travel Grants.

Conference outputs – Poster presentations

1. **Tshisekedi, K.A**., De Maayer P., and Botes A (2023). Exploring Nitrogen-Induced Changes in Barley Microbial Communities through Genome-resolved metagenomics. FEMS Conference on Microbiology 2023, 8th -13th July 2023.
2. **Tshisekedi, K.A**., De Maayer P., and Botes A (2023). Exploring Nitrogen-Induced Changes in Barley Microbial Communities through Genome-resolved metagenomics. Ubuntu Proteomics Summer School 2023. 19th February 2023. **Award:** Best poster

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List of abbreviations

AA: Auxiliary Activities

AB InBev: Anheuser-Busch InBev

AI: Artificial intelligence

ANI: Average Nucleotide Identity

ANOSIM: ANalysis Of SIMilarities

BLASTP: Basic Local Alignment Search Tool for Proteins

CAZymes: Carbohydrate-Active Enzymes

CBM: Carbohydrate-Binding Modules

CDS: Coding DNA Sequences

CE: Carbohydrate Esterases

COGs: Clusters of Orthologous Groups

CSIR: Council for Scientific and Industrial Research

DDA: Data-Dependent Acquisition

DNA: Deoxyribonucleic Acid

dsDNA: Double-Stranded DNA

DTT: Dithiothreitol

E.C.: Enzyme Commission numbers

FDR: False Discovery Rate

FHB: *Fusarium* Head Blight

GH: Glycoside Hydrolases

GO: Gene Ontology

GT: Glycosyl Transferases

GTDB-Tk: Genome Taxonomy Database Toolkit

HH: Harvest, High grain nitrogen content

HL: Harvest, Low grain nitrogen content

KEGG: Kyoto Encyclopedia of Genes and Genomes

KO: KEGG Orthology terms

LAB: Lactic Acid Bacteria

LCA: Lowest Common Ancestor

LC-MS: Liquid Chromatography-Mass Spectrometry

MAGs: Metagenome-Assembled Genomes

Mb: Megabases

mDNA: Metagenomic DNA

MIMAG: Minimum Information about a Metagenome-Assembled Genome

MPA: MetaPhlAn Profile Analysis

mRNA: Messenger RNA

NCBI: National Center for Biotechnology Information

NGS: Next-Generation Sequencing

NMDS: Non-metric Multidimensional Scaling

OMPs: Outer Membrane Proteins

ORFs: Open Reading Frames

PAGE: Polyacrylamide Gel Electrophoresis

PBS: Phosphate-Buffered Saline

PCoA: Principal Coordinates Analysis

PCR: Polymerase Chain Reaction

PHI: Pathogen Host Interaction database

PL: Polysaccharide Lyases

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rRNA: ribosomal RNA

S3H: 3 months storage, High grain nitrogen content

S3L: 3 months storage, Low grain nitrogen content

S6H: 6 months storage, High grain nitrogen content

S6L: 6 months storage, Low grain nitrogen content

S9H: 9 months storage, High grain nitrogen content

S9L: 9 months storage, Low grain nitrogen content

SCOs: Single-Copy Orthogroups

SDS: Sodium Dodecyl Sulphate

tRNA: Transfer RNA

Abstract

Barley (*Hordeum vulgare* L.) is a critical cereal crop, particularly in beer production, where it plays a significant role in the economy, especially in South Africa. Despite its importance, the barley seed microbiome, which affects seed storage and quality, is not well understood. This research addresses two key questions: (1) how microbial composition and function evolve during storage and (2) how the inherent nitrogen content of the grain affects these dynamics. Using metagenomic and metaproteomic approaches, eight barley samples from the Kadie cultivar, stored for various durations (harvest, three, six, and nine months) with high and low nitrogen content, were analysed. Metagenomic sequencing revealed a predominance of bacterial sequences and minimal fungal presence, with storage time having a greater impact on microbial diversity than nitrogen content. However, specific bacterial genera such as *Erwinia*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas* showed nitrogen-dependent prevalence. Metagenome-assembled genomes (MAGs) were reconstructed, representing 26 bacterial genera, with minimal shared orthologues, highlighting taxonomic diversity. Functional analysis identified key metabolic pathways and carbohydrate-active enzymes (CAZymes) essential for microbial adaptation during storage. Metaproteomic analysis further showed the active expression of proteins related to nutrient transport and stress response, indicating functional changes over storage time. Overall, this research enhances the understanding of the barley seed microbiome, providing valuable insights into storage practices that could improve brewing quality and agricultural sustainability.

Preface

Barley (*Hordeum vulgare* L.) ranks as the fourth most widely cultivated cereal crop globally. Characterised by its short growth period and robust adaptability, barley is predominantly used in beer production, boosting the global economy through revenue generation and employment. In South Africa, the bulk of the barley harvest supports the brewing sector, making a substantial contribution to the nation's gross domestic product (GDP). Despite its economic relevance, the barley microbiome is the least explored among cereal crops. Two pivotal unresolved questions were addressed in this study using metagenomic and metaproteomic approaches 1) how the microbial composition and functional capabilities change with prolonged storage and 2) how the microbiome is influenced by the inherent nitrogen content of the barley grain. The microbiome of eight barley samples from the Kadie cultivar (obtained from Anheuser-Busch InBev storage facilities in the Western Cape Province, South Africa) were analysed. These samples were derived at four distinct time points, at harvest and after three, six and nine months of silo storage. Further, at each time point, one barley batch each with low or high grain nitrogen content were analysed.

Chapter 1 provides a review of the existing literature related to beer production, focusing on the role of barley as a crucial ingredient. It begins with an overview of the beer brewing process and explains why barley is particularly suited for this purpose. The chapter also discusses the impact of grain nitrogen content on beer quality. Special attention is given to the barley seed microbiome, examining its characteristics both before and during storage. The dynamic changes in the microbiome throughout these stages are explored to highlight their implications for barley preservation.

Chapter 2 presents the metagenomic sequencing of the barley seed microbiomes, which was conducted using the Illumina short read sequencing. Approximately 365.3 million reads were derived across all eight samples. Initial taxonomic classification of these metagenomic reads revealed a predominance of bacterial sequences, with only a minimal presence of fungal reads. Diversity analysis suggested that the duration of storage, rather than the nitrogen content of the grain, was the primary factor influencing microbial diversity associated with the barley seed.

Chapter 3 describes the reconstruction of 82 high quality metagenome-assembled genomes (MAGs). These MAGs encompass four bacterial phyla and 26 genera, which form part of the core microbiome of the barley seeds. Comparative genomic analysis revealed a limited number

of shared single-copy orthologues across the MAGs, with only 66 common orthogroups detected. Additionally, a survey of plant pathogenicity-related proteins highlighted several MAGs that possess a substantial proportion of these proteins, offering insights into potential plant-microbe interactions.

In **Chapter 4** the metagenomes were assembled and annotated using a variety of *in silico* tools. While metagenomics can provide insights into the functional potential of the microbial community associated with barley seeds, it does not reflect their actual activity. Hence, metaproteomic analysis was undertaken. The findings of this thesis enrich our understanding of the barley seed microbiome and lay a foundation for future meta-omics studies of seed microbiomes.

Chapter 5 synthesises the findings of the previous chapters, focusing on how the microbial dynamics of barley seeds are influenced by nitrogen content and storage duration. While the diversity analysis showed that storage time had a more significant impact than nitrogen content on microbial composition, nitrogen still played a role in shaping specific community structures. This chapter discusses the broader implications of these findings, particularly for storage practices and brewing quality. Additionally, it highlights the distinct behaviour of the six-month low nitrogen (S6L) sample, which presented unique assembly metrics and challenges for interpretation. Chapter 5 concludes by suggesting future research directions, emphasising the importance of biological replication, deeper investigation into microbial interactions, and the application of advanced techniques such as metametabolomics and machine learning to better understand and optimise storage conditions for barley seeds.

Chapter 1 Literature review

1.1 What is beer?

Beer is deeply ingrained in human culture and ranks as the third most favoured drink globally, following water and tea (Bamforth, 2017; Collins *et al.*, 2023). Its popularity can be attributed to its distinct taste, aroma, and appearance, all of which result from a brewing process with ancient origins, dating back more than 8,000 years (Hornsey, 2003).

1.2 Overview of the beer making process

This process begins with the selection of grains, most notably barley, because of its high enzyme content (Magliano *et al.*, 2014); however, wheat, rye, oats, and corn, can also be used, adding distinct flavours and qualities to the beer (Wunderlich and Back, 2009). Once selected, the grain is malted, which involves soaking it in water, allowing it to germinate, and then drying it in a kiln (Figure 1). The malted grain is then mashed, a process where it is soaked in hot water to facilitate the enzymatic breakdown of starches into sugars (Schwarz and Li, 2010). The resulting liquid, known as wort, is boiled with hops for flavour before cooling (Briggs, 1998). Finally, yeast is introduced to the wort for fermentation, which converts the sugars into alcohol and carbon dioxide (CO_2), resulting in beer (Boulton, 2006). The quality of the grain, water, hops, and yeast, as well as any additional additives known as adjuncts, all affect the taste, aroma, and appearance of beer (Schwarz and Li, 2010). This process, although seemingly straightforward in concept, requires accuracy and attention to detail in order produce a product of high quality.

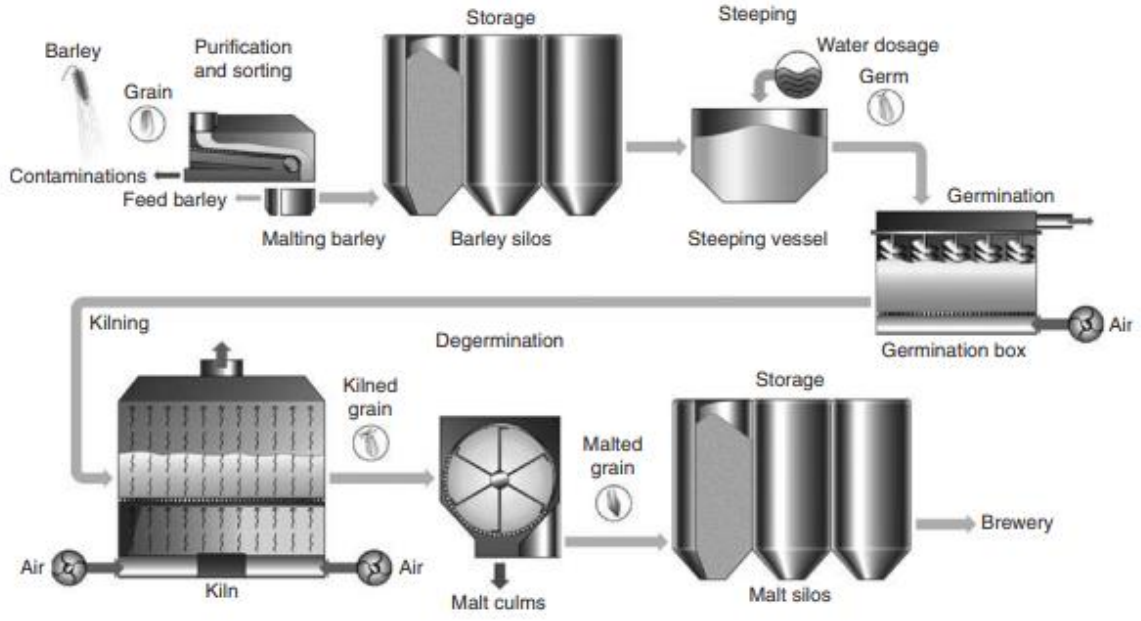


Figure 1.1 Diagram depicting the beer making process adapted from Wunderlich & Back (2009).

1.2.1 The selection and grading of grains

Prior to malting, high quality grains such as barley, rice, maize, oats and wheat must be selected (Wunderlich and Back, 2009). This is achieved by assessing properties of the grain such as husk condition, odour, and uniformity (Burger and LaBerge, 1985). Additionally, researchers conduct germination energy (GE) and germinative capacity (GC) tests to ensure the grains are suitable for use (Bera *et al.*, 2018). The GE test quantifies the percentage of seeds that begin to germinate within the first three days, specifically looking for the emergence of the radicle or the initial root. This test serves as a precursor to the GC test, which further evaluates the viability of the seeds (Bera *et al.*, 2018). In the GC test, a hydrogen peroxide solution is used to determine whether non-germinating seeds are dormant or have sustained damage, by assessing their response to oxidative stress (Bera *et al.*, 2018; Rani and Bhardwaj, 2021). For optimal results, GC should reach 95% in preliminary assessments and 96% following cleaning and storage (Bera *et al.*, 2018; Wunderlich and Back, 2009). Suitable grains are then preserved in refrigerated silos for four to eight weeks. During this dormancy period, the grains are protected from premature stem germination (Shu *et al.*, 2015).

1.2.2 The malting processes

Malting involves converting barley or other cereal grains into an important ingredient for brewing (Burger and LaBerge, 1985). This process consists of three essential stages: steeping, germination, and kilning, with each contributing uniquely to the formation of the final malt product (Rani and Bhardwaj, 2021).

1.2.2.1 Steeping and Germination

The steeping process involves a series of water soakings spanning 48 to 96 hours, followed by an air rest period (Brookes *et al.*, 1976). During this rest period, water is drained and CO_2 is released through aeration (Rani and Bhardwaj, 2021). The goal of steeping is to increase the moisture content of the grain to a range between 42 and 48%, while retaining seed viability (Brookes *et al.*, 1976). The development of the coleorhiza, a protective sheath for the emerging root, after successful steeping indicates that the grain is ready for germination (Schwarz and Li, 2010). The germination process begins at approximately 35% moisture content, but greater steep moisture levels are often required to achieve consistent enzyme diffusion throughout the endosperm, resulting in uniform alteration of the endosperm components (Schwarz and Li, 2010).

Germination is the process by which a seed sprouts and grows into a seedling (Domin *et al.*, 2020). It begins with the seed absorbing water, which causes it to swell and become soft (Shu *et al.*, 2015). This activates the metabolic activity of the seed, which had previously been dormant or reduced (Shu *et al.*, 2015). Germination changes the endosperm, promoting enzyme activity that converts nutrients for the embryo, while helping to limit water loss through respiration (Ahmed *et al.*, 2018). This stage is characterised by embryo development, usually observed through rootlet growth and an increase in shoot length (Shu *et al.*, 2015). During the germination phase, soaked grains are spread over the floor and must be flipped over on a frequent basis to remain loose (Ahmed *et al.*, 2018; Burger and LaBerge, 1985). This is a critical step, as it allows for adequate airflow and prevents matting and excessive heat production by the grains (Ahmed *et al.*, 2018; Shu *et al.*, 2015). Germination takes three to six days in aerobic and humid conditions at 16 - 20°C, depending on the technique and raw material used (Poutanen, 2020).

During germination, gibberellic acid (GA) penetrates the aleurone layer, triggering the production of hydrolytic enzymes that break down polysaccharides and proteins in the

endosperm (Kumar *et al.*, 2013; Armitage *et al.*, 2004; Schwarz, 2020). This breakdown allows starch-converting enzymes, primarily α-amylase and β-amylase, to access the endosperm (Kumar *et al.*, 2013). β-amylase converts starch into fermentable sugars like maltose, which later form alcohol and CO_2, while α-amylase produces non-fermentable sugars such as maltodextrins, contributing to the texture and malty flavour of beer (Hudson, 1986; Bera *et al.*, 2018; Rani and Bhardwaj, 2021).

Concurrently, proteases produced break down 40 to 50% of the proteins into peptides and free amino acids, producing free amino nitrogen (FAN), important for yeast metabolism during fermentation (Fox, 2010; Kumar *et al.*, 2013). These nutrients are transferred to the embryo via the scutellum, providing energy and building blocks for growth (Fox, 2010). Grain modification is considered optimal when the shoot reaches three-quarters of its length, marking readiness for the kilning phase (Schwarz and Li, 2010).

1.2.2.2 Kilning and Storage of malt

Kilning consists of three stages: free drying (50-60°C), falling rate (65-75°C), and curing (above 80°C for up to two hours), reducing grain moisture from 44 to 4-5% while preserving enzymatic activity and developing the malt's aroma and colour (Schwarz and Li, 2010; Rani and Bhardwaj, 2021).This process also removes unwanted flavours and prevents enzyme denaturation (Hudson, 1986). After kilning, rootlets (culms) are removed, and the malt is matured for at least three weeks to ensure even moisture distribution (Briggs *et al.*, 1981). Proper control of moisture and temperature during storage prevents microbial growth and enzyme degradation (Rani and Bhardwaj, 2021; Guine and Correia, 2013).

1.2.3 The brewing processes

The brewing process of beer comprises three main phases: milling and mashing the malted grains to convert starches into fermentable sugars, followed by lautering, boiling the wort with hops, and fermenting with yeast to produce alcohol and CO_2 (Briggs *et al.*, 1981). The final steps involve maturation to enhance flavour, filtration to remove solids, and carbonation before packaging in bottles, cans, or kegs. Each phase contributes to the distinct flavour of beer (Stewart *et al.*, 2017).

1.2.3.1 Milling and mashing

Milling is the process of crushing dried malted grains into a coarse powder called grist, which is crucial for the subsequent stages of brewing, such as mashing and lautering, as it affects the quality of the final beer (Briggs *et al.*, 1981; Stewart *et al.*, 2017). A finer milling increases the surface area, facilitating enzymatic reactions and the dissolution of ingredients (Stewart *et al.*, 2017). During mashing, the grist is mixed with warm water, initiating the conversion of starch into simple sugars and proteins into peptides, a process influenced by pH and temperature control (Mallett, 2014). Starch breakdown involves gelatinisation, liquefaction, and saccharification, converting starch into fermentable sugars, primarily maltose, while proteolysis breaks down proteins into amino acids, which are vital for flavour and foam stability (Schwarz and Li, 2010; Briggs, 1998). The mashing process culminates in the formation of wort, a liquid containing sugars, proteins, and minerals essential for fermentation, which is prepared in a mash tun, where water aids in enzymatic reactions and the extraction of key organic molecules (Schwarz and Li, 2010).

1.2.3.2 Lautering, wort boiling and fermentation

The lautering process separates the liquid wort from the solid grist using a lauter tun, where the grain husk acts as a natural filter (Briggs *et al.*, 1981). After draining the wort, the grist is rinsed with hot water to extract as much sugar as possible (Schwarz and Li, 2010; Stewart *et al.*, 2017). The wort is then boiled to halt enzymatic activity and extract bitterness from hops, which add flavour, aroma, and preservative qualities (Briggs *et al.*, 1981; Stewart *et al.*, 2017). After boiling, the wort is cooled and prepared for fermentation, with yeast converting the sugars into alcohol and CO_2 over 14 days (Briggs *et al.*, 2004; Stewart *et al.*, 2017). Top fermentation, using *Saccharomyces cerevisiae*, produces ales with a rich taste, while bottom fermentation with *Saccharomyces pastorianus* results in lagers with a cleaner, crisper flavour (Bamforth, 2017). Throughout the process, acids are added to lower the pH and prevent bacterial growth, ensuring a stable final product (Bamforth, 2017).

1.2.3.3 Maturation, filtration and packaging

Following fermentation, the freshly generated “green” beer goes through a maturation and carbonation phase (Briggs *et al.*, 1981). The beer is stored at 0°C for several weeks or months, dependent on the beer type. It is during this period that yeast, resin, proteins and other unwanted components are filtered out, resulting in a cleaner beer (Stewart *et al.*, 2017). This maturation

further improves flavour and aroma through the formation of esters (Wunderlich and Back, 2009). The desired level of carbonation is then introduced by infusing CO_2 into the beverage (Schwarz and Li, 2010). The beer is pasteurised at 60°C to stop yeast activity and eliminate any other microorganisms in the beer, consequently increasing shelf life (Stewart *et al.*, 2017). Finally, the beer is packaged in clean and pasteurised containers (Schwarz and Li, 2010).

1.3 The microbiology of beer making

Most of the steps involved in making beer are designed to stop the growth of undesirable microorganisms (Bamforth, 2017). Inevitably, microbial contamination in beer production can occur at any stage of the supply chain, from the field to the manufacturing plant to the point of consumption (Van Nierop *et al.*, 2006). Generally, microbial activity is known to enhance many sensory characteristics that define high-quality beer (Bamforth, 2017). However, at certain stages, the presence of specific microorganisms may be undesirable and can adversely affect the quality of the beer (Bokulich and Bamforth, 2013). This is alarming for the brewing industry since microbial spoilage causes an estimated economic loss of millions of US dollars each year (Bokulich and Bamforth, 2013).

1.3.1 Microbial impact on malt and its quality

The malting phase creates an environment conducive to microbial growth due to several factors (Briggs, 1998; Doran and Briggs, 1993; Kelly and Briggs, 1992). For instance, malting involves soaking barley, which substantially increases its moisture content, creating ideal conditions for microbial proliferation (Armitage *et al.*, 2004). Additionally, the availability of nutrients in barley, or any other grains used for brewing, supports this growth (Bamforth, 2017). The sugars, proteins, and minerals released during the malting process provide a rich food source for microorganisms (Agribook, 2021). This stage is also characterised by several temperature-controlled steps, which may promote microbial growth (Bamforth, 2017). The pH levels during malting are typically neutral to slightly alkaline, optimal for many types of microorganisms (Bamforth, 2017). Furthermore, the malting process includes aerating the grains, supplying the oxygen necessary for aerobic microorganisms (Bokulich and Bamforth, 2013).

In general, microbial growth during germination negatively affects malt quality (Bokulich and Bamforth, 2013). For instance, the microorganism on barley grain surfaces can compete for oxygen with the embryo, slowing germination, and lowering rootlet growth and α-amylase activity (Briggs, 1998; Doran and Briggs, 1993; Kelly and Briggs, 1992). Furthermore, certain bacteria and fungi present in barley are capable of producing the plant hormone indole-3-acetate, GA and abscisic acid, which may alter germination and enzyme synthesis (Tuomi *et al.*, 1995).

The proliferation of the fungal population, particularly *Fusarium* species, is promoted by moisture conditions during the steeping and germination stages (Boeira *et al.*, 1999). However, this population declines as the malt undergoes drying during the kilning phase (Van Nierop *et al.*, 2006). Specific fungal genera, including *Aspergillus*, *Cladosporium*, and *Penicillium*, exhibit resistance to elevated temperatures however, and may persist in their growth processes on malt (Gyllang *et al.*, 1977; Van Nierop *et al.*, 2006).

Similarly, the bacterial population grows considerably during the malting process when compared to the community found in the dried seed (pre-malting seed) (Flannigan, 2003). This increase is dominated by the genus *Pseudomonas* and lactic acid bacteria (LAB), specifically those of the genus *Lactobacilli* (Van Nierop *et al.*, 2006). The growth is predominantly observed from the steeping phase to the completion of the germination process, and then it subsequently drops dramatically during the kilning stage (Bokulich and Bamforth, 2013; Van Nierop *et al.*, 2006). However, certain bacteria may persist on the final malt. This includes members of the genera *Lactobacilli* and *Pediococci*, which are recognised to play are role in beer spoilage (Booysen *et al.*, 2002; O'Sullivan *et al.*, 1999).

1.3.2 Microbial influence on wort quality

Microbial populations decline during the mashing stage, although thermotolerant microorganisms, especially LAB can survive (O'Sullivan *et al.*, 1999). The presence of bacteria during the mashing phase can provide benefits. For example, the use of LAB to acidify the mash improves nitrogen content, and stability of the foam, in addition to enhancing the colour and character of the beer (Lowe *et al.*, 2005). However, bacterial proliferation can also present major obstacles during prolonged mashing periods (Lowe *et al.*, 2005). For instance, *Bacillus* species can cause over-acidification and the production of nitrosamines by reducing nitrate to nitrite (Calderbank and Hammond, 1989; Smith *et al.*, 1992). Additionally, the

growth of *Clostridium* in the mash or wort can result in elevated levels of butyric acid, imparting a cheese-like aroma to the beer (Hawthorne *et al.*, 1991). Bacterial overgrowth on malt can also impede mash filtering through the production of dextrans (Haikara and Home, 1991). Dextrans are long branching polysaccharides that increase the viscosity of the mash, making it difficult to filter (Laus *et al.*, 2022). Furthermore, fungi on malt may produce β-glucanases and xylanases, which reduce wort viscosity and increase mash filtration (Laitila *et al.*, 2007). This decrease in viscosity negatively affects beer foam quality (Evans *et al.*, 1999).

Following the mashing process, the wort is boiled for an extended period, essentially sterilising it (O'Sullivan *et al.*, 1999). However, its high nutritional content makes it susceptible to opportunistic spoiling microorganisms unless fermentation is started immediately. Gram-negative enterobacteria, such as members of the genus *Citrobacter*, *Escherichia*, *Klebsiella*, *Obesumbacterium* and *Pantoea* are responsible in wort spoiling (Laitila *et al.*, 2018). These bacteria are known to produce large amounts of 2,3-butanediol, organic acids, and dimethyl sulphide in the wort, which can give the beer an unpleasant fruity odour (Laitila *et al.*, 2018; Priest *et al.*, 1974). *Enterobacteria* also inhibit the growth of *Saccharomyces* (Bokulich and Bamforth, 2013; Laitila *et al.*, 2018).

1.3.3 Impact of microbial influences on fermentation

Microorganisms on the barley husk produce enzymes that break down the cell walls into various components, some of which are then used in the brewing process (van Nierop *et al.*, 2004). This enzymatic activity results in the generation of various breakdown products, including acidic polysaccharides of substantial molecular weight, which are hypothesised to enhance the cross-linking of lectin-like proteins on yeast cell surfaces, thereby forming significant clusters or flocs of yeast cells (Gorjanović *et al.*, 2004). Moreover, during the maturation of grains and the malting process, microbial stress induces a defensive immunological response in barley, leading to the accumulation of plant defensins, including antimicrobial peptides (Kaur *et al.*, 2012). These peptides have been implicated in premature yeast flocculation (PYF) by disrupting yeast cell metabolism, respiration, and cell membrane integrity, causing irreversible cellular damage (van Nierop *et al.*, 2004; Panteloglou *et al.*, 2012; Qin *et al.*, 2023). Studies have also shown that fungi, rather than bacteria, are more closely associated with PYF throughout the brewing process (Kaur *et al.*, 2012; MacIntosh *et al.*, 2014). Fungal contamination in the field barley, specifically by *Fusarium graminearum* and *Cochliobolus sativus*, are known to cause PYF behaviour (MacIntosh *et al.*, 2014).

Furthermore, it has been reported that treating malt husk with extracellular fungal enzymes before mashing can cause PYF (Kaur *et al.*, 2012).

1.4 Barley as a key ingredient in beer production

1.4.1 Introduction and adaptability

Barley (*Hordeum vulgare* L.) is an important cereal crop globally and is used for both human and animal consumption (Newman, 2006). Globally, it occupies the fourth position in terms of both quantity produced and area of cultivation, following maize, rice and wheat (Bera *et al.*, 2018). Its importance in early agricultural cultures is demonstrated by the fact that it was one of the earliest plants domesticated and played a key part in the transition from nomadic to sedentary lifestyles (Hornsey, 2003).

Barley is regarded as the most adaptable cereal because of its ability to grow in a wide range of climates and geographical regions (Quan *et al.*, 2016; Kumar *et al.*, 2013). It thrives in both temperate and tropical climates and can withstand harsher circumstances than many other cereals (Arendt and Zannini, 2013; Kumar *et al.*, 2013). Barley can grow well on poor soils and tolerate droughts, making it a dependable crop in areas where other cereals may fail (Hao *et al.*, 2022). It is widely grown in countries such as Canada, Germany and Russia, but also thrives in high-altitude countries such as Nepal and Tibet, where it is grown on mountain slopes at higher elevations than any other cereal crops (Kumar *et al.*, 2013; Bianco *et al.*, 2018; Hao *et al.*, 2022). Barley also has a short growing season which allows it to evade seasonal challenges such as late spring frosts or summer droughts (Hao *et al.*, 2022). This adaptability makes barley an essential crop in many parts of the world, contributing to food security and agricultural sustainability (Kumar *et al.*, 2013).

1.4.2 The benefits of using barley in beer production

As mentioned previously, beer can be made from a number of grains, such as barley, rice, millet, corn, and sorghum (Hornsey, 2003). However, the barley seed has unique enzymatic and physical characteristics, making it the preferred grain for the brewing industry (Newman, 2006; Schwarz and Li, 2010). The multilayer seed structure of barley is important, notably the aleurone layer, which is rich in malting enzymes (Rani and Bhardwaj, 2021). Furthermore, the

endosperm of barley seeds contains a substantial starch reserve that is required after germination (Rani and Bhardwaj, 2021).

Although other cereals have an aleurone layer, barley is the most effective at breaking down the endosperm and converting starch to simpler sugars (Arendt and Zannini, 2013). Barley enzymes, particularly α-amylase, are extremely thermally stable, which is important for mashing as temperature changes can impede enzyme activity (Laus *et al.*, 2022). This stability ensures consistent performance across brewing temperatures, allowing for a uniform conversion of starch to sugar (Parés Viader *et al.*, 2021; Laus *et al.*, 2022). Barley enzyme effectiveness is also influenced by temperature and pH; α-amylase and β-amylase perform best at specific temperatures and pH ranges, allowing for a more successful and regulated conversion process (Parés Viader *et al.*, 2021).

The composition and structure of barley provides further evidence of its suitability for beer production. With starch accounting for more than 70% of its dry mass, barley has a high starch-to-husk ratio which is a benefit in the mashing process (Yu *et al.*, 2017). The barley husk not only protects the kernel during malting, but it is also detachable, producing a natural filter bed during the lautering stage of brewing, which aids in the filtration process (Yangcheng *et al.*, 2016). This attribute is not found in other grain used for brewing. Furthermore, barley has a higher diastatic power (DP) than other grains (Rani *et al.*, 2022). This suggests that it has high starch-converting efficiency, which adds to its suitability as the grain of choice for beer production.

1.5 Protein and nitrogen content of barley and their impact on beer production

1.5.1 The impact of grain protein content on beer quality

Barley has a protein content ranging from 9 to 12%; this provides an ideal balance for yeast nutrition, effective fermentation, and the development of beer body and foam stability (Zhu, 2017). This balance is essential since too much protein will generate haze and undesirable flavours, whilst too little can limit mouthfeel and foam retention (Xie *et al.*, 2023). In contrast, grains such as wheat, maize, and rice have a wide range of protein levels (Arendt and Zannini, 2013). This can impact the brewing process and beer quality in a variety of ways. Wheat, for example, may improve foam stability while increasing the probability of haze formation,

whereas maize and rice, with lower protein content, may be preferred for brewing lighter beer styles due to their impact on body and foam stability (Briggs *et al.*, 2004). Considering the importance of grain protein content in defining the taste and quality of the finished beer, it is essential to select barley grains with adequate protein content (Briggs *et al.*, 2004). An effective approach to assess this is through a measurement of the nitrogen content of the grain.

1.5.2 Nitrogen as an indicator of protein levels

1.5.2.1 Introduction and sources of nitrogen in barley

Nitrogen in barley grains originates from two primary sources, the soil and the atmosphere (Pohlman and McColl, 1982). The initial source is soil nitrogen (Toews and Soper, 1978). Through their roots, barley plants assimilate nitrogen available in the soil, which exists in several forms, such as nitrate (NO_3^-) and ammonium (NH_4^+) (Söderberg and Bååth, 2004). Alternatively, barley benefits from the conversion of atmospheric nitrogen (N_2) into a usable form for plants (either ammonium or nitrate) by certain bacteria (Pohlman and McColl, 1982).

Nitrogen is a key component of plant growth, and has a considerable impact on germination rate (Finkel, 2011). The correlation between the nitrogen content of seeds and their protein levels is based on the premise that proteins are composed of amino acids (Kibite and Evans, 1984; Beauregard and Hefford, 2006). All amino acids incorporate nitrogen, which is an essential component of their structure and accounts for approximately 16% of their weight (Yu and Fukagawa, 2020). Given this composition, nitrogen in seeds can serve as a reliable indicator for calculating protein content.

1.5.2.2 Techniques for nitrogen content determination in barley grains

Assessing the nitrogen content in barley grains can be achieved using several methods such near-infrared spectroscopy (NIRS) (Angelino and Convention, 1996), the Kjeldahl method (Buckee, 1994) and the Dumans method (Ebeling, 1968). NIRS is a non-invasive technique that illuminates a sample of barley with near-infrared light and measures the amount of light absorbed by the sample. Different compounds in the seed, including protein, absorb different amounts of light, making it easier to determine protein and, by extension, nitrogen content (Sileoni *et al.*, 2015). The NIRS process is swift, bypasses the need for sample preparation and allows for the evaluation of numerous barley quality aspects beyond protein content, such as moisture, starch, and oil content (Sileoni *et al.*, 2015). However, it requires calibration using

known samples, and its accuracy can be impacted by factors such as the temperature of the grain and the equipment employed (Sileoni *et al.*, 2015).

In parallel, the Kjeldahl method is a standard technique for estimating the total nitrogen (and by proxy the protein content) of a sample (Buckee, 1994). The sample is digested in sulfuric acid to convert organic nitrogen to ammonium, after which it is converted to ammonia and distilled. The resultant ammonia is then titrated to determine its nitrogen content (Marcó *et al.*, 2002). Another laboratory analysis to measure nitrogen content encompasses the Dumas method, which involves the combustion of a barley grain sample to release nitrogen gas (Ebeling, 1968). The nitrogen content is then quantified based on the volume of nitrogen gas produced (Ebeling, 1968).

It is important to note that while these methodologies can provide an approximation of protein content, they do not reveal information about the specific proteins present or their function, which impacts malting quality. Thus, additional tests, such as those for enzymatic activity, GC and grain rigidity, must be conducted to determine the suitability of barley grains for malting (Kumar *et al.*, 2013).

1.5.2.3 Nitrogen grain content impact on brewing.

In brewing, the preferred nitrogen content in barley grains is typically between 1.4 and 1.8% on a dry weight basis (Kassie and Tesfaye, 2019). Barley with a nitrogen content below 1.4% might lead to poor yeast nutrition and inadequate beer head formation, whereas nitrogen levels above 1.8% can jeopardise beer clarity and stability (Kassie and Tesfaye, 2019). Low nitrogen levels can stress yeast cells, resulting in off-flavours, whereas high nitrogen levels might cause excessive fermentation and undesirable byproducts (Kassie and Tesfaye, 2019). Brewers often tailor nitrogen levels to suit the specific beer style, striving for the desired balance of flavour, aroma and physical characteristics (Stewart *et al.*, 2017). For example, ales often require a higher nitrogen content to support their robust flavour and fuller body (Stewart *et al.*, 2017). Accurate measurement and management of nitrogen levels is therefore essential for ensuring the suitability of barley grains for high-quality malt and beer production, with the required nitrogen content varying according to brewing specifications (Agu and Palmer, 2001). Maltsters and brewers meticulously manage nitrogen levels by carefully selecting barley varieties and modifying the conditions of malting and brewing. This precise control maximises protein utilisation, thereby minimising its impact on the quality of beer (Schwarz and Li, 2010).

1.6 Processing barley for brewing from harvest to storage

Barley is generally ready for harvest approximately four months after sowing, though some varieties may mature in about two months (Henry, 2016). It is advisable to harvest barley when the grain moisture level is between 16-18% (w/w) (Henry, 2016). This moisture range is optimal for direct combining, a process that integrates harvesting and threshing into a single step (Khokonova, 2011). Following harvest, it is important to clean the grain to eliminate foreign materials and then dry it to a desired moisture content of 12% (Henry, 2016). Subsequently, the barley is stored in silos, which offer effective protection against external air and pest contamination (Henry, 2016; Khokonova, 2011). Silos are also designed to avert moisture and mould growth through their high-quality sealing, yet they provide the option to ventilate to maintain air quality (Ghali, 2014). With proper temperature and moisture management, barley can be stored for extended periods (Mallett, 2014). The duration of storage can, however, vary based on the moisture content of the grain and its intended use (Khokonova, 2011). For instance, barley intended for brewing, with a moisture content of 10.5% or lower, can be stored for up to 18 months at temperatures ranging from 10 to 20°C (Khokonova *et al.*, 2015; Henry, 2016).

Monitoring the internal temperature of the silos is important; the optimal temperature ranges are recommended to be 20–23°C during summer and below 15°C in winter (Ghali, 2014). If these temperature guidelines are not adhered to, barley may experience accelerated ageing or a decline in viability and GC (Angelino and Convention, 1996). Managing humidity levels is equally important; changes in humidity can affect the moisture content of barley, leading to microbial growth or spoilage (Henry, 2016; Khokonova *et al.*, 2015). The duration of storage distinctly impacts the quality of barley (Flannigan, 1978; Henry, 2016). As storage time increases, barley grains may gradually lose their vitality, leading to slower germination rates and, in some cases, seed decay (Mwazha *et al.*, 2022). Prolonged storage can also negatively affect enzyme activity within the barley (Felšöciová *et al.*, 2021; Mwazha *et al.*, 2022).

Several microorganisms are found in different parts of the seed (Bokulich and Bamforth, 2013). Changes in storage conditions, such as moisture, temperature, oxygen levels and storage duration, can alter the microbial dynamics of the grain or promote the proliferation of microorganisms that may have detrimental effects, such as the growth of pathogenic microorganisms (Bokulich and Bamforth, 2013). (Khokonova *et al.*, 2015).

1.7 Introduction to the seed microbiome

The term microbiome refers to a community of microorganisms, including archaea, bacteria, fungi and viruses, that reside within a specific environment (Marchesi and Ravel, 2015). This environment may vary, extending from the human body to seed, soil, or a body of water (Berg *et al.*, 2020). Each microbiome is unique, with its composition influenced by the environment it occupies and other external factors (Berg *et al.*, 2020). The seed microbiome refers to the community of microorganisms that live in, on and around seeds (Wassermann *et al.*, 2021). These microorganisms can transfer to subsequent plant generations, thereby playing a critical role in plant health (Abdelfattah *et al.*, 2023). The seed microbiome comprises both pathogenic and beneficial microorganisms and includes microorganisms that colonise seeds through two primary modes: endophytic and epiphytic colonisation (Abdelfattah *et al.*, 2023). Endophytes are microorganisms, typically bacteria or fungi, that reside in the internal tissues of seeds, where they vertically transmit to their progeny seedlings (Díaz Herrera *et al.*, 2016; Hodgson *et al.*, 2014). In contrast, epiphytes are microorganisms that live on the surface of seeds (Hodgson *et al.*, 2014). These epiphytes may penetrate the seed tissues and be passed from parents to offspring through vertical transmission, or they may spread between distinct seeds or plants via horizontal transmission (Hodgson *et al.*, 2014; War *et al.*, 2023).

1.7.1.1 Beneficial role of the seed microbiome

Seed-associated microorganisms contribute to the improvement of seedling establishment as well as growth and development, especially in soils lacking nutrients and under harsh conditions (Compant *et al.*, 2019; War *et al.*, 2023). Firstly, they improve nutrient uptake and assimilation through various processes such as phosphate solubilisation, siderophore production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and nitrogen fixation (Bashir *et al.*, 2022, Pohlman and McColl, 1982; Chimwamurombe *et al.*, 2016; Truyens *et al.*, 2015). Secondly, they play a role in biological control, offering protection against pathogens and competing against rival plant species (Díaz Herrera *et al.*, 2016; Saeed *et al.*, 2021). Thirdly, they boost plant metabolic processes such as modulating reactive oxygen species (ROS) levels and regulating phytohormones such as auxin, cytokinin and gibberellin (Pitzschke, 2016; Shahzad *et al.*, 2016; Scott *et al.*, 2007). Furthermore, they confer resistance to abiotic stresses including drought, salinity and heavy metal contamination (Oukala *et al.*, 2021; Trivedi *et al.*, 2020).

1.7.1.2 The detrimental effects of the seed microbiome

Not all microorganisms associated with the seed microbiome are beneficial. The susceptibility of seeds to microbial pathogens can be attributed to their high nutrient content and protective nature, which provides pathogens an attractive habitat and a food source (Turner *et al.*, 2020). Microbial pathogens may produce spores and toxins known to impair seed quality and GC (Noots *et al.*, 1999). Seed germination and growth can be further hindered by the depletion of nutrients, which can occur because of the enzymatic breakdown of complex compounds by the microbial pathogens. Microbial enzymes work together to degrade plant defences, making plant tissues more susceptible to pathogen invasion (War *et al.*, 2023). Endoglucanases and exoglucanases, for example, are enzymes that target cellulose, a key component of seed cell walls (Kubicek *et al.*, 2014; de Vries and Visser, 2001). Pectinases are enzymes that break down pectin, which is required for the plant cell wall to remain intact (Walton, 1994; Bonnin *et al.*, 2014). Lipases damage the lipid components of the plant cell membrane, compromising the structural integrity of the seed (Walton, 1994).

1.7.2 The barley seed microbiome

The barley microbiome refers to the community of microorganisms that live in association with roots, stems, leaves and seeds of barley plants (Noots *et al.*, 1999). Recent studies employing advanced sequencing technologies, such as metagenomics and amplicon sequencing, have revealed the vast microbial diversity associated with barley seeds (Bziuk *et al.*, 2021; Saeed *et al.*, 2021). These studies have identified a wide range of bacterial taxa, including Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes, as well as diverse fungal communities dominated by Ascomycota and Basidiomycota (Bziuk *et al.*, 2021; Felšöciová *et al.*, 2020; Felšöciová *et al.*, 2021).

Among the bacteria, *Pseudomonas* is a predominant genus within the barley seed microbiome (Bziuk *et al.*, 2021; Tshisekedi *et al.*, 2024). Members of this genus are known for their roles in suppressing soil-borne diseases, enhancing stress tolerance, and promoting plant growth by producing phytohormones (Pham *et al.*, 2017; Fröhlich *et al.*, 2012). *Bacillus* species, also found abundantly, offer protection against various pathogens through the production of antibiotics and antifungal compounds and are involved in nutrient mobilisation, which is crucial for seedling development (Smith *et al.*, 1992; Lowe *et al.*, 2005). Additionally, genera such as *Enterobacter* and *Pantoea* are often found in barley seeds, where they promote plant

growth by fixing nitrogen, solubilizing phosphate, and producing growth-promoting substances (Lv *et al.*, 2022; Singh *et al.*, 2021).

The fungal taxa associated with barley seeds include species such as *Fusarium, Alternaria,* and *Cladosporium* (Felšöciová *et al.*, 2021). While some *Fusarium* species can cause diseases in barley, others may be neutral or beneficial, competing with pathogenic microbes and contributing to the microbial balance (Díaz Herrera *et al.*, 2016). *Alternaria,* commonly associated with the phyllosphere, also inhabits barley seeds, with roles varying from pathogenic to symbiotic depending on environmental conditions and the presence of other microorganisms (Felšöciová *et al.*, 2021). *Cladosporium,* known for its ubiquitous presence, can be part of the normal seed microbiota, aiding in biotic and abiotic stress tolerance (Felšöciová *et al.*, 2021).

1.7.3 Bacterial communities in barley

Bacteria are the most abundant microorganisms in soil, playing pivotal roles in nutrient cycling and disease suppression (Felšöciová *et al.*, 2020; Noots *et al.*, 1999; Shala *et al.*, 2023). Commonly found genera include *Actinobacteria*, *Bacillus*, *Paenibacillus, Pantoea*, and *Pseudomonas* (Felšöciová *et al.*, 2020; Noots *et al.*, 1999; Shala *et al.*, 2023). These bacteria are fundamental in promoting seed growth by facilitating processes improving mineral nutrition, nitrogen fixation and phosphate solubilization (Yulan Chen *et al.*, 2022; Wassermann *et al.*, 2021; Bziuk *et al.*, 2021).

Several strains within the barley seed microbiome are particularly beneficial, enhancing plant growth and providing resistance against pathogens (Felšöciová *et al.*, 2020; Noots *et al.*, 1999; Shala *et al.*, 2023). Notably, certain strains of *Bacillus* and *Pseudomonas* produce plant growth-promoting substances such as indole acetic acid (IAA) and siderophores, which not only enhance plant growth but also suppress disease-causing pathogens (Bziuk *et al.*, 2021). For example, strains of *Paenibacillus* produce biofilms that effectively control *Fusarium graminearum* (Taheri *et al.*, 2022), while strains of *Pseudomonas* offer resistance against barley leaf disease caused by *Rhynchosporium secalis* and suppress the growth of the root pathogen *Gaeumannomyces graminis* (Dutilloy *et al.*, 2022; Fröhlich† *et al.*, 2012). *Pantoea agglomerans* inhibits the proliferation of *Pseudomonas syringae pv. syringae*, which causes basal kernel blight (Braun-Kiewnick *et al.*, 2000). Additionally, *Duffyella gerundensis* has been shown to inhibit the growth of *Fusarium tricinctum* and reduce toxin levels associated with this fungus (Gnonlonfoun *et al.*, 2023). However, not all microorganisms in barley seeds

are beneficial; *Erwinia persicina*, for instance, causes a decline in seed quality due to the pink staining it produces (Kawaguchi *et al.*, 2021).

The bacterial communities in stored barley play a role in determining the quality and safety of the grains (Felšöciová *et al.*, 2020). At the onset of storage, *Pantoea agglomerans* is typically predominant, but over time, a diverse array of bacteria including *Alcaligenes spp.*, *Arthrobacter globiformis*, *Clavibacter iranicum*, *Kosakonia cowanii*, *Lactobacillus*, *Pseudomonas fluorescens*, *Pseudomonas libanensis*, *Pseudomonas poae*, *Pseudomonas synxantha*, and *Staphylococcus haemolyticus* can also be found in barley seeds, though the functional roles of these bacteria during storage remains largely unexplored (Felšöciová *et al.*, 2020; Noots *et al.*, 1999). Research indicates that while the abundance of coliform bacteria tends to increase, LAB, which are vital for the malting process, tend to decrease as storage progresses (Felšöciová *et al.*, 2020). Additionally, spore-forming bacteria such as *Bacillus altitudinis* may degrade seed quality and complicate the management of microbial populations in stored grains (Dar *et al.*, 2021).

1.7.4 Fungal communities in barley

Fungi are crucial for organic matter decomposition and nutrient cycling, with common genera including *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Rhizoctonia*, involved in interactions affecting seed health and storage quality (Wen Chen *et al.*, 2022; Felšöciová *et al.*, 2020; Flannigan, 1978; Flannigan, 1969; Laitila *et al.*, 2007). For instance, *Fusarium* species are known for producing mycotoxins that can lead to diseases such as *Fusarium* head blight (Alisaac and Mahlein, 2023). *Alternaria* spp. are responsible for leaf spot diseases that diminish crop yields and degrade barley seed quality (Agostinetto *et al.*, 2020). Additionally, the conditions required for the proliferation of fungi such as *Penicillium* and *Cladosporium*, which thrive with a water activity (*aw*) above 0.90 corresponding to moisture levels of 20 to 25%, are common in fields and storage areas, highlighting the need for effective environmental management to maintain barley quality (Felšöciová *et al.*, 2021).

Fungi proliferate post-harvest and throughout the storage of barley, often associated with the occurrence of pathogens that can deteriorate grain quality (Wen Chen *et al.*, 2022). The mycobiome of stored grains predominantly includes mould genera such as *Aspergillus*, *Alternaria*, *Cladosporium*, *Fusarium*, *Mucor*, *Rhizopus*, and *Penicillium*, which are capable of surviving long periods under varying storage conditions (Noots *et al.*, 1999; Flannigan, 2003;

Blackburn, 2006; Felšöciová *et al.*, 2021). These fungi begin to germinate and expand once the moisture content in barley surpasses 15%, with certain species such as *Aspergillus* and *Fusarium* being particularly detrimental as they invade seeds and degrade essential nutrients, thereby depleting the energy reserves necessary for seed germination and early plant development (Blackburn, 2006; Boeira *et al.*, 1999; Birr *et al.*, 2020).

Moreover, the diversity of fungal species detected in barley changes throughout the storage period, adapting to lower water activity levels, thus showing resilience even in drier conditions (Felšöciová *et al.*, 2021; Laitila *et al.*, 2007; Noots *et al.*, 1999). Yeasts such as *Candida*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sporobolomyces*, and *Torulopsis* also colonise stored barley grains, adding a layer of microbial interaction (Flannigan, 2003; Noots *et al.*, 1999). While some yeasts, like *Saccharomyces*, play a protective role through biocontrol activities that suppress pathogenic filamentous fungi, their presence can also lead to spoilage by increasing moisture content and the accumulation of unwanted metabolites (Bokulich and Bamforth, 2013).

1.7.5 The unexplored aspect of barley microbiomes for brewing

Our current knowledge of the barley seed microbiome hints at a complex web of interactions that are crucial to the storage life and quality of barley seeds, yet the specifics of these relationships need to be elaborated (Bziuk *et al.*, 2021; Felšöciová *et al.*, 2020; Felšöciová *et al.*, 2021). Short-term nitrogen fertilisation has been proven in studies to have an impact on agricultural soil by influencing microbial community composition and nitrogen mineralization processes (Ouyang and Norton, 2020). In addition, increasing nitrogen intake has been shown to increase the complexity of microbial networks in soil (Ouyang and Norton, 2020). These findings demonstrate the impact of nitrogen on the microbial community composition in agricultural settings.

The impact of nitrogen content in barley grain on seed quality is widely understood (Agu and Palmer, 2001). However, the intricacies of how nitrogen content impacts the microbial population in barley grain, and thus seed quality, are largely unresolved. It has been reported that nitrogen levels in the barley rhizosphere influence the selective enrichment of microbial taxa, with low nitrogen levels improving the genetic control of the host over its microbiota (Alegria Terrazas *et al.*, 2022). Furthermore, taxonomic diversity plays a role in functional

specialisation, which promotes plant development in nitrogen-limited environments (Alegria Terrazas *et al.*, 2022).

One key unsolved aspect is how grain nitrogen content affects the microbiome of barley stored for beer production, compared to that of harvested seeds. Although the changes in the barley microbiome during storage are known, its functional capacities during this period remain unclear (Felšöciová *et al.*, 2020; Felšöciová *et al.*, 2021). To address this gap, a more specific strategy could include identifying microbial signatures or indicator species in the microbiome that are capable of predicting grain quality or signal potential shortcomings, allowing for more precise interventions. Furthermore, having a functional profile of what these taxa are capable of would be quite beneficial. This would be best performed through meta-omics analysis, which has potential for expanding our understanding (Fadiji and Babalola, 2020).

1.8 Meta-omics approaches to study temporal microbiome changes

Meta-omic techniques, particularly metagenomics and metaproteomics, have revolutionised our understanding of microbial communities by providing comprehensive insights into both the taxonomic composition and functional potential of these communities (Zhang *et al.*, 2019). In the context of barley seed storage, these techniques could offer an opportunity to delve into the dynamics of the microbiome over time, especially under varying nitrogen conditions, though the application of such approaches remains largely unexplored for barley (Bziuk *et al.*, 2021; Tshisekedi *et al.*, 2023).

Metagenomics allows researchers to capture a snapshot of the genetic potential of microbial communities by sequencing their collective DNA (Desai *et al.*, 2012). This approach provides information on the presence and relative abundance of different microbial taxa, offering insights into how microbial communities shift over time and in response to environmental changes, such as the nitrogen content of barley seeds (Fadiji and Babalola, 2020). For example, studies on other crops like wheat and maize have demonstrated how metagenomics can reveal microbial shifts during storage (Kaushal *et al.*, 2020; Akinola *et al.*, 2021; Aswini *et al.*, 2023). In these systems, it was observed that storage conditions, including temperature and moisture content, can significantly alter microbial community structure (Solanki *et al.*, 2019; Li *et al.*, 2023). Nitrogen fertilisation has similarly been shown to drive changes in microbial diversity and composition in maize rhizospheres (Miranda-Carrazco *et al.*, 2022; Liu *et al.*, 2024). These

findings suggest that metagenomics is an ideal tool for investigating microbial changes in stored barley seeds, particularly in relation to nitrogen content.

Metaproteomics, on the other hand, takes this analysis a step further by linking genetic potential to actual protein expression (Abraham *et al.*, 2014). While metagenomics provides information on what microorganisms could achieve on their genetic repertoire, metaproteomics reveals what microorganisms are doing by analysing the proteins they express (Heyer *et al.*, 2015; Abraham *et al.*, 2014). This technique is especially effective for understanding functional changes within microbial communities over time. For instance, studies on rice storage have used metaproteomics to identify microbial proteins involved in starch degradation and protein synthesis, functions crucial for the preservation of seed quality (Salvato *et al.*, 2021). In nitrogen-fertilised environments, metaproteomics has been used to track how microbial communities adjust their metabolic processes in response to nitrogen availability (Pan *et al.*, 2024). These examples highlight the potential of metaproteomics to provide functional insights into how microbial communities associated with barley seeds may respond to varying nitrogen levels over prolonged storage periods.

While these techniques have been applied to a range of crops, the use of meta-omics to explore temporal changes in microbial communities during the storage of barley seeds remains uncharted. The integration of metagenomics and metaproteomics can provide a comprehensive understanding of both the taxonomic shifts and functional adaptations of microbial communities over time, particularly in response to nitrogen modulation (Zhang *et al.*, 2019; Graw *et al.*, 2021). This study, therefore, aims to fill this gap by applying both approaches to investigate the dynamics of the barley seed microbiome under different nitrogen conditions and storage durations, providing novel insights into how these factors influence microbial composition and function.

1.9 Research Aims and Objectives

The role of nitrogen in shaping microbial communities, particularly in the context of seed storage, remains an underexplored area in microbial ecology. While several studies have examined the effects of nitrogen on soil microbiomes, there is a limited understanding of how nitrogen levels within seeds influence microbial taxa and function over time. This knowledge gap is particularly evident in malting barley, where the effects of varying nitrogen concentrations on the seed microbiome during long-term storage have yet to be fully characterised.

Addressing this gap is essential as nitrogen plays a key role in microbial metabolic activities, which directly affect seed health, preservation, and ultimately malting quality. This research aims to explore these dynamics by employing advanced metagenomic and metaproteomic techniques to analyse how nitrogen content and storage periods influence both the composition and functionality of microbial communities in barley seeds.

The study will achieve this aim through the following objectives:

Objective 1: Assessment of microbial taxonomic abundance

This objective aims to estimate microbial taxa abundance in the barley seed microbiome with varying nitrogen contents across different storage times. By doing so, this research will determine whether certain microbial taxa are favoured or suppressed under specific nitrogen conditions. This will provide insights into how nitrogen shapes the microbial ecosystem over time, allowing for the identification of taxa that may play beneficial or detrimental roles in seed health and storage.

Objective 2: Reconstruction of metagenome-assembled genomes (MAGs)

Through the reconstruction of MAGs, this study will explore the genomic composition of microbial communities in relation to seed nitrogen content and storage conditions. By examining the presence of distinct genomic features, the research will determine if nitrogen-modulated communities harbour specific genetic capabilities, such as nitrogen fixation or enhanced metabolic versatility. This analysis will help pinpoint the nitrogen-responsive microoganisms, potentially including nitrogen-utilising bacteria or fungi, that contribute to seed preservation or deterioration.

Objective 3: Assembly and annotation of metagenomic reads

The assembly and annotation of metagenomic reads will create a comprehensive protein profile of the microbiome, linking genetic potential to microbial metabolic capabilities. By doing so, this research will uncover how the microbial community's metabolic potential shifts in response to nitrogen content, providing a deeper understanding of which metabolic pathways or if functions are enriched or diminished under different nitrogen conditions. This is particularly relevant for understanding microbial contributions to seed health and nitrogen cycling during storage.

Objective 4: Analysis of protein expression through metaproteomics

Metaproteomic analysis will offer direct insights into the functional roles that microoganisms play under varying nitrogen levels and storage conditions by assessing actual protein expression. This objective is critical for identifying which microbial functions are activated or repressed in response to nitrogen modulation, offering a real-time view of microbial activity in the stored seeds. Such insights could reveal key proteins involved in processes like nutrient cycling, seed preservation, or even spoilage, linking microbial function to practical outcomes in barley storage and malting.

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Chapter 2 Microbial community dynamics of stored barley seeds with high and low nitrogen content

2.1 Abstract

The impact of storage conditions on barley quality is pivotal for beer production, with the microbial composition during storage being a critical factor. This study aimed to elucidate the shifts in microbial communities associated with barley seeds, with high and low nitrogen grain contents, stored for varying periods. To achieve this, whole metagenomes of barley seeds were sequenced under different nitrogen and storage time conditions, and the resulting reads were taxonomically classified using the Kraken2 and Bracken pipelines. A notable bacterial predominance was observed, particularly with taxa from the genera *Erwinia*, *Pantoea*, *Pseudomonas* and *Stenotrophomonas*. Distinct microbial genera were more prevalent in seeds with high nitrogen content compared to those with low nitrogen content. Furthermore, notable changes in the microbial community structure correlated with the duration of storage. The detection of lytic phages suggests possible impacts on bacterial populations and overall community structures. Fungi were absent from seeds at harvest and those with high nitrogen content. These findings could provide brewers with insights for optimising storage conditions and time to potentially enhance malt quality.

2.2 Introduction

The quality of barley grain plays a pivotal role in determining the flavour profile of both malt and beer (Bamforth, 2017). Compromised barley quality, due to suboptimal cultivation and storage, directly affects the brewing process, yielding beer with poor flavour (Briggs *et al.*, 1981; Briggs *et al.*, 2004; Bamforth, 2017). This poses major financial risks to the brewing industry (Langridge, 2018). Following harvest, barley seeds may be stored for a period of time before malting to break seed dormancy (Felšöciová *et al.*, 2021). Storage conditions such as aeration level, integumentary grain tissue integrity, moisture content and temperature, are strictly monitored and controlled (Felšöciová *et al.*, 2021; Abubakar, 2019).

Seed spoilage during storage can be caused by a variety of microorganisms, including bacteria, yeasts, and filamentous fungi (Suzuki, 2020; Pitt and Hocking, 1997). These microorganisms

can be introduced to the seed at various stages, including during cultivation, harvesting and postharvest handling (Tyc *et al.*, 2020). The presence of fungi is especially problematic as they not only causes spoiling but can also introduce mycotoxins, which pose a health risk to both humans and animals (Bingham *et al.*, 2012; Felšöciová *et al.*, 2021). Previous studies have identified a wide range of fungi associated with barley at various stages, including storage and malting (Laitila, 2015; Suzuki, 2020). These fungi include *Alternaria*, *Aspergillus*, *Fusarium*, *Geotrichum* and *Penicillium* (Laitila, 2015; Laitila *et al.*, 2007; Felšöciová *et al.*, 2021). The presence of these toxigenic fungi emphasises the importance of strict storage conditions and times to preserve the quality of barley grain (Laitila, 2015).

Bacteria play an important role in seed deterioration during storage with certain species having a particularly strong influence. Among these, *Pseudomonas syringae* is well known for impairing seeds through the production of phytotoxins and extracellular polysaccharides that promote infection and colonisation (Afkhamifar *et al.*, 2023). Its pathogenicity is further enhanced by ice nucleation activity, predisposing seeds to frost damage under cold storage conditions (Lindow *et al.*, 1982). Similarly, *Xanthomonas campestris*, with its diverse pathovars, is implicated in causing black rot in seeds (Batista *et al.*, 2021). This bacterium facilitates disease through the secretion of extracellular enzymes and xanthan gum, which collectively contribute to the disintegration of seed tissues and obstruction of the plant vascular systems (Nejadmansouri *et al.*, 2020). *Pectobacterium carotovorum*, though primarily associated with soft rot in vegetables, also poses a risk to seeds stored in humid environments by releasing a suite of plant cell wall-degrading enzymes, such as cellulases, pectinases, and proteases, leading to the deterioration of seed integrity (Wasendorf *et al.*, 2022; Ranjan and Singh, 2020).

Studies have shown that bacterial communities associated with barley seed undergo substantial changes from pre-harvest to post-storage conditions (Angelino and Convention, 1996; Bziuk *et al.*, 2021). In pre-harvest barley, *Pantoea agglomerans* is the most abundant and commonly reported species within the culturable microbial community (Flannigan, 2003). Several studies have also tracked changes within the bacterial community of stored barley and have noted increases in coliform bacteria and decreases in lactic acid bacteria (LAB) (Felšöciová *et al.*, 2020; Abubakar, 2019). These shifts, however, are largely influenced by factors such as the initial microflora composition, storage conditions and duration (Abubakar, 2019).

The nitrogen content of barley grains is a critical factor in determining the quality of beer (Adeboye *et al.*, 2011). A grain with lower nitrogen content is preferable as grains with a higher nitrogen content may have higher protein levels (Agu and Palmer, 2001) which can result in undesirable foam stability and off-flavours in the beer (Ahmed *et al.*, 2018). Microorganisms play an important part in the nitrogen cycle, turning atmospheric nitrogen into usable forms and recycling it through ecosystems, thus impacting the nitrogen content of crops such as barley (Agu and Palmer, 2001). Their activities ensure the availability of appropriate nitrogen sources, which is essential for optimising barley grain quality for beer production (Baslam *et al.*, 2021). While the impact of nitrogen content on beer quality is well-documented, the relationship between barley nitrogen content and microbial dynamics during storage is less understood.

This knowledge gap presents an opportunity for further research, which could provide valuable insights into improving barley storage and the beer production process. Here, using metagenomics approaches, the microbial community composition associated with low and high nitrogen content barley seeds from harvest through to nine months of storage was determined.

2.3 Methodology

2.3.1 Sample collection and processing

Malting barley (*Hordeum vulgare* L.) samples of a single cultivar (Kadie) were sourced from Anheuser-Busch InBev (AB InBev) storage facilities in the Western Cape Province, South Africa. The collected samples were derived from four distinct time points, including barley seeds at harvest as well as those stored in silos for three, six and nine months, respectively (Table S1). At each time point, barley seeds were selected based on their nitrogen content; those with nitrogen contents < 1.5% and > 1.5% were classified as having low and high nitrogen contents, respectively. Nitrogen content classification was determined by AB InBev. The low and high nitrogen samples collected at different time points originated from a single harvest but were stored in different locations. Despite this, storage conditions were standardised across facilities to minimise variability and maintain comparability. All samples were aseptically collected and stored at -20°C to inhibit microbial growth.

2.3.2 DNA isolation, library preparation and sequencing

A sterile mortar and pestle were used to crush ~10 g of each barley sample. The residue was suspended in 40 ml of phosphate buffered saline (PBS; pH7.4, 0.137M NaCl, 0.01M Na_2HPO_4 and 0.0018M KH_2PO_4). The samples were then sonicated for 7 min at 18 W amplitude in a 30 second on-off pulsating schedule in a Microson Ultrasonic Cell Disruptor (Wazobia Enterprise, USA). Following sonication, the mixtures were centrifuged for 1 min at 4000 x g to separate the supernatant from the solid residue. The supernatants were transferred to a filtration system comprised of an autoclaved polycarbonate filter holder and a 0.45 µm pore Sartorius-Stedim Biotech filter membrane. The microbial cells collected on the filter paper were transferred to sterile Eppendorf tubes. Metagenomic DNA (mDNA) extraction was performed on these samples using the ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol.

Library preparation and sequencing were carried out at Molecular Research LP (MR DNA, Texas, USA). Briefly, the mDNA concentration was evaluated using the Qubit® dsDNA HS Assay Kit (Life Technologies, USA). A total of 50 ng mDNA per sample was used to prepare the libraries, where samples underwent simultaneous fragmentation and addition of adapter sequences (Nextera, Illumina, USA). These adapters were used during a limited-cycle polymerase chain reaction (PCR) in which unique indices were added to the sample. Following the library preparation, the final concentration of the libraries was measured using the Qubit® dsDNA HS Assay Kit, and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The libraries were then pooled in equimolar ratios of 0.6nM and sequenced using the Illumina NovaSeq 6000 system with paired end configuration (2 × 250 bp) for 500 cycles.

2.3.3 Quality trimming and host DNA contamination assessment

Raw sequence reads were evaluated for quality using FastQC (v0.12.1)(Andrews, 2010) and MultiQC (v1.15) (Ewels *et al.*, 2016). Trimmomatic v. 0.36 (Bolger *et al.*, 2014) was used to filter out reads shorter than 36 bp or with an average quality score lower than 15. Host DNA contamination in metagenomic sequences from barley seed was assessed by alignment against the barley reference genome (*H. vulgare* L., NCBI assembly accession number: GCF_904849725.1). The latter reference genome was indexed and the paired-end metagenomic reads were aligned against this index using Bowtie2 (v2.5.1) and SAMtools

(v1.19) (Langmead and Salzberg, 2012; Li *et al.*, 2009). Reads that did not align were classified as microbial, whereas aligned reads were considered as host contamination.

2.3.4 Taxonomic classification with Kraken2-Bracken pipeline

Taxonomic classification of the trimmed paired-end metagenomic reads was conducted using the Kraken2-Bracken pipeline (Lu *et al.*, 2022). Kraken2 is a pipeline for classifying genetic material from microbial communities, employing a k-mer-based approach to assign taxonomic labels to short DNA sequences, which are then matched against a comprehensive database (Wood *et al.*, 2019). This method is well-suited for handling the diverse range of sequences in metagenomic datasets. Kraken2 (v2.1.3) was used with default parameters (k = 31) with NCBI RefSeq (Release 202) reference database (https://www.ncbi.nlm.nih.gov/refseq/), which includes complete genomes of archaea, bacteria, fungi and viruses.

Bracken (Bayesian Reestimation of Abundance with Kraken) (v2.7.0), complementing Kraken2, was employed for more accurate taxonomic abundance estimation at different taxonomic ranks, especially at the species level. It functions by applying a statistical model to distribute reads with precision (Lu *et al.*, 2017). Post-classification, Bracken reports were converted to the MetaPhlAn format using the kreport2mpa.py script of KrakenTools (Lu *et al.*, 2022). This process involves aggregating reports for a comprehensive overview, extracting specific taxonomic ranks for clarity and optimizing file headers to remove redundant extensions. The output from Bracken, in MetaPhlAn Profile Analysis (MPA) format, was used for representing and analysing microbial community composition.

2.3.5 Diversity and statistical analysis

Alpha diversity refers to the variety and abundance of microbial species within individual barley grain samples, measuring the internal complexity of each sample (Xia and Sun, 2023). Beta diversity, on the other hand, compares microbial communities from different samples, emphasising the differences in microbial makeup (Maziarz *et al.*, 2018). Microbial diversity was analysed using R version 4.2.2 (RStudio Team, 2020). The MPA-formatted data were combined with sample metadata after being transformed into a long format. The Shannon Diversity Index was employed to quantify alpha diversity, reflecting species richness and evenness for each individual sample (Shannon, 1948). Calculations were performed using the diversity function in the *vegan* package (Dixon, 2003), with data categorized by sample ID, nitrogen content and storage duration. The impact of barley seed nitrogen content and storage

duration on diversity was assessed using the Kruskal-Wallis test (Kruskal and Wallis, 1952), a non-parametric method comparing diversity indices across groups to identify statistically significant differences. These results were visualized as boxplots, highlighting diversity trends in relation to barley nitrogen content and storage duration.

To investigate beta diversity, the Bray-Curtis dissimilarity matrix was used, followed by Non-metric Multidimensional Scaling (NMDS) for graphical representation (Bray and Curtis, 1957). The Kruskal-Wallis test and ANalysis Of SIMilarities (ANOSIM) were employed to evaluate the effects of barley nitrogen content and storage duration on beta diversity (Clarke, 1993). Results were presented through boxplots and ordination plots, offering insights into how these environmental factors influenced microbial diversity.

2.3.6 Taxonomic data analysis

The MPA data underwent filtration at both the phylum and genus levels, with the goal of calculating the relative abundance of each microorganism (Truong *et al.*, 2015). Given the negligible number of reads attributed to the domain Archaea, the study primarily concentrated on studying bacteria, fungi and viruses. The bacterial MPA data were divided into two groups. The first group, **core**, includes taxa found in all samples. The second group, **non-core**, includes taxa not found in all samples. We then identified the top ten core and non-core genera for bacteria, fungi and viruses. To better understand how different factors affect the samples, we first sorted the samples by their nitrogen content, either high or low, and created plots. Then, the same samples were grouped by storage duration, from harvest up to nine months. All data analyses and visualizations were conducted in RStudio version 4.2.2 (RStudio Team, 2020). Essential packages used included *dplyr* for data manipulation, *tidyr* for data tidying, *ggplot2* for plot creation, and *RColorBrewer* for enhancing colour schemes in visual representations.

2.4 Results and discussion

2.4.1 Metagenomic diversity and read distribution across different storage duration

A total of eight metagenomes were sequenced (Table S2.1), with a total yield of ~365.3 million reads, averaging around 45.7 million reads for each sample. The sample stored for three months with high nitrogen content (S3H) produced the highest number of reads (~76 million), while

the sample stored for nine months with low nitrogen content (S9L) yielded the lowest number (~20 million) of reads (Table S2.1). It should be noted that the average number of reads decreased by nearly 50% over the nine month storage period.

Kraken2 was used to classify the metagenomic reads for each sample (Wood *et al.*, 2019). Sequences that had a match in the database were categorised as classified, while sequences without a match were referred to as unclassified (Fig. 2.1). In most samples > 70% of the reads were classified. Sample S9L (nine months storage, low nitrogen), had the highest percentage of classified reads at 81.41%. In contrast, Sample S3L (3 months storage, low nitrogen) had the lowest percentage at 68.19%. The relatively high level of unclassified sequences may be inherent of the current microbial databases available for classification, which may not be representative of the unculturable majority (Xu *et al.*, 2023).

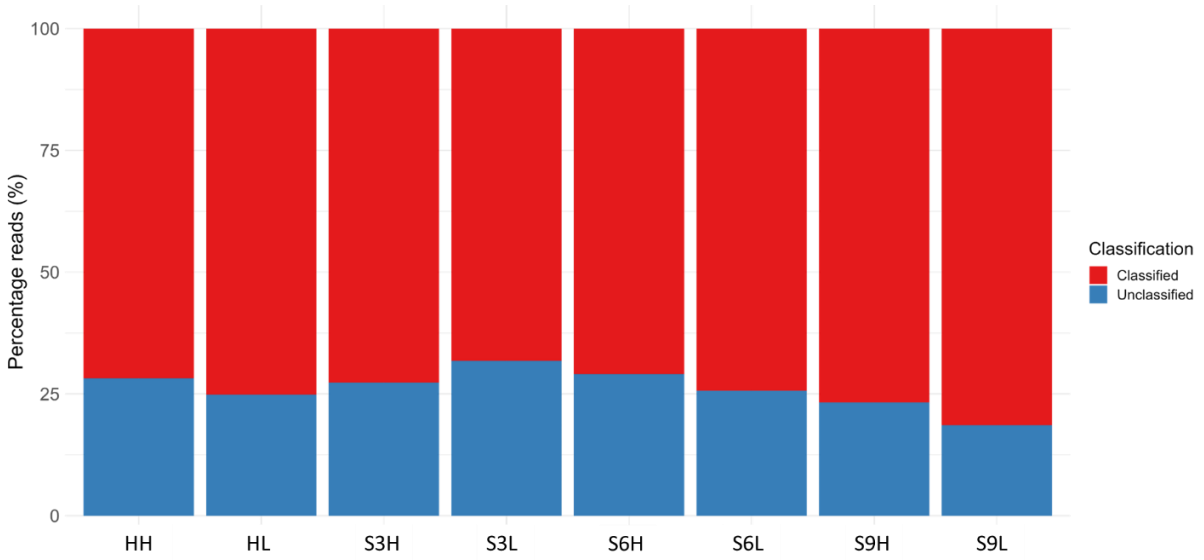


Figure 2.1 Proportional taxonomic classification of reads using the Kraken2-Bracken suite (Lu *et al.*, 2022). Each bar visually contrasts the share of classified reads (in red) against those that remained unclassified (in blue). HH/HL = harvest high/low nitrogen; S3L/S3H, S6L/S6H, S9L/S9H = three, six, nine months storage low/high nitrogen.

2.4.2 Microbial diversity of barley seeds is influenced by storage duration

The Shannon Diversity Index (Shannon, 1948) was used to evaluate the microbial diversity of barley grain samples with high and low nitrogen content (Fig. 2.2A). Minimal difference in mean diversity between high and low nitrogen content was observed, suggesting that nitrogen

content may not substantively impact overall microbial diversity. The Kruskal-Wallis rank sum test (Kruskal and Wallis, 1952) further confirmed that the differences in microbial diversity between high and low nitrogen content barley seeds were not statistically significant (chi-squared: 0.0833, p = 0.7728), indicating that the observed variations are likely not attributable to nitrogen content alone. However, intra-sample variability is greater among the low nitrogen samples compared to the high nitrogen samples (Fig. 2.2A; Table S2.2), pointing to greater variability in microbial composition on low nitrogen content barley seeds.

The Bray-Curtis dissimilarity metric was further applied to assess the composition of microbial communities of the barley grain samples (Bray and Curtis, 1957) (Fig. 2.2B). This showed that the microbial compositions of seeds collected at harvest were comparatively similar. Over time however, this similarity diminished, suggesting a shift in the microbial community structure with increased storage duration in seeds with different nitrogen contents. The Bray-Curtis PCoA highlighted that after six months of storage, microbial communities were significantly distinct, primarily due to a dysbiosis observed in sample S6L (six months storage and low nitrogen) (Fig. 2.2B; Table S2.3). The ANOSIM test for Bray-Curtis dissimilarity by storage indicated an R statistic of 0.5417, with a significance level of 0.034.

The diversity analyses demonstrate that storage duration, rather than nitrogen content, significantly influences the microbial diversity of barley seeds, as evidenced by minimal differences between high and low nitrogen content but notable shifts in microbial communities over time. The Bray-Curtis dissimilarity and ANOSIM tests indicate that microbial compositions become significantly distinct with extended storage, pointing to changes in community structure (Cao *et al.*, 2022; Felšöciová *et al.*, 2021). Importantly, the S6L sample, which exhibited dysbiosis, was included in all downstream analyses to maintain consistency in the study design. This sample was not discarded, as it provided valuable insight into the potential variability within the microbial communities over time. However, it is acknowledged that dysbiosis in S6L could have introduced some bias into the results, and further replication would strengthen the conclusions regarding community shifts over time. This underlines the importance of conducting microbial ecology studies with adequate replication to account for such variability (Felšöciová *et al.*, 2021). Nevertheless, the observed shifts in microbial composition suggest that factors associated with storage duration play a critical role in shaping the microbial ecosystem within barley seeds, overshadowing the impact of nitrogen content.

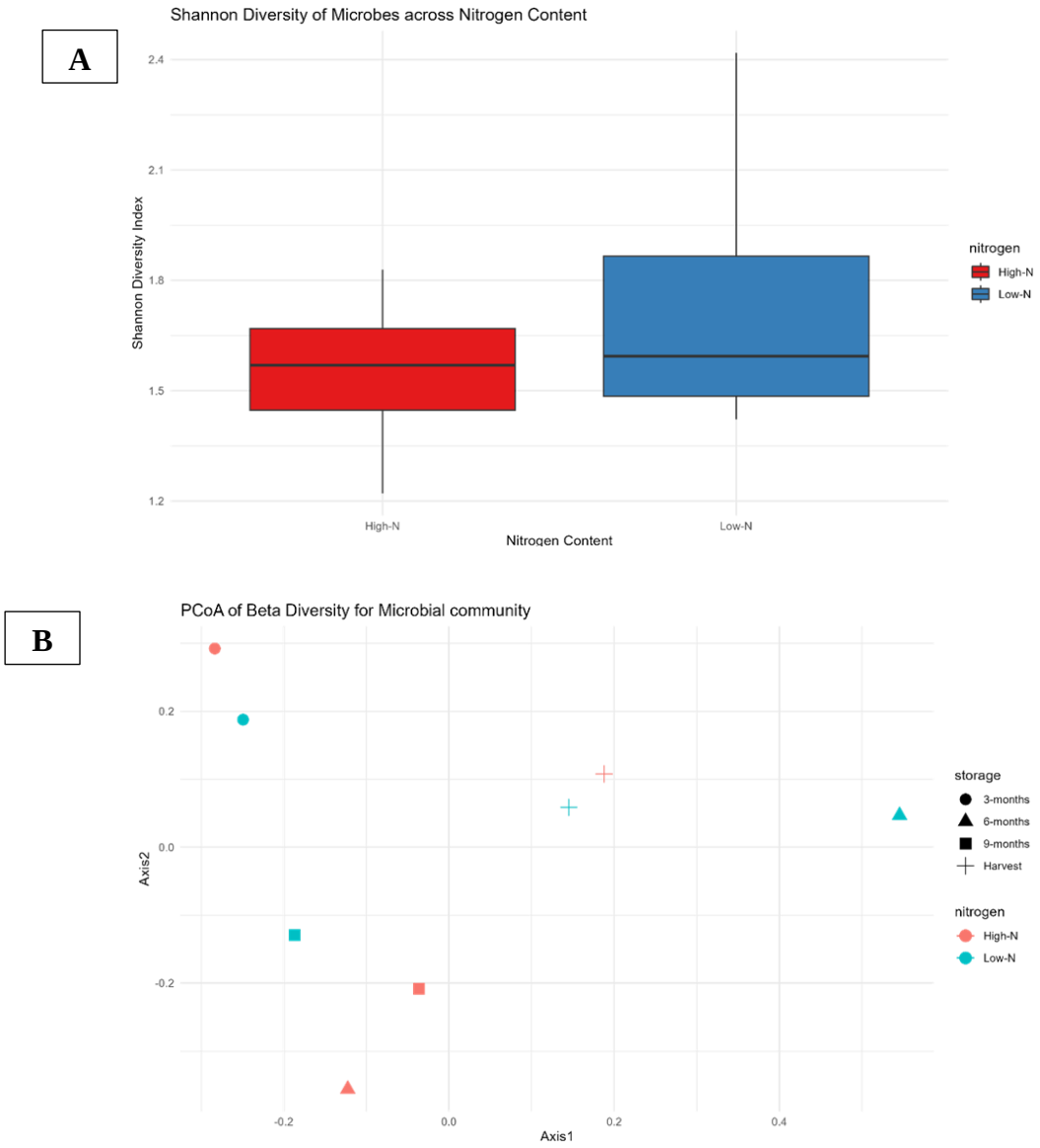


Figure 2.2 Microbial diversity and community dissimilarity in barley grains with high and low nitrogen content and at different storage durations. (A) Box plot on the basis of the Shannon diversity index reflecting microbial diversity in barley grains with high (red box) and low (blue box) nitrogen content. The central line in each box denotes the median diversity index, and the whiskers show the full range of the data. (B) Principle Component of Analysis (PCoA) plot of beta diversity measured by Bray-Curtis dissimilarity (Bray and Curtis, 1957) across the different storage sampling time points.

2.4.3 The seed microbiome is dominated by bacterial communities

Bacteria represented the dominant microorganisms associated with the barley seeds, contributing 99.7% of all classified reads (~111 million reads) (Fig. 2.3A, Table S2.4). A total of 381 distinct bacterial genera were observed across the eight barley metagenomic samples, encompassing 103,266,174 reads (Table S2.5). Fungi and phages were less prevalent, comprising 0.0707 and 0.1903% of the reads, respectively. Archaea, represented mainly by the Euryarchaeota, were scantily detected, contributing 0.0001% (112 reads) of the total classified reads. While this might suggest a higher bacterial abundance, it is crucial to consider the nuances of sequencing technology and experimental design. The use of short-read Illumina sequencing, particularly with 250 bp paired-end reads, inherently favours the recovery of bacterial genomes over eukaryotic ones (Jo *et al.*, 2020). This is due to the smaller, often less complex genomes of bacteria, which are more likely to be efficiently sequenced and assembled with short-read technologies. Eukaryotic genomes, being larger and more complex due to introns and repetitive sequences, might not be as thoroughly sampled or accurately assembled, leading to underrepresentation in the dataset (Borodovsky and Lomsadze, 2011; Richard, 2020). Additionally, the overall coverage of the metagenome may play a critical role; insufficient metagenomic coverage can lead to incomplete sampling of the community, disproportionately affecting organisms with larger genomes or those present in lower abundance (Knight *et al.*, 2018).

A total of twelve bacterial phyla were identified. The phyla Pseudomonadota, Bacteroidota, Actinomycetota, and Bacillota have the most widespread distribution across the barley seed samples. Pseudomonadota dominates, contributing more than 80% of the total reads in all samples. The least represented phyla are Deinococcus-Thermus, Fusobacteria, Tenericutes and Verrucomicrobia, (Fig. 2.3B, Table S2.4). The dominant phyla are not unique to barley but have previously been identified in various other seed microbiomes, including *Brassicaceae*, pumpkin, rice and wild cabbage seeds (Tyc *et al.*, 2020; Raj *et al.*, 2019; Adam *et al.*, 2018; Matsumoto *et al.*, 2021). Microorganisms associated with these phyla have been detected on the surface of seeds from a wide range of plants, including barley (Truyens *et al.*, 2015). Similar to our findings, the abundance of these phyla in barley seed was reported in a study that examined the microbiome of different barley genotypes (Bziuk *et al.*, 2021).

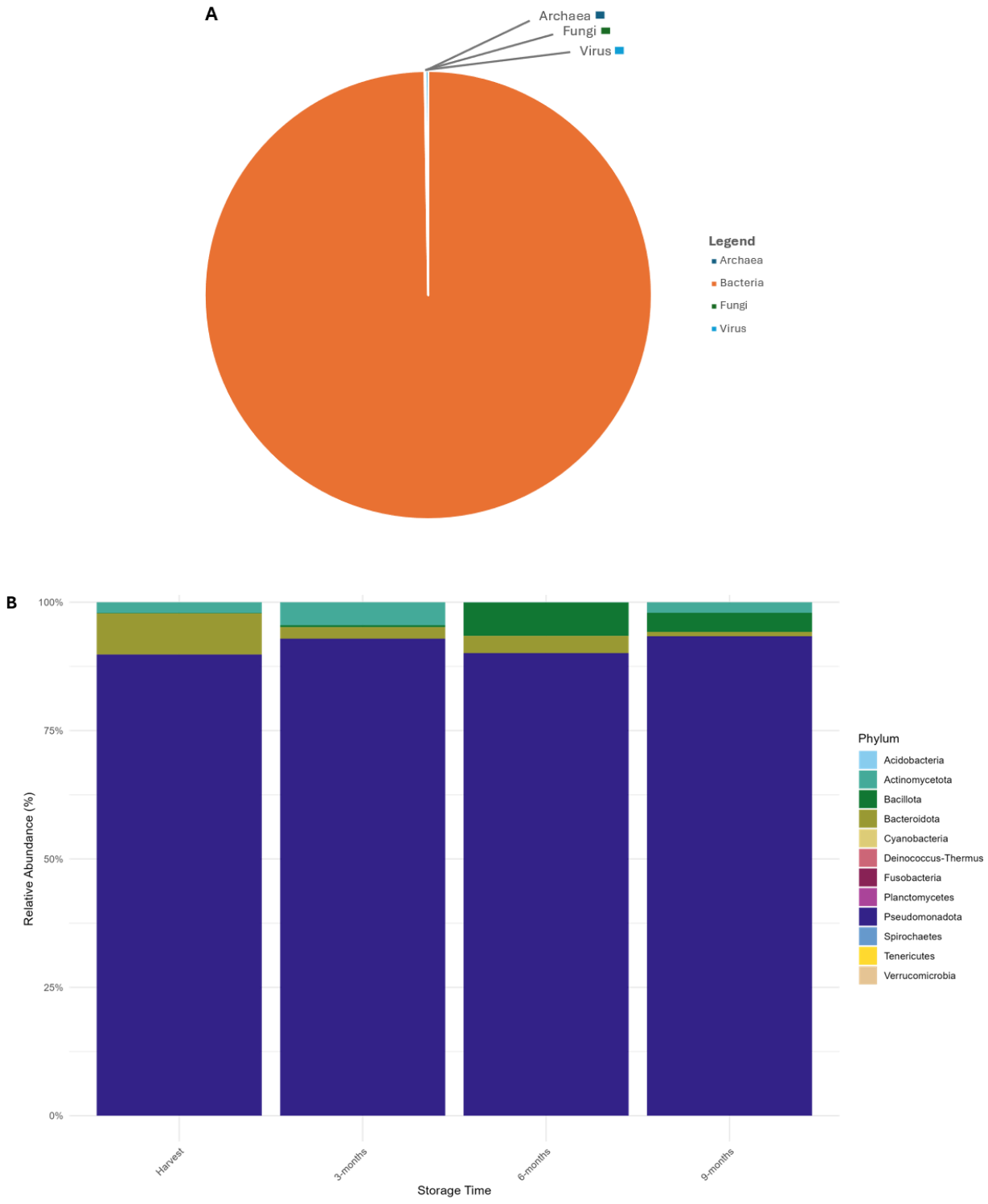


Figure 2.3 (A) Taxonomic composition of classified reads using Kraken2-Bracken suite (Lu *et al.*, 2022). (B) Relative abundance of bacterial phyla within the barley samples. Samples are grouped by storage duration (Harvest, three, six, and nine months).

2.4.4 The core microbial community of barley seed

This study delineates the core microbiome of barley seed samples, highlighting the top ten bacteria, which vary based on storage duration and grain nitrogen content (Fig. 2.4A and B; Table S2.5). Members of the genera *Erwinia*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas* comprised over 50% of the taxa (Fig. 2.4A and B). Additionally, other genera identified among the top ten include *Chryseobacterium*, *Klebsiella*, *Massilia*, *Citrobacter*, *Leclercia*, and *Xanthomonas* (Fig. 2.4A and B). The distribution of these genera varies with both storage duration and grain nitrogen content. For example, genera such as *Erwinia*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas* are consistently present across all storage times but show variations in their relative abundances (Fig. 2.4A). In contrast, other genera such as *Citrobacter*, *Klebsiella*, and *Leclercia* only increase in abundance at later stages of storage (Fig. 2.4A). This suggests the core microbial community may respond to changing conditions within the storage environment (Felšöciová *et al.*, 2020). Conversely, the distribution of the top ten core genera relative to grain nitrogen content exhibits a degree of stability (Fig. 2.4B), suggesting that variations in nitrogen content has minimal impact on the core bacterial communities.

An ideal plant core microbiome is made up of microbial communities consistently found in different plants grown across various locations and soil types (Shade and Handelsman, 2012). The microbial genera identified in this chapter are also found in the rhizosphere, roots, bulk soil, and across different barley genotypes (Yang *et al.*, 2017; Bziuk *et al.*, 2021; Bulgarelli *et al.*, 2015; Rahman *et al.*, 2018). This suggests they play an important role as stable components of the core barley microbiome. Members of the core genera, such as *Erwinia*, *Pantoea*, and *Pseudomonas* are known to exhibit a range of interactions with barley seed (Yang *et al.*, 2017; Bziuk *et al.*, 2021; Rahman *et al.*, 2018). For instance, *Erwinia gerundensis* acts as a biological control agent in barley malt (Gnonlonfoun *et al.*, 2023). Conversely, *Erwinia persicina* is associated with pink seed disease in barley (Kawaguchi *et al.*, 2021). Members of the genus *Pantoea* are ubiquitous and known for their adaptability and ecological versatility (De Maayer *et al.*, 2017; Sabra and Ahmed, 2022). For example, *Pantoea agglomerans* has been reported to inhibit basal kernel blight, typically caused by *Pseudomonas syringae pv. Syringae* (Braun-Kiewnick *et al.*, 2000). Additionally, members of the genus *Pseudomonas* are involved in several beneficial roles in plants, including nitrogen fixation and phosphorus solubilization (Yan *et al.*, 2008; Pham *et al.*, 2017). However, it is important to acknowledge that genus-level

identification may encompass species with both beneficial and detrimental effects, highlighting the importance of species-specific analyses to fully grasp their potential impacts (Chen *et al.*, 2020).

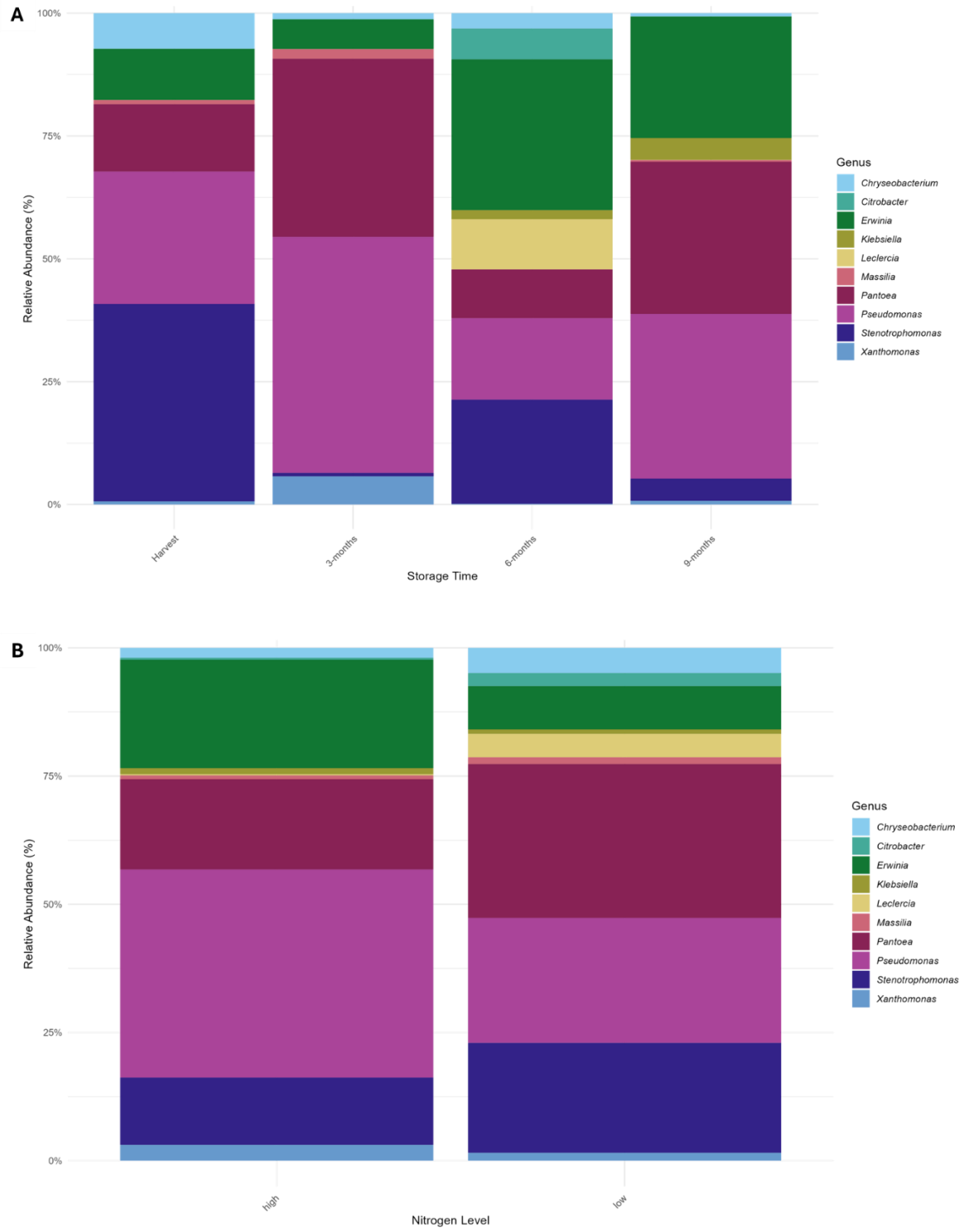


Figure 2.4 Relative abundance of core bacterial community within barley samples, categorised by, (A) Storage duration (harvest, three, six and nine months). (B) grain nitrogen content (high and low).

2.4.5 The non-core microbial community of barley seed

Non-core taxa contribute to the ecological flexibility and functional diversity of microbial communities, enabling them to adapt to a range of environmental conditions (Shade and Handelsman, 2012). The composition of non-core genera changes with storage duration and grain nitrogen content (Fig. 2.5 A; Table S2.5). Initially, at harvest and three months, genera such as *Pedobacter*, *Plantibacter*, and *Pseudarthrobacter* were most abundant, while *Enterococcus*, *Lactococcus*, and *Saccharibacillus* were more abundant in the later stages of storage (six and nine months) (Fig. 2.5A). A distinct pattern emerges when comparing microbial composition in environments with high versus low grain nitrogen content (Fig. 2.5 B). In samples with high nitrogen, *Plantibacter*, *Pseudarthrobacter*, and *Saccharibacillus* collectively represent over 50% of the microbial population (Fig. 2.5B; Table S2.5). In contrast, in low nitrogen samples, *Enterococcus* and *Lactococcus* are the predominant genera, also exceeding 50% combined (Fig. 2.5B). This pattern suggests that changing conditions selectively favour these distinct microbial communities.

The genera identified in the early stages of storage and high nitrogen grain content are known to colonise the endosphere, phyllosphere and rhizosphere (Liao *et al.*, 2024; Park *et al.*, 2020; Woo *et al.*, 2024). In contrast, the genera prevalent in later storage phases and low grain nitrogen content, such as *Enterococcus* and *Lactococcus*, are lactic acid bacteria commonly found in barley malt (Doi, 2018; Song *et al.*, 2017). Although they have benefits as inoculants for enhancing malt quality and safety, their uncontrolled proliferation during germination can lead to the synthesis of organic acids, which can compromise beer quality by modifying its flavour and stability (Laitila *et al.*, 2018; Mokoena, 2017).

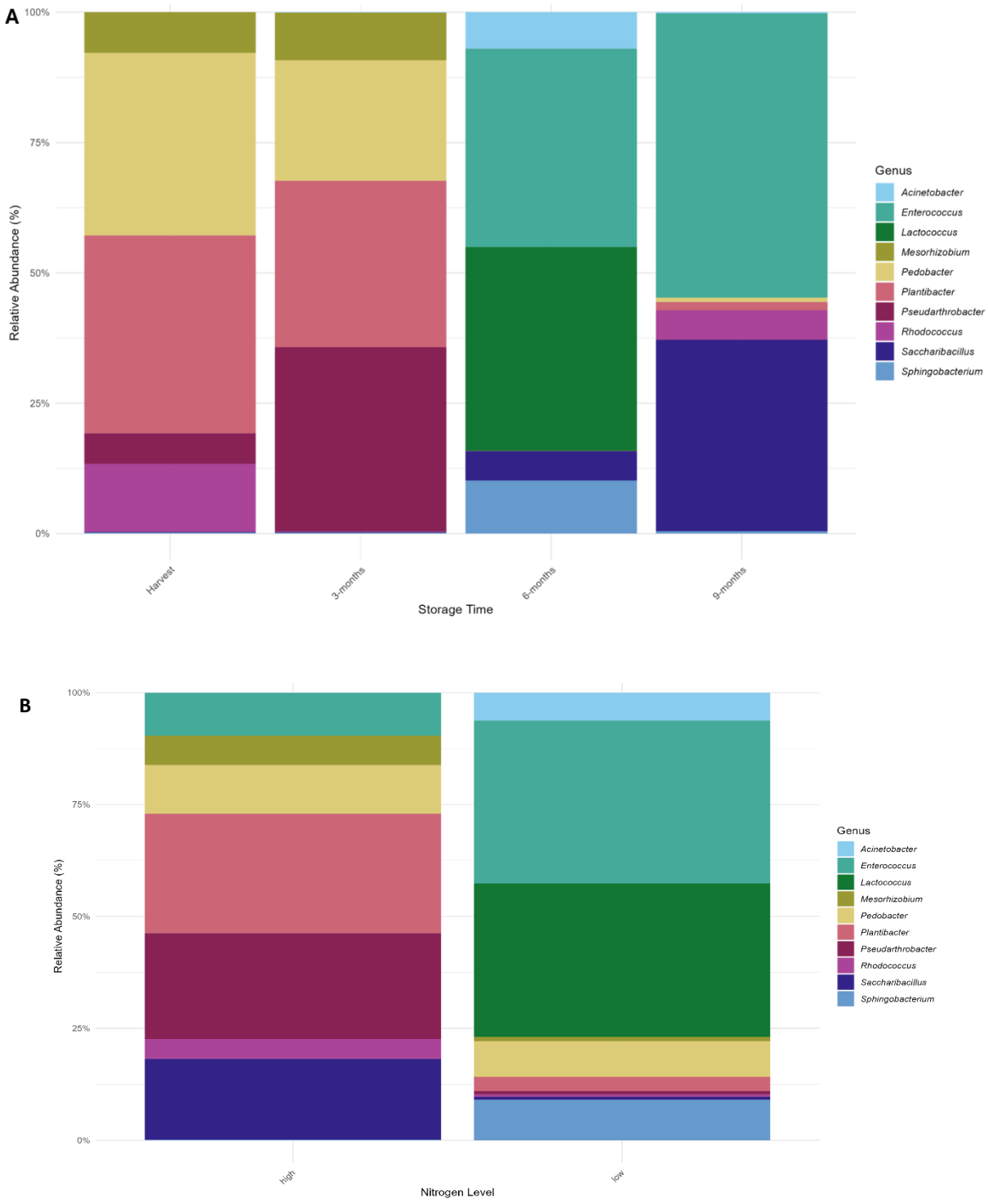


Figure 2.5 Relative abundance of the non-core bacterial community within barley samples, categorised by. (A) Storage duration (harvest, three, six and nine months). (B) Grain nitrogen content (high and low).

2.4.6 Microbial richness variations in barley seeds across storage duration

A total of 192 distinct bacterial genera were identified but not universally, showing a varied prevalence depending on storage duration. Also, a total of 140 genera were unique to each

storage time point. In total, 17, 80, 41 and two genera were unique to the harvest, three month, six month and nine-month storage duration, respectively (Fig. 2.6). The variation in genus richness at different storage time points underlines a complex interaction between the storage environment and microbial dynamics. Particularly, the increase in species richness up to the six-month storage suggests that the early and mid-storage periods are critical for microbial diversity.

The decline in diversity observed between the six- and nine-month intervals points towards a shift in the microbial ecosystem, potentially driven by competitive interactions among microbial species or changes in the nutritional landscape of the barley seeds as they undergo physiological changes in preparation for germination. This suggests that the storage environment, while controlled, does not remain static in its influence on microbial communities (Abubakar, 2019). Although the storage system is highly regulated, interactions within the microbial community, such as competition for nutrients or the production of antimicrobial compounds, likely play a role, alongside abiotic factors like moisture and temperature fluctuations (Chandel *et al.*, 2021). However, it is important to recognise that this controlled environment may not entirely reflect a natural ecological setting. These findings underscore the importance of considering microbial interactions in managing microbial dynamics in stored grain, although further research is needed to fully understand the ecological relevance of these dynamics in artificial storage environments.

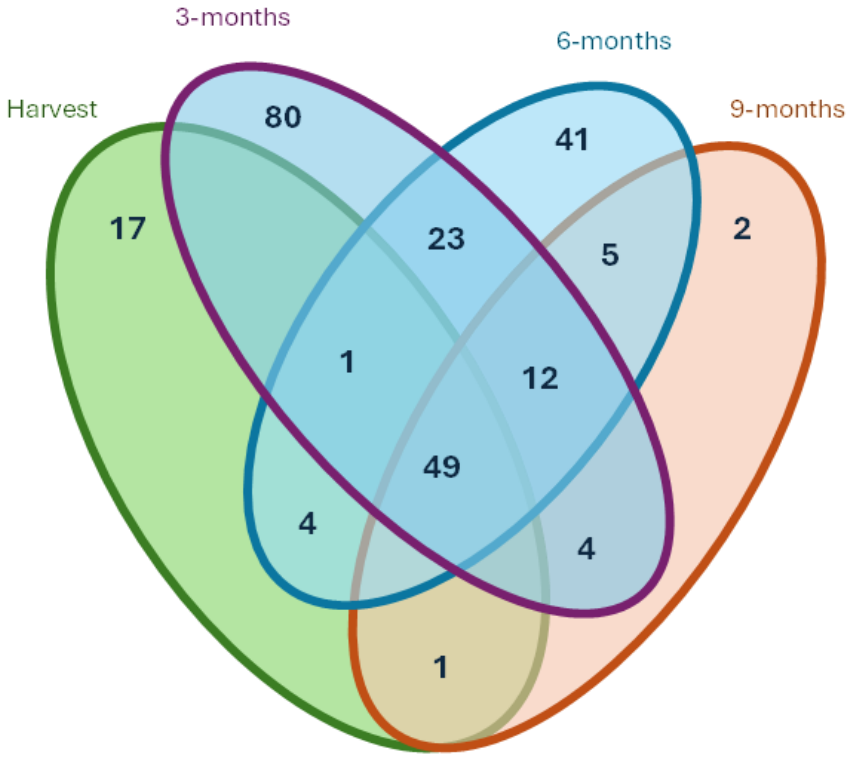


Figure 2.6 The number of shared and unique bacterial genera associated with barley seeds over storage duration.

2.4.7 Differential distribution of phage genera linked to nitrogen content and storage duration in barley seed

A total of 211,526 viral reads were identified, representative of 59 distinct bacteriophage genera. The abundance of the top ten phage genera was calculated by combining samples first by storage duration (Fig. 2.7A) then by grain nitrogen content (Fig. 2.7B). As storage duration increases, a greater diversity of genera becomes evident (Fig. 2.7A). At harvest, the phage population was predominantly comprised of members of the genus *Mimasvirus*, accounting for 91%, with *Petsuvirus* making up the remaining 9%. *Mimasvirus* is noteworthy for including phage GAP32, which targets the opportunistic pathogen *Cronobacter sakazakii* (Abbasifar *et al.*, 2014). The genus *Cronobacter* was observed in our samples (Table S2.6). Three months into storage, there was a shift towards a dominance of *Feofaniavirus*, which constituted 99% of the population. By six months, diversity began to increase, with *Skunavirus* at 60% and *Cornellvirus* at 20%, alongside the emergence of four additional genera (Fig. 2.7B). Phages belonging to the genus *Skunavirus* have a known association with species of *Lactococcus*, especially *Lactococcus lactis* (Lemay *et al.*, 2020; Doré *et al.*, 2021). The highest number of

sequences reads identified as *Lactococcus* (772,822) was found in the sample (S6L) coinciding with the detection of *Skunavirus* (Fig. 2.8A; Table S2.6). This phage is strictly lytic and has been shown to infect and kill *L. lactis* strains (Lemay *et al.*, 2020; Doré *et al.*, 2021). The genus *Cornellvirus* encompasses the phage *Cornellvirus* SLAM_phiST1N3, known for its capacity to infect multidrug-resistant strains of *Salmonella enterica serovar Typhimurium* (Choi *et al.*, 2024). Consistent with the presence of this phage, the highest number of *Salmonella* sequence reads, totalling 29,787, was recorded in sample (S6L) where *Cornellvirus* was detected (Table S2.6). At nine months of storage, five genera were observed with *Mosigvirus* (32%), *Kanagawavirus* (29%), and *Feofaniavirus* (28%) being the most abundant (Fig 2.7A). The evolving diversity over time could reflect changes in the microbiome associated with the seeds, host availability, or environmental conditions during storage facilitating the proliferation of different viral genera.

In samples with high nitrogen content, the distribution among phage genera was notably uniform, with *Sauletekiovirus*, *Feofaniavirus*, and *Ravinvirus* each contributing approximately 17-18% to the viral community (Fig. 2.7B). *Sauletekiovirus*, identified as a genus of lytic phages, are associated with *P. agglomerans* (Žukauskienė *et al.*, 2021), aligning with the finding that *Pantoea*, one of the top ten core genera in this study (Table S2.6;Fig. 2.8B). *Feofaniaviruses*, also lytic in nature, targets the genus *Erwinia* (Kim *et al.*, 2021), present across all samples. *Ravinvirus*, known for infecting *Escherichia coli* (Ravin, 2011), corresponds with the detection of the genus *Escherichia* in our samples (Table S2.6). In contrast, the phages associated with barley seed samples with low nitrogen contents were distinct, with the genera *Skunavirus* and *Cornellvirus* (64 and 21% of viral reads, respectively) dominating (Fig. 2.7B).

The presence of lytic phages in this study supports phage-bacterial host interaction. The presence of lytic phages can lead to the lysis of specific bacterial populations, thereby potentially altering community compositions and nutrient cycling processes (Hobbs and Abedon, 2016; Bertani, 1953). Bacterial genera such as *Cronobacter*, *Escherichia*, and *Salmonella*, showed a relatively low number of reads (Table S2.6). It is possible that their associations with lytic phages in the study could be responsible for their low read number. Further investigation into the interactions between these lytic phages and their bacterial hosts will be crucial in understanding the extent of their impact on microbial communities.

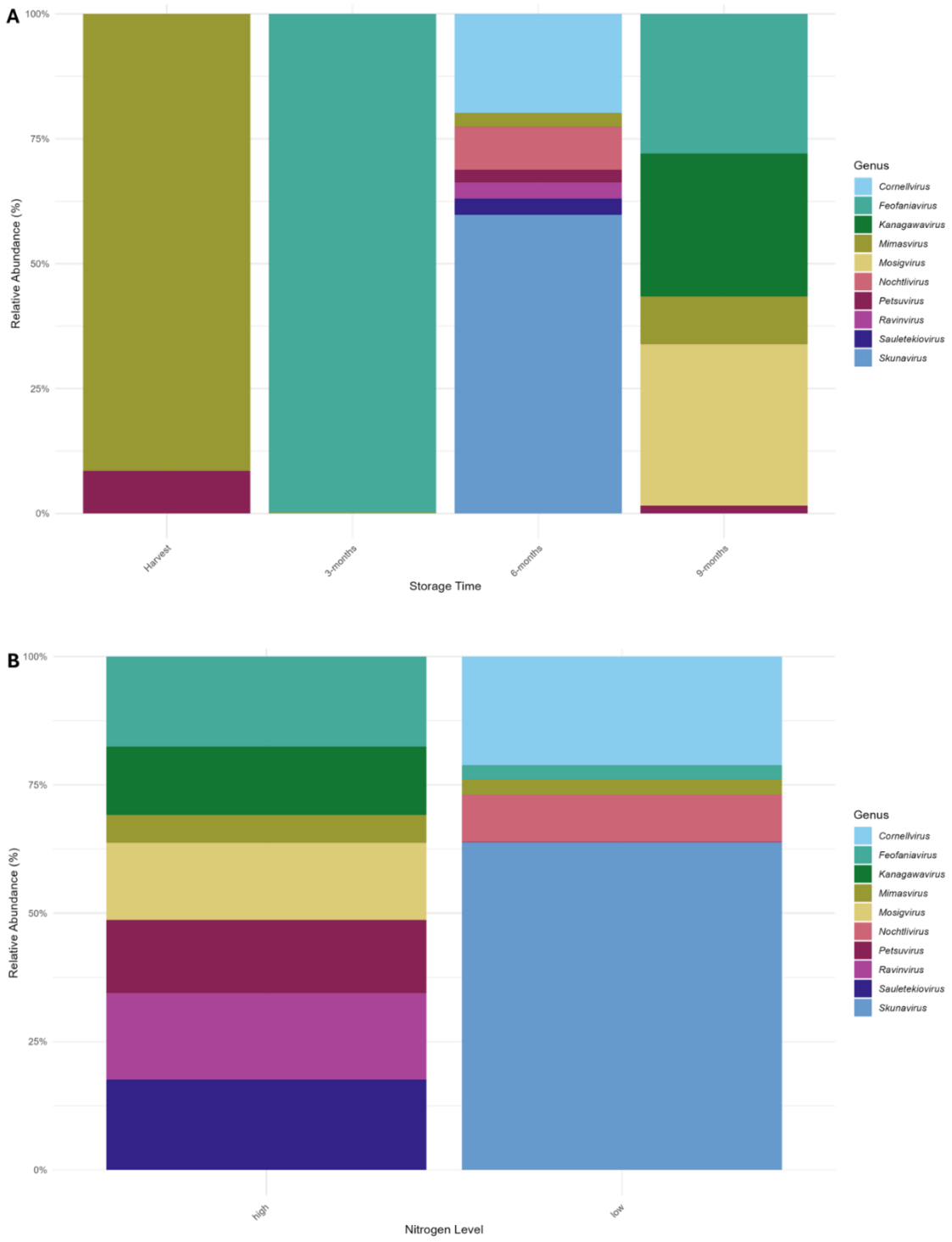


Figure 2.7 Relative abundance of phages within the barley metagenome. (A) Storage duration samples (B) Nitrogen content samples.

2.4.8 The barley seed microbiome exhibits no fungal presence at harvest or under conditions of high nitrogen content

A total of 46,979 fungal reads were identified, with an absence of such reads in samples from the initial harvest and those from barley seeds with high nitrogen content (Table S2.7). Among the low nitrogen content barley seed samples, a total of 19,052 fungal reads were observed at three months, 7,030 reads at six months and 20,897 reads at nine months of storage (Table S2.5). At three months of storage, the genera *Fusarium* (41%), *Candida* (21%), *Aspergillus* (17%) and *Thermothelomyces* (13%) were observed (Fig. 2.9). There was a shift at six months of storage, with *Eremothecium* accounting for all the detected fungal reads. At nine months in storage, the fungal distribution shifted again, including *Cryptococcus* (36%), *Fusarium* (20%), *Botrytis* (16%) and *Thermothelomyces* (11%) (Fig. 2.8).

Fungi exploit the storage phase to establish and proliferate (Laitila, 2015). This pattern suggests that the environmental conditions and microbial interactions within these specific contexts may inherently deter fungal colonisation and growth (Wang and Kuzyakov, 2024). High nitrogen content appears to create conditions less conducive to fungal presence, potentially through the competitive exclusion of nitrogen-using bacteria (Masuda *et al.*, 2024). This notion is supported by the exclusive detection of fungal species in low-nitrogen samples, where the reduced bacterial competition may allow fungi to utilise the available nutrients more freely.

The observed fungal communities were dominated by the genus *Fusarium*, which is recognised for its role in spoilage and mycotoxin production, with profound implications for agriculture. *Fusarium* is also associated with *Fusarium* Head Blight (FHB), a disease that significantly affects wheat and barley crops (Alisaac and Mahlein, 2023). The exclusive colonisation by *Eremothecium* at the six-month storage time point, may be indicative of an environment that possibly favours its rapid growth, potentially due to unique substrate characteristics or a state of dysbiosis. This shift correlates with the observed dysbiosis in bacterial communities at the same storage duration (Fig 2.6B), suggesting a broader ecological disruption. The presence of *Eremothecium* in storage environments is important, as this genus is known for its pathogenic potential, including causing yeast-spot disease in soybean seeds, often in association with the stink bug *Riptortus clavatus* (Kimura *et al.*, 2009; Kimura *et al.*, 2008; Zečević *et al.*, 2023).

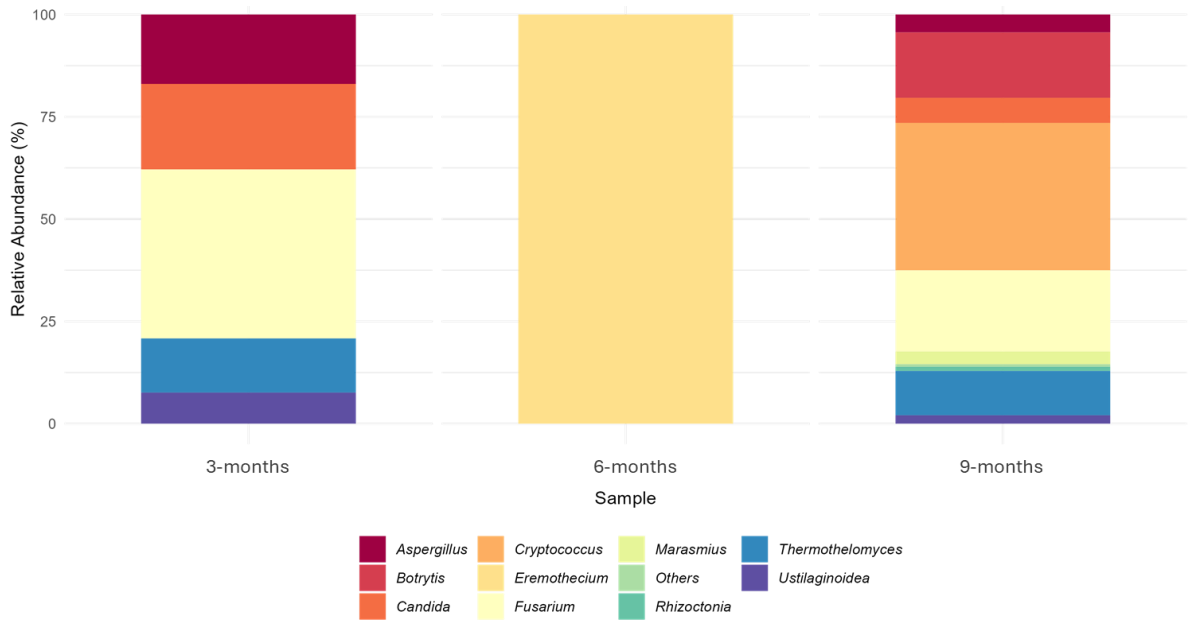


Figure 2.8 Relative abundance of fungal genera within the barley metagenome over a nine month storage period.

2.5 Conclusions

Almost 400 million metagenomic reads were generated from eight barley seed samples with high and low nitrogen content, stored for four different periods of time. More than 70% of the reads per sample were classified, leaving a substantial fraction unclassified due to the existing limitations within various metagenomic databases. The Shannon diversity metrics have revealed that seed nitrogen content does not significantly affect overall diversity. This observation was further supported by Bray-Curtis dissimilarity analyses, which also highlighted a shift in community structure over time, particularly noting a dysbiosis at the six-month storage duration. The microbial composition was predominantly bacterial, with both core and non-core bacteria shedding light on how different genera respond to nitrogen content and storage duration. Phages were closely associated with bacterial genera, with a noted decline in bacterial diversity corresponding with an increase in phage populations. Of note, fungal reads were absent from harvest barley seed samples and those characterised by high nitrogen content, suggesting conditions that may inhibit fungal growth. These findings contribute to the existing body of knowledge of the barley seed microbiome and could provide insight for optimal storage practices and microbial dynamics.

While these findings highlight important microbial shifts during storage, it is important to acknowledge that the storage environment represents an artificial system, and thus, the microbial dynamics observed here may not fully reflect those found in natural ecosystems. Nonetheless, competitive interactions, resource availability, and abiotic factors such as moisture and temperature are still relevant within this controlled system. Further research could help clarify how these factors influence microbial communities under storage conditions and how these dynamics may differ from those in natural settings. Future chapters will delve into genome reconstruction from these metagenomic data and further explore the functional profiles at the community level.

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Chapter 3 Reconstruction of metagenome assembled genomes of microorganisms associated with stored barley seeds with varied nitrogen contents

3.1 Abstract

Seed microbiomes play important roles in the physiological processes required for effective crop development. The barley seed microbiome influences not only crop yield but also the quality of barley-derived products. The diversity, organisation and functional capacities of the barley seed microbial community are largely unexplored. In this study metagenome-assembled genomes (MAGs) were derived from metagenomes obtained from barley seeds of varied nitrogen grain content sampled across different storage times. The majority (91%) of the MAGs had a completeness score above 75%. The MAGs were classified into 26 bacterial genera belonging to four distinct phyla: Pseudomonadota, Bacteroidota, Actinomycetota, and Bacillota, revealing a diverse microbial community associated with stored barley seeds. The MAGs were structurally and functionally annotated using a range of *in silico* tools. OrthoFinder analysis revealed a limited number of single-copy orthologues and only 66 core orthogroups across the 82 MAGs analysed in this study, reflecting the substantial taxonomic diversity observed within the sample set. Additionally, Cluster of Orthologous Groups (COG) analysis via eggNOG revealed that these COGs play could be involved in critical cellular functions, particularly in translation, ribosomal structure, and biogenesis (14%), and replication, recombination, and repair (14%), highlighting their importance in adapting to environmental shifts in barley seed microbiomes. Plant pathogenicity-related proteins were also identified in MAGs from the genera *Duffyela*, *Erwinia*, and *Klebsiella*. This study enriches our understanding of the genomic content and potential functions of the predominant microbial species in barley seeds, offering insights into their roles within the barley seed microbiome.

3.2 Introduction

The barley seed microbiome comprises a diverse assembly of bacteria, fungi and other microorganisms located on and within the seeds (Noots *et al.*, 1999; Bianco *et al.*, 2018). While many of these microorganisms are beneficial, some contribute to pathogenicity (Noots *et al.*,

1999). In the context of beer production, unwanted microbial activity can extend seed dormancy, impair germination and decrease malt yield (Bokulich and Bamforth, 2013). These microbial influences may degrade wort quality, induce yeast flocculation and, ultimately, affect beer quality (Bamforth, 2017; Guo *et al.*, 2021).

In recent years, the reduced costs of next-generation sequencing (NGS) has catalysed a surge in microbiome sequencing projects across various cereal crops (Goodwin *et al.*, 2016; Grant, 2022). Research has primarily focused on the wheat microbiome, with maize and rice also receiving substantial attention (Michl *et al.*, 2023). By contrast, the barley microbiome has not been studied as extensively. Our current understanding is based on culture-dependent and culture-independent studies that mainly report on the taxonomy of microorganisms associated with the barley seed (Bziuk *et al.*, 2021; Felšöciová *et al.*, 2021; Noots *et al.*, 1999). These studies have successfully identified key microbial taxa within the core barley microbiome, including beneficial microorganisms and pathogens (Bziuk *et al.*, 2021; Felšöciová *et al.*, 2021; Noots *et al.*, 1999). However, the primary limitation of purely taxonomic studies lies in their limited ability to evaluate the functional capacities of these microorganisms (Muhamad Rizal *et al.*, 2020). A more effective strategy would involve the reconstruction of metagenome-assembled genomes (Setubal, 2021).

Metagenome-assembled genomes (MAGs) represent an advance in the reconstruction of microbial genomes directly from metagenome data (Setubal, 2021). In recent years, the scientific community has reported several thousand MAGs across diverse environments and in association with a range of different hosts, markedly enhancing our understanding of microbial populations and their interactions with their respective habitats (He *et al.*, 2021; Kayani *et al.*, 2021; Chen *et al.*, 2020). Notably, a considerable proportion of these MAGs are attributed to novel species, thereby contributing to the reduction of so-called microbial “dark matter” (Jiao *et al.*, 2021; Lok, 2015). The construction of metagenome-assembled genomes (MAGs) begins with metagenomic sequencing of mixed-species samples to generate a vast array of sequence data (Wu *et al.*, 2022). Subsequently, this data is assembled into longer fragments known as contigs, a challenge compounded by the mixture of sequences from various organisms (Nurk *et al.*, 2017). Following assembly, the contigs are sorted into bins, with each bin approximating the genome of an individual microorganism (Alneberg *et al.*, 2014). After binning, the MAGs undergo a rigorous quality assessment, typically involving the identification of single-copy marker genes to evaluate their completeness and accuracy (Parks *et al.*, 2015). Finally, the

MAGs are taxonomically classified and their genes functionally annotated using gene and protein databases, to uncover their potential roles (Chaumeil *et al.*, 2020; Zhou *et al.*, 2022; Stewart *et al.*, 2019).

Our understanding of plant microbiomes has substantially improved through reconstruction of MAGs (Fadiji and Babalola, 2020). For instance, MAGs have shed light on the microbial networks that underpin plant health, revealing how microbiomes build networks to preserve functionality across many plant-associated variables (Singh *et al.*, 2020). Additionally, MAGs have shed light on techniques for improving plant health and stress tolerance, providing insights into the biochemical, physiological and molecular aspects of plant-microbe interactions under stress (Gagliano *et al.*, 2022; Dindhoria *et al.*, 2024). While several studies have documented the taxonomic composition of microbial communities associated with barley seeds (Felšöciová *et al.*, 2020; Felšöciová *et al.*, 2021; Bziuk *et al.*, 2021), comprehensive knowledge of the functionality of the barley seed microbiome remains limited. Building upon the metagenome sequence of barley samples in Chapter 2, here we constructed MAGs from these metagenomic datasets and functionally annotated the resultant genomes. As such, the genomes of prevalent bacterial taxa from the barley seed communities could be reconstructed. Furthermore, these MAGs give insight into the functional potential of the taxa associated with barley seeds.

3.3 Methods

3.3.1 Metagenomic assembly, binning, and refinement

The metagenomic read datasets processed in Chapter 2 (section 2.3.2) were used for MAG reconstruction. A *de novo* assembly of the reads was performed using metaSPAdes v3.15.3 (Nurk *et al.*, 2017) with default parameters. The integrity and quality of the final assemblies were evaluated using QUAST v5.2.0 (Gurevich *et al.*, 2013). Subsequently, three binning tools, employing distinct algorithms, namely MaxBin v2.0 (Wu *et al.*, 2016), MetaBAT2 (Kang *et al.*, 2019) and CONCOCT v1.0.0 (Alneberg *et al.*, 2014), were used to assign the resultant contigs to bins. MaxBin groups contigs together using an Expectation-Maximization algorithm and Markov Chain Monte Carlo methods, focusing on tetranucleotide frequency and differential coverage (Wu *et al.*, 2016). It also improves the accuracy of bins by searching for highly conserved marker genes within the assembled contigs, serving as reliable genetic

markers to differentiate microbial species or strains (Wu *et al.*, 2016). MetaBAT2 adopts an adaptive binning algorithm based on tetranucleotide frequency and differential coverage, with a unique approach for contig distance measurement to optimise accuracy and minimise misclassification (Kang *et al.*, 2019). CONCOCT employs a Gaussian mixture model to cluster contigs, considering nucleotide composition and coverage across samples, effectively partitioning data into genetically related groups suitable for short-read sequencing data analysis (Alneberg *et al.*, 2014). The completeness and contamination levels of the generated bins were evaluated using CheckM v1.2.2 (Parks *et al.*, 2015).

Following the preliminary binning, the resultant bins were processed using the *MetaWRAP-Bin_refinement* module of the MetaWRAP v1.3 pipeline (Uritskiy *et al.*, 2018) for further refinement. The parameters for this module were set to -c 70 and -x 10 (completeness > 70 % and contamination < 10%). The module enhances the bin quality by integrating the outputs from the initial binning algorithms, thereby improving genome completeness and reducing contamination (Uritskiy *et al.*, 2018). The refinement process involves a detailed assessment of the quality of each bin, reallocating contigs between bins and selecting bins with completeness and contamination criteria outlined above (Uritskiy *et al.*, 2018). Subsequently, the bins were reassembled using the *MetaWRAP-reassemble_bins* module aiming to rectify misassemblies and bolster genomic integrity.

After refinement and reassembly, the bins were dereplicated to reduce redundancy and improve the uniqueness of the MAGs. Using dRep v2.6.2 (Olm *et al.*, 2017), with a 95% average nucleotide identity (ANI) threshold, closely related bins were identified and consolidated, resulting in a collection of 82 non-redundant MAGs.

3.3.2 Phylogenetic analysis and classification of MAGs

For taxonomic assignment of the MAGs, the *classify_wf* workflow from GTDB-Tk v2.3.2 was executed using default settings, (Parks *et al.*, 2022) with the reference database GTDB release 207v2 (Parks *et al.*, 2022). A comprehensive phylogenetic tree encompassing 82 species-level bacterial MAGs was derived from 120 bacterial marker genes using the *gtdbtk_infer* module in GTDB-Tk (Parks *et al.*, 2022). To improve interpretation and visualisation, the tree was annotated using iTOL v5 (Letunic and Bork, 2021). While GTDB is among the most robust databases available for taxonomic classification, it is important to recognise that reliance on a single database may limit the detection of novel or less-represented taxa. Future work could

incorporate additional databases or complementary methods to address these potential gaps and enhance taxonomic discovery in underexplored microbial environments.

3.3.3 Structural and functional annotation

Structural annotation of MAGs was performed using Prokka v1.14.5 (Seemann, 2014), a pipeline that integrates multiple algorithms and tools to accurately predict genomic features. Prodigal was utilised for coding sequence identification (Hyatt *et al.*, 2010), while Aragorn facilitated the detection of tRNA and tmRNA genes (Laslett and Canback, 2004).

3.3.4 Analysis of orthologous gene clusters in the MAGs

The protein sequences from structurally annotated MAGs were analysed using OrthoFinder v2.5.5 (Emms and Kelly, 2015). OrthoFinder groups proteins into orthogroups, which are sets of proteins descended from a single ancestral gene shared by all included species (Emms and Kelly, 2015). OrthoFinder starts by performing an all-against-all protein sequence similarity search. By default, it uses DIAMOND (Buchfink *et al.*, 2015), a sequence aligner that is considerably quicker than BLAST for large datasets. Based on the results from DIAMOND, OrthoFinder employs a graph-based approach to infer orthogroups. Essentially, these are groups of orthologues and closely related paralogues. OrthoFinder constructs a graph where each node represents a protein, and an edge between any two genes indicates a similarity score from DIAMOND. It then uses various graph clustering algorithms to delineate orthogroups, grouping together genes that are more similar to each other than to genes in other groups (Emms and Kelly, 2015).

The results from OrthoFinder identified which orthogroups were core (shared by all taxa), unique to a single taxon, or shared by at least two species within the MAGs. This differentiation was based on specific criteria such as nitrogen grain content (high or low) and storage time (harvest, three, six and nine months) of the samples. The output from OrthoFinder was then fractioned into different segments. Proteins shared by all MAGs were classified as the “core orthogroups”. Next the orthogroups were grouped by nitrogen grain content and storage time to identify orthogroups that are shared and unique to these specific groups.

3.3.5 Functional annotation of Orthogroups

A single representative from each identified orthogroup was selected and analysed using a local implementation of eggNOG mapper v2.1.12 (Cantalapiedra *et al.*, 2021). Functional annotation was carried out using the HMMER sequence similarity search tool for protein family profile searches (Eddy, 2011), targeting the eggNOG database v5.0 (Huerta-Cepas *et al.*, 2019). The sequences that show similarity are mapped to their corresponding orthologous groups in the eggNOG database. These groups are based on evolutionary relationships and are assumed to have retained similar functions across species (Koonin, 2005). Once the sequences are mapped to specific orthologous groups, the tool assigns functional annotations to the input sequences. These annotations are derived from the known functions of the genes or proteins within each orthologous group. The eggNOG annotations also categorised the proteins based on their Clusters of Orthologous Groups (COGs) functional categories (Yin *et al.*, 2012). This approach enabled the exploration of the potential biological roles of these proteins across different MAGs, providing insights into the functional capabilities of each group.

3.3.6 Pathogenicity proteins in the reconstructed MAGs

To assess the presence of genes related to plant pathogenicity in the MAGs, the protein sequences from all MAGs were aligned against the Pathogen-Host Interactions Database (PHI-base version 5.0) (Urban *et al.*, 2020). This database is a collection of well-studied genes and proteins that have been experimentally confirmed to be involved in pathogen-host interactions (Urban *et al.*, 2015; Urban *et al.*, 2020). Proteins related to pathogenicity were sourced from the PHI-base (available at http://www.phi-database.org) and subjected to a BLASTP analysis (Shiryev *et al.*, 2007) against the annotated MAGs. Proteins displaying >70% amino acid identity and an alignment coverage of > 70% were identified as potential pathogenicity protein orthologues in the MAGs. Additionally, the proportion of proteins matching PHI-base homologs that exceeded 70% was calculated for each MAG. This was calculated by dividing the number of PHI-base hits per MAG by the total number of proteins in that MAG with greater than 70% amino acid identity. These ratios were depicted using bar plots and incorporated into a phylogenetic tree through iTOL to facilitate data interpretation and visualisation (Letunic and Bork, 2021).

3.4 Results and discussion

3.4.1 Genome reconstruction of the barley microbial community

In this study, a total of 82 metagenome-assembled genomes (MAGs) were recovered from the metagenome data generated from eight barley seed samples stored for different lengths of time (harvest, three, six and nine months) and with different nitrogen contents (high or low) (Table 3.1). The highest number of MAGs were derived from the metagenomes of barley seeds stored for three months (29 MAGs), followed by those sampled at harvest (27 MAGs). The lowest number of MAGs could be assembled form the barley stored for six months (10 MAGs). Typically, a greater number of MAGs were derived from the low-nitrogen content barley seeds (46 MAGs) than high-nitrogen content barley seeds (36 MAGs), with the exception of the harvest samples, where similar numbers of MAGs could be assembled (Table 3.1).

Table 3.1 Number of metagenome-assembled genomes (MAGs) recovered per barley metagenome samples.

Sample	Storage duration	Grain nitrogen content	Number of MAGs
HHN	Harvest	High	14
HLN	Harvest	Low	13
T1HN	3 months	High	11
T1LN	3 months	Low	18
T2HN	6 months	High	4
T2LN	6 months	Low	6
T3HN	9 months	High	7
T3LN	9 months	Low	9

3.4.2 Quality metrics of the barley seed MAGs

Genome completeness determines the accuracy and reliability of the genomic data acquired (Albanese and Donati, 2021), allowing for better functional inference and taxonomy classification (Eisenhofer *et al.*, 2023). Conversely, lower completeness levels may result in gaps in the genome, potentially leading to the omission of essential genes and pathways (Chen *et al.*, 2020). Studies have shown that accurate genomic analysis requires high-quality MAGs, which are defined by completeness levels greater than 90% and contamination levels less than 10% (He *et al.*, 2021; Liu *et al.*, 2021). All MAGs demonstrated completeness > 75%, with

50/82 being >90% complete (Fig. 3.1A and B; Table S3.2). These completeness values are in alignment with the high-quality draft criterion of the Minimum Information about a Metagenome-Assembled Genome (MIMAG) standards for Bacteria and Archaea (Bowers *et al.*, 2017). Further, most of the MAGs (~91%; 75/82) registered contamination levels < 5%, whereas the remaining seven MAGs exhibited contaminant levels between 5 and 10%, indicating the reliability and integrity of our dataset (Fig. 3.2A-D, Table S3.2). We identified a notable negative correlation between genome completeness and contamination ($r = -0.498$, $p < 0.00001$; Fig. 3.2A). In parallel, our data demonstrated a positive relationship between genome size and N50 metric ($r = 0.251$, $p = 0.023$; Fig. 3.2 B), indicating that larger genomes are often associated with superior assembly contiguity.

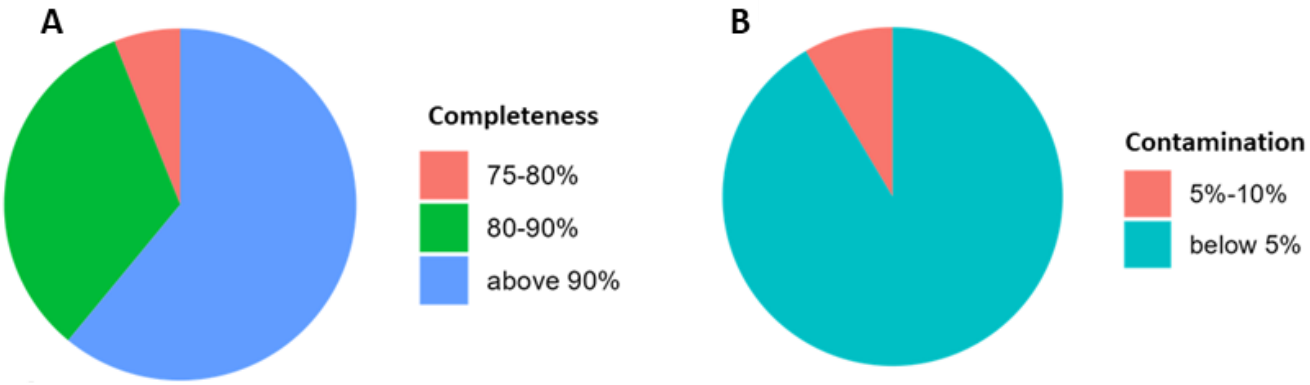


Figure 3.1 Comparative analysis of phylum distribution, MAGs completeness, and contamination. (A) Depicts the completeness of MAGs reconstructed from the metagenomic data, categorised into three ranges: 75-80%, 80-90%, and above 90%, which represent the percentage of the expected genome content present. (B) Illustrates the estimated contamination in the reconstructed MAGs, with categories indicating the percentage of sequences identified as foreign or contaminant, split into two ranges: 5-10% and below 5%.

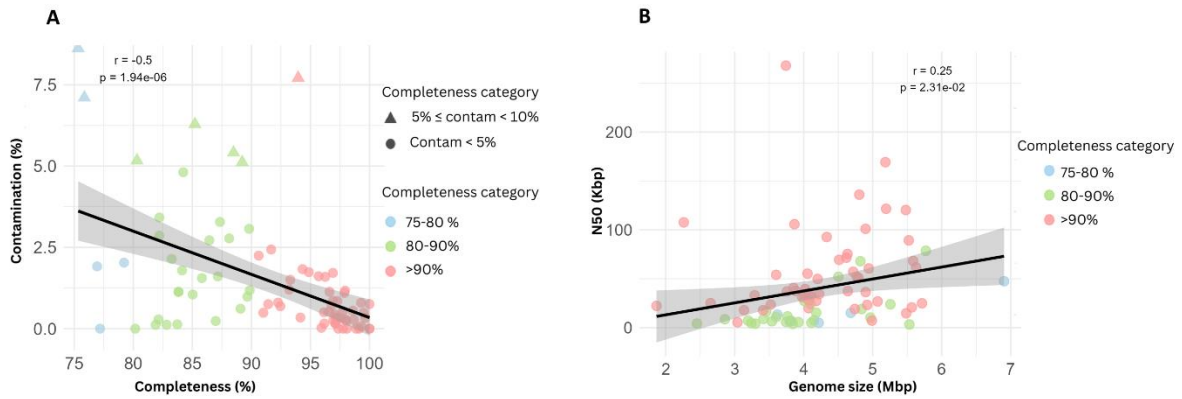


Figure 3.2 Correlations in metagenome-assembled genomes (MAGs). (A) relationship between completeness and contamination of MAGs (B) Correlation between genome size (Mbp) and N50 values (Kbp).

3.4.3 Taxonomic composition the MAGs

Taxonomic evaluation using the Genome Taxonomy Database Toolkit (GTDB-Tk) (Chaumeil *et al.*, 2020) revealed that the barley-associated MAG dataset was dominated by members of the phylum Pseudomonadota, comprising 53.7% (44/82) of the total MAGs (Fig. 3.3).The remainder of the MAGs belong to the phyla Bacteroidota 19.5% (16/82), Bacillota 17% (14/82) and Actinomycetota 9.7% (8/82) (Fig. 3.3, Table S3.2). These findings are consistent with our observations in Chapter 2 (Fig. 2.4, and Table S2.3), in which these four phyla were the most abundant, with Pseudomonadota accounting for the greatest number of sequences reads. Furthermore, these phyla have been observed to dominate the seed microbial communities of various plants, including barley (Bziuk *et al.*, 2021; Tyc *et al.*, 2020; Raj *et al.*, 2019; Adam *et al.*, 2018; Matsumoto *et al.*, 2021).

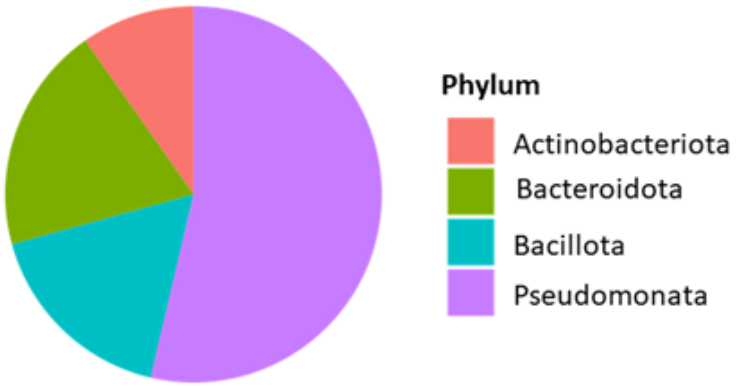


Figure 3.3 Relative abundance of microbial phyla identified in the metagenomic dataset.

MAGs from the phylum Pseudomonadota were found in abundance in all samples, except for the low-nitrogen content barley seeds stored for six months, where the Bacillota dominated. MAGs belonging to the phylum Bacillota were more common after six and nine months, with four and six MAGs, respectively, whereas only one MAG was detected from barley at harvest and barley seeds stored for three months (Table S3.2). This is consistent with the findings in Chapter 2 (Table S2.3) which show that the six month samples (S6L and S6H) included the highest number of Bacillota sequence reads.

The barley-seed-derived MAGs were further classified at the genus and species levels where possible. A total of 26 bacterial genera (Fig. 3.3, Table S3.2) were observed across the barley

metagenomes. The most abundant genera included *Pseudomonas*_E (9 MAGs), *Chryseobacterium* (7 MAGs), *Duffyella* (7 MAGs) and *Erwinia* (6 MAGs) (Fig. 3.4). It is noteworthy that sixteen MAGs could not be classified at the species level, highlighting the underexplored microbial diversity associated with barley seeds (Table S3.2). Most of the MAGs not assigned to a species belong to the phylum Bacteroidota (Table S3.2).

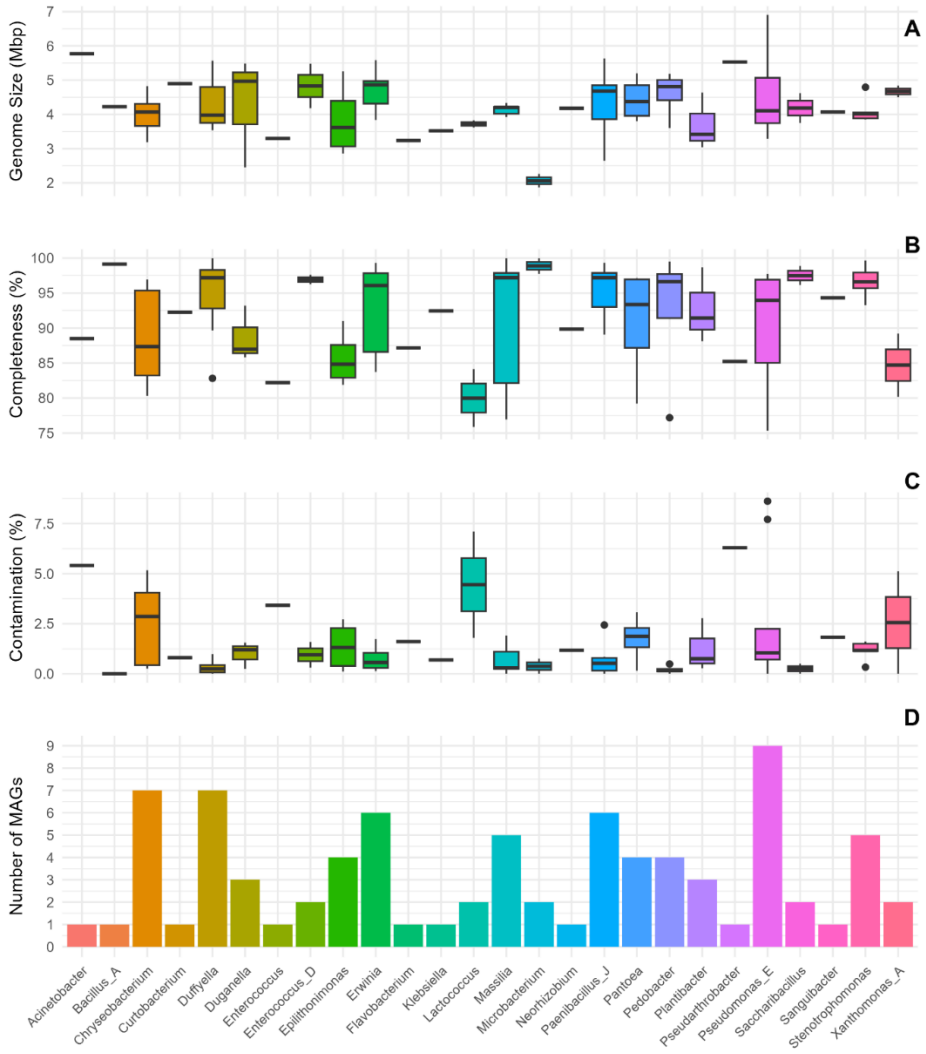


Figure 3.4 Genomic metrics of the identified bacterial genera**.** (A) Genome size distributions across genera. (B) Representation of genome completeness (C) contamination levels in assembled genomes (D) Number of Metagenome-assembled genomes (MAGs) identified per genus. The letter following the genus names indicates a novel genus as predicted by the GTDB (Genome Taxonomy Database) (Parks *et al.*, 2022) classification system.

3.4.3.1 Distribution of MAG genera per nitrogen content and storage duration

The distribution of MAGs by genus, as well as their abundance patterns grouped based on barley seed nitrogen grain content, exhibit trends closely aligning with the metagenomic read-based taxonomy previously reported in Chapter 2 (Table 2.1). For instance, in Chapter 2 (Figure 2.4), reads assigned to the genus *Pseudomonas* were abundant in barley seeds from both high and low nitrogen environments. Similarly, reads associated with the genus *Pantoea* and *Stenotrophomonas* were more abundant in barley samples with low nitrogen, a trend that is consistent with the higher number of MAGs observed in this study (Fig. 3.4A).

The abundance of MAGs at the different storage time point also mirrors the patterns seen in Chapter 2 (Figure 2.5). For example, at harvest in reads assigned to the genus *Chryseobacterium* and *Stenotrophomonas* were abundant (Chapter 2 Fig. 2.4); the same trend is also observed in this chapter (Fig.3.5A). *Pseudomonas*_E MAGs were found in all storage samples, while *Chryseobacterium* was most abundant in the harvest samples. Similarly, relative abundance profiles of the classified metagenomic reads showed that *Pseudomonas* (*Pseudomonas*_E) is not included in the kraken2 database (Lu *et al.*, 2022) recorded the highest read count across all samples and *Chryseobacterium* was predominantly found at harvest (Chapter 2; Fig. 2.4A). *Lactococcus*, which had the highest number of reads in Chapter 2 (Fig. 2.6B) at six months, mirrored this with *Lactococcus* MAGs only recovered from metagenomic DNA at the six month storage time point (Fig. 3.5B). Additionally, *Pedobacter*, *Plantibacter* and *Pseudarthrobacter*, primarily observed at harvest and six months of storage based on relative metagenomic read abundance (Fig. 3.5B), showed a similar presence in MAGs at these storage time points (Fig. 3.5B).

The congruence between taxonomic classifications derived from sequence reads and those from MAGs implies that taxa with higher read abundance are more likely to be assembled into bins, whereas those with lower abundance often fail to meet completeness criteria (Alneberg *et al.*, 2014). While the MAGs discussed in this chapter do not provide extensive insights into the overall microbial community composition, further exploration through annotation and comparative genomics will offer at least a preliminary understanding of the genomic content of dominant taxa within the barley samples.

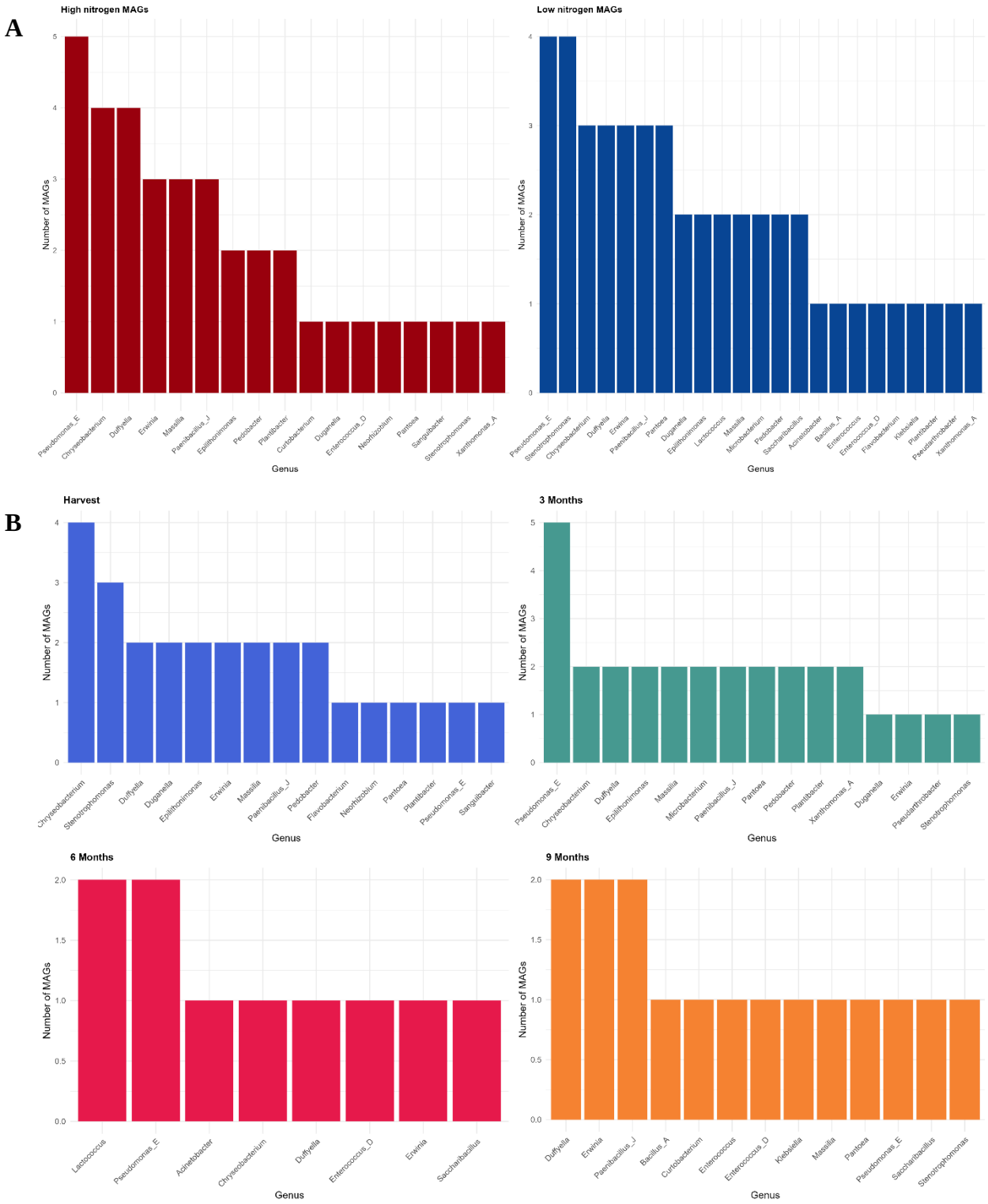


Figure 3.5 Distribution of metagenome-assembled genomes (MAGs) genera. (A) MAGs count per grain nitrogen content (high or low) and (B) storage duration (harvest, three, six and nine months).

3.4.4 The number of MAGs identified decreased over storage time

A decrease in the number of MAGs throughout the storage periods was observed (Fig. 3.6). This is consistent with what was observed in Chapter 2 (Section 2.4.1, Table S2.1), where the number of sequence reads obtained per storage period also decreased (Fig. 3.6). The binning process of MAGs involves assembling microbial genomes from raw sequencing data, which requires that the number of reads for a particular microorganism exceed a certain threshold to ensure accurate and reliable genome assembly (Alneberg *et al.*, 2014). This suggests that the microorganisms were sufficiently prevalent to be detected through their genetic material. The MAG assembly process improves our understanding of microbial communities by identifying which taxa or strains predominate at specific nitrogen grain content and storage time. The reduction in MAG counts over storage duration indicates that fewer microbial species maintain the abundance necessary for detection and assembly into MAGs.

This observation suggests a decline in microbial diversity and abundance, which could be attributed to several factors. One possible explanation is Gause's law of competitive exclusion, which states that two species competing for the same limited resources cannot coexist at constant population levels (Gause, 1934; Hening and Nguyen, 2020). Additionally, even under controlled silo storage conditions, subtle changes in nutrient availability are inevitable over time (Abubakar, 2019). As nutrients deplete, competition among microorganisms intensifies (Wieder *et al.*, 2015; Zhu *et al.*, 2017). This competition may impact previously abundant species, making it difficult for them to thrive. Consequently, their reduced abundance hinders the assembly of their reads into MAGs.

The reduction in microbial diversity with prolonged storage could have both positive and negative implications for the health and resilience of the barley seeds. A diverse microbial community is often linked to improved resistance against pathogens, enhanced nutrient availability and overall plant health (Harman *et al.*, 2021; Trivedi *et al.*, 2020). A decline in microbial diversity might expose the seeds to a higher risk of colonisation by opportunistic pathogens or lead to a decrease in beneficial microbial interactions (Hardoim, 2019; Rosenblueth and Martínez-Romero, 2006). Empirical validation of these hypotheses through *in vitro* assays is essential to elucidate the specific impacts and guide effective seed storage practices.

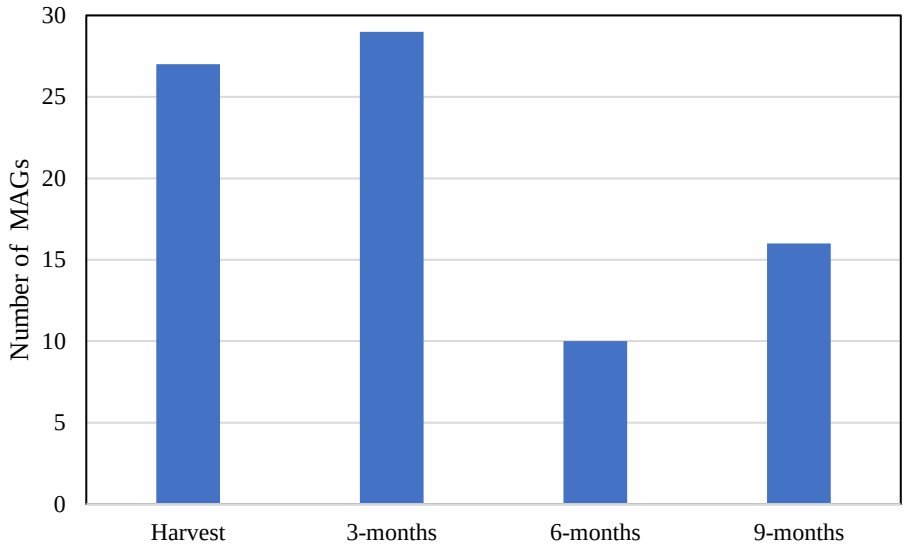


Figure 3.6 The abundance of metagenome-assembled genomes (MAGs) recovered per storage time.

3.4.5 Functional analysis of the MAGs hints at taxonomic diversity

Structural annotation of the 82 MAGs yielded a total of 317,760 proteins. Of these, 95.5% (317,760 proteins) were assigned to orthogroups, while the remaining 4.5% (14,847 proteins) were singletons, occurring in a single MAG (Table 3.3). The proteins were distributed across 34,233 orthogroups. Notably, the analysis identified 707 orthogroups that are distinct to each MAG, comprising 1,682 proteins, which represent merely 0.5% of the total (Table 3.3). The presence of these MAG-specific orthogroups, indicates both functional conservation and a degree of specialisation (Table 3.3) (Plett *et al.*, 2023). Such distinct adaptations improve the generalist functions seen in shared and core orthogroups, establishing a collaborative community in which diverse microorganisms play different roles, ultimately benefiting the group as a whole (Yajima *et al.*, 2023). The low incidence of single-copy orthogroups (SCOs) observed in the dataset is primarily a consequence of taxonomic diversity, reflecting a population-wide orthology analysis. The level of taxonomic diversity among the taxa analysed is a critical factor; as it increases, there is a natural decline in both SCOs and shared orthogroups (Steenwyk *et al.*, 2022; Emms and Kelly, 2015). This pattern illustrates the genetic distance, which significantly escalates among microorganisms from different phyla.

Table 3.2 OrthoFinder general statistics (Emms and Kelly, 2015).

Number of species (MAGs)	82
Number of proteins	332607
Number of proteins in orthogroups	317760
Number of unassigned proteins	14847
Percentage of proteins in orthogroups	95,5
Percentage of unassigned proteins	4,5
Number of orthogroups	34233
Number of MAG-specific orthogroups	707
Number of proteins in MAG-specific orthogroups	1682
Percentage of proteins in MAG-specific orthogroups	0,5
Mean orthogroup size	16,4
Median orthogroup size	5
G50 (assigned proteins)	56
G50 (all proteins)	51
O50 (assigned proteins)	1281
O50 (all proteins)	1420
Number of orthogroups with all species present	66
Number of single-copy orthogroups	1

3.4.6 Diversity of orthogroups in MAGS from barley seeds with different nitrogen grain contents and stored for different durations

The classification of orthogroups in MAGs based on nitrogen grain content (high or low) and over storage time (harvest, three, six and nine months) revealed differences in orthogroup diversity. In low nitrogen samples, 11,128 distinct orthogroups were identified, with 1,331 being shared by at least two species and 9,797 singletons (Figure 3.7A). In contrast, high-nitrogen samples exhibited a lower diversity, with only 6,182 orthogroups, including 425 shared and 5,757 as singletons (Figure 3.7A). The greater diversity in low-nitrogen MAGs can be attributed to a larger sample size (46 MAGs in low nitrogen versus 36 in high nitrogen), which provides a broader genetic base, enhancing the likelihood of identifying unique orthogroups and taxonomic diversity. Therefore, the observed differences in orthogroup diversity reflects underlying taxonomic diversity, pointing to variations in the microbial community that are yet to be fully explored.

Further analysis over different storage durations also demonstrated a dynamic pattern in the diversity of orthogroups (Figure 3.7B). Initially, at harvest, 5,281 orthogroups were identified, including 526 shared and 4,755 singletons. After three months of storage, the number modestly increased to 5,578 distinct orthogroups, comprising 718 shared and 4,860 singletons (Fig. 3.7B). However, a noticeable decrease was observed by six months, with only 2,334 unique orthogroups remaining, including 91 shared and 2,243 singletons. Similar results were observed in nine months storage including 85 shared and 3,360 (Fig 3.7). This trend aligns with the observed decrease in the number of MAGs and raw sequence reads over time, reflecting a similar pattern to the high-singleton prevalence seen under different nitrogen conditions.

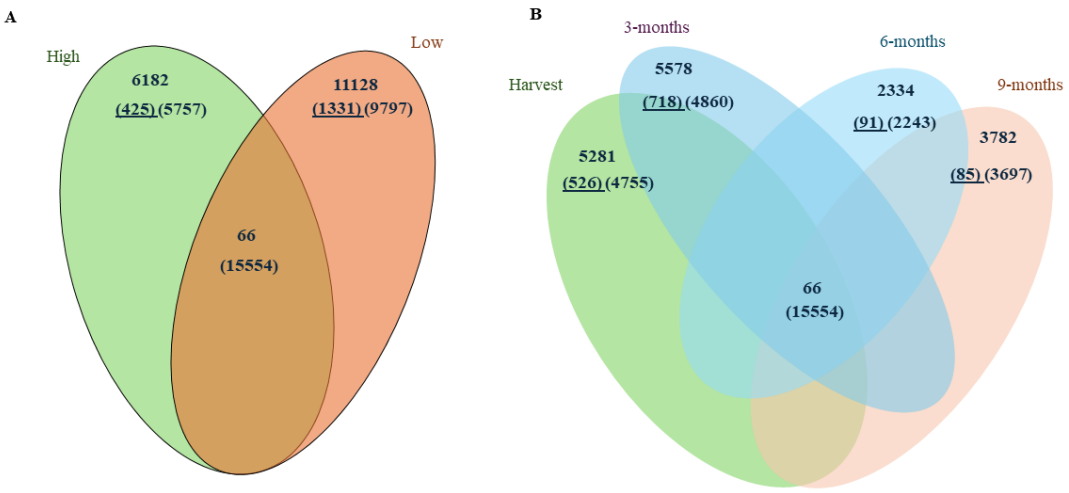


Figure 3.7 Shared and unique orthogroups in metagenome-assembled genomes (MAGs). (A) Shared and unique orthogroups of MAGs based on grain nitrogen content (high or low) (B) Shared and unique orthogroups of MAGs based on storage duration (harvest, three, six and nine months). The underlined number represent orthogroups shared by at least two species.

3.4.7 Functional insights into core and unique orthogroups in barley seed microbiome through COG analysis

Approximatively 35% of proteins shared by at least two MAG taxa from the OrthoFinder output did not have orthologues in the eggNOG database and were classified in the COG category ‘function unknown’ (S) (Fig. 3.8). Analysis of the core proteins COG data revealed that the highest proportion of proteins belong to the COG functional categories ‘translation,

ribosomal structure, and biogenesis' (J; 14%) and 'DNA replication, recombination, and repair' (L;14%). The high representation of proteins involved in these categories in the barley seed microbiome underscores the fundamental cellular functions necessary for growth and adaptation to environmental conditions (Truyens *et al.*, 2015) (Fig. 3.8).

The COG functional categorisation of unique proteins from MAGs derived from barley seeds with differing nitrogen content reveals a distinct functional adaptation within the seed microbiome. In MAGs from low nitrogen conditions, the dominant COG category (aside from category S) is 'transcription' (K; 10%). In contrast, MAGs from high nitrogen conditions show a prevalence in the category of 'cell wall/membrane/envelope biogenesis' (M; 11%) (Fig. 3.8)

The analysis of COG categories across different storage times for MAGs derived from barley seeds reveals an intriguing shift in the dominant metabolic functions of the microbial communities (Fig. 3.8). At harvest and continuing through three months of storage, the predominant functional category is 'carbohydrate transport' and 'metabolism' (G; 11% and 10% respectively). This trend changes by nine months, where 'amino acid transport and metabolism' (E; 22%) becomes the dominant function. Initially, the microbial communities exploit the abundant carbohydrate resources, but as these decrease in availability, they may increasingly turn to amino acids derived from the breakdown of seed proteins or from nitrogenous compounds in the seed environment (Pan *et al.*, 2022). This shift could impact the nutritional quality and shelf life of the grain, potentially affecting its viability and economic value (Ziegler *et al.*, 2021).

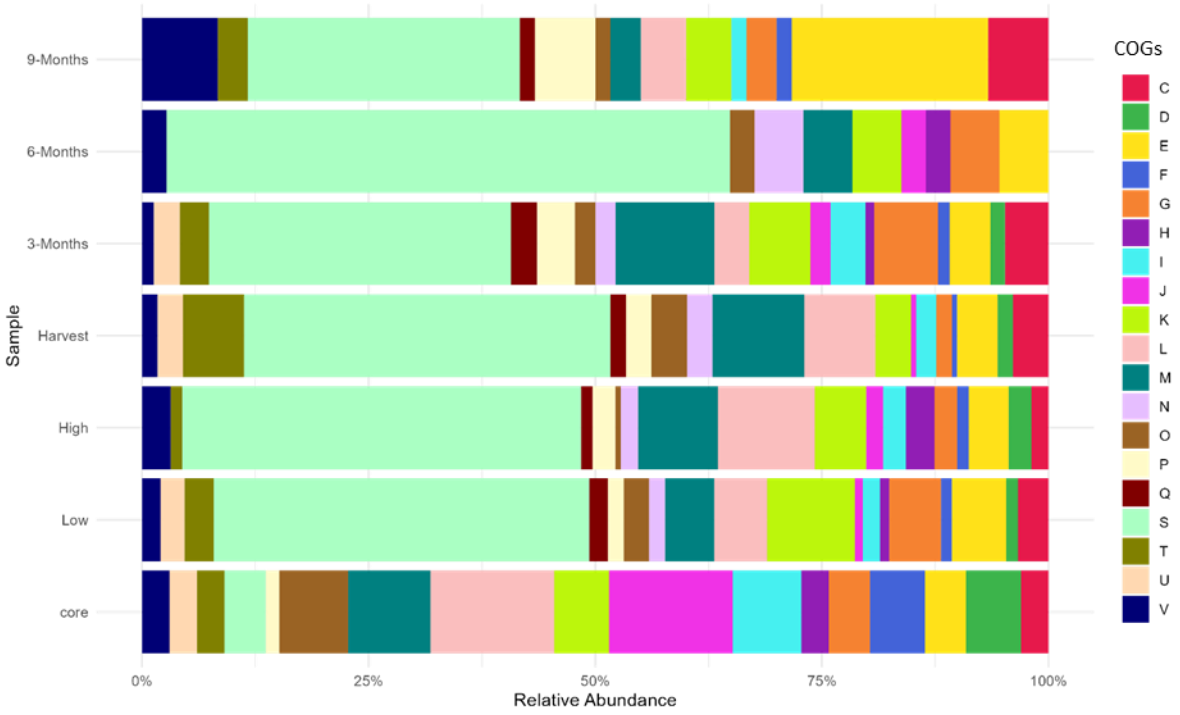


Figure 3.8 Functional annotation of unique proteins in metagenome assembled genomes (MAGs). Cluster of orthologous function (COGs) (Huerta-Cepas *et al.*, 2019). of shared and unique orthogroups of MAGs based on nitrogen grain content and storage time. **A:** RNA processing and modification; **B:** Chromatin structure and dynamics; **C:** Energy production and conversion; **D:** Cell cycle control, cell division, chromosome partitioning; **E:** Amino acid transport and metabolism, **F:** Nucleotide transport and metabolism; **G:** Carbohydrate transport and metabolism; **H:** Coenzyme transport and metabolism; **I:** Lipid transport and metabolism; **J:** Translation, ribosomal structure and biogenesis; **K:** Transcription; **L:** Replication, recombination and repair; **M:** Cell wall/membrane/envelope biogenesis; **N:** Cell motility; **O:** Posttranslational modification, protein turnover, chaperones; **P:** Inorganic ion transport and metabolism; **Q:** Secondary metabolites biosynthesis, transport and catabolism; **S:** Unknown; **T:** Signal transduction mechanisms; **U:** Intracellular trafficking, secretion, and vesicular transport; **V:** Defence mechanisms; **W:** Extracellular structures; **Y:** Nuclear structure; **Z:** Cytoskeleton.

3.4.8 Survey of proteins involved in pathogenicity in the MAGs

The barley seed microbiome is populated by a varied range of microorganisms, some of which are known to play a role in pathogenicity (Bziuk *et al.*, 2021; Noots *et al.*, 1999). The PHI database (Urban *et al.*, 2020) was searched to identify proteins in each MAG exhibiting over 70% homology with known pathogenic proteins. Of the 82 MAGs analysed, 20 exhibited a pathogenicity ratio exceeding 50%. Notably, MAGs from the genera *Erwinia*, *Duffyella*, *Klebsiella* and *Pantoea*, showed particularly high pathogenicity ratios Fig 3.9. For instance, *Erwinia persicina* MAGs (6 MAGs) exhibit pathogenicity ratios ranging from 0.67 to 0.74. Members of the genus *Erwinia* are implicated in causing plant diseases, with *E. persicina* specifically known for causing pink seed disease in barley (Kawaguchi *et al.*, 2021; Wasendorf *et al.*, 2022). Similarly, *Duffyella gerundensis* MAGs (6 MAGs) displayed ratios above 0.73

(Fig. 3.9). The species *D. gerundensis*, previously classified as *E. gerundensis*, was reclassified following taxonomic analyses that determined it did not belong to the *Erwinia* species group (Soutar and Stavrinides, 2022).

Pantoea agglomerans MAGs (2 MAGs) demonstrated a pathogenicity ratio of 0.86, reflecting its dual functionality within plant ecosystems as both a pathogen and a plant growth promoter (Lv *et al.*, 2022). Specifically, *P. agglomerans* has been implicated in reducing seed germination, seedling growth and overall seed quality in oats (Wang *et al.*, 2022). Additionally, *Klebsiella grimontii* MAG70 displayed the highest ratio of 0.87 (Fig. 3.9). This species is known for its antifungal activity against *Fusarium oxysporum*, a pathogen that impacts soybean plants (Wang *et al.*, 2023). In contrast, genera such as *Chryseobacterium* (7 MAGs), *Microbacterium* (2 MAGs) and *Pedobacter* (4 MAGs) exhibited much lower pathogenicity ratios (Fig. 3.9). Member of these genera are mainly associated with plant growth promotion roles in plants (Hayat *et al.*, 2010).

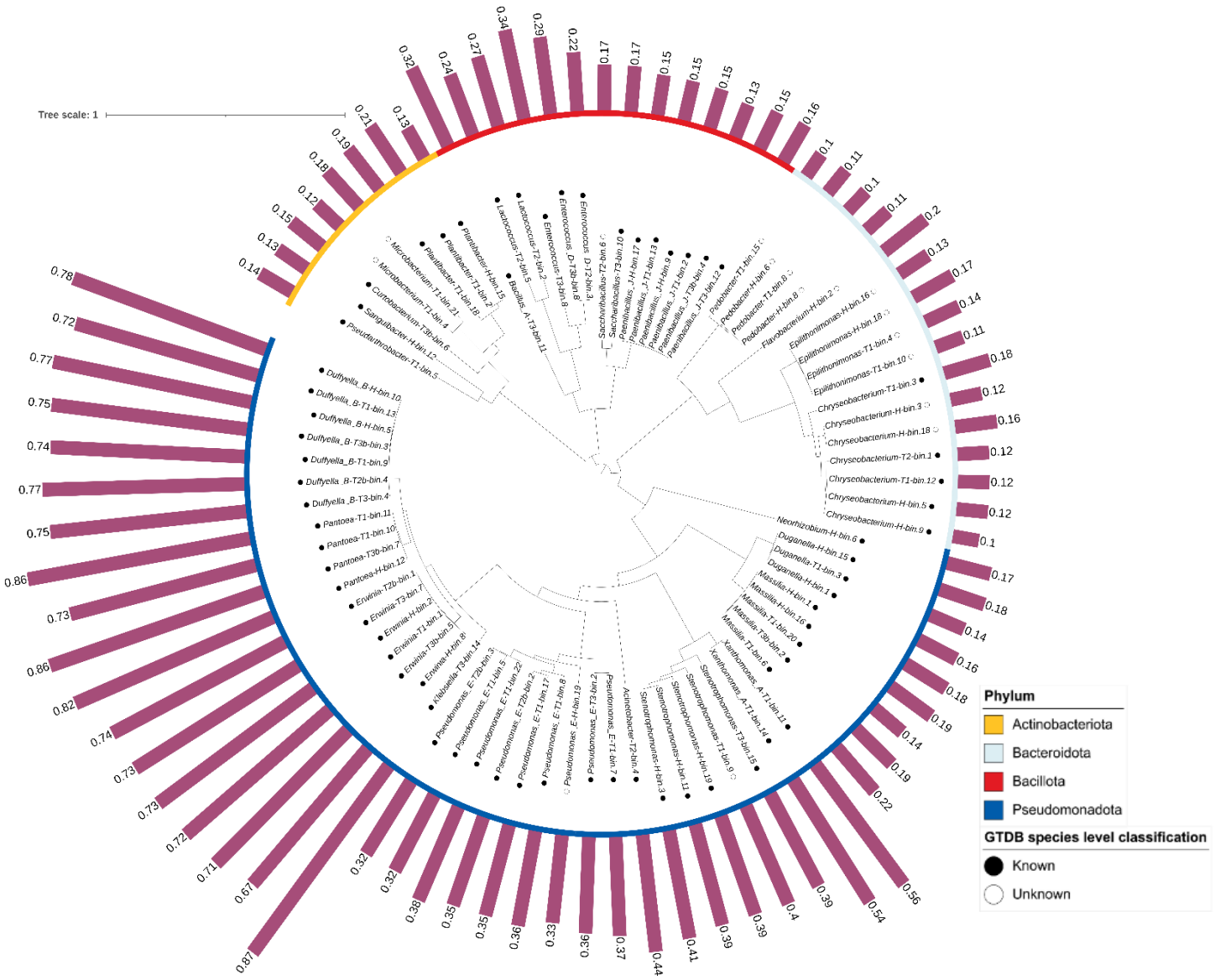


Figure 3.9 Phylogenetic relationships of bacterial metagenome-assembled genomes (MAGs). Phylogenetic tree of 82 species-level MAGs based on 120 conserved bacterial marker genes. Bar plot displaying the ratio of pathogenicity-related proteins, each with homology greater than 70% to entries in the Pathogen Host Interaction (PHI) database (Urban *et al.*, 2020), identified in each MAG.

3.5 Conclusions

This study has reconstructed 82 high-quality MAGs from barley seeds of varying nitrogen content and storage durations, providing detailed insights into the genomic content of the seed-associated microbiome. The analysis revealed substantial taxonomic diversity, with MAGs classified into 26 bacterial genera from four distinct phyla. Despite this diversity, functional conservation was evident in the presence of core orthogroups related to essential cellular functions, including translation, ribosomal structure, and DNA repair. These functions suggest that the barley seed microbiome plays a critical role in maintaining microbial viability and activity during extended storage, potentially contributing to the overall stability of the seed environment.

Notably, the identification of proteins associated with plant pathogenicity from genera such as *Erwinia* and *Klebsiella* underscores the complex role of these microorganisms. While these findings do not directly indicate a pathogenic impact on barley seeds during storage, they suggest that these microorganisms may possess latent pathogenic capabilities, which could become relevant under specific conditions. This highlights the need for further investigation into the ecological roles of these microbes within the stored seed microbiome.

While this study offers valuable genomic insights into the barley seed microbiome, it is important to acknowledge its limitations. The reconstructed MAGs represent only a portion of the microbial community, focusing primarily on higher-abundance taxa. Future studies should incorporate deeper sequencing and complementary approaches, such as single-cell genomics, to capture the full complexity of the microbial community, including low-abundance species that may play crucial roles in barley seed health and preservation.

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Chapter 4 Functional insights into the barley seed microbiome using a meta-omics approach

4.1 Abstract

The barley seed microbiome plays a key role in agricultural sustainability and quality of this crop. While the taxonomic landscape of the barley microbiome is well documented, particularly its implications for brewing quality influenced by storage practices and nitrogen content, the functional dynamics of its microbial constituents are not fully understood. This study employed a comprehensive meta-omics strategy, integrating metagenomics and metaproteomics, to delineate the functional capabilities and active biological processes of the barley seed microbiome during storage. A total of eight samples were investigated in this study. The metagenome assemblies ranged in size from 78 to 180 megabases, comprising a total of 274,778 contigs, averaging 34,347 contigs per sample. Structural annotation identified 952,951 protein-coding sequences (CDS), while functional annotation mapped these sequences to a broad array of metabolic pathways. Clusters of Orthologous Groups (COGs) analysis suggested functions such as transcription, amino acid metabolism, and ion transport that are essential for microbial adaptation to the storage environment. Additionally, the detection of carbohydrate-active enzyme (CAZyme) families, including glycosyl transferases and glycoside hydrolases, indicates a microbial proficiency in modifying plant-derived substrates. The nutrient cycling pathways that were reconstructed from the metagenomic data hinted at potential anaerobic activities that increased with storage duration. Complementarily, metaproteomic analysis showed that a broad range of outer membrane proteins, porins and catalases are expressed by the microbial community, suggesting active roles in nutrient transport, cellular defence and oxidative stress mitigation. To our knowledge, this is the first study that integrates metagenomics with metaproteomics to investigate the barley seed microbiome during storage.

4.2 Introduction

Analysis of the microbial population associated with barley seeds offers insights into seed storage longevity, contamination hazards and the potential effects of these microorganisms on beer production (Cao *et al.*, 2022; Felšöciová *et al.*, 2021; Felšöciová *et al.*, 2020). Despite

substantial advancements, there is still a limited understanding of the barley seed microbiome in comparison to other cereals (Michl *et al.*, 2023). This is due in part to a shortage of comprehensive metagenomic datasets focusing on these microbial communities, as well as a previous reliance on culture-dependent methods (Rahman *et al.*, 2018; Tshisekedi *et al.*, 2024). While these strategies provide useful insights, their applicability is limited (Yulan Chen *et al.*, 2022). Currently, only a number of studies have adopted culture-independent approaches, with a primary focus on taxonomic classification (Bziuk *et al.*, 2021). Crucially, the functional capacity of microorganisms associated with the barley seed remains elusive.

The assembly of metagenomic sequencing reads into longer contiguous sequences (contigs) makes it possible to acquire a thorough picture of the functional profile of microbial communities (Fadiji and Babalola, 2020). This is supplemented by *in silico* functional analysis, which involves gene prediction to identify open reading frames (ORFs) in the assembled sequences (van der Walt *et al.*, 2017). These predicted genes are then aligned against those maintained in databases such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COGs) or CAZy (Carbohydrate-Active enZymes) databases to determine functional roles based on gene similarity (Ogata *et al.*, 1999; Park *et al.*, 2018; Tatusov *et al.*, 2003). While metagenomics identifies the genetic material in environmental samples, shedding light on the potential roles of the microbiome (Desai *et al.*, 2012), it does not verify active gene expression or protein production (Fadiji and Babalola, 2020). Metaproteomics fills this gap by analysing proteins extracted from environmental samples (Abraham *et al.*, 2014). In metaproteomics, it is common practice to match mass spectrometry spectra against annotated metagenomic databases, enhancing specificity and accuracy in protein identification. This approach allows for a direct link between protein function and genetic context (Erickson *et al.*, 2012; Heyer *et al.*, 2019).

In earlier chapters (Chapters 2 and 3), the taxonomic diversity of the barley microbiome at different storage time points in silos (at harvest and after three, six and nine months of storage) and reconstructed metagenome-assembled genomes (MAGs) were explored. Building on this taxonomic foundation, the current chapter adopted a meta-omics approach to investigate the functional profiles of the barley seed microbiome. The annotated metagenome sequences were studied and then used as a database for metaproteomic protein identification by comparing mass spectral data to proteins (Fig. 4.1). This combination of metagenomic and metaproteomic

data attempts to close the gap between genetic potential and actual biological activity, providing additional insight into the barley seed microbiome.

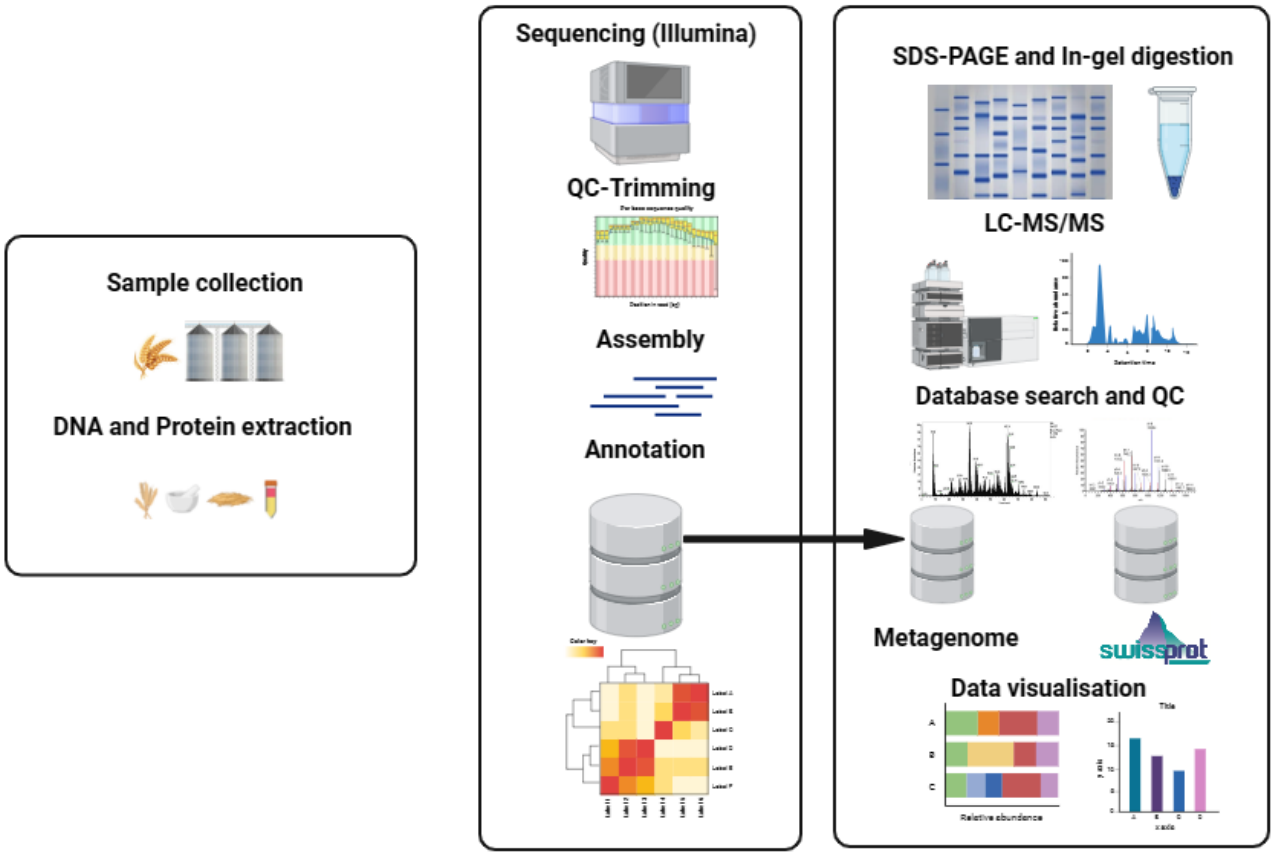


Figure 4.1 Methodology workflow of the meta-omics approach used in the study.

4.3 Methodology

4.3.1 Metagenomic analysis

4.3.1.1 Sample collection, DNA extraction and metagenome sequencing

Barley seeds (Kadie cultivar) were collected at harvest and after three, six and nine months of storage at Anheuser-Busch InBev (AB InBev) storage facilities in the Western Cape Province, South Africa (Chapter 2; Section 2.3.1). At each time point barley seeds with high and low nitrogen content were collected (Chapter 2, Table S2.1). Subsequently, metagenomic DNA (mDNA) was extracted (Chapter 2; Section 2.3.2) and sequenced using the Illumina NovaSeq 6000 system with paired end configuration (2 × 150 bp) for 500 cycles (Chapter 2; Section

2.3.2). The resultant metagenomic sequence data were quality trimmed and curated (Chapter 2; Section 2.3.3).

4.3.1.2 Metagenome assembly

The metagenomic data were assembled using MetaSPAdes version 3.15.3 (Nurk *et al.*, 2017). This tool has been developed specifically for the complex nature of metagenomes, which comprise genetic sequences from a diverse range of microorganisms found in environmental samples (Nurk *et al.*, 2017). MetaSPAdes utilises a de Bruijn graph algorithm as its foundation, starting with the rectification of errors in reads to improve accuracy (Compeau *et al.*, 2011). Subsequently, it merges overlapping reads into elongated sequences and then constructs de Bruijn graphs using various k-mer sizes (21, 33, and 55). This multi-k-mer strategy facilitates the assembly of genomic regions of varying complexity by accommodating different structures and coverage depths inherent in metagenomic datasets (Nurk *et al.*, 2017). The integrity and quality of the final assemblies was evaluated using QUAST v5.2.0 (Gurevich *et al.*, 2013).

4.3.1.3 Structural and functional annotation

Structural annotation of the metagenomic assemblies was conducted using the Prokka v1.14.5 pipeline (Seemann, 2014). Prodigal identifies protein-coding sequences (Hyatt *et al.*, 2010). Concurrently, Aragorn is used for the prediction of tRNA genes and tmRNA, while Barrnap is employed for ribosomal RNA (rRNA) gene prediction (Laslett and Canback, 2004). phaseFor functional annotation, BLAST+ was used to match the predicted protein sequences against known proteins in the UniProtKB (SwissProt) database (UniProt Consortium, 2018). Additionally, HMMER was used to identify protein domains via hidden Markov models against the Pfam and TIGRFAM databases (Prakash *et al.*, 2017). Subsequently, the protein sequences generated by Prokka were processed using eggNOG-mapper v5.0, which employs the DIAMOND algorithm for rapid sequence alignment (Huerta-Cepas *et al.*, 2019) against the EggNOG database v.5.0 (Huerta-Cepas *et al.*, 2019).

While eggNOG-mapper was a core tool for functional annotation, it draws upon multiple underlying databases, ensuring comprehensive functional insights. These include the Clusters of Orthologous Groups (COGs) database (Tatusov *et al.*, 2003), KEGG Orthology (KO) terms (Ogata *et al.*, 1999), Pfam domains (Finn *et al.*, 2014), Gene Ontology (GO) terms (Harris *et al.*, 2004) to functionally annotate each protein. Eggnog-mapper also includes the dbCAN tool (Yin *et al.*, 2012) for the identification of carbohydrate-active enzymes (CAZymes). This

multi-database approach captures a broad array of functional attributes and avoids the limitations of relying on a single database, providing a robust and well-rounded annotation of the metagenomic data.

4.3.1.4 Predicted coding sequences (CDS) coverage analysis

Paired-end reads were mapped to coding DNA sequences (CDS) within .ffn files, previously annotated using Prokka, by employing Bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012). Coverage for each CDS was determined by computing the average depth of coverage, which involves dividing the accumulated per-base read depth by the total length of the CDS. This calculation was performed using the bedcov function of SAMtools v1.9 (Li *et al.*, 2009). The coverage data were then integrated with functional annotations obtained from eggnog-mapper (Huerta-Cepas *et al.*, 2019).

4.3.1.5 Metabolic pathway reconstruction and metagenome mining

Genes known to enhance plant growth in barley and related seeds have been documented in previous studies (Akinola *et al.*, 2021; Robertson-Albertyn *et al.*, 2022). The presence of similar CDS was determined by manually searching within the functional annotation files. To reconstruct important metabolic pathways, the KO identifiers associated with key metabolic functions, such as amino acid utilisation, carbon degradation, fermentation, and nitrogen metabolism, were searched. These KO identifiers served as markers to locate and verify the corresponding genes in the functional annotation files, thus facilitating the reconstruction of relevant metabolic pathways based on the metagenomic data. The refined pathways were then redrawn to incorporate the additional information obtained from the genome mining process. The final visualisations of the nutrient cycling pathways were created using Microsoft PowerPoint.

4.3.2 Metaproteomic analyses

4.3.2.1 Microbial cells separation

Approximately 10 g of barley seeds per sample were ground using a sterilized pestle and mortar. The powdered material was suspended in 40 ml of phosphate-buffered saline (PBS, pH 7.4). This solution was mixed by brief vortexing to ensure uniform consistency.

Subsequently, the solution was sonicated (Microson XL 200, United States of America) at 18 W, with a pattern of 30 s active and 30 s inactive, for a total of 7 min. After sonication, the suspension was centrifuged at 4,000 x g for 1 min. The clear supernatant was filtered through a 0.45 µm pore filter (Sartorius-Stedim Biotech).

4.3.2.2 Protein Extraction

Microbial cells obtained from the filter paper were subjected to protein extraction using the sodium dodecyl sulphate (SDS) and acetone method, adhering to the protocol detailed by Hall and coworkers (2012). Initially, cells were resuspended in an extraction buffer containing 50 mM Tris (pH 7.5, adjusted with NaOH) and 1% (w/v) SDS. The mixture was subjected to vigorous vortexing for 2 min, sonication for 5 min on ice at 20% amplitude (Q125 Sonicator, Qsonica, Connecticut, USA), boiling for 15 min at 95 °C, and then gentle agitation on an orbital shaker (Reflecta Laboratories, South Africa) at 20 rpm for 1 h at 4 °C. Following centrifugation (1,000 x g for 5 min at 4°C), the supernatant, which contained the extracted proteins, was transferred into a sterile tube. Proteins in the supernatant were concentrated and purified through acetone precipitation, maintaining a 5:1 ratio of acetone to sample, using 1 ml of sample. The mixture was left to precipitate at −20°C overnight. The samples were centrifuged (1,000 x g for 1 min at 4°C), the supernatant was discarded, and the pellet was allowed to dry on ice for 10 min. The resulting pellets were redissolved in 50 mM Tris buffer (pH7.4), and a standard 30 µl volume of the protein solution was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970), using 12% (w/v) polyacrylamide gels stained with Coomassie Brilliant Blue (G-250, Bio-rad). Subsequently, the gels were sent to the Council for Scientific and Industrial Research (CSIR) in Pretoria, South Africa, for in-gel digestion and liquid chromatography-mass spectrometry (LC-MS) analysis.

4.3.2.3 In-gel digestion

Protein lanes were divided into five separate sections based on band density and molecular weight range. From each sample, five gel bands were selected, excised and subjected to in-gel digestion, followed by analysis as described by Shevchenko and colleagues (2006). Proteins within the gel sections were reduced using 10 mM dithiothreitol (DTT) mixed with 25 mM of ammonium bicarbonate (NH_4HCO_3) and the mixture was maintained at 60°C for 1 h. After cooling to ambient temperature, gel sections were immersed in 100% acetonitrile for 10 min. After discarding the supernatant, the gel sections were exposed to 55 mM iodoacetamide

combined with 25 mM NH_4HCO_3. The reaction proceeded for 20 min under light-protected conditions. The supernatant was discarded, and the gel sections were dehydrated using a mixture of 25 mM NH_4HCO_3 and 50% (v/v) acetonitrile. Once completely dry, fresh trypsin solution (0.25% v/v) was introduced. Digestion was performed overnight at 37°C. The addition of 0.1% (v/v) formic acid halted digestion, after which the samples were dried under vacuum. The dried specimens were then prepared in a solution of 2% (v/v) acetonitrile and 0.2% (v/v) formic acid for liquid chromatography–mass spectrometry (LC-MS)

4.3.2.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

Peptide fragments derived from each gel section were analysed using a Dionex Ultimate 3000 RSLC system (ThermoFisher, USA) coupled with an AB Sciex 5600 TripleTOF mass spectrometer (Sciex, USA). The peptides were initially purified on an Acclaim PepMap C18 trap column (ThermoFisher, USA) and subsequently subjected to gradient elution on a Waters NanoEase MSH C18 column (ThermoFisher, USA). LC-MS analysis was conducted on the 5600 TripleTOF platform, employing a positive ion mode and data-dependent acquisition (DDA) mode. Precursor (MS) scans were collected from m/z 400-1500, followed by 40 fragment ion (MS/MS) scans, ranging from m/z 100-1800.

4.3.2.5 Database generation

Database searching is a fundamental component of metaproteomic analysis (Saito *et al.*, 2019). It serves as the primary method for identifying proteins from the complex mixtures present in environmental samples. In a metaproteomic experiment, peptides and consequently the proteins from which they originate can be identified by matching the generated mass spectra against theoretical spectra derived from a protein sequence database (Heyer *et al.*, 2019). In this study, a customised database was generated for the metaproteomics analysis to enhance protein identification accuracy. The database incorporated protein sequences from the host organism, *Hordeum vulgare,* potential contaminants identified in-house (CSIR), and annotated sequences obtained earlier (Section 4.4.3). This ensured the database specificity to the samples, increasing the likelihood of accurate protein identification. A target-decoy approach was implemented to control the False Discovery Rate (FDR) (Aggarwal and Yadav, 2016). This is a statistical measure used to control the rate of Type I errors (false positives), ensuring the reliability of the results (Elias and Gygi, 2007). The total database consisted of 372,508 proteins (745,016 in the concatenated target-decoy protein database).

4.3.2.6 Protein search

The raw LC-MS datasets (.wiff files) and generated database were analysed using the Sciex software Protein Pilot V5.010 (Seymour and Hunter, 2015). This software used the Paragon Database Search algorithm to identify peptides from the MS/MS spectra (Shilov *et al.*, 2007). Subsequently, the Pro Group algorithm was used to organise these identified peptides into a coherent lists of reliable peptide identifications. FDR analysis provided a confidence level for the results and a report was generated for each peptide identification result. The LC-MS datasets were segmented into ten parts for processing to enhance the sensitivity and coverage of the analysis. Each segment underwent two separate searches, culminating in a total of 20 sub-searches. A reversed search function was applied, which is a common strategy in proteomics used to estimate the FDR (Gupta *et al.*, 2011). The multi-precursor search function was enabled during the Fraglet and Taglet stages of mass spectrometry data analysis to accommodate different precursor masses, improving the identification of peptides, especially in complex mixtures (Shilov *et al.*, 2007). When the precursor charge state (z) was undetermined, the first three charge states were considered, a common practice in proteomics to account for the fact that peptides can carry multiple charges, increasing the likelihood of correctly identifying the peptide (Sharma *et al.*, 2010).

4.3.2.7 Protein quantification and grouping

Protein grouping was conducted using Bayesian protein confidence calculations alongside peptide FDR analysis (Seymour and Hunter, 2015). The criteria for grouping proteins were based on Intersection ProtScore and Unused ProtScore values. In Protein Pilot, ProtScore is a scoring metric whereby the total ProtScore aggregates all peptide evidence associated with a specific protein and the Unused ProtScore represents the total unique peptide evidence for that protein (Seymour and Hunter, 2015). Protein-level ratios were derived from the median of peptide ratios, mitigating the influence of extreme values or outliers (Maia *et al.*, 2020). For peptide quantification, filtering based on signal-to-noise ratios and peptide confidence thresholds was used to enhance the accuracy of peptide identifications (Cox *et al.*, 2014). The signal-to-noise ratio contrasts the level of desired signal against background noise, with a higher ratio indicating minimal background noise (Maia *et al.*, 2020). The peptide confidence threshold serves as a cut-off value, beyond which peptides are deemed confidently identified. Additionally, specific considerations for discordant and shared peptides were applied to decide their inclusion or exclusion in the analysis. Discordant peptides, which deviate from the

majority, and shared peptides, common to several proteins, were evaluated through this logic to ensure the inclusion of only the most reliable peptide identifications in the final analysis.

4.3.2.8 Protein data filtering

The range of spectral counts found in the eight samples varied from 17,702 to 29,299 (Table S4.2). To control false positive identifications, we used a protein FDR of 1 % (Seymour and Hunter, 2015). Protein hits obtained from the databases were filtered based on the criterion that their Unused ProtScore satisfied the 1 % FDR. This was followed by host and contaminant removal. In addition to filtering at 1 % FDR and host removal, only those data points that exhibited a confidence score of ≥ 95 %, coupled with a contribution score of ≥ 0.1 (10 %), were retained. Subsequently, the curated peptides belonging to the identified proteins were parsed through the Unipept web application (https://unipept.ugent.be/) for protein search and functional annotation (Verschaffelt *et al.*, 2021).

4.3.2.9 Functional annotation and taxonomy of peptides

Functional annotation of peptides and their taxonomic classification involved several steps. Peptides from the proteins identified by Protein Pilot V5.010 (Seymour and Hunter, 2015) were further analysed using the Unipept application (Verschaffelt *et al.*, 2021). This platform receives peptide sequences, typically from metaproteomic experiments, and provides both taxonomic and functional annotations. For taxonomic classification, Unipept uses the Lowest Common Ancestor (LCA) algorithm, which identifies the deepest node, or the lowest common ancestor, in the taxonomic tree that includes all organisms sharing a specific peptide. This process draws on taxonomic information from the NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy). For functional annotation, Unipept uses data from multiple databases, including Enzyme Commission (E.C.) numbers, GO terms, and InterPro entries (Harris *et al.*, 2004; Jones *et al.*, 2014). This data helps elucidate the functions of the peptides and the roles of the organisms in a specific environmental context.

4.4 Results and discussion

4.4.1 Metagenome assembly metrics

The metagenomes of the eight barley seed samples in this study were assembled using MetaSpades (Nurk *et al.*, 2017). Assembly sizes of samples ranged from 78 to 180 megabases (Mb). The largest assembly was observed in the high nitrogen content barley seeds sampled at harvest (HH), whereas the smallest assembly was obtained from the low nitrogen content grain maintained in storage for nine months (S9L) (Table 4.1). The assembly sizes correlate with the number of reads obtained from each sample (Chapter 2; Table 2.1) and the number of MAGs recovered per sample (Chapter 3; Table 3.1). The observed trend of decreasing assembly size with increased storage time raises intriguing questions about the stability and longevity of microbial communities in stored grain (Wisnoski and Lennon, 2021). It is possible that microbial population is initially diverse but reduces as storage conditions become less favourable or as microbial competition and succession lead to the dominance of a few adapted species (Dutta *et al.*, 2022).

Metagenome assembly produced a total of 274,778 contigs across all samples, averaging 34,347 contigs per sample (Table 4.1). The highest count was observed in sample S6L with 36,111 contigs, while the lowest was in sample S9L with 17,032 contigs. The N50 values ranged from 137,89 bp in sample S3H to 468,6 bp in sample S6L (Table 4.1). The N50 value is a statistical metric that indicates the median length of the contigs, where the shortest contig equals half of the overall genome length (Parra *et al.*, 2009). Higher N50 values suggest a higher-quality assembly characterised by longer contigs (Parra *et al.*, 2009; Wang and Wang, 2023). All samples exhibited N50 values exceeding 1,000 bp, indicative of robust assembly quality, a result consistent with the superior outcomes reported using SPAdes for complex microbial communities (van der Walt *et al.*, 2017).

The number of contigs per sample does not necessarily correlate with the assembly sizes (Papudeshi *et al.*, 2017). While a high number of contigs can indicate extensive coverage of a metagenome, it can also imply fragmentation during the assembly process (Papudeshi *et al.*, 2017; van der Walt *et al.*, 2017). Notably, sample S6L, which had the highest number of contigs (36,111), also demonstrated the lowest N50 value (Give the value), suggesting a fragmented assembly, possibly due to the presence of many unique, short sequences that could not be fully assembled (van der Walt *et al.*, 2017). This fragmentation may also explain the lower number

of MAGs recovered from sample S6L (Chapter 3). This highlights the bioinformatic challenges faced in metagenomic sequencing, particularly in complex environmental samples where genomic overlap among different species can complicate the accurate assembly of complete genomes (Berg *et al.*, 2020).

Samples with a high nitrogen content generally exhibited larger assembly sizes, with the exception of the barley seeds stored for six months (S6H and S6L) (Table 4.1). A higher nitrogen content within the seeds likely enhances the availability of nitrogen for microorganisms residing in or colonising the seed (Liu *et al.*, 2023). As these microorganisms consume nitrogen, they proliferate, thereby increasing the overall microbial biomass (Arduini *et al.*, 2006). This increase may be directly reflected in the amount of microbial DNA present, contributing to larger metagenomic assembly sizes upon sequencing of the seed microbiome. The six months storage samples did, however, display signs of dysbiosis (Chapter 2; Section 2.4.1), which could explain the deviation observed in these samples.

Table 4.1 Metagenome assembly and structural annotation metrics.

Sample	**HH**	**HL**	**S3H**	**S3L**	**S6H**	**S6L**	**S9H**	**S9L**
Grain nitrogen content	High	Low	High	Low	High	Low	High	Low
Storage duration	Harvest	Harvest	3-months	3-months	6-months	6-months	9-months	9-months
#contigs	26,069	23,100	23,526	17,737	13,574	36,111	20,368	17,032
Total_length (Mb)	159.01	129.63	146.25	109.46	80.11	134.23	108.08	78.17
GC (%)	54.06	56.24	57.92	52.52	53.91	53.98	54.44	55.68
N50	130,49	118,78	137,89	137,88	100,21	468,6	921,5	724,1
CDS	74,436	151,214	169,009	75,592	103,736	136,775	135,364	106,825
rRNA	36	28	42	15	17	27	19	21
tRNA	720	1,273	1,138	357	940	1,003	1,028	1,067
mRNA	17	21	12	13	16	17	11	6
eggNOGs-query	135,443	124,932	85,980	116,142	67,085	108,812	87,574	62,211
COG_category	120,401	111,592	76,654	103,974	61,441	97,379	79,196	56,316
KEGG_ko	71,134	66,609	46,162	61,747	41,171	61,105	50,710	35,874
CAZy	1726	1539	1084	1454	1015	1525	1123	820

4.4.2 Structural and functional annotation metrics

Structural annotation using Prokka identified a total of 952,951 CDS, averaging 119,119 CDS per sample (Table 4.1). The number of CDS varied among the samples, with the highest count of 169,009 CDS observed in sample HH and the lowest count of 75,592 CDS in sample S6H (Table 4.1). Notably, the distribution of CDS across samples did not consistently align with metagenome size or contig counts; for example, sample S9L, which had one of the smallest metagenome sizes and lowest contig counts, displayed one of the highest numbers of CDS (Table 4.1). The number of CDS might not correspond to the assembly size or the number of contigs because the assembly size contains both coding and non-coding regions and contigs may contain several genes or incomplete genes, resulting in multiple CDS (Papudeshi *et al.*, 2017).

The number of CDS also appeared to vary with nitrogen content and storage duration (Table 4.1). At harvest, the high nitrogen content samples contained a markedly lower number of CDS (74,436) compared to the other samples. However, after three months of storage, the CDS count in the high nitrogen content samples surged to 169,009, the highest across all samples, before slightly decreasing at six months and then increasing again at nine months. In contrast, the low nitrogen content samples exhibited a different trend, starting with a high CDS count at harvest (151,214), which then dramatically dropped to 75,592 after three months of storage, increased again at six months and remained relatively stable at nine months of storage. The variation in CDS numbers across different storage times and nitrogen content suggests potential adaptations of the microbial communities to the environmental conditions within the barley seeds (Agu and Palmer, 2001).

4.4.3 Functional annotation of the metagenome assemblies

4.4.3.1 Clusters of Orthologous Groups (COG) functions

COGs are collections of homologous proteins that are used to study evolutionary relationships (Tatusov *et al.*, 2003). Each COG consists of individual proteins or groups of paralogues that are assumed to have evolved from an ancestral protein. This assumption allows for the functional annotation of uncharacterised proteins based on their evolutionary relationships, thus providing a robust framework for exploring the genetic potential and functional diversity of microbial communities in metagenomic studies (Tatusov *et al.*, 2003; Tatusov *et al.*, 1997). Functional annotation of CDS using the eggNOG mapper tool (Huerta-Cepas *et al.*, 2019)

identified 706,953 COGs across all samples (Table S4.2). The distribution of these COGs showed variation in the number of COGs based on storage time and grain nitrogen content (Table S4.2). For instance, the high nitrogen harvest sample (HH) exhibited the highest count at 120,401 COGs, while sample S9L recorded the lowest at 56,316 COGs.

The protein coding sequences belonged to a total of 23 distinct COG categories (Table S4.2). The COG category function unknown (S) was the most represented, comprising approximately 21 % (167,146 entries) of the proteins with a COG category assignment (Fig. 4.2; Table S4.2). Metagenomic studies often uncover genes/proteins of unknown function from uncultured microorganisms that are underrepresented in current databases (Lok, 2015).

In terms of COGs with known functions, the COG category with the largest number of CDS was transcription (K; 67,581 total entries), closely followed by the category for amino acid transport and metabolism (E; 60,147 total entries) (Fig. 4.2; Table S4.2). Transcription is a fundamental biological process essential for all living organisms, as it initiates the pathway of gene expression by converting genetic information from DNA into RNA (Mejía-Almonte *et al.*, 2020). Similarly, the category for amino acid transport and metabolism includes genes involved in the uptake, transport, synthesis, and breakdown of amino acids; these are the building blocks of proteins (Marin and Krämer, 2007).

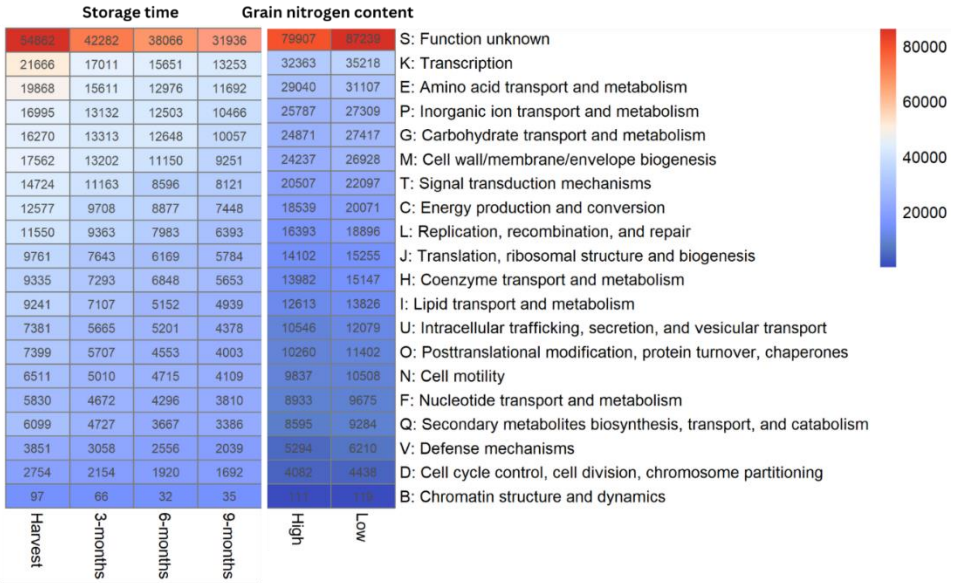


Figure 4.2 Heatmap illustrating the distribution of Clusters of Orthologous Groups (COGs) (Tatusov *et al.*, 2003) across different variables. (Left) Distribution by storage times: harvest, three months, six months, and nine months. (Right) Distribution by grain nitrogen content: high and low.

The third most populous COG category was inorganic ion transport and metabolism (P; 53,096 total entries) (Fig. 4.2). This category includes proteins that manage the uptake and utilisation of inorganic ions, which are vital for various cellular processes such as osmoregulation, electron transport and nutrient assimilation (Shabala and Shabala, 2011). Effective ion management can help microorganisms maintain cellular balance and energy production(Chandel *et al.*, 2021). This was followed by the COG category carbohydrate transport and metabolism (G; 52,288 total entries) (Fig. 4.2). This category encompasses enzymes and transport systems involved in the degradation and synthesis of carbohydrates, which are essential for energy production and storage (Biely, 2012). The presence of these proteins suggests that bacteria may impact the carbohydrate economy of the seed environment, potentially influencing seed decay or preservation during storage (Boraston *et al.*, 2004).

When grouped based on storage time and grain nitrogen content, it was observed that the number of COGs per category decreased with increasing storage duration (Fig. 4.2). The decline in COGs could suggest a simplification of the microbial community as the storage period extends (Chandel *et al.*, 2021). This trend may also be linked to the initially lower read counts obtained, making recovery less likely, particularly given that the highest number of CDS was observed at harvest and after three months of storage, indicating a potential decline as storage progresses.

4.4.3.2 Distribution of CAZymes and carbon degradation proteins across the metagenomic samples

Carbohydrate-Active Enzymes (CAZymes) play a role in the formation and breakdown of complex carbohydrates and glycoconjugates (Cantarel *et al.*, 2009). These enzymes are fundamental in plant-microbial interactions, facilitating the degradation of complex plant biomass into simpler components (Deveau *et al.*, 2008). As such, analysis of the CAZyme composition can provide insight into the microbiome-barley seed interactions.

Across all samples, a total of 10,286 CAZymes were identified, spanning six enzyme classes and 90 families (Table S4.3). The highest number of CAZymes was observed at harvest in the high nitrogen content barley seeds (HH) and the lowest in sample S9L, with 1,726 and 820 representatives, respectively. Overall, a decrease in the total number of CAZymes correlates with extended storage times (Fig.4.3A and B). Additionally, samples with lower nitrogen content consistently exhibited higher counts of CAZymes (Fig.4.3A and B).

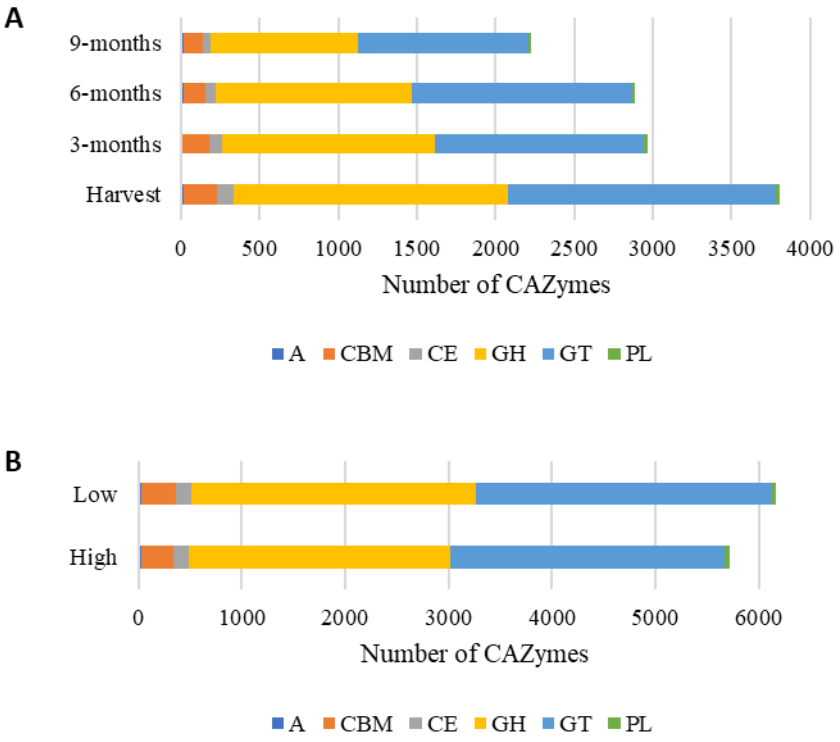


Figure 4.3 Distribution of CAZymes families (Park *et al.*, 2018) in barley seed microbiome metagenomes. The bar charts illustrate the quantitative representation of different CAZyme class: Auxiliary Activities (AA), Carbohydrate-Binding Modules (CBM), Carbohydrate Esterases (CE), Glycoside Hydrolases (GH), Glycosyl Transferases (GT), Polysaccharide Lyases (PL). (A) Distribution across storage times (harvest, three, six, and nine months). (B) Distribution by grain nitrogen content (high and low).

More than half (56.6%) of the identified CAZymes belong to the enzyme class glycosyl transferases (GTs). Within this class, the GT2 family was the most abundant (1,580 total entries), followed by the GT4 family (1,130 total entries) (Table S4.3). Glycosyl transferases are involved in the biosynthesis of glycosidic bonds (Zhu *et al.*, 2013). The GT2 family includes enzymes such as cellulose synthase, which synthesises beta-glucan chains integral to microbial biofilms and extracellular matrices (Schmid *et al.*, 2016). The GT4 family consists largely of glycogen synthases, required for microbial glycogen biosynthesis and energy management (Zhu *et al.*, 2013).

Glycoside hydrolases (GH) form the second-largest CAZyme class, encompassing 5,274 CAZymes, with the GH13 family accounting for 1,246 CAZymes (Fig. 4.4; Table S4.3). This family, often referred to as the alpha-amylase family, includes enzymes that break down starch into simpler sugars such as maltose and glucose (Stam *et al.*, 2006; Franceus and Desmet, 2020). This suggests the microbiome integrates strategies for utilising starch and other energy-

rich compounds within the seeds, which are needed for microbial energy acquisition, but could reduce the starch reserves essential for malting in beer production (Parés Viader *et al.*, 2021). Metagenome mining of glycosyl hydrolases also identified beta-glucosidases (BglX, BglB), which are pivotal for cellulose degradation, and beta-galactosidases (LacZ), which facilitate the conversion of beta-galactosides into monosaccharides, aiding in the metabolism of plant-derived sugars (Rosenberger *et al.*, 2012). Enzymes of the classes Auxiliary activities (AA - primarily involved in lignin degradation), carbohydrate esterases (CE - involved in modifying esterified plant cell components) and polysaccharide lyases (PL - degrade pectin) were found in lower abundance in the barley seed metagenomes. This may be due to the less complex barley seed cell wall, which further has negligible amounts of pectin and lignin (Shafiei *et al.*, 2023; Sidar *et al.*, 2020; Langenaeken *et al.*, 2020).

Overall, analysis of the CAZYmes highlights a robust system for carbohydrate manipulation. These enzymes could prepare the seeds for more efficient malting by breaking down complex carbohydrates into simpler sugars required for fermentation (Parés Viader *et al.*, 2021).

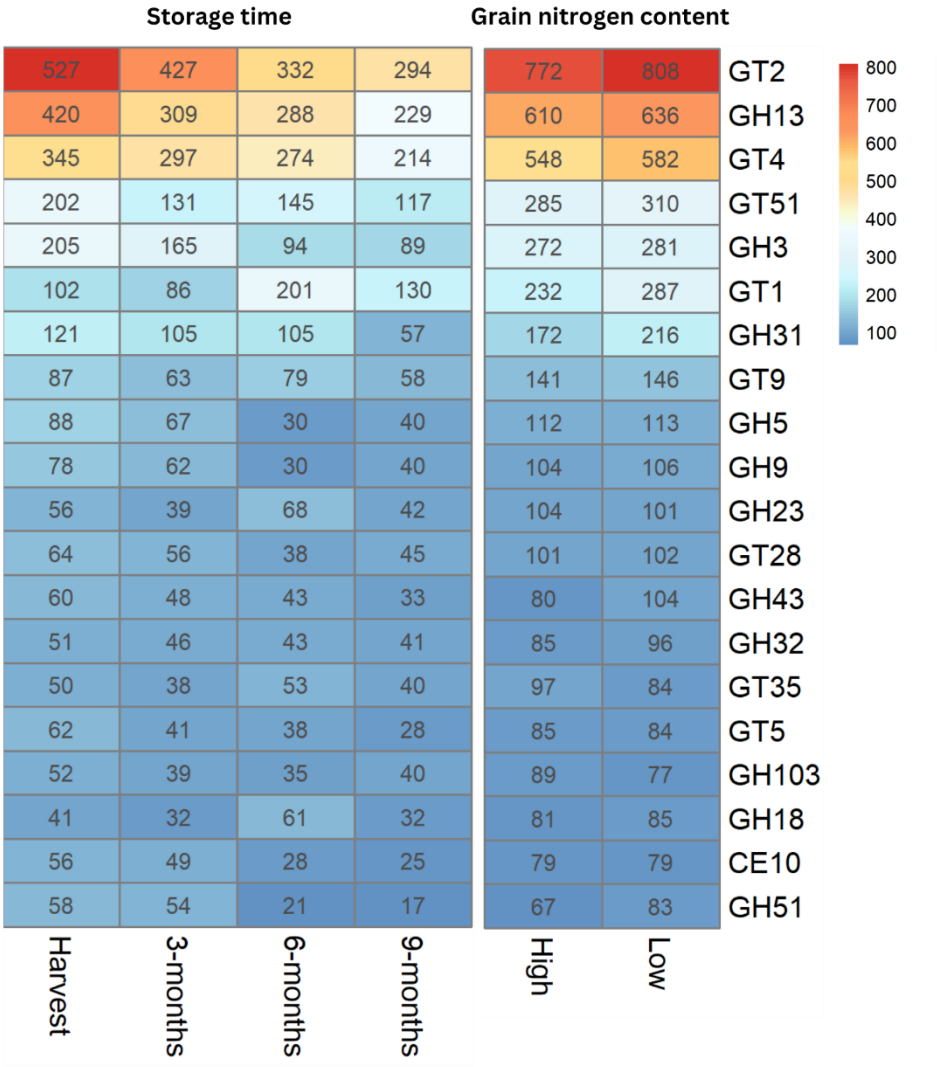


Figure 4.4 Comparative analysis of Carbohydrate-Active enZYmes (CAZymes) (Park *et al.*, 2018) family abundance in barley seed metagenomes. The heatmaps display the abundance counts of various CAZyme subfamilies. (Left) Distribution across storage times (harvest, 3, 6, and 9 months). (Right) Distribution by grain nitrogen content (high and low). Carbohydrate Esterases (CE), Glycoside Hydrolases (GH) and Glycosyl Transferases (GT).

4.4.4 Reconstruction of metabolic pathways from the barley microbiome

Reconstruction of the metabolic pathways of seed microbiomes facilitates an understanding of the complex interactions between plants and their associated microbial communities (Zhou *et al.*, 2022). This section details metabolic pathways related to amino acids, carbohydrate degradation, fermentation, and nitrogen (Table S4.4). Examining these pathways elucidates how seed storage conditions and nitrogen content may influence the metabolic activities of the seed microbiome. Additionally, it provides insights into how these metabolic processes contribute to the nutritional quality of barley seed (Bziuk *et al.*, 2021; Rahman *et al.*, 2018).

4.4.4.1 Survey of proteins involved in fermentation

Fermentation processes in the barley seed microbiome may support microbial survival and activity under anaerobic or low-oxygen conditions (Bokulich and Mills, 2012). These conditions often occur in environments where seeds are densely packed, limiting the availability of oxygen (Chandel *et al.*, 2021). Under such circumstances, traditional aerobic metabolic pathways are not feasible, prompting microorganisms to rely on anaerobic respiration or fermentation for energy production (Chandel *et al.*, 2021; Hirko *et al.*, 2023). Additionally, the lactate fermentation pathway, facilitated by enzymes such as L-lactate dehydrogenase, becomes particularly advantageous in these anaerobic settings, helping microorganisms efficiently produce energy while contributing to the acidity and potential flavour profiles of the grains used in brewing(Gaspar *et al.*, 2007; Laitila *et al.*, 2018).

Metagenomic mining of the barley seed microbiome identified key proteins involved in fermentation, such as acetyl-CoA synthetase (AcS) and formate C-acetyltransferase (PflD) (Table S4.4). Acetyl-CoA synthetase converts acetate into acetyl-CoA, thus enhancing the utilisation of acetate and influencing the carbon cycle in the seed environment (Krivoruchko *et al.*, 2015). Formate C-acetyltransferase catalyses the reversible transformation of pyruvate and CoA into acetyl-CoA and formate (Bar-Even, 2016). The presence of these enzymes indicates a microbiome adapted to survive under anaerobic or low-oxygen conditions (Chandel *et al.*, 2021).

Additionally, orthologues of L-lactate dehydrogenase (Idh) were observed in all samples, with particularly high read coverage depth in samples S6H and S9H (Table S4.4). This enzyme catalyses the conversion of pyruvate to lactate in the lactate fermentation pathway (Gaspar *et al.*, 2007). Notably, samples S6H and S9H also showed a high abundance of reads assigned to

the genera *Lactococcus* and *Enterococcus* (Chapter 2; Section 2.4.5) that are known for their ability to produce lactate via lactate dehydrogenase (Song *et al.*, 2017; Cruz *et al.*, 2013).

4.4.4.2 Survey of proteins involved in amino acid metabolism

Amino acids are an essential source of nitrogen, vital for growth of almost all living organisms (Reineke and Schlömann, 2023). Consequently, the metabolism of amino acids by the seed microbiome could impact the nitrogen cycle within the plant-microorganism ecosystem (Jetten, 2008). Metagenomic analysis identified several proteins involved in amino acid metabolism, primarily aminotransferases/transaminases (Table S4.4). These enzymes catalyse the transfer of an amino group from a donor to a recipient molecule, thereby transforming amino acids into other forms usable by the plant or its associated microorganisms (Givan, 1980; Dold *et al.*, 2016). These enzymes included histidinol-phosphate aminotransferase (HisC), involved in histidine biosynthesis; phosphoserine aminotransferase (SerC), which converts 3-phosphohydroxypyruvate to 3-phosphoserine; and branched-chain amino acid aminotransferase (IlvE), required for the synthesis and degradation of leucine, isoleucine and valine (Nelson, 2018; Egle *et al.*, 2015; Rudat *et al.*, 2012). These activities may facilitate the conversion of seed nitrogen into forms that are more accessible for use by both the microorganisms and the plant (Marin and Krämer, 2007).

4.4.4.3 Survey of proteins involved in nitrogen metabolism

Metagenomic analysis of the barley seed microbiome identified a range of nitrogen cycling processes. The reconstruction of nitrogen cycling within the barley seed microbiome revealed that key processes were present in all metagenome samples. These processes included assimilatory and dissimilatory nitrate reduction to ammonium, denitrification, nitrite reduction to ammonium, while nitrogen fixation was restricted to only two barley metagenome samples (Table S4.4).

Nitrite ammonification transforms nitrite into ammonia, a less toxic form, and nitrate reduction converts nitrate into nitrite, both of which are essential for nitrogen assimilation in microbial metabolism (Egas *et al.*, 2024; Tiso and Schechter, 2015). The ubiquity of these pathways could suggest a fundamental role in enabling the microbial community to manage nitrogen compounds effectively. Denitrification is primarily an anaerobic process where microorganisms reduce nitrate to gaseous nitrogen forms, functioning as an alternative respiratory pathway when oxygen is scarce (Zumft, 1997). Silos and storage facilities often create low-oxygen environments due to dense packing and limited air circulation (Ghali, 2014).

The ubiquity of these pathways could suggest a fundamental role in enabling the microbial community to manage nitrogen compounds effectively.

Nitrogen fixation refers to the biological conversion of atmospheric nitrogen into a more reactive form that can be used biologically, typically ammonia (Table S4.4) (Burris and Roberts, 1993). Proteins related to nitrogen fixation (NifD, NifH, and NifK) were observed in only two samples, namely S6L and S9H (Table S4.4). The appearance of nitrogen fixation genes later in the storage period could result from the observed succession in the microbial community (Chapter 2; Section 2.4.7). As storage conditions change, the environment may become more favourable for nitrogen-fixing bacteria to proliferate (Jetten, 2008; Fani *et al.*, 2000).

4.4.5 Metaproteomic analysis

4.4.5.1 General metrics

A total of 3,267 proteins were identified across all barley seed samples at a 1% FDR, with an average of 408 proteins per sample (Table S4.5). The number of proteins identified is lower than the CDS obtained from the metagenome of the same samples (Fig. 4.5; Table S4.5). This discrepancy can be attributed to the fact that not all genes are expressed simultaneously or at the same level (Desai *et al.*, 2012). Some genes may not be expressed under the specific conditions at the time of sampling, leading to no detectable proteins (Salvato *et al.*, 2021). Additionally, challenges in protein identification in metaproteomics contribute to underreporting (Saito *et al.*, 2019). As an emerging field, metaproteomics faces hurdles such as the complexity of the sample, the presence of low-abundance proteins and the limitations of analytical methods, all of which can reduce protein identification rates (Abraham *et al.*, 2014; Saito *et al.*, 2019). Secreted proteins were underrepresented in the data, likely due to losses incurred during protein isolation steps that may fail to effectively capture or preserve them. Given their less stable and more soluble characteristics, these proteins are prone to degradation or loss throughout the extraction and purification processes (Saito *et al.*, 2019).

The highest number of proteins (604 proteins) were identified in sample S9H (Fig. 4.5; Table S4.5). This sample also recorded a high number of CDS (135,364). Conversely, sample S6L had the lowest number of identified proteins (93), despite having one of the highest numbers of CDS (136,775). The number of proteins per storage period does not follow a clear ascending

or descending trend; instead, it fluctuates. The samples stored for nine months consistently show the highest counts of identified proteins in the dataset (Figure 4.5). This suggests that as storage time progresses, the microbial community likely undergoes changes in response to storage conditions (Mordant and Kleiner, 2021), which could lead to variations in the types and quantities of proteins produced.

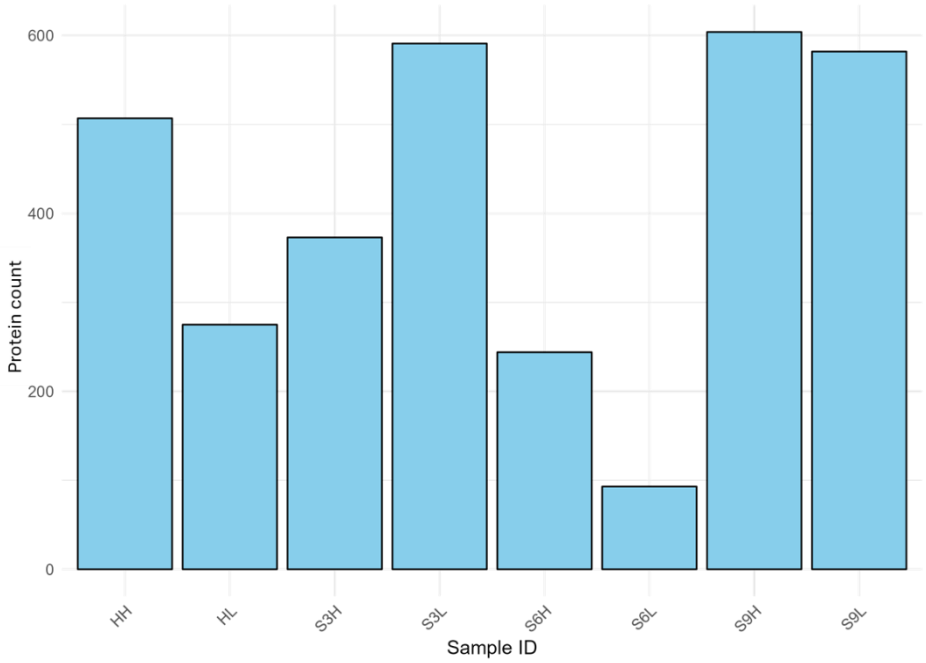


Figure 4.5 Protein count of metaproteome samples identified using Protein Pilot (Seymour and Hunter, 2015) and Unipept (Verschaffelt *et al.*, 2021). HH: Harvest, High grain nitrogen content; HL: Harvest, Low grain nitrogen content; S3H: 3 months storage, High grain nitrogen content; S3L: 3 months storage, Low grain nitrogen content; S6H: 6 months storage, High grain nitrogen content; S6L: 6 months storage, Low grain nitrogen content; S9H: 9 months storage, High grain nitrogen content; S9L: 9 months storage, Low grain nitrogen content.

4.4.5.2 Taxonomic classification of peptides

Taxonomic classification of peptides against the NCBI database via Unipept revealed that the proteins belong to four bacterial phyla, namely the Pseudomonata, Bacteroidota, Bacillota, and Actinomycetota, as well as the viral phylum Uroviricota (Fig. 4.6). Seed-associated bacteria across a wide range of plant taxa predominantly belong to the bacterial phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, as documented in several studies (Bulgarelli *et al.*, 2015; Bziuk *et al.*, 2021; Rahman *et al.*, 2018). The dominance of these phyla in soil likely explains their frequent encounters with seeds during development and post-harvest periods (Fierer *et al.*, 2012).

The phylum Pseudomonata accounted for 92% of the total peptides identified (Fig 4.6), aligning with the taxonomy of metagenomic reads (Chapter 2; Section 2.4.1), which suggests not only their abundance but also the substantial activity linked to this phylum within the barley seed microbiome (Bziuk *et al.*, 2021). Archaea and fungi were detected in the metagenomic data in very low proportions but were not observed in the protein data. This absence might indicate that their protein concentrations are too low for detection, given the sensitivity of protein extraction methods (Abraham *et al.*, 2014).

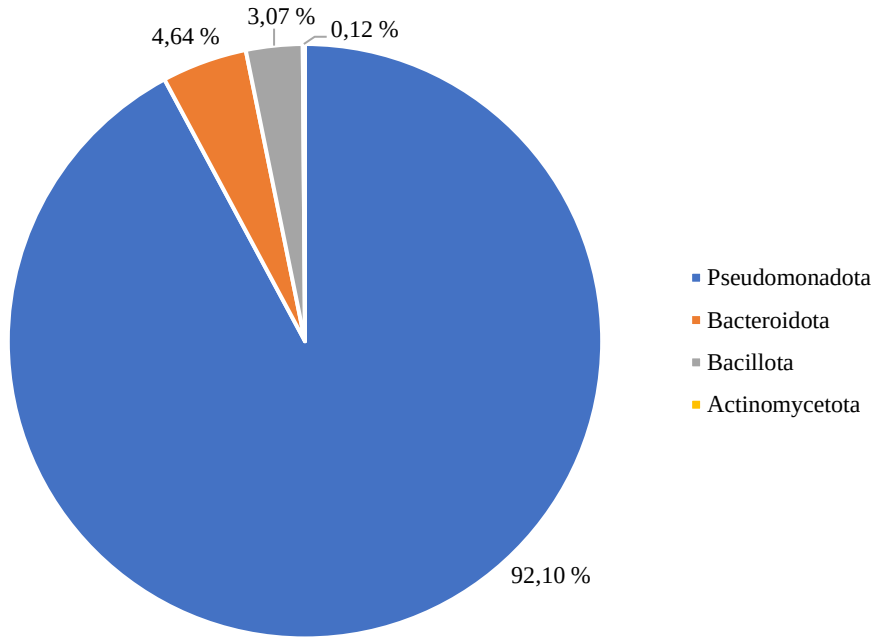


Figure 4.6 Proportional distribution of proteins, categorised by microbial phyla, based on identifications from the InterPro protein database (Jones *et al.*, 2014).

The metaproteomic analysis identified bacterial proteins associated with 22 genera (Fig. 4.7). Proteins from the genus *Pseudomonas* were the most prevalent, comprising 45% of the total protein count, followed by *Pantoea* (16%), *Xanthomonas* (14%) and *Duffyella* (11%). These genera were consistently identified in both the metagenomic reads and account for the majority of the assembled MAGS (chapter 2 and 3 respectively) Previous studies using RNA and DNA analyses have noted the frequent occurrence of *Paenibacillus*, *Pantoea*, *Pseudomonas* and *Stenotrophomonas* within the barley seed microbiome (Yang *et al.*, 2017; Rahman *et al.*, 2018; Bziuk *et al.*, 2021). The identification of proteins related to these bacterial genera cements their

status as core components of the barley seed microbiome. Additionally, given that the proteomic analysis reflects active protein expression, it suggests that they play a role in the functioning of the microbiome and its interaction with the barley seed (Erickson *et al.*, 2012). Notably, proteins from other prevalent genera identified from the metagenomic data (Chapter 2; Section 2.4.4), such as *Citrobacter*, *Klebsiella* and *Leclercia*, were not detected in the proteomic data, suggesting that these may be less active participants in the seed microbiome.

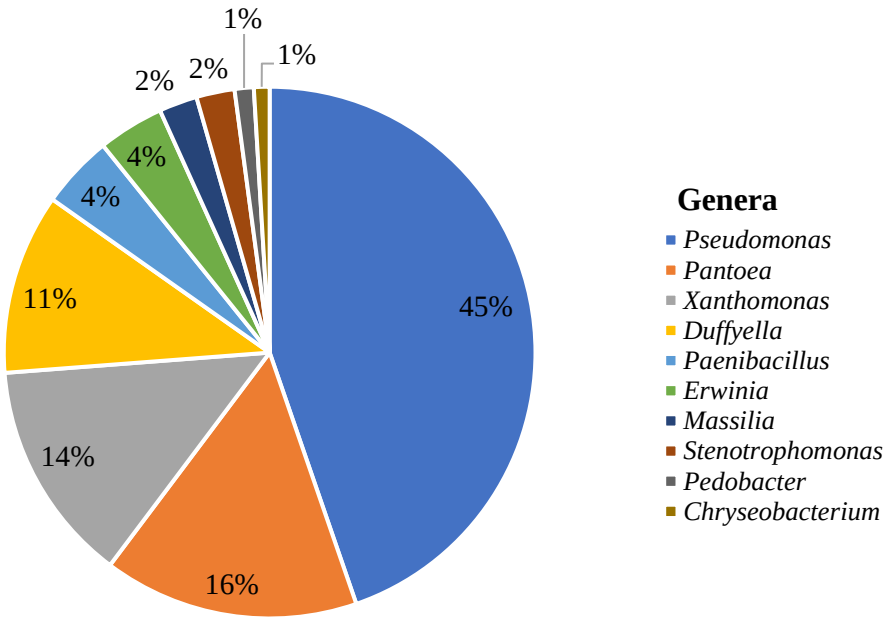


Figure 4.7 Distribution of the top ten microbial genera identified based on InterPro protein identifications (Jones *et al.*, 2014).

4.4.5.3 Distribution of proteins of the dominant genera based on storage time

The taxonomic profiling of proteins from barley seeds at different storage durations reveals deviations from the metagenomic data (Fig. 4.8). At harvest, proteins predominantly linked to *Pseudomonas* (33%), *Pantoea* (22%), and *Erwinia* (11%) were observed (Fig. 4.8). As storage progressed, a shift in the dominant genera was noted; after three months of storage, *Xanthomonas* proteins were most abundant (40%), surpassing *Pseudomonas* (25%) and *Pantoea* (15%) (Fig. 4.8). After six months of storage, *Pseudomonas* (48%) regained dominance, followed closely by *Pantoea* (28%) and *Paenibacillus* (14%). At nine months of storage, *Pseudomonas* proteins further increased in prevalence (69%), followed by *Duffyella* (13%) and *Pantoea* (9%) (Fig. 4.8).

These observations confirm that the barley seed microbiome undergoes changes over time (Felšöciová *et al.*, 2020). Additionally, it highlights disparities between the taxonomic abundance observed in metagenomic data and metaproteomic data (Fig. 4.8). For example, while metagenomic data at harvest highlighted that the genus *Stenotrophomonas* was prevalent, proteomic data indicated members of the genus *Pantoea* and *Pseudomonas* as the most active. Similarly, while *Xanthomonas* showed a higher protein count at the three month storage point, it had a lower read count in metagenomic data, suggesting varied microbial activities. This disparity continues at six and nine months, where the proteomic data show *Pseudomonas* (48, 69%, respectively), *Pantoea* (28, 9%, respectively), and *Paenibacillus* (14, 14%, respectively) as the more active genera, contrasting with the metagenomic prevalence of *Erwinia* and *Stenotrophomonas* (Chapter 2; Section 2.4.4). These findings underscore the differences between genomic and proteomic data in detecting taxa presence within a community. The observed differences suggest that proteomic data more directly reflects microbial activity, whereas metagenomic data primarily indicates the presence of taxa (Schiebenhoefer *et al.*, 2019). This distinction could result in varied conclusions regarding the functional impact of the microbiome.

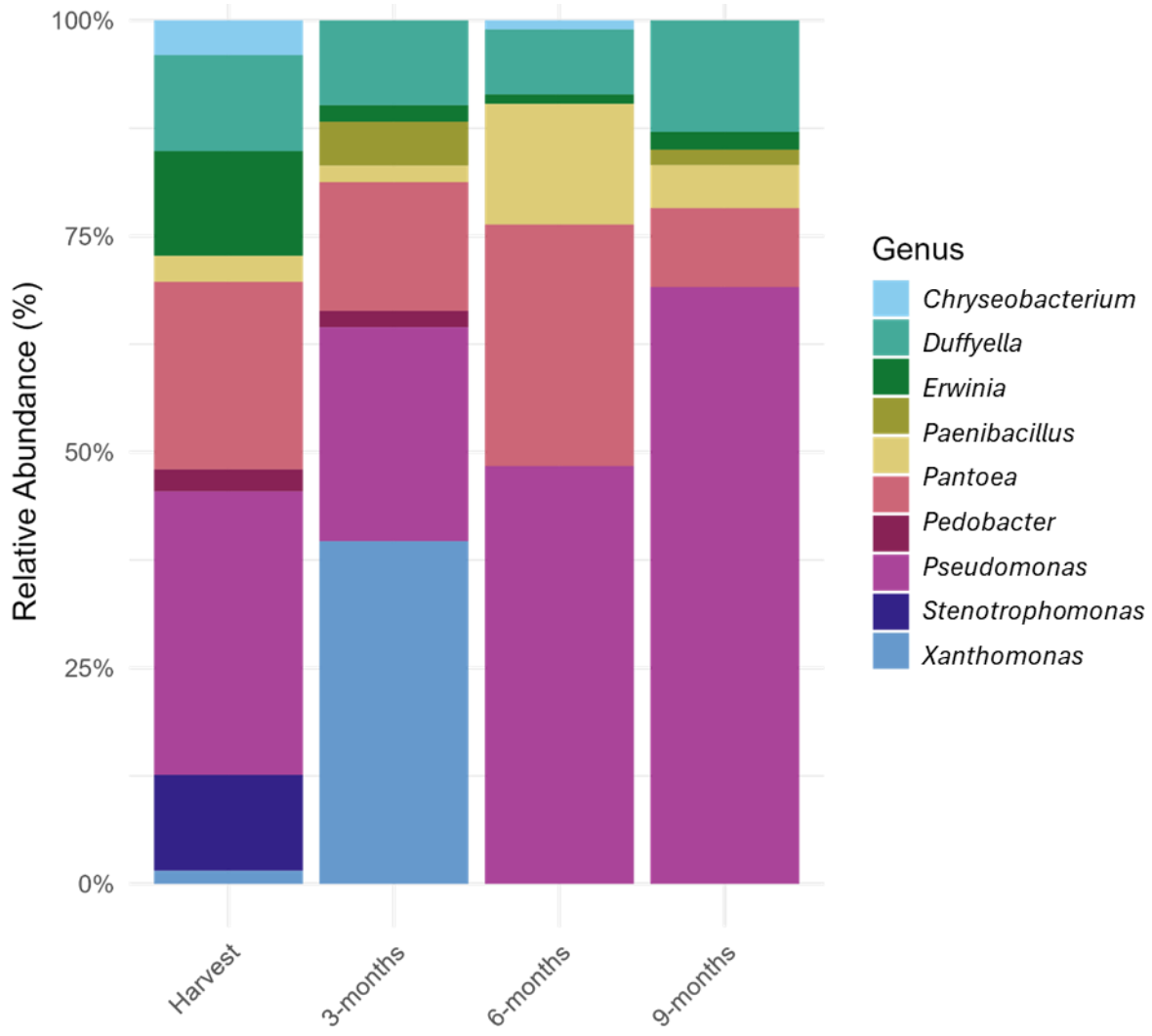


Figure 4.8 Bar plot of relative abundance showing the distribution of proteins of the top ten microbial genera in relation to storage time (harvest, three, six, and nine months).

4.4.5.4 Distribution of proteins of the dominant genera based on grain nitrogen content

Variations in the taxonomic prevalence of peptides were observed between the high and low nitrogen content barley seeds (Fig. 4.9). The genus *Pseudomonas* exhibited the highest protein counts in both high (51%) and low (39.6%) nitrogen content samples, consistently dominating the protein profile (Fig. 4.9). In the high nitrogen samples, *Pantoea* was the second most abundant (15%) in terms of protein abundance, while in the low nitrogen samples, *Xanthomonas* proteins were more prevalent (22.5%) (Fig. 4.9). *Paenibacillus* showed a marked preference for high nitrogen conditions, making up 7% of proteins compared to only 2.1% in low nitrogen samples. *Stenotrophomonas* was found exclusively in high nitrogen samples (5%) and *Massilia* was only detected in low nitrogen samples (4.2%) (Fig. 4.9).

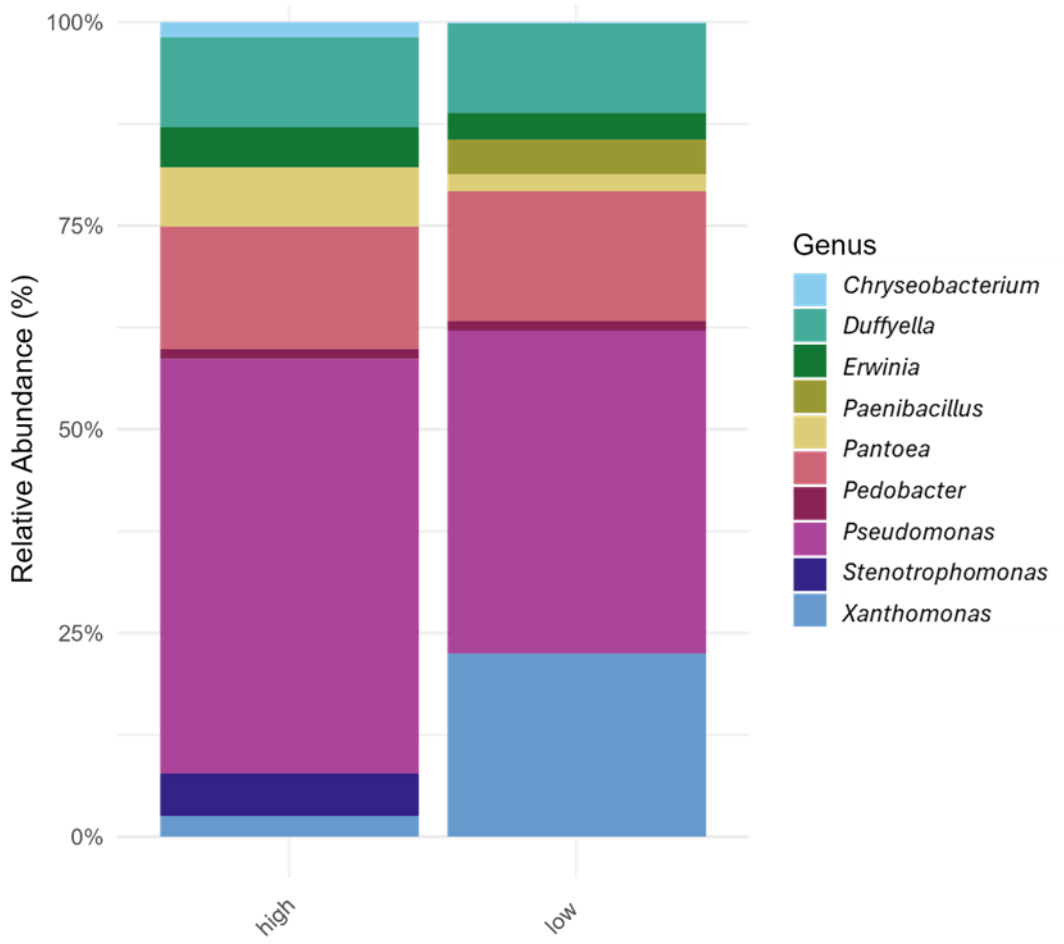


Figure 4.9 Bar plot of relative abundance showing the distribution of proteins of the top ten microbial genera in relation to grain nitrogen content (high vs low).

The metaproteomic data from barley seeds suggests that the nitrogen content may influence the activity of certain microbial genera. Genera such as *Paenibacillus*, *Pantoea* and *Pseudomonas* thrive regardless of nitrogen levels. *Stenotrophomonas*, however, exhibits a high protein count in high nitrogen conditions, suggesting that it may be more active when nitrogen is abundant, even though it appears in both high and low nitrogen metagenome samples. This indicates that its activity is likely enhanced in nitrogen rich environments (Reineke and Schlömann, 2023).

4.4.5.5 Functional analysis of metaproteomic data

A total of 3,268 proteins were assigned to InterPro functions across 371 families (Fig. 4.10). The most prevalent bacterial protein families identified included outer membrane proteins (OMPs, 29%), catalases (18%), porins and TonB-dependent receptors (each at 13%), pyridine

nucleotide-disulfide oxidoreductases (7%), and solute-binding protein family (6%). Smaller proportions were observed for proteins involved in flagellar motility (flagellin), FAD/NAD(P)-binding domains (4%), OmpA-like domains (3%), and glucose metabolic processes (3%) (Fig. 4.10).

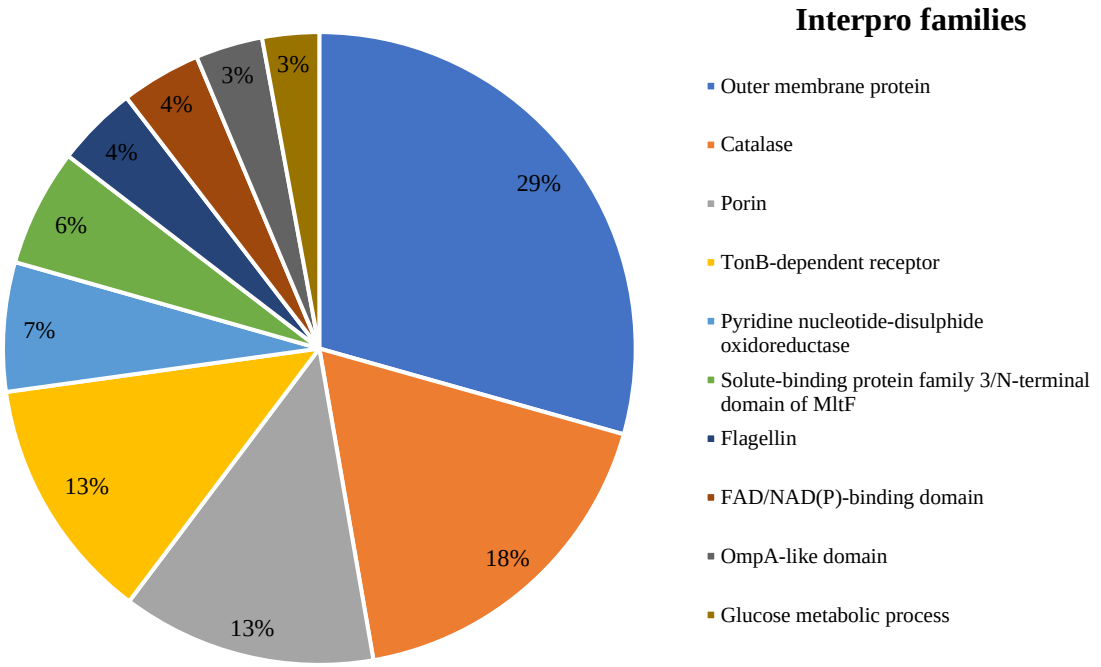


Figure 4.10 Abundance of the top ten bacterial proteins identified based on InterPro (Jones *et al.*, 2014).

The abundance of membrane-associated proteins underscores a primary focus on maintaining structural integrity. OMPs regulate the permeability of microbial cell envelopes, allowing selective transport of nutrients and metabolites and protecting against toxins, thereby shaping cellular structure and morphology (Nikaido, 2003). In Gram-negative bacteria, OMPs are particularly versatile, engaging in a range of functions, including signalling, host cell adhesion, catalysing key reactions, and facilitating both active and passive solute transport (Shearer *et al.*, 2019). Additionally, porins play a role in passively transporting hydrophilic molecules across membranes (Achouak *et al.*, 2001). TonB-dependent receptors, typically located in the outer membrane of Gram-negative bacteria, play a role in the uptake and transportation of substrates, including iron siderophore complexes, vitamin B12, nickel complexes and carbohydrates (Noinaj *et al.*, 2010).

Catalases are the second most prevalent function within the microbial community (Fig 4.10). They play a pivotal role in defending against oxidative stress by catalysing the decomposition of hydrogen peroxide into water and oxygen (Aebi, 1984). This crucial reaction protects microbial cells from the damaging effects of reactive oxygen species (ROS), which can originate from environmental stresses or metabolic activities (Glorieux and Calderon, 2017). By mitigating oxidative damage, catalases sustain the resilience and health of the microbial community (Aebi, 1984). They also support vital processes such as cell development, differentiation, and metabolite production (Glorieux and Calderon, 2017).

4.5 Conclusions

This study delved into the functional capabilities of the barley seed microbiome using metagenomic and metaproteomic approaches. Functional annotation of the proteins encoded on the metagenomes identified that the microbiome encompasses a broad array of key functions, including metabolic functions (e.g., carbohydrate and amino acid metabolism) and nutrient cycling pathways that could ensure their succession in the barley seed environment throughout long-term storage. This analysis also highlighted how variations in storage duration and nitrogen content influence the microbial dynamics within the barley seed microbiome. Variations in the microbiome taxonomy and protein profiles over different storage durations and grain nitrogen content can reveal how specific microbial communities and their metabolic activities are optimised for barley seed preservation and preparation for brewing.

These findings were complemented by metaproteomic investigations, which provided insights into protein expression by the barley seed microbiome. The presence of proteins linked to key microbial genera, as discussed in Chapters 2 and 3, affirmed their active roles within the microbiome. The proteins identified were predominantly involved in functions related to outer membrane proteins and catalases, crucial for bacterial survival and adaptation under various conditions.

While this integrated meta-omics approach provides insights into the microbial ecology of stored barley seeds, there are inherent limitations to these techniques, particularly in the context of complex microbial communities. For instance, functional annotation often relies on reference databases that may not comprehensively cover the microbial diversity present, potentially leading to incomplete or biased functional profiles. Additionally, metagenomic

sequencing can fail to capture the full scope of low-abundance microbial species, which may still play critical roles in the ecosystem. Furthermore, while metaproteomics can reveal expressed proteins, it does not capture transient interactions or dynamic functional changes within the community over time. These limitations must be considered when interpreting the results, particularly regarding the broader ecological interactions and their real-time impact on barley seed health and preservation.

Overall, the integration of metagenomic and metaproteomic analyses enhances our understanding of the microbial dynamics within the barley seed microbiomes, effectively bridging the gap between genomic potential and actual protein expression under real-time conditions. However, future studies incorporating more refined approaches, such as single-cell sequencing or real-time functional assays, may help overcome these limitations and provide deeper insights into the functional interactions within complex microbial communities.

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Chapter 5 Summary

The barley seed microbiome plays a critical role in determining the storage life and quality of seeds, however, the intricate interactions within this microbial community and their precise influence on seed health and longevity remain unknown. This research explored the microbiome of South African barley seeds, with either high or low nitrogen content, at harvest and after three, six and nine months of storage in silos. Metagenome sequencing and taxonomic classification of the resultant reads revealed a predominance of bacterial reads with minimal fungal presence. Lytic dsDNA phages were also detected within the sequence reads. An in-depth analysis of read distribution across different storage periods (harvest, three, six and nine months) and grain nitrogen contents (high and low) showed that each phase had a distinct microbial composition, with varying relative abundances. Diversity analysis indicated that the duration of storage, rather than the nitrogen content of the grain, impacted the microbial diversity associated with the barley seeds. These findings directly address the research question concerning the role of nitrogen in microbial dynamics by demonstrating that while nitrogen content did not significantly impact overall microbial diversity, it did influence specific community compositions. This advances the understanding of how nitrogen modulates the microbial ecosystem within barley seeds, particularly in relation to community functionality over time. Core microbial genera identified in this study align with previous research looking at the barley microbiome. Binning of the metagenomic reads facilitated the reconstruction of 82 high-quality metagenome-assembled genomes (MAGs) representative of the core microbial taxa. Comparative genomic analysis showed that these MAGs contained few shared single-copy orthologues, with an extensive accessory genome observed for each distinct MAG, suggesting they function as a consortium playing complementary roles within the barley seed environment. Additionally, certain MAGs harboured pathogenicity determinants that could be detrimental to the health of the barley seeds, and ultimately malt quality.

Metagenome assembly, followed by structural and functional annotation identified a broad array of metabolic pathways and carbohydrate active enzymes highlighting the key role of the barley seed microbiome in nutrient cycling and by extension the health, germination and growth of the barley seed. Metaproteomic analysis identified a broad array of expressed microbial proteins associated with the barley seed microbiome, with a large proportion of these

associated with membrane protection and oxidative stress, potentially triggered by changes in environmental conditions during prolonged storage.

The application of metagenomic and metaproteomic analyses revealed that the South African barley microbiome comprises a diverse array of microorganisms, which is anything but static. Instead, the microbiome shifts in response to prolonged storage and the different nitrogen content of the barley grains, both in terms of microbial community and their projected activities. This may be used to inform both on the storage practices and usage of barley grains with specific nitrogen contents to ensure barley grains of the highest nutritional and microbial quality for downstream applications, such as beer brewing. These findings not only contribute to our understanding of microbial dynamics in barley storage but also have broader implications for agricultural practices, particularly in managing nitrogen levels during barley storage. By optimising nitrogen content, it may be possible to promote beneficial microbial communities that enhance seed quality and preservation, further supporting sustainable storage practices in the brewing and grain industries.

One notable observation from the study was the distinct behaviour of the S6L sample, which consistently presented different quality and assembly metrics compared to other samples. S6L displayed signs of dysbiosis and had the highest number of contigs but the lowest N50 value, indicating a fragmented assembly. These unique characteristics suggest that S6L may represent an outlier, which poses challenges for interpreting the microbial community dynamics during storage. Replication could have confirmed the status of S6L as an outlier, and the inclusion of replicates in future studies could help ensure robust conclusions.

While the metagenomic and metaproteomic analyses shed light on who is associated with the barley seeds and what they are doing, further insights into how the bacterial community is interacting with the barley seed would be of benefit. Future research should build upon these findings by exploring microbial interactions and functional dynamics in greater detail. Expanding the scope to include metametabolomics and metatranscriptomics would provide more comprehensive insights into the metabolic exchanges and gene expression patterns within the barley seed microbiome during storage. In addition, studies that employ greater biological replication, including the use of multiple cultivars and environmental conditions, would help validate the current findings and account for variability such as the dysbiosis observed in the S6L sample. It would also be valuable to investigate how nitrogen levels influence microbial interactions, particularly focusing on nitrogen cycling, functional activity, and its broader

impact on barley seed preservation. Exploring the combined effects of nitrogen and environmental factors such as temperature and moisture would also provide a more comprehensive understanding of microbial dynamics in storage. Finally, studies that integrate machine learning or artificial intelligence (AI) driven models to predict microbial behaviour and optimise storage conditions would be highly beneficial for the brewing industry.

Appendix

Chapter 2 Appendix

Table S2.1. Overview of sample collection, sequencing metrics, and genomic data for barley seed microbiome study.

Sample ID	Nitrogen grain content	Depot location	Storage-time	Reads	Contamination Ratio (%)	accession	Average quality of reads (PHRED score)
HH	High (>1.5%)	Klipdale	Harvest	50840506	0.0128	SRR27317622	36
HL	Low (< 1.5%)	Caledon	Harvest	44861190	0.0827	SRR27317623	36
S3H	High (>1.5%)	Caledon	3-months	76518028	0.0751	SRR27317620	36
S3L	Low (< 1.5%)	Napier	3-months	56163948	0.8122	SRR27317621	36
S6H	High (>1.5%)	Protem	6-months	37911140	0.0069	SRR27317618	36
S6L	Low (< 1.5%)	Krige	6-months	52681492	0.0059	SRR27317619	36
S9H	High (>1.5%)	Krige	9-months	25566560	0.2103	SRR27317616	36
S9L	Low (< 1.5%)	Protem	9-months	20722664	2.7368	SRR27317617	36

Table S2.2. Summary of alpha diversity statistics for barley seed metagenomic samples.

Sample ID	Nitrogen	Storage	Shannon Diversity
HHN	High-N	Harvest	1.62
HLN	Low-N	Harvest	1.68
T1HN	High-N	3-months	1.52
T1LN	Low-N	3-months	1.42
T2HN	High-N	6-months	1.22
T2LN	Low-N	6-months	2.42
T3HN	High-N	9-months	1.83
T3LN	Low-N	9-months	1.51

Kruskal-Wallis test for Shannon diversity by storage

Kruskal-Wallis chi-squared = 1.1667, df = 3, p-value = 0.761

Kruskal-Wallis test for Shannon diversity by nitrogen

Table S2.3. Summary of beta diversity statistics for barley seed metagenomic samples.

Sample ID	HLN	T1LN	T2LN	T3LN	HHN	T1HN	T2HN	T3HN
HLN	0.0	0.46	0.49	0.42	0.18	0.57	0.57	0.45
T1LN	0.46	0.0	0.84	0.42	0.5	0.33	0.62	0.54
T2LN	0.49	0.84	0.0	0.8	0.51	0.88	0.81	0.72
T3LN	0.42	0.42	0.8	0.0	0.59	0.55	0.45	0.41
HHN	0.18	0.5	0.51	0.59	0.0	0.6	0.63	0.45
T1HN	0.57	0.33	0.88	0.55	0.6	0.0	0.7	0.64
T2HN	0.57	0.62	0.81	0.45	0.63	0.7	0.0	0.44
T3HN	0.45	0.54	0.72	0.41	0.45	0.64	0.44	0.0

ANOSIM Test for Bray-Curtis Dissimilarity by Nitrogen

ANOSIM statistic R: -0.07292, Significance: 0.64, Number of permutations: 999

ANOSIM Test for Bray-Curtis Dissimilarity by Storage

ANOSIM statistic R: 0.5417, Significance: 0.034, Number of permutations: 999

Table S2.4 Kingdom and phylum distribution of metagenomic reads.

Kingdom	**Phylum**	**HL**	**HH**	**S3L**	**S3H**	**S6L**	**S6H**	**S9L**	**S9H**
Bacteria	Pseudomonadota	12986078	13968330	14631301	21177324	13574863	11033650	6610882	7392268
Bacteria	Bacteroidota	1311673	1094279	582331	289454	918729	421	85698	41518
Bacteria	Actinomycetota	124141	502281	171710	1549330	14118	1294	256967	51588
Bacteria	Bacillota	9679	15098	50230	93903	1629501	142251	90762	466506
Phages	Uroviricota (Virus)	1338	1957	29562	153258	4024	14792	2195	4400
Fungi	Ascomycota (Fungi)	7352	8024	3535	7047	15066	15	6856	18373
Fungi	Basidiomycota (Fungi)	423	0	0	0	5949	0	5118	780
Bacteria	Spirochaetes	48	51	60	211	0	0	0	0
Bacteria	Cyanobacteria	19	0	57	46	24	0	20	0
Bacteria	Planctomycetes	0	13	20	18	21	10	0	0
Archea	Euryarchaeota (Archea)	0	10	0	11	0	0	20	17
Bacteria	Verrucomicrobia	0	0	13	0	0	0	0	0
Bacteria	Fusobacteria	0	0	0	12	0	0	0	0
Bacteria	Acidobacteria	0	0	0	0	12	0	0	0
Bacteria	Deinococcus-Thermus	0	0	10	0	0	0	0	0
Bacteria	Tenericutes	0	0	10	0	0	0	0	0

Table S2.5 Core and non-bacterial genera of barley seed microbiome.

Category	**Genus**	**HL**	**HH**	**S9H**	**S6L**	**S9L**	**S6H**	**S3H**	**S3L**
Core	*Achromobacter*	136	395	41	96159	27	12	304	222
Core	*Aeromonas*	24	33	46	647	96	32	96	69
Core	*Azotobacter*	14	19	32	21	10	11	94	43
Core	*Bacillus*	684	176	190235	174	989	11989	4157	167
Core	*Brenneria*	15	17	19	274	50	85	23	22
Core	*Burkholderia*	223	309	93	313	92	43	970	774
Core	*Cedecea*	57	293	151	1287	151	597	114	124
Core	*Chryseobacterium*	1120110	853586	32663	660035	56823	165	99934	323654
Core	*Citrobacter*	5634	1566	2769	1108262	3077	196765	2655	3668
Core	*Clavibacter*	20047	20001	6828	1280	88156	519	82865	13289
Core	*Clostridium*	11	11	468	878	75	11	35	84
Core	*Cronobacter*	129	63	115	3903	56	526	317	184
Core	*Curtobacterium*	40975	14270	15426	1545	108754	491	281302	30530
Core	*Dickeya*	384	89	195	865	220	265	202	232
Core	*Edwardsiella*	467	131	148	187	167	120	245	445
Core	*Enterobacter*	2671	1745	30145	601963	3330	175755	2874	2223
Core	*Erwinia*	1280489	1553131	1978325	109655	1352391	6303737	1073965	971993
Core	*Escherichia*	438	244	2215	14472	302	3595	499	3743
Core	*Flavobacterium*	6386	2001	89	742	192	61	2435	3019
Core	*Frigoribacterium*	1122	8451	948	66	14992	30	4104	4038
Core	*Hafnia*	107	59	110	197	18	523	53	73
Core	*Klebsiella*	1365	1802	596008	374996	2591	5548	2486	1625
Core	*Kluyvera*	16	13	38	1553	20	179	38	31
Core	*Kosakonia*	262	157	514	97892	316	1276	633	2037
Core	*Leclercia*	174	128	4205	2016197	543	126885	325	1618
Core	*Lelliottia*	259	102	61567	31765	3256	713	829	231

Core	*Lonsdalea*	32	10	64	62	10	25	157	26
Core	*Massilia*	138916	98933	3136	259	43941	615	278352	393871
Core	*Microbacterium*	5393	31750	333	2101	1339	13	505238	9228
Core	*Mixta*	368	105	316	852	238	1989	363	300
Core	*Morganella*	44	13	35	95	23	55	66	42
Core	*Nissabacter*	425	32	62	59	20	132	137	50
Core	*Paenibacillus*	5216	8541	27451	5178	4960	14915	45730	42449
Core	*Pantoea*	2635287	1095015	969409	271573	3199546	1800410	5218757	7091193
Core	*Paraburkholderia*	762	701	26	46	47	11	9169	1730
Core	*Pectobacterium*	1100	412	1680	1438	2046	2754	627	941
Core	*Pluralibacter*	87	380	101	929	40	68	107	24
Core	*Pseudomonas*	3032583	4309770	2885783	1417522	1633126	2047298	11685145	4639277
Core	*Rahnella*	826	341	1408	16445	1885	665	1095	660
Core	*Ralstonia*	242	310	16	279	13	46	1901	462
Core	*Raoultella*	415	215	1921	208824	1159	392	275	227
Core	*Salmonella*	141	364	1182	29787	263	2979	118	118
Core	*Serratia*	3241	2485	2545	9528	4383	5557	2483	1258
Core	*Sphingomonas*	30257	62832	2441	396	31051	608	128709	84458
Core	*Stenotrophomonas*	4841231	6102215	514716	4429884	92911	69	163857	71292
Core	*Tatumella*	12	12	38	89	88	42	28	19
Core	*Vibrio*	11	12	32	348	91	355	115	137
Core	*Xanthomonas*	99170	82002	68276	29748	33589	588	1435416	510985
Core	*Yersinia*	866	1305	1496	2314	645	1138	1015	266
Non-Core	*Acetobacter*	0	0	0	0	16	0	0	0
Non-Core	*Acidithiobacillus*	0	0	0	14	0	0	0	0
Non-Core	*Acidovorax*	124	376	0	12892	14	0	155	148

Non-Core	*Acinetobacter*	0	10	169	138942	556	12	466	264
Non-Core	*Actinomyces*	0	19	0	0	0	0	0	0
Non-Core	*Actinoplanes*	0	21	0	0	0	0	21	0
Non-Core	*Actinotalea*	0	20	0	0	0	0	0	0
Non-Core	*Advenella*	0	0	0	52	0	0	0	0
Non-Core	*Aequorivita*	0	10	0	0	0	0	12	0
Non-Core	*Aeromicrobium*	0	146	10	0	0	0	0	0
Non-Core	*Afipia*	0	0	25	0	55	0	12	0
Non-Core	*Agreia*	945	2121	50	41	76	0	1964	933
Non-Core	*Agrobacterium*	15502	10144	302	577	33	0	1923	1042
Non-Core	*Agrococcus*	0	47	0	0	22	0	61	0
Non-Core	*Agromyces*	22	314	25	12	31	0	363	134
Non-Core	*Alcaligenes*	10	11	37	724	0	0	13	0
Non-Core	*Alcanivorax*	0	0	0	18	0	0	25	0
Non-Core	*Alicycliphilus*	0	0	0	31	0	0	14	10
Non-Core	*Alistipes*	0	0	0	11	0	0	0	0
Non-Core	*Allosphingosinicella*	0	0	0	0	0	0	17	0

Non-Core	*Altererythrobacter*	0	0	0	0	0	0	15	0
Non-Core	*Aminobacter*	0	0	0	0	0	0	44	0
Non-Core	*Amniculibacterium*	36	49	0	0	0	0	36	31
Non-Core	*Amycolatopsis*	0	0	0	0	0	0	21	0
Non-Core	*Amylolactobacillus*	0	0	0	19	0	0	0	0
Non-Core	*Anoxybacillus*	0	0	0	0	0	0	15	0
Non-Core	*Apibacter*	13	0	0	0	0	0	0	0
Non-Core	*Aquitalea*	0	0	0	14	0	0	22	13
Non-Core	*Arachidicoccus*	0	0	0	29	0	0	0	17
Non-Core	*Arenimonas*	10	0	0	18	0	0	0	0
Non-Core	*Aromatoleum*	0	0	0	0	0	0	17	15
Non-Core	*Arsenophonus*	11	59	0	0	0	0	0	14
Non-Core	*Arthrobacter*	118	196	0	0	14	0	3401	119
Non-Core	*Asticcacaulis*	0	0	0	0	0	0	10	0
Non-Core	*Atlantibacter*	15	0	46	8796	15	40	12	23
Non-Core	*Aureimonas*	0	44	0	0	0	0	37	29
Non-Core	*Azoarcus*	17	13	29	14	0	0	37	64

Non-Core	*Azospira*	0	0	0	0	0	0	0	11
Non-Core	*Azospirillum*	13	18	0	16	0	0	31	16
Non-Core	*Bacteroides*	11	13	0	18	0	0	14	31
Non-Core	*Bergeyella*	0	0	0	0	0	0	0	12
Non-Core	*Bifidobacterium*	0	0	0	0	0	0	17	10
Non-Core	*Blastochloris*	0	0	0	0	0	0	12	0
Non-Core	*Blastomonas*	0	0	0	0	0	0	16	0
Non-Core	*Blattabacterium*	0	0	0	0	0	0	12	11
Non-Core	*Blautia*	0	0	0	23	0	0	0	0
Non-Core	*Bordetella*	72	148	0	474	13	0	110	148
Non-Core	*Bosea*	56	49	265	0	716	0	288	46
Non-Core	*Brachybacterium*	0	36	0	45	0	0	25	0
Non-Core	*Bradyrhizobium*	1454	1216	36	61	65	0	6207	1141
Non-Core	*Brevibacillus*	0	0	0	11	0	0	11	0
Non-Core	*Brevibacterium*	0	23	0	251	0	0	44	14
Non-Core	*Brevundimonas*	7922	12789	561	32172	115	0	1196	4863
Non-Core	*Brochothrix*	0	0	0	43	0	0	0	0

Non-Core	*Brucella*	0	35	0	31302	25	0	20	17
Non-Core	*Buttiauxella*	37	0	27	1446	14	1586	31	32
Non-Core	*Caballeronia*	0	0	0	0	0	0	33	18
Non-Core	*Candidatus_Symbiopectobacterium*	28	18	12	46	0	27	0	0
Non-Core	*Capnocytophaga*	11	16	0	0	0	0	20	12
Non-Core	*Carnobacterium*	0	0	14	105	9471	0	12	0
Non-Core	*Caulobacter*	16	25	10	80	37	0	85	49
Non-Core	*Cellulomonas*	0	532	53	2652	16	0	159	43
Non-Core	*Cellulophaga*	17	0	0	0	0	0	0	20
Non-Core	*Cellulosimicrobium*	0	133	0	0	0	0	42	0
Non-Core	*Cereibacter*	0	11	0	0	0	0	0	0
Non-Core	*Chania*	0	0	0	16	0	12	0	22
Non-Core	*Chitinophaga*	0	0	0	109	0	0	0	13
Non-Core	*Chromobacterium*	20	17	0	20	0	0	61	48
Non-Core	*Cloacibacterium*	125	309	0	52	0	0	154	352
Non-Core	*Cnuibacter*	0	10	0	0	0	0	13	13
Non-Core	*Cobetia*	0	0	0	0	0	0	12	0

Non-Core	*Cohnella*	0	0	0	44	23	0	22	0
Non-Core	*Collimonas*	108	109	0	0	17	0	463	279
Non-Core	*Comamonas*	25	36	0	51686	14	0	79	118
Non-Core	*Corynebacterium*	0	48	0	39	0	0	37	23
Non-Core	*Croceicoccus*	0	0	0	0	0	0	15	14
Non-Core	*Cruoricaptor*	0	0	0	0	0	0	10	10
Non-Core	*Cryobacterium*	21	172	13	0	13	0	116	61
Non-Core	*Cupriavidus*	292	447	27	304	83	0	2978	1395
Non-Core	*Curvibacter*	0	0	0	14	0	0	0	0
Non-Core	*Cutibacterium*	43	20	23	0	0	0	25	65
Non-Core	*Dechloromonas*	0	0	0	11	0	0	0	14
Non-Core	*Delftia*	48	38	19	32131	0	0	86	86
Non-Core	*Denitratisoma*	0	0	0	37	0	0	0	0
Non-Core	*Dermacoccus*	0	0	0	0	0	0	12	0
Non-Core	*Desulfohalobium*	0	0	0	0	0	15	67	0
Non-Core	*Devosia*	0	20	0	0	0	0	17	0
Non-Core	*Diaminobutyricimonas*	0	16	0	0	0	0	0	0

Non-Core	*Diaphorobacter*	15	20	0	311	0	0	49	47
Non-Core	*Dokdonella*	33	0	0	0	0	0	0	0
Non-Core	*Duganella*	2235	2752	39	20	363	0	11722	2938
Non-Core	*Dyella*	18	0	0	76	0	0	27	14
Non-Core	*Dysgonomonas*	0	0	0	205	0	0	0	0
Non-Core	*Echinicola*	0	0	0	0	0	0	10	0
Non-Core	*Elizabethkingia*	191	348	53	204	29	0	301	277
Non-Core	*Empedobacter*	0	28	17	26113	19511	0	32	16
Non-Core	*Ensifer*	32	88	0	15	0	0	47	37
Non-Core	*Enterococcus*	0	0	115967	751067	66701	579	53	13
Non-Core	*Epilithonimonas*	5918	9252	244	1117	541	0	7572	6461
Non-Core	*Erythrobacter*	0	10	0	0	0	0	27	13
Non-Core	*Ewingella*	57	22	19	30	0	16	15	129
Non-Core	*Faecalibacter*	0	0	0	218	111	0	0	0
Non-Core	*Falsirhodobacter*	0	0	0	0	0	0	0	12
Non-Core	*Ferribacterium*	0	0	0	0	0	0	10	0
Non-Core	*Fictibacillus*	0	0	0	0	0	12	0	0

Non-Core	*Flammeovirga*	0	0	0	0	0	0	0	18
Non-Core	*Frateuria*	15	0	0	44	0	0	15	0
Non-Core	*Frondihabitans*	10	118	10	0	127	0	98	35
Non-Core	*Fusobacterium*	0	0	0	0	0	0	11	0
Non-Core	*Georgenia*	0	27	0	0	0	0	0	0
Non-Core	*Gibbsiella*	0	0	0	97	0	15	95	14
Non-Core	*Glaciihabitans*	0	16	0	0	0	0	19	0
Non-Core	*Glaciimonas*	0	0	0	0	0	0	12	11
Non-Core	*Glutamicibacter*	0	0	0	22	0	0	0	0
Non-Core	*Glycocaulis*	0	0	0	0	0	0	10	0
Non-Core	*Gordonia*	0	20	0	0	0	0	26	0
Non-Core	*Gryllotalpicola*	0	11	0	0	0	0	19	0
Non-Core	*Haematobacter*	0	0	0	12	0	0	0	0
Non-Core	*Halanaerobium*	12	10	0	0	0	0	0	0
Non-Core	*Halomonas*	25	33	392	80	28	0	107	122
Non-Core	*Halopseudomonas*	31	25	31	15	0	13	133	158
Non-Core	*Halotalea*	0	18	0	0	0	0	0	0

Non-Core	*Herbaspirillum*	206	242	0	14	86	0	989	728
Non-Core	*Herbiconiux*	14	101	0	0	0	0	56	21
Non-Core	*Humibacter*	0	30	0	0	0	0	26	0
Non-Core	*Hydrogenophaga*	58	47	0	129	37	0	84	80
Non-Core	*Hymenobacter*	223	302	110	0	435	16	2123	447
Non-Core	*Isoptericola*	0	38	0	0	0	0	10	0
Non-Core	*Janthinobacterium*	2324	4507	55	131	2588	0	9672	3704
Non-Core	*Jejubacter*	0	0	40	196	0	30	0	0
Non-Core	*Jeongeupia*	0	0	0	0	0	0	17	25
Non-Core	*Jeotgalibaca*	0	0	0	146	0	0	0	0
Non-Core	*Kaistella*	908	1697	56	109	80	0	1178	1389
Non-Core	*Ketogulonicigenium*	0	0	0	14	0	0	0	0
Non-Core	*Kineococcus*	0	10	0	0	0	0	0	0
Non-Core	*Kingella*	0	20	0	0	0	0	0	0
Non-Core	*Kinneretia*	13	15	0	0	0	0	32	20
Non-Core	*Kocuria*	0	17	0	0	15	0	25	0
Non-Core	*Kordia*	0	0	0	0	0	0	0	12

Non-Core	*Lacinutrix*	0	0	0	0	0	0	10	0
Non-Core	*Lacrimispora*	0	0	3016	0	0	0	0	0
Non-Core	*Lacticaseibacillus*	0	0	0	84	0	0	0	0
Non-Core	*Lactiplantibacillus*	0	0	0	62	0	0	0	0
Non-Core	*Lactobacillus*	0	0	0	11	0	0	0	0
Non-Core	*Lactococcus*	0	0	0	772822	0	0	12	0
Non-Core	*Laribacter*	0	0	0	70	0	0	0	0
Non-Core	*Latilactobacillus*	0	0	10	1806	0	0	0	0
Non-Core	*Lawsonella*	0	17	0	0	0	0	0	0
Non-Core	*Leeuwenhoekiella*	0	0	0	0	0	0	20	0
Non-Core	*Leifsonia*	86	290	13	0	30	0	389	171
Non-Core	*Lentilactobacillus*	0	0	0	87	0	0	0	0
Non-Core	*Leptospira*	48	47	0	0	0	0	207	46
Non-Core	*Leucobacter*	13	84	24	733	0	0	115	32
Non-Core	*Leuconostoc*	0	0	139	12448	0	0	0	0
Non-Core	*Levilactobacillus*	0	0	0	137	0	0	0	0
Non-Core	*Ligilactobacillus*	0	0	0	65	0	0	0	0

Non-Core	*Limnohabitans*	0	0	0	40	0	0	0	0
Non-Core	*Limosilactobacillus*	0	0	0	95	0	0	0	0
Non-Core	*Listeria*	0	0	11	3197	0	11	10	0
Non-Core	*Loigolactobacillus*	0	0	0	12	0	0	0	0
Non-Core	*Luteibacter*	159	41	0	23	0	0	40	58
Non-Core	*Luteimonas*	540	1226	52	842	210	0	239	570
Non-Core	*Lysinibacillus*	0	0	0	0	0	0	19	0
Non-Core	*Lysobacter*	1400	1365	131	4615	105	0	641	209
Non-Core	*Macrococcus*	0	0	0	0	0	0	17	0
Non-Core	*Maribacter*	0	0	0	0	0	0	12	0
Non-Core	*Mariniflexile*	0	0	0	10	0	0	0	0
Non-Core	*Marinobacter*	0	15	0	23	0	0	40	14
Non-Core	*Marisediminicola*	0	0	0	0	0	0	16	0
Non-Core	*Martelella*	0	34	0	15	0	0	13	0
Non-Core	*Melaminivora*	0	0	0	43	0	0	0	13
Non-Core	*Mesorhizobium*	14160	13100	17	37	18	0	66617	7181
Non-Core	*Metakosakonia*	11	0	0	1259	0	21	0	0

Non-Core	*Methylobacterium*	317	608	29	0	33	0	2722	257
Non-Core	*Methylorubrum*	731	670	712	0	1259	12	4213	441
Non-Core	*Methylosinus*	0	0	0	0	0	0	12	0
Non-Core	*Microbulbifer*	15	0	0	0	0	0	17	0
Non-Core	*Microcella*	0	38	0	0	0	0	30	0
Non-Core	*Micrococcus*	0	18	0	0	0	0	40	0
Non-Core	*Microlunatus*	0	0	0	0	0	0	18	0
Non-Core	*Micromonospora*	0	0	0	0	0	0	13	0
Non-Core	*Micropruina*	0	0	0	0	0	0	0	11
Non-Core	*Microterricola*	0	64	0	0	0	0	47	23
Non-Core	*Mongoliitalea*	0	0	0	0	0	0	17	0
Non-Core	*Moraxella*	0	0	0	35	0	0	0	0
Non-Core	*Mucilaginibacter*	96	129	0	20	0	0	200	452
Non-Core	*Musicola*	0	0	0	15	0	0	0	16
Non-Core	*Mycetocola*	10	137	12	0	0	0	89	18
Non-Core	*Mycobacterium*	0	17	0	0	0	0	22	11
Non-Core	*Mycobacteroides*	0	0	16	0	84	0	57	0

Non-Core	*Mycolicibacterium*	27	61	0	10	0	0	157	43
Non-Core	*Myroides*	0	0	0	9893	79	0	15	13
Non-Core	*Neisseria*	0	0	0	0	0	0	11	0
Non-Core	*Neorhizobium*	288	2138	24	344	10	0	522	544
Non-Core	*Niallia*	0	0	0	0	0	0	11	0
Non-Core	*Niastella*	0	0	0	0	0	0	0	10
Non-Core	*Nitratireductor*	0	0	0	0	0	0	0	12
Non-Core	*Nitrobacter*	0	0	0	0	0	0	17	0
Non-Core	*Nitrosomonas*	0	0	0	14	0	0	0	0
Non-Core	*Niveispirillum*	0	0	0	0	0	0	11	0
Non-Core	*Nocardia*	0	23	0	0	0	0	10	0
Non-Core	*Nocardioides*	0	60	0	0	12	0	105	21
Non-Core	*Nocardiopsis*	0	17	0	0	0	0	0	0
Non-Core	*Noviherbaspirillum*	13	12	0	0	11	0	60	62
Non-Core	*Novosphingobium*	40	53	11	643	26	0	167	94
Non-Core	*Obesumbacterium*	0	0	0	17	10	0	26	0
Non-Core	*Ochrobactrum*	0	49	0	121	0	0	0	0

Non-Core	*Oerskovia*	0	53	0	57	0	0	12	0
Non-Core	*Olivibacter*	0	0	0	133	0	0	10	0
Non-Core	*Orrella*	11	0	0	15	0	0	31	19
Non-Core	*Ottowia*	0	0	0	27	0	0	19	10
Non-Core	*Paenarthrobacter*	13	19	0	0	0	0	344	0
Non-Core	*Paludibacterium*	0	0	0	0	0	0	0	11
Non-Core	*Pandoraea*	44	50	0	48	12	0	132	158
Non-Core	*Pannonibacter*	0	14	0	0	0	0	28	0
Non-Core	*Parabacteroides*	0	0	0	0	0	0	32	0
Non-Core	*Paracoccus*	0	28	0	38	12	25	34	219
Non-Core	*Paraliobacillus*	0	0	0	0	0	0	10	0
Non-Core	*Paraoerskovia*	0	25	0	0	0	0	0	0
Non-Core	*Pasteurella*	0	0	0	0	53	0	21	25
Non-Core	*Pediococcus*	0	0	0	24	0	0	0	0
Non-Core	*Pedobacter*	56220	65836	1859	52	908	0	65229	121195
Non-Core	*Peribacillus*	0	0	0	0	0	0	11	0
Non-Core	*Peteryoungia*	0	15	0	0	0	0	0	0

Non-Core	*Photobacterium*	0	0	0	24	0	0	0	0
Non-Core	*Photorhabdus*	0	0	0	10	0	0	16	0
Non-Core	*Phyllobacterium*	0	18	0	0	0	0	0	0
Non-Core	*Phytobacter*	0	21	26	3733	0	260	78	33
Non-Core	*Pigmentiphaga*	1295	5903	39	51	0	0	1445	483
Non-Core	*Pistricoccus*	0	0	0	10	0	0	0	0
Non-Core	*Planococcus*	0	0	0	11	0	0	32	0
Non-Core	*Plantibacter*	16112	116189	3386	1000	1900	0	205109	52319
Non-Core	*Plesiomonas*	0	0	0	29	0	0	0	0
Non-Core	*Polaribacter*	13	27	0	15	0	0	25	40
Non-Core	*Polaromonas*	0	15	0	24	0	0	41	43
Non-Core	*Polymorphobacter*	0	0	0	0	0	0	26	16
Non-Core	*Polynucleobacter*	0	0	0	17	0	0	0	13
Non-Core	*Porphyrobacter*	0	0	0	0	15	0	11	0
Non-Core	*Prevotella*	0	0	0	0	0	0	15	21
Non-Core	*Priestia*	0	0	0	0	0	0	24	0
Non-Core	*Protaetiibacter*	0	46	0	0	10	0	63	21

Non-Core	*Proteus*	0	0	30	1360	12	33	0	473
Non-Core	*Providencia*	0	27	16	75	38	85	24	30
Non-Core	*Pseudarthrobacter*	10917	9440	0	0	0	0	278952	6407
Non-Core	*Pseudescherichia*	0	0	0	3560	0	104	0	0
Non-Core	*Pseudoalteromonas*	0	0	0	16	0	234	10	327
Non-Core	*Pseudochrobactrum*	0	0	0	156	0	0	0	0
Non-Core	*Pseudocitrobacter*	31	0	69	867	35	73	23	10
Non-Core	*Pseudogulbenkiania*	0	0	0	0	0	0	10	0
Non-Core	*Pseudolabrys*	0	0	0	0	0	0	10	0
Non-Core	*Pseudolysobacter*	21	0	0	0	0	0	0	0
Non-Core	*Pseudonocardia*	0	0	0	0	0	0	22	0
Non-Core	*Pseudopedobacter*	10	0	0	116	0	0	10	14
Non-Core	*Pseudorhizobium*	12	103	0	0	0	0	12	16
Non-Core	*Pseudoxanthomonas*	575	157	56	2204	0	0	197	57
Non-Core	*Psychrobacter*	11	5407	5148	0	160	64	18	468
Non-Core	*Pulveribacter*	0	0	0	21	0	0	0	0
Non-Core	*Pusillimonas*	0	0	0	0	0	0	0	10

Non-Core	*Qipengyuania*	0	0	0	0	0	0	14	0
Non-Core	*Ramlibacter*	0	0	0	0	0	0	16	0
Non-Core	*Rathayibacter*	2146	21424	1079	51	2033	0	13398	2946
Non-Core	*Rhizobium*	869	4697	73	703	23	0	1016	1041
Non-Core	*Rhizorhabdus*	17	20	0	35	0	0	125	22
Non-Core	*Rhodanobacter*	43	17	0	183	0	0	17	0
Non-Core	*Rhodococcus*	1002	44552	8657	148	10326	0	690	224
Non-Core	*Rhodoferax*	21	30	0	131	10	0	88	82
Non-Core	*Rhodoplanes*	0	0	0	0	0	0	26	0
Non-Core	*Rhodopseudomonas*	39	50	0	0	0	0	258	53
Non-Core	*Riemerella*	0	19	0	0	0	0	27	13
Non-Core	*Roseomonas*	10	399	0	0	0	0	70	26
Non-Core	*Rouxiella*	12	0	0	79	22	11	34	22
Non-Core	*Ruania*	0	22	0	0	0	0	11	0
Non-Core	*Runella*	0	0	0	0	0	0	0	11
Non-Core	*Saccharibacillus*	0	452	120747	11621	2169	99307	219	1030
Non-Core	*Salegentibacter*	0	0	0	0	0	0	0	15

Non-Core	*Salinibacterium*	0	73	0	0	11	0	62	25
Non-Core	*Sanguibacter*	0	30900	2723	59	443	0	3928	404
Non-Core	*Scandinavium*	0	0	17	379	0	57	10	21
Non-Core	*Serpentinimonas*	0	140	0	63	0	0	0	0
Non-Core	*Shewanella*	0	10	31	33	18	1048	38	315
Non-Core	*Shigella*	0	0	0	33	0	0	39	0
Non-Core	*Shimwellia*	51	0	0	144	41	45	20	24
Non-Core	*Shinella*	19	60	0	43	0	0	70	31
Non-Core	*Simplicispira*	0	0	0	11	0	0	0	11
Non-Core	*Sinomonas*	0	0	0	0	0	0	22	0
Non-Core	*Sinorhizobium*	15	85	0	25	0	0	28	33
Non-Core	*Sodalis*	0	0	14	109	219	31	31	27
Non-Core	*Solimonas*	0	0	0	0	0	0	15	0
Non-Core	*Solitalea*	0	12	0	0	0	0	11	15
Non-Core	*Sphingobacterium*	429	234	0	201486	1533	0	832	348
Non-Core	*Sphingobium*	216	273	38	421	127	0	1245	336
Non-Core	*Sphingopyxis*	51	104	0	14	32	0	404	114

Non-Core	*Sphingorhabdus*	0	0	0	0	0	0	11	0
Non-Core	*Sphingosinicella*	0	0	0	0	0	0	40	23
Non-Core	*Sphingosinithalassobacter*	14	18	0	0	13	0	144	43
Non-Core	*Spirosoma*	0	0	0	0	0	0	12	0
Non-Core	*Spongiibacter*	0	0	0	0	0	0	66	0
Non-Core	*Sporosarcina*	0	0	0	0	0	0	24	0
Non-Core	*Staphylococcus*	13	0	12	240	26	18	491	27
Non-Core	*Streptococcus*	0	0	0	3919	386	15	39	10
Non-Core	*Streptomyces*	77	435	66	60	123	0	630	214
Non-Core	*Subtercola*	15	60	0	0	0	0	54	23
Non-Core	*Sulfuriferula*	0	0	0	11	0	0	28	14
Non-Core	*Sulfuritortus*	0	0	0	0	0	0	11	0
Non-Core	*Swingsia*	0	0	0	0	0	12	0	0
Non-Core	*Tardibacter*	0	0	0	0	0	0	15	0
Non-Core	*Tardiphaga*	0	0	0	0	0	0	11	0
Non-Core	*Tenacibaculum*	0	10	0	0	0	0	0	15
Non-Core	*Tessaracoccus*	0	0	0	0	0	0	25	0

Non-Core	*Tetragenococcus*	0	0	0	35	0	0	0	0
Non-Core	*Thalassospira*	0	0	0	33	0	0	0	0
Non-Core	*Thauera*	0	0	16	31	0	0	23	21
Non-Core	*Thermodesulfobium*	0	0	0	0	0	0	18	0
Non-Core	*Thermomonas*	279	85	18	818	0	0	70	20
Non-Core	*Thiopseudomonas*	0	0	0	13	0	0	0	0
Non-Core	*Tsuneonella*	0	0	0	0	0	0	0	11
Non-Core	*Undibacterium*	22	22	0	0	13	0	72	134
Non-Core	*Usitatibacter*	0	0	0	0	0	0	0	13
Non-Core	*Vagococcus*	0	0	10	138	0	0	0	0
Non-Core	*Variovorax*	1512	1513	18	142	37	0	6576	1695
Non-Core	*Verminephrobacter*	0	0	0	147	0	0	17	16
Non-Core	*Vitreoscilla*	0	0	0	0	0	0	0	10
Non-Core	*Weeksella*	0	0	0	21	0	0	0	0
Non-Core	*Weissella*	0	0	0	6560	0	0	0	0
Non-Core	*Wenzhouxiangella*	0	0	0	0	0	0	0	12
Non-Core	*Winogradskyella*	0	0	0	0	0	0	0	13

Non-Core	*Xenorhabdus*	0	0	16	27	15	135	0	15
Non-Core	*Xylella*	0	0	0	52	0	0	0	0
Non-Core	*Yokenella*	10	0	0	424	0	49	19	0
Non-Core	*Zhihengliuella*	0	0	0	0	0	0	118	0
Non-Core	*Zunongwangia*	0	0	0	0	0	0	17	13
Non-Core	*Zymobacter*	0	0	0	0	0	0	51	0

Table S2.6 Distribution Phages genera.

Genus	**HL**	**HH**	**S3H**	**S3L**	**S6H**	**S6L**	**S9H**	**S9L**
Aarhusvirus	0	0	0	325	0	0	40	0
Agricanvirus	0	0	0	0	0	0	1174	0
Asteriusvirus	0	0	0	0	0	11	10	0
Cbunavirus	0	0	0	90	0	331	0	0
Ceduovirus	0	0	0	0	0	82	0	0
Certrevirus	1188	0	0	40	71	0	11	387
Cornellvirus	0	0	0	0	0	30199	0	0
Dhakavirus	0	0	0	0	0	0	80	0
Dibbivirus	0	0	0	0	0	0	23	0
Eneladusvirus	0	91	0	0	0	1442	598	0
Feofaniavirus	0	0	2073	3154	0	0	2874	800
Flaumdravirus	0	21	0	0	0	0	0	13
Gelderlandvirus	0	0	0	0	0	0	40	0
Ghunavirus	0	0	0	0	0	0	78	213
Henunavirus	0	0	0	0	77	0	244	0
Ithacavirus	0	0	0	0	0	212	0	0
Jedunavirus	0	0	0	0	0	1817	0	0
Jerseyvirus	0	0	0	0	0	176	0	0
Jiaodavirus	0	0	0	0	0	0	1887	0
Johnsonvirus	0	0	0	0	10	0	0	0
Kagunavirus	0	0	0	0	0	2094	0	0
Kanagawavirus	0	0	0	0	0	0	3762	0
Karamvirus	0	0	0	0	0	0	158	0
Kuttervirus	0	0	0	0	0	28	0	0
Littlefixvirus	0	0	0	0	0	0	21	0
Loessnervirus	0	0	0	0	0	587	2871	0

Marfavirus	0	0	0	0	0	0	50	0
Mimasvirus	0	279	0	10	0	4259	1251	0
Moabitevirus	0	0	0	0	0	23	0	0
Moonvirus	0	0	0	0	0	0	987	0
Mosigvirus	0	0	0	0	0	0	4244	0
Mydovirus	0	0	0	0	13	411	424	0
Naesvirus	0	0	30	0	0	140	0	0
Nochtlivirus	0	0	0	0	0	13077	0	0
Otagovirus	30	0	0	38	0	0	0	29
Peatvirus	0	0	0	0	0	2263	0	0
Petsuvirus	15	11	0	0	3891	16	125	80
Phutvirus	0	13	0	0	0	0	0	12
Pifdecavirus	0	106	0	0	0	23	0	48
Plaisancevirus	0	0	0	0	0	23	0	0
Pseudotevenvirus	0	0	0	0	0	0	386	0
Pylasvirus	0	0	0	0	0	19	0	0
Ravinvirus	0	0	0	0	4749	95	0	0
Risingsunvirus	0	0	0	0	29	0	0	0
Riverridervirus	0	0	0	0	0	0	0	21
Sauletekiovirus	0	0	0	0	4960	0	0	0
Seunavirus	0	0	0	0	726	554	1628	0
Shizishanvirus	0	0	0	0	0	0	59	0
Skunavirus	0	0	0	0	0	91068	0	0
Sugarlandvirus	0	0	0	0	0	0	21	0
Tegunavirus	0	0	0	0	0	0	56	0
Tequatrovirus	0	0	0	0	0	0	23	0
Uetakevirus	0	0	0	0	0	23	0	0
Uliginvirus	0	0	0	39	0	92	69	1490
Vedamuthuvirus	0	0	0	0	0	673	0	0

Waedenswilvirus	0	0	0	0	0	16	0	0
Winklervirus	0	0	0	0	0	0	123	0
Yonginvirus	0	0	0	0	31	0	0	0
Zurivirus	0	0	0	0	0	0	0	151

Table S2.7 Distribution of fungal genera.

Genus	**S3L**	**S6L**	**S9L**
Aspergillus	3232	0	912
Botrytis	0	0	3342
Candida	3981	0	1281
Cercospora	0	0	123
Cryptococcus	0	0	7532
Eremothecium	0	7030	0
Fusarium	7872	0	4150
Marasmius	0	0	647
Rhizoctonia	0	0	222
Thermothelomyces	2522	0	2263
Ustilaginoidea	1445	0	425

Chapter 3 Appendix

Table S3.2 Detailed assembly and annotation metrics for barley seed microbiome metagenome-assembled genomes (MAGs).

Sample id	Nitrogen-content	Bin-name	Completeness (%)	Contamination (%)	Total length	GC (%)	N50	Phylum	Genus	Species
T2LN	Low	MAG58-bin2	97.77	0.0	1865236	38.18	22371	Firmicutes	*Lactococcus*	*Lactococcus garvieae_A*
T2LN	Low	MAG61-bin5	100.0	0.757	2260998	34.98	118370	Firmicutes	*Lactococcus*	*Lactococcus lactis*
T2LN	Low	MAG60-bin4	85.81	1.556	2451992	43.03	4356	Pseudomonadota	*Acinetobacter*	*Acinetobacter variabilis*
T3LN	Low	MAG75-bin8	97.07	0.436	2648119	37.53	25727	Firmicutes	*Enterococcus*	*Enterococcus faecalis*
HHN	High	MAG20-bin18	81.89	0.118	2856754	39.91	10588	Bacteroidota	*Epilithonimonas*	
T3HN	High	MAG82-bin8	91.42	0.754	3039522	42.99	5647	Firmicutes	*Enterococcus_D*	*Enterococcus_D casseliflavus*
HLN	Low	MAG4-bin16	91.01	0.490	3133833	39.55	25933	Bacteroidota	*Epilithonimonas*	
T3HN	High	MAG80-bin6	84.23	4.812	3192441	71.33	8733	Actinobacteriota	*Curtobacterium*	*Curtobacterium flaccumfaciens_A*
HLN	Low	MAG8-bin2	87.16	1.607	3239357	39.87	4923	Bacteroidota	*Flavobacterium*	
T2LN	Low	MAG59-bin3	97.35	0.0	3287730	43.14	48287	Firmicutes	*Enterococcus_D*	*Enterococcus_D casseliflavus*
T1HN	High	MAG49-bin2	82.21	3.420	3297731	69.80	4591	Actinobacteriota	*Plantibacter*	*Plantibacter sp015351235*
T1LN	Low	MAG38-bin21	90.62	2.247	3411048	69.06	17946	Actinobacteriota	*Microbacterium*	
HHN	High	MAG18-bin15	88.12	2.777	3417083	70.06	17891	Actinobacteriota	*Plantibacter*	*Plantibacter sp015351235*
HHN	High	MAG23-bin3	82.20	2.859	3455050	34.81	22649	Bacteroidota	*Chryseobacterium*	
T1LN	Low	MAG44-bin9	92.45	0.691	3522157	66.62	25013	Pseudomonadota	*Stenotrophomonas*	
T1HN	High	MAG47-bin12	82.82	0.122	3538543	34.27	7567	Bacteroidota	*Chryseobacterium*	*Chryseobacterium indoltheticum*
T1LN	Low	MAG29-bin10	99.50	0.490	3600078	39.11	54766	Bacteroidota	*Epilithonimonas*	
T3HN	High	MAG81-bin7	89.08	0.613	3601193	56.28	13890	Pseudomonadota	*Pantoea*	*Pantoea agglomerans*
T1LN	Low	MAG35-bin18	75.86	7.108	3617542	69.26	13948	Actinobacteriota	*Plantibacter*	*Plantibacter cousiniae*
T1HN	High	MAG51-bin4	100.0	0.0	3740955	38.87	267888	Bacteroidota	*Epilithonimonas*	
T1HN	High	MAG46-bin11	85.03	1.051	3743315	68.29	6415	Pseudomonadota	*Xanthomonas_A*	*Xanthomonas_A translucens*
T1LN	Low	MAG41-bin4	96.14	0.505	3754105	67.87	37408	Actinobacteriota	*Microbacterium*	
T1LN	Low	MAG30-bin11	89.66	0.983	3764179	56.22	13016	Pseudomonadota	*Pantoea*	*Pantoea agglomerans*

T3LN	Low	MAG69-bin12	79.22	2.030	3798932	39.59	6488	Firmicutes	*Paenibacillus_J*	*Paenibacillus_J nuruki*
T3HN	High	MAG76-bin2	83.82	1.133	3804786	64.81	10259	Pseudomonadota	*Massilia*	*Massilia aurea_B*
T1LN	Low	MAG32-bin14	84.13	1.793	3821329	68.39	8412	Pseudomonadota	*Xanthomonas_A*	*Xanthomonas_A translucens*
T1HN	High	MAG53-bin6	83.73	0.133	3838592	65.23	4645	Pseudomonadota	*Massilia*	*Massilia aurea_B*
HLN	Low	MAG9-bin3	93.29	1.505	3852006	65.94	42222	Pseudomonadota	*Stenotrophomonas*	*Stenotrophomonas sp014621775*
HHN	High	MAG24-bin5	94.15	0.343	3865160	34.12	108584	Bacteroidota	*Chryseobacterium*	*Chryseobacterium indoltheticum*
HLN	Low	MAG7-bin19	95.69	1.613	3885098	66.41	43286	Pseudomonadota	*Stenotrophomonas*	*Stenotrophomonas sp002836635*
HHN	High	MAG19-bin16	82.14	0.286	3924242	64.95	8808	Pseudomonadota	*Massilia*	*Massilia aurea_B*
HHN	High	MAG15-bin10	98.03	0.245	3979189	55.75	51484	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
HLN	Low	MAG2-bin12	89.81	3.073	4008689	55.44	30922	Pseudomonadota	*Pantoea*	*Pantoea sp002419935*
T3HN	High	MAG77-bin3	96.61	1.136	4014262	56.04	37421	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
T2HN	High	MAG66-bin4	97.18	0.245	4021096	55.43	51456	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
HHN	High	MAG16-bin11	99.64	0.330	4054845	65.80	72028	Pseudomonadota	*Stenotrophomonas*	*Stenotrophomonas sp014621775*
T1LN	Low	MAG31-bin13	96.93	0.260	4069491	55.76	35365	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
HHN	High	MAG17-bin12	94.33	1.830	4070944	70.83	20846	Actinobacteriota	*Sanguibacter*	*Sanguibacter inulinus*
HLN	Low	MAG10-bin5	97.17	0.327	4080115	55.66	62804	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
HLN	Low	MAG13-bin9	87.34	3.284	4097961	34.00	25722	Bacteroidota	*Chryseobacterium*	*Chryseobacterium indoltheticum*
T3LN	Low	MAG73-bin4	96.78	0.714	4105162	55.65	57133	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
T3LN	Low	MAG68-bin11	83.25	2.143	4110094	34.99	5938	Firmicutes	*Bacillus_A*	*Bacillus_A bombysepticus*
T1LN	Low	MAG37-bin20	83.84	1.133	4150027	64.71	14133	Pseudomonadota	*Massilia*	*Massilia aurea_B*
HHN	High	MAG25-bin6	89.86	1.180	4180807	60.69	23553	Pseudomonadota	*Neorhizobium*	*Neorhizobium soli*
T1HN	High	MAG56-bin9	97.60	0.309	4181855	55.70	35876	Pseudomonadota	*Duffyella*	*Duffyella gerundensis*
T1HN	High	MAG50-bin3	97.87	1.102	4201248	39.58	49864	Bacteroidota	*Chryseobacterium*	*Chryseobacterium sp001421435*
HLN	Low	MAG1-bin1	76.94	1.916	4216299	64.66	9399	Pseudomonadota	*Massilia*	*Massilia aurea_B*
T1LN	Low	MAG42-bin5	99.12	0.0	4223639	66.34	38378	Actinobacteriota	*Pseudarthrobacter*	*Pseudarthrobacter equi*
T2LN	Low	MAG57-bin1	100.0	0.0	4332316	33.55	117162	Bacteroidota	*Chryseobacterium*	*Chryseobacterium piscium*
T3HN	High	MAG79-bin5	80.17	0.0	4502603	55.98	82812	Pseudomonadota	*Erwinia*	*Erwinia persicina*
T2HN	High	MAG63-bin1	96.57	0.519	4512341	55.87	96926	Pseudomonadota	*Erwinia*	*Erwinia persicina*
T1LN	Low	MAG36-bin2	98.86	0.0	4617697	39.64	80281	Firmicutes	*Paenibacillus_J*	*Paenibacillus_J nuruki*

T3LN	Low	MAG74-bin7	98.68	0.273	4634138	55.65	91004	Pseudomonadota	*Erwinia*	*Erwinia persicina*
HLN	Low	MAG5-bin17	98.08	0.0	4640035	39.66	37393	Firmicutes	*Paenibacillus_J*	*Paenibacillus_J nuruki*
T3LN	Low	MAG72-bin2	77.19	0.0	4681908	63.75	26823	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E sp003253735*
HHN	High	MAG27-bin9	99.31	0.069	4717584	39.64	57215	Firmicutes	*Paenibacillus_J*	*Paenibacillus_J nuruki*
T3HN	High	MAG78-bin4	96.90	1.716	4740412	39.44	19164	Firmicutes	*Paenibacillus_J*	*Paenibacillus_J nuruki*
T1LN	Low	MAG28-bin1	96.90	0.860	4768914	55.97	64761	Pseudomonadota	*Erwinia*	*Erwinia persicina*
T1LN	Low	MAG45-bin10	97.93	1.170	4792467	55.28	59038	Pseudomonadota	*Pantoea*	*Pantoea agglomerans*
HHN	High	MAG22-bin2	97.30	0.338	4805805	55.63	152377	Pseudomonadota	*Erwinia*	*Erwinia persicina*
HLN	Low	MAG6-bin18	80.32	5.172	4821760	34.43	73923	Bacteroidota	*Chryseobacterium*	
T3LN	Low	MAG71-bin15	89.22	5.119	4838580	67.28	19361	Pseudomonadota	*Stenotrophomonas*	*Stenotrophomonas sp003086775*
T1LN	Low	MAG43-bin8	91.67	2.435	4896167	59.56	101115	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E syringae*
T1HN	High	MAG54-bin7	92.26	0.803	4897798	63.55	37031	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E sp003253735*
T1HN	High	MAG48-bin13	98.00	0.275	4919275	39.48	27429	Firmicutes	*Paenibacillus_J*	*Paenibacillus_J nuruki*
HHN	High	MAG26-bin8	97.10	0.159	4942342	39.06	74375	Bacteroidota	*Pedobacter*	
HLN	Low	MAG3-bin15	86.98	0.237	4967183	64.14	11980	Pseudomonadota	*Duganella*	*Duganella phyllosphaerae*
T3LN	Low	MAG67-bin10	94.86	1.739	4988107	59.53	8126	Firmicutes	*Saccharibacillus*	*Saccharibacillus brassicae*
T2HN	High	MAG65-bin3	97.75	0.537	5070665	61.48	29080	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E poae*
HLN	Low	MAG11-bin6	96.17	0.159	5183976	38.97	193357	Bacteroidota	*Pedobacter*	
T1HN	High	MAG55-bin8	97.12	0.159	5194491	38.99	148394	Bacteroidota	*Pedobacter*	
T2HN	High	MAG64-bin2	86.44	2.718	5255736	59.62	25025	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E viridiflava*
T1LN	Low	MAG33-bin15	96.25	1.594	5480430	39.01	122493	Bacteroidota	*Pedobacter*	
HHN	High	MAG14-bin1	93.20	1.201	5485102	64.32	17745	Pseudomonadota	*Duganella*	*Duganella phyllosphaerae*
T3LN	Low	MAG70-bin14	98.58	0.553	5523418	56.06	113978	Pseudomonadota	*Klebsiella*	*Klebsiella grimontii*
T2LN	Low	MAG62-bin6	85.22	6.291	5532076	56.67	3460	Firmicutes	*Saccharibacillus*	
T1LN	Low	MAG40-bin3	95.93	0.026	5566168	64.22	31007	Pseudomonadota	*Duganella*	*Duganella phyllosphaerae*
HLN	Low	MAG12-bin8	99.28	0.796	5586454	54.51	92296	Pseudomonadota	*Erwinia*	*Erwinia persicina*
T1HN	High	MAG52-bin5	97.28	0.847	5629308	60.65	64684	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E rhodesiae*
T1LN	Low	MAG39-bin22	93.96	7.712	5714451	61.45	25000	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E poae*
T1LN	Low	MAG34-bin17	88.49	5.414	5770567	59.19	85504	Pseudomonadota	*Pseudomonas_E*	*Pseudomonas_E viridiflava_C*

HHN	High	MAG21-bin19	75.34	8.620	6904257	61.22	51262	Pseudomonadota	*Pseudomonas_E*	

Chapter 4 Appendix

Table S4.2 Distribution of clusters of orthologous groups (COG) across metagenomics sample.

COGs	**HH**	**HL**	**S3H**	**S3L**	**S6H**	**S6L**	**S9H**	**S9L**
S: Function unknown	28395	26467	18271	24011	14201	23865	19040	12896
K: Transcription	11522	10144	7021	9990	6052	9599	7768	5485
E: Amino acid transport and metabolism	10523	9345	6435	9176	5263	7713	6819	4873
P: Inorganic ion transport and metabolism	9002	7993	5590	7542	5089	7414	6106	4360
G: Carbohydrate transport and metabolism	8660	7610	5297	8016	5162	7486	5752	4305
M: Cell wall/membrane/envelope biogenesis	9000	8562	5744	7458	4180	6970	5313	3938
T: Signal transduction mechanisms	7619	7105	4773	6390	3429	5167	4686	3435
C: Energy production and conversion	6556	6021	4168	5540	3453	5424	4362	3086
L: Replication, recombination, and repair	5856	5694	3944	5419	2851	5132	3742	2651
J: Translation, ribosomal structure and biogenesis	5034	4727	3313	4330	2447	3722	3308	2476
H: Coenzyme transport and metabolism	4751	4584	3122	4171	2810	4038	3299	2354
I: Lipid transport and metabolism	4843	4398	2976	4131	1958	3194	2836	2103
U: Intracellular trafficking, secretion, and vesicular transport	3717	3664	2447	3218	1839	3362	2543	1835
O: Posttranslational modification, protein turnover, chaperones	3755	3644	2498	3209	1689	2864	2318	1685
N: Cell motility	3311	3200	2168	2842	1971	2744	2387	1722
F: Nucleotide transport and metabolism	2962	2868	1993	2679	1750	2546	2228	1582
Q: Secondary metabolites biosynthesis, transport, and catabolism	3246	2853	1913	2814	1436	2231	2000	1386
V: Defense mechanisms	1934	1917	1267	1791	876	1680	1217	822
D: Cell cycle control, cell division, chromosome partitioning	1459	1295	873	1281	757	1163	993	699
B: Chromatin structure and dynamics	54	43	26	40	9	23	22	13
W: Extracellular structures	54	46	27	22	4	37	14	10
Z: Cytoskeleton	42	39	15	20	8	44	25	12
A: RNA processing and modification	18	18	15	14	8	17	12	11

Table S4.3 Distribution of CAZymes and carbon degradation proteins across the metagenomic samples.

CAZy	HH	HL	S3H	S3L	S6H	S6L	S9H	S9L
AA10	8	8	8	4	5	11	12	6
CBM15	0	1	1	0	1	3	0	0
CBM48	106	78	54	84	41	66	53	42
CBM6	2	1	2	1	2	2	1	1
CBM73	8	8	8	4	5	11	10	6
CE1	23	24	16	18	14	24	18	10
CE10	34	22	18	31	15	13	12	13
GH102	18	15	8	12	13	15	12	11
GH103	30	22	19	20	17	18	23	17
GH105	12	17	10	10	14	17	14	7
GH13	230	190	128	181	115	173	137	92
GH130	5	5	1	3	3	4	2	0
GH18	22	19	15	17	22	39	22	10
GH19	26	25	9	20	12	18	15	9
GH20	24	25	20	23	11	14	12	9
GH23	30	26	19	20	29	39	26	16
GH26	9	11	7	9	7	2	6	5
GH29	18	21	21	20	3	12	4	5
GH3	110	95	70	95	42	52	50	39
GH30	15	11	10	8	2	6	3	3
GH31	61	60	38	67	37	68	36	21
GH32	30	21	15	31	21	22	19	22
GH37	14	16	9	13	15	29	14	7
GH38	6	5	2	7	10	16	7	6
GH39	9	8	4	8	3	1	5	4
GH4	1	0	0	0	10	15	9	3
GH43	32	28	17	31	12	31	19	14
GH5	47	41	30	37	11	19	24	16
GH51	34	24	19	35	7	14	7	10
GH52	1	1	1	1	2	0	1	0
GH6	2	2	2	3	1	1	1	0
GH63	0	0	0	0	1	5	2	0
GH65	12	10	9	13	5	19	8	6
GH68	8	10	7	9	6	1	4	6
GH73	0	0	0	0	5	8	2	0
GH77	27	24	17	18	18	16	17	10
GH8	10	5	4	5	8	18	8	5
GH88	2	3	2	2	6	17	8	4
GH9	42	36	27	35	11	19	24	16
GH94	6	6	2	5	3	8	3	5
GH95	14	14	14	13	2	6	0	5
GT1	49	53	39	47	71	130	73	57

GT17	3	2	3	3	6	2	3	3
GT19	25	27	16	20	11	19	14	11
GT2	288	239	176	251	142	190	166	128
GT20	25	22	15	21	13	24	11	11
GT21	1	0	0	0	1	4	1	0
GT25	4	3	3	2	1	9	2	3
GT26	10	13	8	11	9	15	11	8
GT28	33	31	24	32	17	21	27	18
GT30	28	26	14	15	13	21	14	11
GT35	28	22	19	19	27	26	23	17
GT36	3	5	4	6	8	9	6	6
GT39	5	1	2	6	2	2	2	4
GT4	184	161	128	169	116	158	120	94
GT5	30	32	23	18	14	24	18	10
GT51	106	96	58	73	49	96	72	45
GT52	0	0	0	0	1	1	0	0
GT56	4	4	4	3	10	12	6	3
GT73	4	4	1	3	2	5	5	2
GT8	3	5	3	2	10	23	3	2
GT80	0	0	0	0	1	0	0	0
GT81	5	5	2	5	3	1	3	4
GT84	4	4	0	2	1	4	1	2
GT9	44	43	28	35	36	43	33	25
PL11	7	8	8	5	4	1	2	2
PL4	5	6	3	7	4	1	3	2
CBM20	2	5	3	2	0	2	2	0
CBM50	1	2	2	3	0	8	3	2
GH121	2	0	1	0	0	0	0	0
GH15	5	4	3	4	0	1	1	1
GH16	4	3	2	2	0	0	0	0
GH28	1	1	1	1	0	3	2	1
GH33	3	2	1	2	0	2	1	1
GH97	4	9	6	3	0	3	0	1
GT3	3	3	3	1	0	1	1	1
GT70	2	2	2	2	0	0	0	0
PL8	2	1	2	1	0	1	4	2
GH101	1	1	0	1	0	2	1	0
GT66	0	0	0	2	0	0	0	1
GT87	2	0	0	1	0	0	0	1
GT71	2	2	0	1	0	1	0	0
GT14	1	0	0	0	0	0	0	0
GT89	1	0	0	0	0	0	0	0
CBM5	0	0	0	0	0	1	0	0
GH116	0	0	0	0	0	1	0	0
GH47	0	0	0	0	0	1	0	0

GH66	0	0	0	0	0	1	0	0
GH82	0	0	0	0	0	1	0	0
GT11	0	0	0	0	0	1	0	0

Table S4.4 Reconstruction of metabolic pathways from the barley microbiome.

	KEGG_ko	Encoded protein (KEGG annotation)	*Protein*	Protein read coverage per sample							
				HL	HH	S3L	S3H	S6L	S6H	S9H	S9L
Amino acid	K00817	hisC; histidinol-phosphate aminotransferase [EC:2.6.1.9]	*HisC*	663	675	850	471	634	301	513	415
	K00831	serC, PSAT1; phosphoserine aminotransferase [EC:2.6.1.52]	*SerC, PSAT1*	447	413	490	266	303	269	345	240
	K00826	E2.6.1.42, ilvE; branched-chain amino acid aminotransferase [EC:2.6.1.42]	*E2.6.1.42, ilvE*	478	508	453	293	223	231	288	240
	K00821	argD; acetylornithine/N-succinyldiaminopimelate aminotransferase [EC:2.6.1.11 2.6.1.17]	*ArgD*	572	555	485	353	187	104	220	223
	K00832	tyrB; aromatic-amino-acid transaminase [EC:2.6.1.57]	*TyrB*	320	267	294	255	349	288	198	162

	K00812	aspB; aspartate aminotransferase [EC:2.6.1.1]	*AspB*	364	504	344	261	172	20	176	52
	K02619	pabC; 4-amino-4-deoxychorismate lyase [EC:4.1.3.38]	*PabC*	241	327	294	188	210	200	238	174
	K07250	gabT; 4-aminobutyrate aminotransferase / (S)-3-amino-2-methylpropionate transaminase / 5-aminovalerate transaminase [EC:2.6.1.19 2.6.1.22 2.6.1.48]	*GabT*	124	227	237	124	14	153	65	81
	K05825	LYSN; 2-aminoadipate transaminase [EC:2.6.1.-]	*LYSN*	111	143	152	81	240	113	61	88
	K00819	rocD, OAT; ornithine--oxo-acid transaminase [EC:2.6.1.13]	*RocD, OAT*	156	85	112	42	21	0	40	57
	K00823	puuE; 4-aminobutyrate aminotransferase [EC:2.6.1.19]	*PuuE*	89	112	47	66	24	47	71	47
	K03918	lat; L-lysine 6-transaminase [EC:2.6.1.36]	*LaT*	112	122	72	113	23	0	0	19

	K00830	AGXT; alanine-glyoxylate transaminase / serine-glyoxylate transaminase / serine-pyruvate transaminase [EC:2.6.1.44 2.6.1.45 2.6.1.51]	*AgxT*	20	41	0	37	0	20	4	47
Carbon degradation	K05349	bglX; beta-glucosidase [EC:3.2.1.21]	*BglX*	2747	3125,97	2892,954	2114,827	1202,93	1029,927	1035,802	1156,903
	K01190	lacZ; beta-galactosidase [EC:3.2.1.23]	*LacZ*	1110	827,2761	1343,214	997,5625	644,9148	766,4954	429,8287	388,9496
	K01176	AMY, amyA, malS; alpha-amylase [EC:3.2.1.1]	*AmY, AmyA, MalS*	493,3	412,4599	300,8974	330,4118	570,5961	379,8491	196,6601	275,1116
	K01207	nagZ; beta-N-acetylhexosaminidase [EC:3.2.1.52]	*NagZ*	328,1	424,8242	505,0109	285,4906	404,6925	285,4731	215,0501	300,3768
	K01811	xylS, yicI; alpha-D-xyloside xylohydrolase [EC:3.2.1.177]	*XylS, YicI*	239,2	317,5511	544,5119	275,7944	396,9284	339,7735	182,0487	176,9164
	K05989	ramA; alpha-L-rhamnosidase [EC:3.2.1.40]	*RamA*	234,9	371,1447	813,983	276,5019	314,2917	200,7502	13,5231	14,9667
	K01192	E3.2.1.25, MANBA, manB; beta-mannosidase [EC:3.2.1.25]	*E3.2.1.25, MAnbA, ManB*	193,9	430,6751	343,4769	271,345	248	239,9155	139,696	305,9322
	K01214	ISA, treX; isoamylase [EC:3.2.1.68]	*IsA, TreX*	319,4	328,7972	580,2116	180,5562	195,4794	69,7134	101,051	98,8971

	K01209	abfA; alpha-L-arabinofuranosidase [EC:3.2.1.55]	*AbfA*	310	354,0085	337,8002	281,8126	117	94	179,9483	91
	K01183	E3.2.1.14; chitinase [EC:3.2.1.14]	*E3.2.1.14*	134,1	265,5779	112	168,7829	440,8003	226,8115	21	198,9328
	K01779	racD; aspartate racemase [EC:5.1.1.13]	*RacD*	163,1	149,3747	164,9651	109,3593	332,4521	147,1821	65,5148	156,9655
	K01218	gmuG; mannan endo-1,4-beta-mannosidase [EC:3.2.1.78]	*GmuG*	207,6	188,0896	195,932	268,9463	19,9659	170,5474	112	113,7915
	K01198	xynB; xylan 1,4-beta-xylosidase [EC:3.2.1.37]	*XynB*	158	145,6541	161,0879	105,7242	397,4209	27	88,52576	165,8603
	K05343	treS; maltose alpha-D-glucosyltransferase / alpha-amylase [EC:5.4.99.16 3.2.1.1]	*treS*	221,2	251,3303	368,8706	71,5155	97,9485	28	71,7937	70,7452
	K05350	bglB; beta-glucosidase [EC:3.2.1.21]	*BglB*	135,7	242,3799	420,8994	115,9748	115,0754	33,9654	39,90476	25
	K01195	uidA, GUSB; beta-glucuronidase [EC:3.2.1.31]	*uidA, GUSB*	160,8	190,0221	237,7076	120	62,9658	58	58,9484	57,7152
	K01200	pulA; pullulanase [EC:3.2.1.41]	*PulA*	48,8	49	49	48,9831	216,9328	48,9831	33,73832	84,00567
	K01178	SGA1; glucoamylase [EC:3.2.1.3]	*Sga1*	46,63	127,5167	109	88,9831	22,0482		32	21

Fermentation	K01895	ACSS1_2, acs; acetyl-CoA synthetase [EC:6.2.1.1]	ACSS1_2, acs	1 070	1 076	940	779	625	470	407	825
	K00656	E2.3.1.54, pflD; formate C-acetyltransferase [EC:2.3.1.54]	E2.3.1.54, *PflD*	194	193	194	116	958	471	155	461
	K00001	E1.1.1.1, adh; alcohol dehydrogenase [EC:1.1.1.1]	E1.1.1.1, *AdIH*	291	374	381	233	408	300	171	357
	K00925	ackA; acetate kinase [EC:2.7.2.1]	*AckA*	218	207	326	139	212	114	186	165
	K00016	LDH, ldh; L-lactate dehydrogenase [EC:1.1.1.27]	LDH, *LdH*	31	33	33	33	283	82	49	176
	K00625	pta; phosphate acetyltransferase [EC:2.3.1.8]	*PtA*	17	33	33	17	-	-	17	-
Nitrogen metabolism	K00372	nasC, nasA; assimilatory nitrate reductase catalytic subunit [EC:1.7.99.-]	*NasC, NasA*	458	568,7169	494,5649	295,5658	380,9155	369,8825	270,7828	317,7218
	K00367	narB; ferredoxin-nitrate reductase [EC:1.7.7.2]	*NarB*	59	59	116,9206	59	0	0	0	0
	K00366	nirA; ferredoxin-nitrite reductase [EC:1.7.7.1]	*NirA*	0	0	0	0	0	0	0	5
	K00360	nasB; assimilatory nitrate reductase electron transfer subunit [EC:1.7.99.-]	*NasB*	7	61,83193	45,4906	0	0	0	0	0

	K00374	narI, narV; nitrate reductase gamma subunit [EC:1.7.5.1 1.7.99.-]	*NarI, NarV*	65,71	46	63,9823	69,00001	139,9824	153,6227	47,7168	155,965
	K02568	napB; nitrate reductase (cytochrome), electron transfer subunit	*NapB*	4	9	8,98148	9	33,88958	14,57346	0	9
	K02305	norC; nitric oxide reductase subunit C	*NorC*	14,49	0	0	8		0	0	8
	K04561	norB; nitric oxide reductase subunit B [EC:1.7.2.5]	*NorB*	82	0	0	24	39	0	0	24
	K15864	nirS; nitrite reductase (NO-forming) / hydroxylamine reductase [EC:1.7.2.1 1.7.99.1]	*NirS*	28	0	0	28	0	0	16,4164	29
	K19340	nosF; Cu-processing system ATP-binding protein	*NosF*	0	0	0	0	10,6387	0	0	16
	K00376	nosZ; nitrous-oxide reductase [EC:1.7.2.4]	*NosZ*	0	0	0	0	0	0	0	33
	K19341	nosY; Cu-processing system permease protein	*NosY*	0	0	0	0	0	0	0	14
	K00368	nirK; nitrite reductase (NO-forming) [EC:1.7.2.1]	*NirK*	17,98	62,76539	0	0	0	0	0	0

	K00370	narG, narZ, nxrA; nitrate reductase / nitrite oxidoreductase, alpha subunit [EC:1.7.5.1 1.7.99.-]	*NarG, NarZ, NxrA*	255,9	184,05	286,9999	189	242,3205	434,7477	204	520,9665
	K00371	narH, narY, nxrB; nitrate reductase / nitrite oxidoreductase, beta subunit [EC:1.7.5.1 1.7.99.-]	*NarH, NarY, NxrB*	112	78	90,9832	104,8713	299,9829	258,1374	119,9493	220,478
	K02567	napA; nitrate reductase (cytochrome) [EC:1.9.6.1]	*NapA*	115,8	17,84226	83,3293	42	42	67,5264	0	8,99999
	K03385	nrfA; nitrite reductase (cytochrome c-552) [EC:1.7.2.2]	*NrfA*	0	0	0	0	24	13	0	0
	K00362	nirB; nitrite reductase (NADH) large subunit [EC:1.7.1.15]	*NirB*	799,4	807,8825	855,7621	659,3153	604,8901	891,7947	471,5986	662,9792
	K00363	nirD; nitrite reductase (NADH) small subunit [EC:1.7.1.15]	*NirD*	106,5	131,2613	97,65868	73,48071	76,03105	98,88971	57,54516	86,598
	K02586	nifD; nitrogenase molybdenum-iron protein alpha chain [EC:1.18.6.1]	*NifD*	0	0	0	0	22,983	0	0	25
	K02591	nifK; nitrogenase molybdenum-iron protein beta chain [EC:1.18.6.1]	*NifK*	0	0	0	0	31,9827	0	0	27
	K02588	nifH; nitrogenase iron protein NifH	*NifH*	0	0	0	0	0	0	0	15

Table S4.5 Metaproteomic analysis general metrics.

Sample Id	#Peptide	Phylum	Genus	Interpro
HH	507	253	130	399
HL	275	160	79	229
S3H	372	174	100	265
S3L	590	358	220	488
S6H	244	130	81	188
S6L	93	33	13	78
S9H	604	196	124	541