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METAPROTEOMIC ANALYSIS OF BARLEY GERMINATION REGIME UNDER THE INFLUENCE OF INCREASED TEMPERATURE

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Elif Göksu KIZILYAR

Signature

Dedicated to my beloved sister

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I hope this thesis will contribute meaningful insight into the field of barley metaproteomic analysis, facilitating future research.

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Elif Göksu KIZILYAR

TABLE OF CONTENTS

LIST OF SYMBOLS viii
LIST OF ABBREVIATIONS ix
LIST OF FIGURES xi
LIST OF TABLES xiii
ABSTRACT xiv
ÖZET xvi
1 INTRODUCTION 1
1.1 Literature Review 1
1.2 Objective of the Thesis 3
1.3 Original Contribution 4
2 GENERAL INFORMATION 5
2.1 The Cereal Crop “Barley” & Its Use in Malt 5
2.1.1 Components of Barley 7
2.2 The Malting Process 9
2.2.1 Steeping, Germination and Kilning 11
2.3 Accelerated Malting Regime (AMR) 13
2.4 Quality Attributes for Malt 14
2.4.1 Properties of Malting Barley 16
2.5 Malting and Barley Microbiome 18
2.5.1 The Complex Interaction of The Barley Grain and Its Microbiome During Malting 20
2.6 Proteomics 26
2.6.1 Bottom-Up Proteomics 29
2.7 Metaproteomics 29
3 MATERIALS AND METHODS 36
3.1 Sample History 36
3.1.1 Barley Cultivars 37
3.1.2 Pilot Scale Malting Process for Accelerated Malting Regime 37
3.1.3 Sampling Points 38
3.2 Sample Preparation for the Metaproteomic Analysis of Barley Microbiome 39
3.2.1 Microbial Protein Extraction 40

3.2.2 Protein Precipitation 42
3.2.3 Treatment of the Protein Pellet 42
3.2.4 Purification and Resolubilization of the Protein Pellet 42
3.3 Protein Quantification using BCA Assay 44
3.4 Protein Digestion and Purification 45
3.4.1 Protein Digestion using Trypsin 45
3.4.2 C-18 Peptide Clean-up of the samples 46
3.5 Peptide Labelling with TMT Isobaric Mass Tags 48
3.6 Raw Data Processing and Peptide Identification 49
3.7 Microbial Taxonomic Analysis with Unipept 52
3.7.1 Lowest Common Ancestor (LCA) 52
4 RESULTS AND DISCUSSION 54
4.1 Taxonomic Analysis with Identified Peptides via Unipept Search 54
4.2 Quantitative Metaproteome Analysis of AMR 56
4.3 Microbial Metabolism and Its Role in Accelerated Malting Process 63
4.4 Uniquely Identified Microorganisms for AMR 67
4.5 AMR Samples Malt Quality Properties 73
4.6 Malt Quality and Correlation with Microbiome Activity 73
5 CONCLUSION 76
REFERENCES 79
A APPENDIX MATERIAL AND METHODS 88
B APPENDIX RESULTS AND DISCUSSION 90
PUBLICATIONS FROM THE THESIS 93

LIST OF SYMBOLS

α	Alpha
β	Beta
Cu^{+2}	Copper
°	Degree
d.m	Dry matter
E	Exponent (‘10 to the power of’)
g	Gram
μ	Micro
mg	Milligram
mU	Milliunits
%	Percentage

LIST OF ABBREVIATIONS

ACN	Acetonitrile
AmBic	Ammonium bicarbonate
AMR	Accelerated malting regime
APV	Adjusted P-Value
BCA	Bicinchoinic acid
BSA	Bovine serum albumin
CAA	2-chloroacetamide
DP	Diastatic power
EPS	Extracellular polymeric substance / Exopolysaccharide
ESI	Electrospray ionization
FAN	Free amino nitrogen
FDR	False discovery rate
GA	Gibberellic acid
GD1	Germination day 1
GD2	Germination day 2
GD3	Germination day 3
GI	Glycemic index
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HLW	Hectoliter weight
IMR	Industrial malting regime
KI	Kobalch index
LAB	Lactic acid bacteria
LC	Liquid chromatography
lca	Lowest common ancestor
LD	Limit dextrinase
LDL	Low-density lipoproteins
limma	Linear models for microarray data
logFC	Logarithmic fold change
M	Molar

MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS-RUN	Mass spectrometry run (the samples labeled in groups of 12)
N.O.P.	Number of proteins
ppm	Parts per million
PSMs	Peptide spectrum matches
PTM	Post-translational modifications
PUG	Protease units of germination
S	Steep
SDS	Sodium dodecyl sulfate
SPE	Solid phase extraction
ssp.	Species
STP	Steep
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl) phosphine
TFA	Trifluoroacetic acid
TMTs	Tandem mass tags
ToF	Time-of-flight
w/v	Volume/volume
w/v	Weight/volume
WUG	Wheat-like units of germination

LIST OF FIGURES

Figure 2.1 Cultivated barley varieties two-row and six-row type (Rani & Bhardwaj, 2021)……..7

Figure 2.2 The structure of the barley grain showing the husk, pericarp, aleurone layer, and starchy endosperm (Neylon et al., 2020)…..……...8

Figure 2.3 Barley grain and magnified illustration of the grain components; a magnified picture of the layers husk, pericarp, testa and endosperm (Olkku et al., 2005)……...……..8

Figure 2.4 The summary of the steps in the malting and brewing processes…..…10

Figure 2.5 Classification of the principal approaches in quantitative proteomics (Y. Zhang et al., 2013)……...……...27

Figure 2.6 An overview of plant-microbe interactions, including discovered proteins and enzymes at the interface (Sultan et al., 2016)……..…...28

Figure 3.1 Accelerated malting regime steeping and germination information relevant to the sampling points……..……...38

Figure 3.2 Seed morphologies of various malting stages used for proteome analysis for barley variety Faro, samples belonging to malting stages: end of steep, ends of germination day 1 (showing chitting), germination day 2 and germination day 3……..……...39

Figure 3.3 (a) Puncturing the bottom of 5 mL Eppendorfs with a needle; (b) fitting the Eppendorf on top of an empty vessel to collect the rinse solution containing microbial cells and debris……..……...41

Figure 3.4 Samples placed for centrifugation……..…….41

Figure 3.5 Size comparison of the air-dried protein pellets for (a) steep and (b) germination day 2……..……...43

Figure 3.6 Formation of Cu^{+2} and peptide bond complex (together called "biuret reaction") giving a violet-colored complex (Bhagavan, 2002)…....44

Figure 3.7 Preparation of the "C-18 peptide clean-up column" with the C-18 disk plugs……..……...46

Figure 3.8 Centrifugation step in the "C-18 peptide clean-up column" with the C-18 disk plugs……..…….47

Figure 3.9 Experimental scheme illustrating the step of Peptide-labelling with TMT Isobaric Mass-Tags of the submitted thesis. Constructed with BioRender.com for this study.....53

Figure 4.1 Upset-plot of the different LCA set intersections between the different barley cultivars. The set size indicates the number of unique LCA within each barley variety. Dots and lines mark the individual set comparisons……...……..54

Figure 4.2 Upset-plot of the different LCA set intersections between the timepoints of research. Set size indicates the number of unique LCA

within each step of malting. Dots and lines mark the individual set comparisons……..……..55

Figure 4.3 Volcano plots for Fandaga GD1 vs. STP and GD3 vs. STP comparisons……..……..64

Figure 4.4 Volcano plots for Fantex GD1 vs. STP and GD3 vs. STP comparisons…..……..64

Figure 4.5 Volcano plots for Faro GD1 vs. STP and GD3 vs. STP comparisons…..……..65

Figure 4.6 Volcano plots for Planet GD1 vs. STP and GD3 vs. STP comparisons……..……..65

Figure 4.7 Volcano plots of all barley varieties for the time-point contrasts GD1 vs. STP, GD2 vs. GD1, GD3 vs. GD2 and GD3 vs. STP…..……...66

Figure 4.8 Identified species for the barley cultivar Fandaga, showing the dominating Kingdom of the microorganisms…….……67

Figure 4.9 Identified species for the barley cultivar Fantex, showing the dominating Kingdom of the microorganisms…….……68

Figure 4.10 Identified species for the barley cultivar Faro, showing the dominating Kingdom of the microorganisms…….……68

Figure 4.11 Identified species for the barley cultivar Planet, showing the dominating Kingdom of the microorganisms…….……69

Figure 4.12 Identified genera for the barley cultivar Fandaga, showing the dominating Kingdom of the microorganisms…..……70

Figure 4.13 Identified genera for the barley cultivar Fantex, showing the dominating Kingdom of the microorganisms…..……70

Figure 4.14 Identified genera for the barley cultivar Faro, showing the dominating Kingdom of the microorganisms…..……71

Figure 4.15 Identified genera for the barley cultivar Planet, showing the dominating Kingdom of the microorganisms…..……71

Figure 4.16 Comparison of the malt filtration quality of the accelerated malting regime (AMR) with the industrial malting regime (IMR)..……74

Figure 4.17 β-Glucan concentration…..……..74

Figure 4.18 Comparison of the malt friabilimeter value (%) of the accelerated malting regime (AMR) with the industrial malting regime…..……75

Figure 4.19 Endo-Xylanase activity…..……...75

Figure A.1 Resulting % moisture content of green malts of the four barley cultivars……..……...……88

Figure A.2 Experimental procedure for the barley metaproteomics analysis, showing protein precipitation, resolubilization of the protein pellet, protein quantification and digestion steps, briefly. Constructed with BioRender.com for this study……..……..88

LIST OF TABLES

Table 2.1 Ideal malt quality attribute values for two-row and six-row barley kernel varieties (Heisel, 2006) ……..……...…..…16

Table 3.1 Final concentrations of BSA standards……...…..…45

Table 4.1 Top three significantly up/down regulated peptides between stages for the barley variety Fandaga……...……..…57

Table 4.2 Top three significantly up/down regulated peptides between stages for the barley variety Fantex……...……...…58

Table 4.3 Top three significantly up/down regulated peptides between stages for the barley variety Faro……...……...…60

Table 4.4 Top three significantly up/down regulated peptides between stages for the barley variety Planet……...…….……61

Table 4.5 Top three significantly up/down regulated peptides of the comparisons made between the barley varieties for the time point Day 3 in malting step Germination……...…....…62

Table 4.6 Identified common species for the malting steps Steeping and Germination for AMR……...……..……..…72

Table 4.7 Malt quality properties per cultivar……..……..…73

Table A.1 Mechanical and Physicochemical properties of four barley varieties used in the study……...……..……..…89

Table A.2 The accelerated malting regime barley samples for the variety Fantex (FTX) given as an example of MS-RUN TMT-labelling scheme…...89

Table B.1 Identified unique species for the germination days of AMR……..90

Table B.2 Identified unique microorganisms of the malting step “Steeping” for AMR……...……..…91

Table B.3 Identified unique microorganisms of the time-point Germination Day 1 for AMR……...……..…91

Table B.4 Identified unique microorganisms of the time-point Germination Day 2 for AMR……....……92

Table B.5 Identified unique microorganisms of the time-point Germination Day 3 for AMR……...……..…92

ABSTRACT

Metaproteomic Analysis of Barley Germination Regime Under the Influence of Increased Temperature

Elif Göksu KIZILYAR

Department of Bioengineering

Master of Science Thesis

Supervisor: Prof. Dr. Didem BALKANLI

Co- supervisor: Assist. Prof. Dr. Florian WEILAND

Malting is a critical step in beer production that involves steeping, germination, and kilning. This can be explained by the complex interaction between germinating barley and its microbial community. The microbiome has a multifaceted impact where beneficial microorganisms can contribute to the production of starch-degrading enzymes, facilitating germination and subsequent sugar production for fermentation. In contrast, undesirable microbes can produce toxins or interfere with germination, bringing down malt quality. In this study, the dynamic evolution of the accelerated malting regime (AMR) microbiome was investigated using a metaproteomic approach, which characterizes the microbial community's taxonomic composition and functional activities via its proteins. Accordingly, microorganisms present were collected using ultrasonication from barley samples taken during the steeping and germination stages of AMR. Relevant microbial proteins were extracted and trypsinized. The resulting peptides were labeled with TMT (Tandem Mass Tags) and analyzed using LC-MS/MS, with the raw data annotated using the Comet search engine and the Trans-Proteomics Pipeline. By analyzing the peptides further with Unipept, microorganisms associated with the barley and malting regime were identified, their lowest common ancestor was determined, and AMR potential and optimization were interpreted. It was found that the bacterial population

suppresses the fungal population during the malting process. The microbial community similarity observed in AMR malt, despite different barley species, also demonstrated that wort filtration was inhibited. Possible explanations for poor filtration include reduced arabinoxylan degradation due to fungal suppression and/or the presence of biofilm-forming *Pseudomonas* and *Pseudomonadota* in all samples. The study demonstrates the metaproteomic approach's effectiveness at comprehending the dynamics of microbial populations in intricate settings such as malting barley. By understanding these dynamics, control over the microbial community to enhance the quality and efficiency of the malting process can be gained, ultimately leading to improved product.

Keywords: Barley, malting, accelerated malting regime (AMR), taxonomic analysis, metaproteomic.

ÖZET

Arttırılmış Sıcaklık Etkisi Altında Arpa Çimlenme Rejiminin Metaproteomik Analizi

Elif Göksu KIZILYAR

Biyomühendislik Anabilim Dalı

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Maltlama, bira üretiminde ıslatma, çimlenme ve fırınlamadan oluşan çok önemli bir adımdır. Maltlama süreci, çimlenen arpa ve onunla ilişkili mikrobiyal topluluk arasındaki karmaşık etkileşimle açıklanabilir. Mikrobiyomun etkisi çok yönlüdür: Yararlı mikroorganizmalar nişastayı parçalayan enzimlerin üretimine katkıda bulunabilir, bu da çimlenmeyi ve ardından fermantasyon için şeker üretimini kolaylaştırır. İstenmeyen mikroplar ise toksin üretebilir veya çimlenmeye müdahale ederek maltın kalitesinden ödün verebilir. Bu çalışmada, hızlandırılmış maltlama rejim (AMR) mikrobiyomunun dinamik gelişimi, mikrobiyal topluluğun proteinleri aracılığıyla taksonomik kompozisyonunu ve fonksiyonel aktivitelerini karakterize eden metaproteomik yaklaşım kullanılarak araştırılmıştır. Bu doğrultuda AMR'nin ıslatma ve çimlenme aşamalarından alınan arpa örneklerinin mevcut mikroorganizmaları ultrasonikasyon yoluyla toplanmıştır. İlgili mikrobiyal proteinler ekstrakte edilmiş ve tripsinize edilmiştir. Elde edilen peptitler TMT (Tandem Kütle Etiketleri) ile etiketlenerek LC-MS/MS yoluyla analiz edilmiş ve ham veriler, Trans-Proteomik Pipeline ek uygulamasıyla Comet arama motoru kullanılarak anlamlandırılmıştır. Peptitlerin Unipept ile ileri-analiziyle arpa ve maltlama rejimi ile ilişkili mikroorganizmalar tanımlanmış ve bunların en düşük ortak ataları belirlenerek AMR potansiyeli ve optimizasyonu yorumlanmıştır. Bulgular, maltlama işlemi sırasında bakteri popülasyonunun

mantar popülasyonunu baskıladığını göstermektedir. Farklı arpa türlerine rağmen mikrobiyal topluluktaki bu benzerliğin AMR maltında görülen dinamiği şıra filtrasyonunun engellendiğini de göstermiştir. Engellenen filtrelemeye ilişkin varılan olası açıklamalar, mantar baskılanması nedeniyle arabinoksilan bozunmasının azalması ve/veya tüm numunelerde baskın bakterilerin biyofilm oluşturan *Pseudomonas* ve *Pseudomonadota* olması olarak yorumlanmıştır. Çalışma, maltlık arpa gibi karmaşık ortamlardaki mikrobiyal toplulukların dinamiklerini anlamak için metaproteomik yaklaşımın etkinliğini göstermektedir. Bu dinamikleri anlayarak, maltlama sürecinin kalitesini ve verimliliğini artırmak adına mikrobiyal topluluk üzerinde kontrol sağlamak ve sonuçta geliştirilmiş ürün elde etmek mümkün olacaktır.

Anahtar Kelimeler: Arpa, maltlama, hızlandırılmış maltlama rejimi (AMR), taksonomik analiz, metaproteomik.

1 INTRODUCTION

1.1 Literature Review

The barley grains are converted into malt during the malting process, which is a crucial component of beer and other fermented drinks. This is the process of carefully regulating the germination of barley, which releases enzymes that break down proteins and other complex components as well as carbohydrates (Mahalingam, 2018). The varied “microbial population” spontaneously colonizing the barley grains aids in the malting process (Booysen et al., 2002; Laitila et al., 2018). The microbial community may contain numerous species of viruses, bacteria, fungi, micromycetes, and protozoa (Felšöciová et al., 2021). Mainly, the microbial kingdoms bacteria and fungi are of interest, fungi being the main colonizers of barley grains in the field and bacteria found predominant in the germination step of malting (Lenaerts et al., 2011; Sultan et al., 2016). This microbiome helps break down starch and other carbohydrates in barley grains, creating fermentable sugars that yeast can then ferment into alcohol (Fox & Bettenhausen, 2023). The microbial enzymes, thus contribute to the flavor / aroma and color of beer (Van Nierop et al., 2006). The variety of barley, growing circumstances, and malting technique are some of the factors that affect the complex and dynamic system that is the barley microbiome (Chen et al., 2016).

The malting process consists of three steps: steeping, germination, and kilning (Jiang et al., 2023). During the steeping process, barley grains are soaked in water to increase their moisture content to around 45% (Chen et al., 2022; Rani & Bhardwaj, 2021). This enables the grains to germinate, which in turn promotes the breakdown of starch into simpler carbohydrates. During the germination phase, the barley grains are allowed to sprout (Rani & Bhardwaj, 2021). The barley microbiome, which originates from the barley or the malting environment, is essential for determining the fate of malt, particularly during the germination process. Malting is finalized by the kilning process which involves drying and

heating the germinated barley grains to halt the germination process (MacLeod & Evans, 2015). The high temperature and low humidity of this step deplete the microbial load in malt compared to barley and restrict microbial activity (Rani & Bhardwaj, 2021).

Malt, that is, barley which has gone through the process mentioned above steps, is a versatile agricultural product that, although usually associated with its final product, beer, also has various applications in the food and other sectors. While it is beyond the scope of malting, brewing is often mentioned alongside this process. Therefore, understanding the sequential relationship provides valuable context for the overall process. Once malting is over with kilning, water is added to the malt and mashing takes place at 60-72°C to enzymatically break down the starch into sugars, obtained "wort" is then used in the brewing procedures (Rani & Bhardwaj, 2021).

Furthermore, the metabolomics approach is high in demand for plant and food science, and omics research for malting barley is an emerging field (Frank et al., 2011). Plant-microbe interactions with proteins, including enzymes, are of great interest. Metaproteomics is the study of the protein components found in a targeted microbial population, and it is a complementary approach to metabolomics, helping to identify microorganisms associated with the production of metabolites (Van Den Bossche et al., 2021). The metaproteomic approach examines the proteins of a mixed microbial community using the same procedures as proteomics for pure cultures: sample preparation, protein extraction, separation, and MS analysis (Bond & Wexler, 2007).

Many brewers are turning to metaproteomics to investigate the microbiome of barley malt. Trying to examine the changes in the microbiome by experimenting with different malting regimes would be a nice addition to many in the literature that only goes as further as suggesting the idea. Following this context, changes to a malting regime, namely "Accelerated Malting Regime (AMR)" are investigated. The conditions of accelerated malting regimes are advantageous for breweries with increased efficiency, reduced costs, and environmental impact.

In many ways, such a study would be an important step toward developing new strategies to achieve the desired microbial profile in the malting process. A better

understanding of microbial dynamics could guide new approaches for optimizing these processes and improving malt quality, as well as providing a better understanding and management of microbial ecosystems in food production processes.

1.2 Objective of the Thesis

The microbial count demonstrates a significant increase during steeping, with the microbiome subsequently remaining elevated throughout (O'Sullivan et al., 1999). These early findings emphasize the critical role of steeping and germination in shaping the barley microbiome.

In this study, to further explain the dynamic process of the microbiome, a metaproteomic investigation focusing on these two stages of malting, with sample collection during both steeping and germination, with a promising path for research was aimed.

The specific objectives of this study were to:

- Identify the differential bacterial and fungal protein expressions for the adjusted malting regime and present a taxonomic characterization or find out the difference between the temporal changes in the bacterial and fungal protein expression due to increased temperatures during steeping and germination.
- Examine the up/down regulated peptides and their corresponding lowest common ancestor for the different germination time points under the modified malting regime.
- Comment on the impact of higher temperatures applied during steeping and germination on the development of the microbiome associated with barley during its germination.
- Determine the changes in the temporal profile of the microbiome associated with barley during germination as a result of the adjusted malting regime with applied increased temperatures, with microbial profile dynamics.

1.3 Original Contribution

This study represents an establishing effort to characterize the dynamic changes in the barley microbiome under accelerated malting conditions using a metaproteomic approach. While brewers are well aware of the significance of investigating the function of microorganisms in guaranteeing quality malt production, more study is still required to completely comprehend the impacts of certain microbial populations within the framework of a complex environment with alterations. Such as accelerated malting regimes (AMRs) investigated in this study.

This microbial environment was seen as easily identifiable by using the metaproteomics approach, a specialty of proteomics that focuses on the analysis of the proteins generated by a community of microbes. With the correct background database created, the measured proteins from such a wide range of samples are estimated to provide comprehensive findings. It was also anticipated that the conclusions of this research to help the management and maintenance of specific and easily modifiable malting regimes and provide an overall improvement to engineer easier production.

And adequately, with this thesis, it had been possible to investigate how the microbial population may shift substantially over time; the main discovery of this study was its contribution to the narrative of providing insight for a malting regime with modified conditions. This study is the first to comprehensively analyze the metaproteome of barley during an accelerated malting regime. The significant temporal changes in the microbiome that occurred during the malting process, with the fungal population becoming suppressed and a small bacterial population becoming dominant in later stages of malting and pointing out a commonality between the barley varieties:

- offer important insights into the intricate interactions between germinating barley and its associated microbial community
- pose as an encouraging effect for the research with modified conditions / variable parameters to improve process optimization

2
GENERAL INFORMATION

2.1 The Cereal Crop "Barley" & Its Use in Malt

The cereal crop *Hordeum vulgare*, also known as barley, is one of the first domesticated crops worldwide and belongs to the grass family *Poaceae* (Bothmer et al., 2003; Kumar et al., 2016). Along with several important forage crops such as wheat (*Triticum aestivum L.*) and rye (*Secale gravee L.*), belongs to the *Triticeae* tribe (Bothmer et al., 2003). The branch of the family *Poaceae* belongs to this tribe and represents highly successful evolutionary traits. And barley, in contrast to other crops, can survive in a variety of environmental conditions (Kumar et al., 2016).

It is also one of the most grown grains in the world and ranks 4th after maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*) worldwide both for human and animal consumption (Kumar et al., 2016; Taner et al., 2004). Each cereal grain of which accounts for approximately 30% of the total cereal production (Deme et al., 2020).

The closest ancestor of cultivated barley continues to be present as plentiful in nature. While the precise details of this early discovery remain elusive due to the passage of time; the closest ancestor is considered to have been initially discovered in Türkiye by Carl Koch, a German botanist, and identified as "*Hordeum spontaneum*" (Bothmer et al., 2003; Kumar et al., 2023). Today, the mainly cultivated species is called *"Hordeum vulgare L.*" and *Spontaneum* spp name is considered as the wild subgenre.

Türkiye is a significant producer of barley globally (Taner et al., 2004). While not the top one, it ranks 3rd among the seven major barley-growing countries (alongside Russian Federation, Austria, Canada, Spain, Kazakhstan, and Ukraine) with 5.99% and contributes a substantial share to the global total, ranking sixth

(with 5.28%) among the countries responsible for nearly 60% of all barley production (Kumar et al., 2023).

As mentioned earlier, this agricultural crop is known to thrive in temperate to tropical climates, withstanding harsh factors like heat, salt, drought, and frost (Kumar et al., 2023). The grains have high levels of soluble and insoluble dietary fiber (DF), as well as other bioactive components including vitamin E, B-complex vitamins and minerals (Gupta et al., 2010); and contain various other healthy plant chemicals like phenolic acids and lignans (Kumar et al., 2023; Raj et al., 2023).

The barley grains also possess a low glycemic index (GI) and help in lowering the overall GI of the meal they were included in. Eating barley regularly has been linked to a lower risk of high blood pressure, gallstones, and even colon cancer (Kumar et al., 2023).

Cereals are basic products to produce feed, food, and malt, whose by-products are also an important source of biomass (Sultan et al., 2016). All these applications depend on the efficient enzymatic degradation of complex polysaccharides, proteins, or other bioactive components (Sultan et al., 2016).

The dominant worldwide distribution of barley production (60-65%) is utilized in animal feed, followed by 20% use for malt (Deme et al., 2020; Kumar et al., 2023) About ninety-five percent of the produced malt is used for the production of beer, while the rest is used for distillation or in food products (e.g. for breakfast cereals) (Laitila et al., 2011). The preference for using barley in malting practices above other cereals is due to the presence of its husk, the growth of acrospire (“chit”) beneath the husk, and its enzymatic properties (Kumar et al., 2023).

Barley can be categorized as spring or winter types, as two-row or six-row, according to the presence of the outer 'husk' of the barley grain, or by the parameter of hectoliter weight (HLW) that determines the barley to be categorized as suitable for use in malting or feed (Deme et al., 2020; Sullivan et al., 2013).

Several factors, including but not limited to germination, moisture content, water sensitivity, glassiness, and protein content, are related to the quality of the barley grain used in malting (Deme et al., 2020). For instance, barley with a HLW within the European Brewery Convention's (EBC) recommended range of 65–75 kg/hL

is generally considered suitable for malting, other factors like protein content and kernel size also influence malt quality (Rani & Bhardwaj, 2021).

The barley is composed of spikelets that are attached to the rachis (the barley stem), in alternating patterns that define different row types. The arrangement of the kernels on the barley spikes showcases to determine the uniformity and shape of the barley kernels to be used in malting. The domesticated barley is classified by the number of kernel rows, as demonstrated in Figure 2.1.

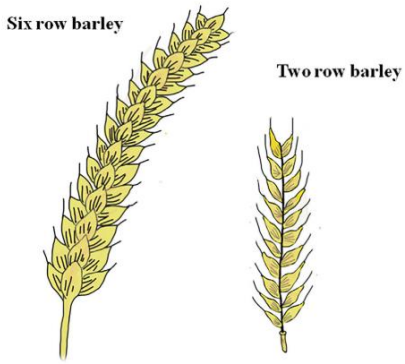


Figure 2.1 Cultivated barley varieties two-row and six-row type (Rani & Bhardwaj, 2021)

Although current barley cultivars vary in spike shape, with both two-row and six-row types, ancient barley was initially two-rowed (Harwood, 2019). The distinction between the two kinds is the fertile node for their spikelets. For the six-row cultivars, all three spikelets are fertile, while only the central spikelet is fertile for two-row barley (Deme et al., 2020; Sullivan et al., 2013). Every kind of barley, whether two- or six-row, has unique qualities for malting and brewing.

2.1.1 Components of Barley

The terms barley “kernel" and "grain" were used interchangeably in this thesis to refer to the whole seed, however, the “kernel” is the complete, unprocessed seed of the barley plant, while the “grain” is the kernel that has been processed to remove the husk.

Barley kernel consists mostly of starch with 60-70% (w/w), followed by a 10-20% protein amount, 5-10% β-glucan and 2-3% free lipids, and the rest 1-2.5% being minerals (Contreras-Jiménez et al., 2019; Zhang et al., 2023). The components of

barley grain are the *husk*, which entirely encases the grain and is the outermost layer, the *pericarp* and the *testa* layers, the *endosperm* which has the outer layer called "aleurone" and *embryo* (Sudheesh et al., 2021). The barley grain and its components are given in Figures 2.2 and 2.3.

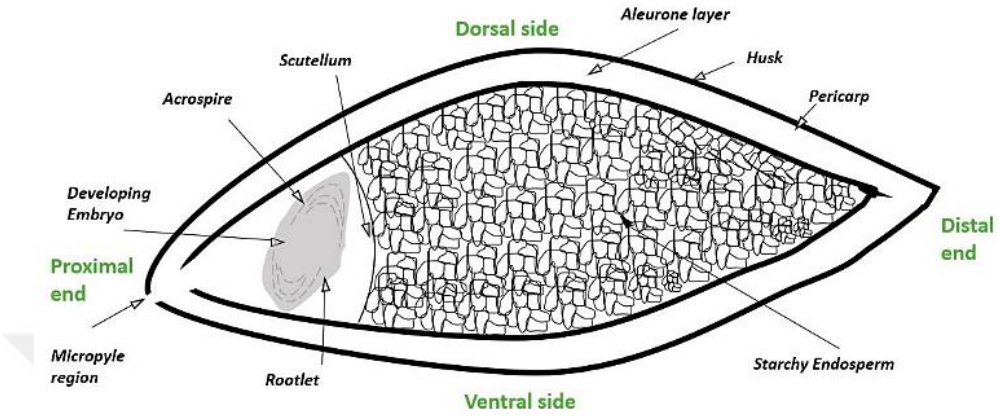


Figure 2.2 The structure of the barley grain showing the husk, pericarp, aleurone layer, and starchy endosperm (Neylon et al., 2020)

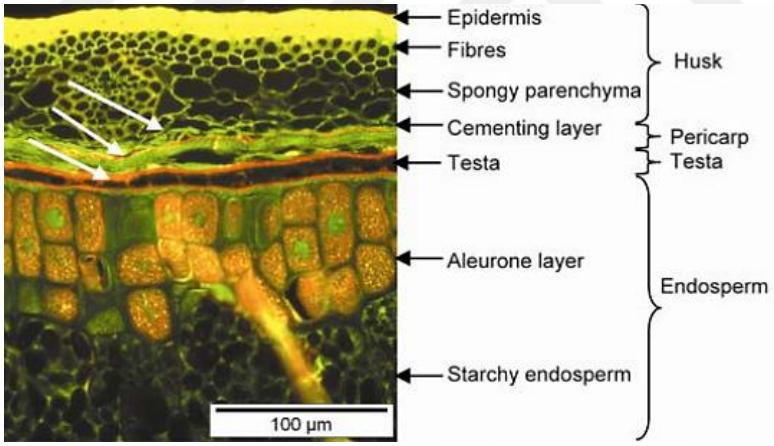


Figure 2.3 Barley grain and magnified illustration of the grain components; a magnified picture of the layers husk, pericarp, testa and endosperm (Olkku et al., 2005)

The husk is composed of cellulose, insoluble arabinoxylans, lignin, polyphenols, and minerals. The layer beneath the husk, the pericarp, contains cellulose, lignin, and arabinoxylan. The endosperm, the largest part of the grain (75-80% of the weight), is composed of starch and a protein matrix (Vasanthan & Hoover, 2009). Approximately 75% of barley endosperm cell walls consist of mixed-linked (1-3), (1-4)-β-D-glucans, 20% arabinoxylans, and of proteins (Gupta et al., 2010; Lowe

et al., 2005; Malfliet et al., 2013). The embryo (germ) makes up the smallest weight in the barley grain and is rich in lipids, proteins, and amino acids (Vasanthan & Hoover, 2009) where eight of the nine necessary amino acids are present (Clifton & Keogh, 2015).

The two main parts of the barley grain are this embryo that grows into a new plant and the *starchy endosperm* (Figures 2.2 & 2.3) that serves as the storage compartment for starch and proteins which are then used by the embryo during germination for its development (Fox & Bettenhausen, 2023).

Among all cereal crops, barley grains possess the highest functional value, the lowest GI, the most β-glucans, resistant starch, and antioxidant characteristics (Newman et al., 2019; Raj et al., 2023). β-glucans are a key soluble fiber that has been linked to reduced plasma cholesterol, improved lipid metabolism, and lower glycemic index (Gupta et al., 2010). These fibers have also been shown to lower "bad" cholesterol (LDL), help regulate blood sugar levels, improve intestinal health, and reduce the risk of heart diseases (Kumar et al., 2023; Raj et al., 2023).

2.2 The Malting Process

Malting is defined as stabilizing the grain through certain changes through a controlled germination and drying phase (Gupta et al., 2010). Industrial malting is composed of three steps: steeping, germination, and kilning (Chen et al., 2022).

These three process steps are required to provide the necessary physical and biochemical changes, starting with "steeping", in which good absorption of water into the grain is achieved, followed by the "germination" of the grain, which is carried out under special conditions that provide maintenance of embryo growth, enzyme synthesis and limited endosperm fragmentation, and finally “kilning” to ensure the stabilization of the product, reducing the effect of microorganisms and plant growth hormones. The differences in the malt type provide the products with a range variety of flavors, colors, and textures (Kaur et al., 2015). A simple visualization of the malting and brewing processes, interpreted by Gupta et al. (2010) is given in Figure 2.4.

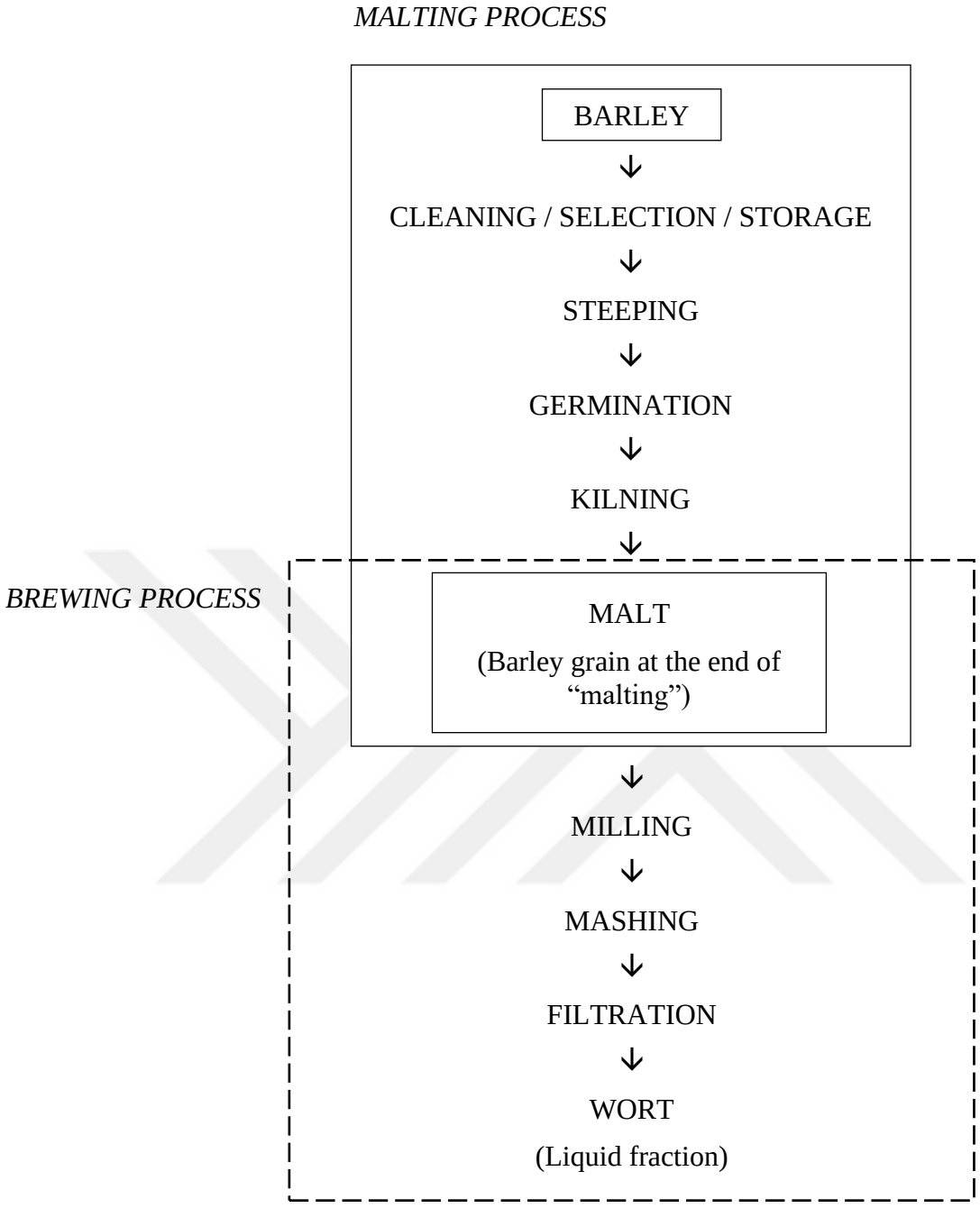


Figure 2.4 The summary of the steps in the malting and brewing processes

The malt (barley grain after kilning) is milled to increase surface area and dissolve it in water. This process separates the husk from the endosperm and exposes the interior starch. The resulting flour then mixed with water is called "mash". In the mashing process, this substance is followed to be heated for saccharification, converting the remaining starches into simple sugars. During mashing the microbial load is reduced (Bokulich & Bamforth, 2013). This process ends up with a liquid separated from the solids called the "wort". It contains fermentable

sugars, nitrogen, oxygen, sulfur, phosphates, vitamin biotin, calcium, magnesium, and trace elements (Fox & Bettenhausen, 2023). Malt is the initial ingredient of wort that is used to ferment beer. Wort is cooled and oxygenated and transferred for the fermentation of maltose and other sugars to ethanol and carbon dioxide via added *Saccharomyces* strains (Bokulich & Bamforth, 2013).

The barley grain is needed for all the steps in the brewing process, except for the fermentation step. The starch that is converted into sugar, the enzymes that break down the starch, and the proteins and amino acids that are needed by the yeast are provided by barley and the grain is then removed after the wort is boiled.

2.2.1 Steeping, Germination and Kilning

It's essential to comprehend how the biology of germination and the various steps of the malting process interact to transform barley into malt fit for beer brewing.

Harvested barley is generally stored at a moisture level of 10% to 14% (MacLeod & Evans, 2015). During steeping, it is aimed to ensure rapid and equal intake of water and oxygen to encourage the embryo to breathe and initiate hormone activity (MacLeod & Evans, 2015). The grains are soaked in water and filtered several times during the steeping stage to increase the moisture content to 42-47%, this step ensures the germination of the grain, and possibly "washes-off" residue and dust on the grains (Chen et al., 2022; MacLeod & Evans, 2015). Immersion with aerated steeping water where air rests at 13–20°C are also applied to improve oxygen availability (Laitila et al., 2011). The grains are dipped more than once into water with air rest periods in between (where the water is drained from the grain). Steeping usually takes approximately 24-48 hours in cylindrical or flat-bottomed tanks large enough to ensure that hydrostatic pressure does not damage the grains and requires turning and aerating the grains to supplement oxygen and release carbon dioxide (Fox & Bettenhausen, 2023; MacLeod & Evans, 2015).

After the "chit" (coleorhiza) appears in the barley grain, the barley is moved to the germination beds for the "germination" step. This step takes place in humid, aerobic conditions at 16–20°C to stimulate the enzymes that initiate the hydrolysis of proteins, lipids, and starch, this can take about 3–6 days (Contreras-Jiménez et

al., 2019; Fox & Bettenhausen, 2023; Laitila et al., 2011). Many enzymes are produced or activated during the germination stage of malting which leads to the breakdown of proteins, starch, and cell-wall polysaccharides (Mahalingam, 2018). Even some microbes get washed out during the steeping period, overall, the microbial population significantly increases and then remains high in the germination step (Østlie et al., 2021). During germination, α- and β-amylase enzymes are released, and the natural barley starch “amylopectin” and “amylose” are broken down. This starch breakdown results in chains of varying lengths including glucose, maltose, maltotriose, and other saccharides for subsequent consumption by yeast during fermentation (Fox & Bettenhausen, 2023; Mahalingam, 2018). However, the hydrolysis of starch does not result in the full conversion to maltose or glucose during malting (Contreras-Jiménez et al., 2019).

Once the grain’s root (acrospire) is of a similar size to the grain, germination is stopped and kilning is aided (Contreras-Jiménez et al., 2019). “Kilning” refers to the drying of germinated barley for 24-30 hours at gradually increasing temperatures between 50 to 85°C or above; the germination process stops, the moisture level of the grain reduces, and malt is hardened in a way that gives a roasted taste (Fox & Bettenhausen, 2023; Laitila et al., 2011). With the kilning step, the product is stabilized, and the final moisture is 3-4% (Laitila et al., 2011). Kilning also influences the color and flavor of the malt (Lukinac & Jukić, 2022).

The term "green malt" refers to the barley grains that have germinated. Green malt is roasted during the kilning process, which stops all biological activity. With kilning, most metabolic proteins remain intact, however, others are broken down by proteases or destroyed by the high-temperature (Mahalingam, 2018), meaning that this step halts microbial activity but preserves the microbial community. As mentioned above, kilning is an important step that determines the final characteristics of malt, such as enhanced flavor and aroma (Contreras-Jiménez et al., 2019).

2.3 Accelerated Malting Regime (AMR)

The KU Leuven-Campus Ghent approach is to propose, investigate, and implement system changes to improve flavor stability as well as reduce total costs. Essentially, brewers want to obtain a combination of increased temperatures and less time. Certain beer styles might benefit from high-temperature usage, and reduced fermentation time can be energy-saving and boosting the brewery's efficiency.

In this study, the applied increase in temperature during steeping and germination in comparison to the pilot scale malting regime conditions, has the steeping time temperature increased for 4-5°C in comparison to the standard industrial malting regime with less time for wet steeps and dry rests. The germination time is also shortened to 61% of the time utilized for the standard industrial regime with a 4°C increase in temperature. And the 35-hour kilning process of the industrial standard remained unchanged.

Accelerated malting regimes (AMRs) are generally beneficial and preferred by breweries for the improvement of efficiency, saving money by reducing the amount of time and energy that is required to malting barley. Reduced time and energy consumption are especially significant for breweries that operate on a low budget or want to lessen their environmental impact. AMRs also provide improved quality control since shorter malting times lower the danger of spoilage while also ensuring that the malt satisfies the required parameters.

The shorter malting period further lowers the loss of starch and other nutrients from barley, resulting in higher yield. It's likely that quality attributes to be affected by AMR conditions. For instance, the shorter steeping time in AMR may result in lower DP, as this process is essential for the production of fermentable sugars. That said, the shorter germination time might give lower enzyme activity causing a drop in the quality of production of flavor and aroma compounds.

This thesis aims to delve into the intricate dynamics of the microbial environment of the malting barley in response to such alterations in the malting regime.

2.4 Quality Attributes for Malt

The selection of barley varieties for brewing is essential, as different beer types demand distinct malt characteristics. Several factors must be considered when selecting barley varieties for malt production and the malt produced is also evaluated based on several quality attributes.

The diastatic power (DP) is one of the most important factors in evaluating malt. This term refers to the capacity to degrade starch, the branching glucose polymer with various forms of connection. As a result, DP demonstrates the combined activity of four starch-hydrolytic enzymes that break these bonds. These enzymes that are active during malting and mashing include α- and β-amylases, limit dextrinase (LD), and α-glucosidase. High values of DP mean higher malt extract and greater malt quality (Davies, 2006; Rani & Bhardwaj, 2021).

Another attribute is the free amino nitrogen (FAN), a measurement of the nitrogen molecules that may be taken up or broken down by yeast during fermentation and therefore expressed in the unit of milligrams per hectoliter (mg/hL). The nitrogen compounds consist of ammonia, short peptides (di- and tri-peptides), and individual wort α-amino acids and the non-α-amino acid proline (Hill & Stewart, 2019).

There's also the significance of color as a quality feature, which stems from the Maillard process in kilning, as well as from the polyphenol oxidation in the brewery (Bamforth, 2006). Differences in the treatment of the malt can cause to production of wort in darker colors which can be taken as an indication of having higher nitrogen content. This would suggest that the applied treatment enhances the proteolysis of the malt, which is a desirable characteristic in brewing (Laitila et al., 2018).

During germination, enzymes break down the cell walls in the barley kernel, making the grain slightly less hard, and the drying process in the kilning step hardens the kernel again. However, the effect of enzymes during germination, combined with drying in the kilning process, creates a brittle structure in barley, which is referred to as malt "friability". It should be highlighted that during the malting process, barley is soaked, germinated, and then dried with hot air, and

starch and proteins are affected by the activation of enzymes. Consequently, the final product called "malt" is the name given to the simple barley grain after going through these processes. This brittleness makes the malt easier to break down, leading to good friability. The friability as a quality attribute is particularly important as this indicator is used to assess the alteration of malt grains' cell walls.

The determination of the grain friability is based on the idea that poorly adjusted malt is steely and difficult to grind (Rani & Bhardwaj, 2021). Barley kernels are mainly composed of arabinose, xylan, ferulic acid, and acetic acid while the inner layer contains the soluble fiber β-glucan (Kumar et al., 2023). In malting, to access the β-glucan inside the grain, the complex sugars found on the outer cell wall of the barley kernels called “arabinoxylans” need to be broken down with enzyme arabinoxylanases. Both the solubility of β-glucans and degradation of arabinoxylans are effective on the wort filtration rate, viscosity, and Kolbach index (Kumar et al., 2023). Poorly modified malts that possess high β-glucan and arabinoxylan concentrations, can increase wort viscosity and significantly hinder extract recovery (wort filterability) (Laitila et al., 2018). However, while β-glucans and arabinoxylans cause problems in viscosity or in mash filtration, Malfliet et al. (2013) states arabinoxylans to be recognized as more likely to cause viscosity problems due to their low degradation compared to β-glucans.

Kolbach index (KI) is the soluble protein amount of the wort divided by the total amount of protein in the malt. It is a crucial sign of proteolysis during malting and mashing, showing the nitrogenous compounds dissolve efficiently. Protein turbidity, reduced malt extract, and wort filtering issues are caused by low KI, indicating inadequate protein hydrolysis (Rani & Bhardwaj, 2021).

Other attributes are; FinalAtte, which is the measure of the total amount of starch that is converted into fermentable sugars during the malting process; WUG (Wheat-like Units of Germination) a metric used to quantify the number of enzymes in barley that convert starch; PUG (Protease Units of Germination) a measurement of the activity of proteases and the Protein Solubility Percentage (%), a measurement of the water-soluble protein content.

While some protein is necessary for yeast growth and healthy fermentation, excessive protein can lead to sluggish fermentations and off-flavors. Limiting

protein content to 12% helps ensure that the yeast can work efficiently and produce a clean-tasting product. For brewing, lower malt-soluble protein levels are often preferable.

Heisel, (2006) provided the ideal malt criteria for the cultivars of two-row and six-row barley kernels, a few of the values according to their study are given in Table 2.1.

Table 2.1 Ideal malt quality attribute values for two-row and six-row barley kernel varieties (Heisel, 2006)

	Two-row barley	**Siz-row barley**
DP	>120	>140
FAN	>180	>190
KI	40-47%	42-47%
Soluble protein	4.4-5.6%	5.2-5.7%
Viscosity (cP)	<1.50	<1.50

The quality of malt, which influences the quality of wort and beer, is also influenced by the microbial population of barley grains (Østlie et al., 2021).

2.4.1 Properties of Malting Barley

The steps that make malting are determined according to facility design, condition, target malt and barley characteristics (MacLeod & Evans, 2015). Understanding the metabolic proteins created during the malting process, which transform the chemicals in the barley into molecules that can be fermented, is necessary to explain the malting barley used in beer production. Especially, the hydrolysis enzymes that play a crucial role by enabling the transformation of the complex carbohydrate components of barley endosperm cell walls and proteins

into soluble molecules like fermentable sugars and peptides (Chen et al., 2022; Sultan et al., 2016).

These were mentioned by their presence inside the components of barley and are the three groups of hydrolysis enzymes:

- Cytolytic acting enzymes or cell wall hydrolases (e.g. β-glucanases and xylanases) which degrade the cell walls and structural substances (β-glucans, cellulose, hemicellulose, and pectin).
- Amylolytic enzymes or starch hydrolases (α-amylases, β-amylases, LD, and α-glucosidase): which take part during mashing to hydrolyze starch to fermentable sugars, (their effect is summarized to indicate the quality parameter DP) (Geißinger et al., 2022).
- Proteolytic enzymes that break down proteins into peptides and amino acids, providing the nutrients for yeast during fermentation (Bretträger et al., 2024).

Larger barley grains have thin cell walls and loose endosperm structure and therefore absorb water quickly; this allows the uniform distribution of water and followingly a uniform distribution of the hydrolytic enzymes, providing consistency and efficiency in malting processes (Rani & Bhardwaj, 2021). The cytolytic and proteolytic enzymatic activity makes the endosperm fragile, and this is favorable for malting. In contrast, amylolytic enzymes are desired to be low during malting and high in the mashing step; as otherwise results in undesirable loss (Geißinger et al., 2022). Similarly, suppose cell walls are not properly broken-down during malting, malt extracts may include high levels of polysaccharides, causing the filtering of extracts (wort) to be problematic and slowing the brewing process considerably (Lowe et al., 2005). Large molecules like arabinoxylans, proteins, and polyphenols are also effective in filtration, causing bad filtration (Gupta et al., 2010).

Kumar et al., (2023) describe β-glucan in barley endosperm cell walls as one of the major quality determination factors for malting. These soluble fibers can come from the cell walls of bacteria, fungi, and yeasts, as much as plants.

β-glucan, which is required to be high in the food industry to obtain significant health benefits, is preferred to be lower in malting as it causes blockages during

wort filtering (Kumar et al., 2023). β-glucans and arabinoxylans form a barrier for hydrolytic enzymes that attack the starch and protein within cell walls. Due to this barrier, barley possesses positive effects on health such as reducing the risk of colorectal cancer, lowering blood cholesterol, and managing diabetes; the wort viscosity and filtration rate are rather affected (Gupta et al., 2010). Malting performance is therefore linked to β-glucanase activity, which depolymerizes β-glucan; low levels of β-glucan in grains and high levels of β-glucanase in malt are expected to lead to better malting performances (Gupta et al., 2010).

The microorganisms coming from the environment, that might be considered as contaminations during the production and/or packaging can actively take part in metabolizing the compounds of the barley grain. And may cause problems such as off-flavors, poor quality, and stabilization (product spoilage) (Oldham & Held, 2023).

2.5 Malting and Barley Microbiome

Microbial communities that live on or inside the barley seeds, whether native to this grain, or introduced to the grain from the field, during transportation, or storage have an impact on the quality of both the grain and the produced malt and are affected by factors such as its growing location, climatic conditions, storage and malting techniques (Chen et al., 2022; Kaur et al., 2015). The microbiome can affect the flavor, aroma, and color of the beer, as well as its stability and shelf life. After harvesting, barley is stored for some time to overcome the state of dormancy, where storage conditions are effective on the active microbial community's growth and interaction with the grain (Bokulich & Bamforth, 2013).

The colonizing microbial community actively interacts with grains through a variety of pathways, including the degradation of enzymes, plant-stimulating hormones, and cell wall components, the production of metabolites essential for nutrient uptake, host colonization, and virulence (Sultan et al., 2016).

The "microbes" are known to contribute to the produced plant hormones; and to various events, such as flowering, transpiration, dormancy and germination of seeds, cold acclimation, and tropical responses in plants that rely on these hormones. Considering the events to be affected by the present microbes that can

provide hormones in sufficient amounts, there were studies specifically focused on this ability of seed-borne bacteria and fungi in malting barley and monitoring the course of germination or seed enzymatic capacity accordingly (Tuomi et al., 1995). To have a successful malting process, the proper balance must be maintained between the plant hormones in the germinating barley kernel (Noots et al., 1999).

These hormones are:

- Gibberellic acid (GA3) which promotes germination and enzymatic activity.
- Abscisic acid (ABA) that maintains grain dormancy and prevents endosperm degradation.
- Indole-3-acetic acid (IAA), which serves as the primary shoot signal hormone in seed germination (Tuomi et al., 1995)

During steeping, the embryo produces enzymes that break down embryonic starch, initiating shoot and root growth (MacLeod & Evans, 2015). In barley germination, the release of gibberellic acid (GA) from the embryo initiates the breakdown of storage products contained in the starchy endosperm by the aleurone layer (Finnie, Sultan, et al., 2011). As mentioned above, some species in the microbial community can produce hormones and enzymes to promote germination, produce hydrolytic enzymes involved in cell wall degradation, or antimicrobial factors among themselves to inhibit other species (Li et al., 2018). Such ways are definitive of how the barley microbiome contributes to the nutritional value of the malt. The effect of the microbiome during germination is firmly relied on.

The microbial community found on or in the barley seeds may contain numerous species from five groups; viruses, bacteria, fungi, micromycetes, and protozoa (Felšöciová et al., 2021). The elimination of this microbiome is neither practically possible nor desired by producers (Kaur et al., 2015). As mentioned, the quantities and types of these microbes largely depend on the species, developmental stage, as well as environmental and post-harvest storage conditions (Sultan et al., 2016). The enzymes produced during germination might originate from the microbial community of the malting ecosystem which includes diverse bacteria, yeast, and

filamentous fungi, in addition to the germinating grains (Kaur et al., 2015). Such research regarding enzyme activity specific to xylanase has shown that only 25% of the contribution is from the germinating grains while the remaining significant percentage is from the microbial community (Malfliet et al., 2013).

2.5.1 The Complex Interaction of The Barley Grain and Its Microbiome During Malting

The microbes can affect the quality of barley and malt in positive or negative ways. The undesired effects of microbes in barley microbiome are causing plant diseases which would cause a reduction of yield, inhibition of the grain germination, the metabolites of the microbial community causing technical problems in the malting regime such as organic acids causing variation in wort pH, slimes causing wort filtration problems or premature yeast flocculations, causing overfoaming of the end product. But it's mainly expected of the microbial community to enhance the grain germination with growth regulators (e.g. gibberellin) or contribute to the malting process with the production of hydrolytic enzymes (Tuomi et al., 1995).

Research indicates that the microbiome seen in germination often is expected to include bacteria from the *lactic acid bacteria LAB*, *Enterobacteria*, and *Pseudomonas* genera as well as fungi from phylum *Ascomycota* (Chen et al., 2022). There are various microbial groups on the barley in the field, which might originate from several sources including air, dust, water, soil, insects, and birds in the environment (Bokulich & Bamforth, 2013; Felšöciová et al., 2021).

The microbial community plays a crucial role in both enhancing and hindering the malting process. During steeping, bacteria, yeasts, and molds proliferate quickly; LAB are especially well-suited to this process since there is less oxygen available. As the barley kernel undergoes significant metabolic changes during germination, the predominant microflora is composed of bacteria, with *Enterobacteriaceae* and *Pseudomonas* species reaching concentrations of 10^8–10^9 cfu/g, *Leuconostoc* species from steeping are replaced by *Lactobacillus* species as the dominating *LAB*, towards the conclusion of germination, *Ascomycetous* yeasts predominate (Lenaerts et al., 2011). During germination, the number of grain-associated bacteria peaks. The level of infection during this time is closely related to the

microbial load on the barley before steeping (Kelly & Briggs, 1992). The grains being dried after harvest to low moisture percentage is to limit further multiplication of field microbes. However, the drying circumstances might favor the growth and spread of storage fungi (Kelly & Briggs, 1992). The microbial community can be beneficial, by producing enzymes and enhancing germination; it can also be detrimental, by producing toxins and hindering the brewing process. The careful control of the malting process and the microbial community is essential for producing high-quality malt and beer. Some studies have also found that suppressing the bacterial community leads to improved germination and extract yield, while the fungal community contributes to enzyme production (Laitila et al., 2007). The plant, harvest, and storage conditions of the cereal have an impact on the grain microbiome (Østlie et al., 2021). As the warm, humid environment and the dissolution of nutrients in boiling water activates bacteria and fungi, stimulating rapid microbial proliferation (Chen et al., 2022).

2.5.1.1 Bacteria

Excessive numbers of aerobic bacteria might compete with the embryo for oxygen, reduce α-amylase activity, and slow down germination (Bokulich & Bamforth, 2013; Felšöciová et al., 2021). During steeping, the dormant microbes on the grain might get stimulated to grow into biofilms, creating an amorphous / fibrous and slimy matrix of extracellular polymeric substance (EPS) which coats the husk and pericarp layers of grains (Tsitko et al., 2018). These biofilms consist mainly of polysaccharides (Mann & Wozniak, 2012). The gram-negative bacteria can produce these exopolysaccharides that might cause biofilm formation and affect nutrient diffusion, leading to filtration problems in mashing (Li et al., 2018). Certain strains of *Pseudomonas* and *Lactic Acid Bacteria (LAB)* are of such exopolysaccharide producers (Chen et al., 2022)

Even small amounts of biofilm components, i.e. bacterial polysaccharides such as gellan, xanthan, and dextran, have shown a strong effect on wort filtration (Li et al., 2018; Tsitko et al., 2018). With the study of Laitila et al., (2018), the genera belonging to the classes *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Bacilli* were characterized as abundant in the filtration problems related to the gelling components. Tsitko et al. (2018) demonstrated in their study that the antibacterial treatments performed

during steeping were effective in restoring the wort filtering performance once reduced due to EPS-producing bacteria.

The wort filtering performance has a considerable impact on the end product, and the factors influencing this performance are relative to one another (Laitila et al., 2018). To explain this connection, even the presence of relatively small numbers of damage occurring on the testa layer in the barley husk during processing or handling of the grains before malting, results in a considerable reduction of the wort filtration (Tsitko et al., 2018). This is associated with increases in various groups of bacteria that produce exopolysaccharides due to the exposed starchy endosperm. That belongs to the groups *Flavobacterium, Leuconostoc, Pseudomonas, Pantoa,* and *Paenibacillus* (Laitila et al., 2018; Tsitko et al., 2018).

LABs are gram-positive, non-spore-forming, facultative anaerobes that are catalase and oxidase-negative. Homofermentative ones mainly produce lactic acid, while heterofermentative ones metabolize carbohydrates and produce a mixture of lactic acid, carbon dioxide, acetic acid, diacetyl, and/or ethanol. These, in turn, can lead to end-product characteristics such as sour taste, vinegar aroma, and oily mouthfeel (Oldham & Held, 2023). After malting, homofermentative LAB remains active in the nutrient-rich, high-moisture environment of the mashing (Bokulich & Bamforth, 2013). The presence of LAB in mashing has been shown to increase fermentability and nitrogen yield of the wort; and improve foam stability, color, and taste of the final product (Bokulich & Bamforth, 2013). Addition of LAB starters during steeping increases microbial β-glucanase and xylanase production, promoting yeast growth (Tsitko et al., 2018). Conversely, according to Oldham & Held (2023), the main spoilage genera are *Pediococcus* and *Lactobacillus* belonging to the genus LAB, having been shown to cause up to 90% of the spoilage incidents.

According to Tsitko et al., (2018), changing bacterial community dynamics during malting simultaneously affects the fungal community consisting of yeasts and filamentous fungi; the suppression of bacterial communities promotes yeast growth.

2.5.1.2 Fungi

Barley is very sensitive to fungal infections due to its high concentration of starch, fatty acids, and other substances that serve as rich nutrients for fungi (Lu et al., 2022). Fungi are the main colonizers after the maturity of cereal grains and have been reported to interfere with grain respiration, reduce grain germination/viability, and produce mycotoxins (Sultan et al., 2016).

The growth of various pathogenic fungi on the grains also affects malt quality negatively. One of the negative effects that the microbial community possesses is the potential of filamentous fungal species *Aspergillus*, *Penicillium*, and *Fusarium* to cause serious food safety problems by producing mycotoxins (Li et al., 2018).

Fungal pathogens can produce hydrolytic enzymes that cause cell wall degradation (Tini et al., 2020). For mashing and filtering, the ability of different filamentous fungi to generate enzymes that degrade plant cell walls is appealing. Filamentous fungi, in particular, are recognized as an important source of barley cell wall-degrading enzymes (e.g. β-glucanase and xylanase), which accelerate wort filtration rates (Li et al., 2018). Additionally, the fungal peptides known as hydrophobins are responsible for the phenomenon of "gushing" where there's excessive release of gas is present, causing over-foaming (Bokulich & Bamforth, 2013; Kaur et al., 2015; Li et al., 2018).

Storage fungi, also called storage molds, are fungi that invade grain or seeds during storage. Fungi belonging to the *Aspergillus* and *Penicillium* genera or the *Mucoraceae* family develop in stored seeds (Majchrzak et al., 2008). These fungi are usually not present in significant numbers before harvest. Small amounts of their spores may be present on grain entering storage or on harvesting, handling, and storage equipment. Under improper storage conditions, these small amounts of spores can rapidly increase and cause significant problems. Fungal species found in barley grains might change over time, particularly during storage, an example of this is the species in the genera *Aspergillus* and *Penicillium*, starting to develop and multiply after harvest, when the water activity of grains drops to low levels during drying and storage (Felšöciová et al., 2021) The temperature and humidity required for malt production are also favorable for fungal spore germination and hyphae development (Lu et al., 2022). Therefore, after harvest

and before storage, barley is usually stored at a moisture content below 14% (Krasauskas, 2017; MacLeod & Evans, 2015).

Even though the cereal grains are colonized by many microorganisms, they are initially infected with the "field fungi", which can be saprotrophs or pathogens (Majchrzak et al., 2008). Common field fungi like *Cladosporium* and *Alternaria* thrive best in environments with relatively high water-activity and are acclimated to occasionally abrupt temperature changes (Østlie et al., 2021). Popular field fungi including *Alternaria spp., Aureobasidium spp., Cladosporium spp., Curvularia spp., Drechslera spp., Epicoccum nigrum, Fusarium spp., Microdochium spp., Nigrospora spp., Septoria spp.,* and *Trichoderma spp.* are present in the microbial community because of their ability to use the developing grain as substrate without damaging or killing the embryo (Felšöciová et al., 2021). The increased incidence of *Fusarium* and *Alternaria* infections, as well as the formation of several mycotoxins in barley grain and malt, are often linked to a variety of natural factors such as weather, storage duration, and conditions (Lu et al., 2022).

Alongside the aforementioned, the growing conditions in the field, agricultural practices, barley varieties, lodging, fertilization, soil, post-harvest storage conditions, and different malting processes, affect the microbiological community of barley. Kaur et al., (2015) presented in their study that among these factors, the geographical region creates the biggest difference and that different barley and therefore malt varieties tend to form separate fungal communities, having differences in *Fusarium* populations and the mycotoxin formation.

Barley is vulnerable to the fungi *Fusarium* spp., which causes a variety of infections throughout crop growth. Even more, is that the most economically harmful disease for barley use is thought to be *Fusarium* Head Blight (FHB), causing major losses in crop production and quality, as well as safety concerns regarding grain contamination with mycotoxins (Inbaia, 2023). It has been observed by Chen et al., (2022) that *Fusarium* species especially proliferate during the steeping stage and followingly inhibit the growth of yeast used in processing stages after malting (such as fermentation). In addition to inhibiting yeast growth during the fermentation process, mycotoxins produced by *Fusarium* species can also be immunosuppressants and may be associated with health

disorders (e.g. cancer, hematological disorders, and endocrine dysfunction, etc.) (Chen et al., 2022). *Fusarium* species are also known to reduce wort β-glucan and viscosity, increase soluble nitrogen and free amino nitrogen, and darken the wort color. These changes in malt properties are indicative of the action of fungal enzymes, such as excessive starch degradation, and higher protease and β-glucanase activities, reported in *Fusarium*-infected grains (Kaur et al., 2015).

While the high moisture content during steeping (around 45%), combined with low temperature might enable the germination of fungal spores and mycelium growth, the high temperature and low humidity level of the kilning step reduces the microbial load in malt compared to barley and restricts microbial activity (Rani & Bhardwaj, 2021). However, heat-resistant fungi like *Rhizopus*, *Mucor*, and some *Ascomycetes* can still thrive in malt, owing to their biofilm-forming ability (Lenaerts et al., 2011). Species that can persist after kilning are generally heat-tolerant *Ascomycetes* species such as *Candida* spp and *Pichia* spp (Chen et al., 2022).

Fungi that colonize cereal grains can also synthesize numerous secondary metabolites, the presence of which can be harmful to humans and animals (Majchrzak et al., 2008). Various strategies, such as fungicide treatment and nitrogen fertilization during malting or changes in temperature and moisture schedule, intensive steeping, and ensuring shorter germination time, can be adopted to prevent fungal growth and mycotoxin production (Lu et al., 2022).

Laitila et al. (2011) examined the applicability of yeast strain commonly present in malting barley as a biocontrol agent to restrict the growth of harmful fungi due to the observed strong antagonistic activity of the yeast. Biocontrol agent applications which suppress harmful microorganisms while simultaneously enhancing the beneficial ones, are suggested in production facilities to improve efficiency.

2.6 Proteomics

One of the most recent methods for evaluating the protein in cereal grains is proteomics (Labuschagne & Igrejas, 2020). The term "proteomics" was first used by Wilkins et al. (1995), who defined it as the study of proteins in terms of their structure, composition, functions, and relationships that control cellular activity (Labuschagne & Igrejas, 2020). Since genomics only provide an approximate estimate of protein expression, proteomics provides deeper insight of an organism.

Proteomics studies began with 2-D gel electrophoresis with mapping protein expression (Finnie & Svensson, 2009). With this method, it was feasible to segregate proteins from complicated mixtures of cellular samples into individual polypeptides, however, the protein identification was slow due to the lack of fast sequencing techniques. With the introduction of high-efficiency peptide ionization techniques in mass spectrometry (MS), quick and extremely sensitive protein identification and bioinformatics tools for analyzing the data of 2-D gels and MS emerged (Maron et al., 2007). While 2-D gel electrophoresis was applied in early barley seed proteins; in the recent decade with a widespread availability of mass spectrometry, proteome analysis has been utilized in this field of research to study the changes in the cereal crop's seeds, aiming to enhance our understanding of their protein composition and function (Maron et al., 2007).

Thanks to biochemical and molecular research, the understanding of the genetics, structure, and form of storage proteins has greatly increased during the past few decades. Although genome sequencing offers a basis for comprehending the function of genes, it falls short of explaining the mechanism by which genes are translated into proteins and the roles that proteins play in biological processes. Gene expression patterns are revealed by transcriptomics; however, because of post-translational modifications (PTMs), mRNA levels may not necessarily correspond to these protein levels or the vast number of proteoforms might be formed (Dupree et al., 2020; Zhang et al., 2013). However, through MS-based proteomics, it is possible to make sequence analysis, investigate protein-protein interactions, and identify post-translational modifications (Dupree et al., 2020).

Proteins may be distinguished using reference protein sequences from publicly accessible databases and separated based on their mass-to-charge ratio by MS, an

effective approach in the proteomics field. The principal approaches of quantitative proteomics can be found in Figure 2.5.

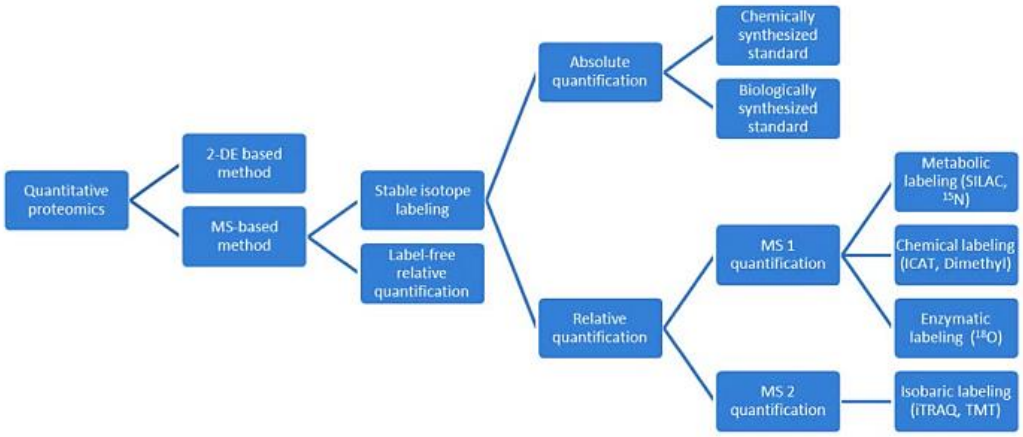


Figure 2.5 Classification of the principal approaches in quantitative proteomics (Y. Zhang et al., 2013)

Omics research for malting barley is an emerging field and especially the metabolomics approach is high in demand for plant and food science (Frank et al., 2011). Metabolites being an indicator of end products coming from the interaction between genome, transcriptome, proteome and the environment explain the biochemical phenotype of the biological system (Frank et al., 2011). Plant-microbe interactions with proteins, including enzymes, are of great interest. Metaproteomics stands as a complementary approach to metabolomics, as it helps identify microorganisms associated with the production of metabolites (Van Den Bossche et al., 2021). By identifying proteins and enzymes that support plant growth, it is possible to develop effective microbial inoculants—beneficial microbes that will increase crop yield and quality. Especially by understanding the proteins and enzymes involved in plant-microbe interactions, new control strategies, and resistant crop varieties have room to grow. In Figure 2.6, such dynamics between the barley crop and its microbiome (bacteria & fungi) are given, showing the present proteins and enzymes stimulated through their recognition of each other.

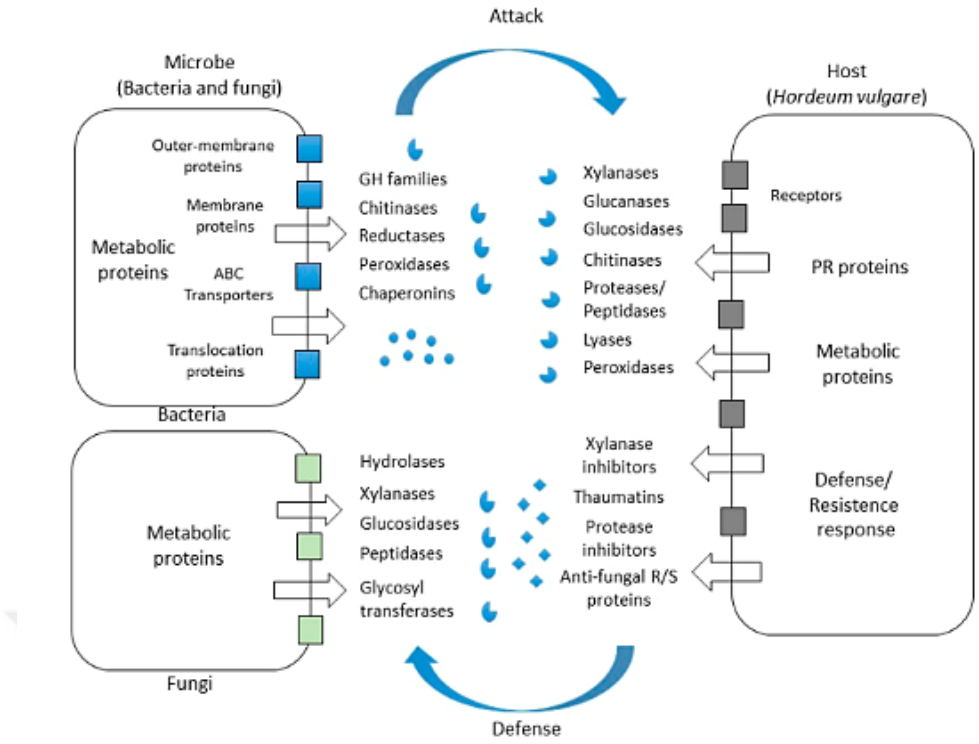


Figure 2.6 An overview of plant-microbe interactions, including discovered proteins and enzymes at the interface (Sultan et al., 2016)

As explained, the germination of seeds is a complex process involving communication between different tissues. Membrane proteins play a crucial role in this process, however, studying them is challenging due to their low abundance and amphiphilic nature (Finnie, Andersen, et al., 2011). It is anticipated that the membrane proteomes of barley seed tissues will differ greatly from one another (Finnie, Andersen, et al., 2011). Separating membrane proteins from the relevant tissues is essential for understanding their function in seed germination.

Almost two-thirds of the proteins that change during malting are present through the stages of steeping and germination (Mahalingam, 2018). This implies that these key metabolic processes are synchronized when malting. In which, there is a particularly high concentration of proteins related to translation, metabolism of carbohydrates, and stress response. Targeting proteins involved in the metabolism of carbohydrates, particularly those that regulate enzyme activity, may help enhance the malting quality. Similarly, finding the proteins that differ in quantity during the different phases of malting can be expected to lead to the development of innovative breeding techniques.

2.6.1 Bottom-Up Proteomics

Bottom-up proteomics involves analyzing peptides from complete protein digests using a protein database to characterize where this peptide originated from.

Sample preparation for this method includes extracting proteins from the biological matrix, removing nonprotein contaminants, such as DNA, sugars, and lipids, removal of the residual salts that may form adducts during ionization, and protein fractionation to reduce sample complexity (Dupree et al., 2020). More cleavage sites for proteases are exposed by unfolded proteins, which are a more thorough breakdown of the protein into peptides; this is useful for the bottom-up approach and helps to generate more peptides for MS analysis. Failure in bottom-up proteomics can occur due to incomplete lysis, incomplete solubilization of proteins, and contamination during sample handling with compounds that interfere with downstream analysis steps (like MS).

The bottom-up approach is used to examine proteolytic peptides while the top-down method is used to measure the intact proteins. Not only these two approaches but a middle-down method is also applicable where larger peptides that are formed by more selective proteases or by limited digestion are evaluated (Y. Zhang et al., 2013).

This thesis utilizes a bottom-up proteomics workflow, including LC-MS/MS and database search, to investigate the barley microbial community.

2.7 Metaproteomics

While DNA sequencing approaches are widely used technologies that can reveal the genes present in a sample, these end up only indicating the potential activities of those organisms (Blakeley-Ruiz & Kleiner, 2022). Metaproteomics, however, serves as an umbrella term for the approaches that take things a step further by identifying and quantifying the proteins that are present in a population of microorganisms. By examining proteins, it is then possible to determine which genes are useful in processes and obtain a more comprehensive understanding of molecular activities. The term proteome refers to the set of proteins in a single organism and “metaproteome” describes the complete protein collection of the various microorganisms found in the target community (Van Den Bossche et al.,

2021). Metaproteomics is the study of the protein components found in an ambient microbial population at a certain time. This approach examines the proteins of a mixed microbial community using the same procedures as proteomics for pure cultures: sample preparation, protein extraction, separation, and MS analysis (Bond & Wexler, 2007).

In metaproteomics, complex protein mixtures from environmental samples are often separated using 2D gel electrophoresis or liquid chromatography (LC). After protein separation, high-throughput mass spectrometry (MS)-based analytical tools are used to analyze fractions of interest (Dupree et al., 2020; Panigrahi et al., 2019). The protein prediction and subsequent identification are greatly facilitated by available relevant metagenomic sequence data (Panigrahi et al., 2019). The findings of the research are combined with data from microbial ecology, genetics, plant physiology, and computational biology to create complete metabolic pathways that offer a clear and simple picture of how the ecosystem functions (Srivastava et al., 2019).

The analytical chain of LC/MS can be divided into three stages: separation, interface, and mass analysis (Blasco & Picó, 2007). Peptides are further broken down by a process known as dissociation before being analyzed by mass spectrometry. There are types of dissociation such as collision-induced dissociation (CID), electron capture dissociation (ECD), electron transfer dissociation (ETD), and high-energy collision dissociation (HCD) (Dupree et al., 2020).

Mass spectrometry includes:

- an ion source to ionize target substances; with ionization, the sample molecules are converted into charged particles (ions).
- a mass analyzer to measure mass-to-charge ratio (m/z) since the ions get separated inside the analyzer based on this ratio and
- a detector to detect the number of ions at each m/z value (Dupree et al., 2020; Hernychova & Hubalek, 2011).

For the last 20 years, the use of MS for biological sample analysis has grown significantly due to the development of electrospray (ESI) and matrix-assisted

laser desorption/ionization (MALDI). In MS analysis, these techniques are used to ionize peptides/proteins (Dupree et al., 2020; Hernychova & Hubalek, 2011).

There are different types of mass analyzers used in proteomics: ion trap, quadrupole, time-of-flight (TOF) and Fourier transform ion cyclotron (FT-MS) (Dupree et al., 2020). Thanks to these analyzers, sensitivity, mass accuracy, and resolution are maintained and information-rich ion mass spectra, i.e. MS/MS spectra, are obtained from peptide fragments (Dupree et al., 2020).

Another example of a mass analyzer is the Orbitrap which works based on the principle of ion trapment. Orbitrap in brief explanation, traps ions and measures their m/z based on their behavior in the trap. The Orbitrap analyzer can be coupled with either ESI or MALDI sources depending on the specific needs of the analysis. Since ESI ionizes the molecules is rather gentle with minimized fragmentation and is often used for analyzing biological samples like proteins and peptides. ESI is a common choice for Orbitrap mass spectrometers.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) can be used for proteomics profiling, especially in the identification of large biological samples (Oldham & Held, 2023). Through this technique, with MALDI, mass profiles of ribosomal proteins or whole cells are detected and measured by the spectrometer. The MS spectra are then produced by measuring the mass-to-charge (m/z) ratio of peptides (Labuschagne & Igrejas, 2020). The resulting spectra are compared to mass fingerprints to determine the source of the proteins or cells. Comparison of mass spectra is achieved by matching with reference databases (Oldham & Held, 2023).

In the ESI technique, the liquid solution of the sample is ionized, therefore it also works compatible with LC for separation (Hernychova & Hubalek, 2011). ESI is a very soft ionization technique, especially well suited for ionic compounds and compounds with a high molecular weight (Blasco & Picó, 2007).

TMT labeling is the use of isobaric tags to label peptides from different samples, with this method, the ability to distinguish closely located mass-to-charge ratios (m/z), e.g., isotopic peaks of high-mass ions, and thus to determine their charge z, allowed researchers to measure their masses “m” with relative accuracies at the level of $< 10^{-7}–10^{-6}$ (Zubarev & Makarov, 2013). TMT isobaric mass tags and

LC-ESI-MS/MS were used in this thesis study. The benefit of using an isobaric label is that it doesn't contribute more to the complexity of the MS1 scan or decrease the precursor signal sensitivity since all the tags lead to the same mass increase for each labeled peptide (Y. Zhang et al., 2013). While isobaric labeling is a powerful tool in quantitative proteomics, it suffers from a lack of quantification accuracy, which is largely influenced by the co-fragmentation of co-eluting peptides with similar m/z (Y. Zhang et al., 2013). This refers to that, sometimes, peptides from different samples (labeled with different isobaric tags) might have very similar masses (m/z) and elute (come out of the chromatography column) together. When this happens, these peptides can fragment together in the MS/MS, creating a mixed spectrum. This co-fragmentation can make it difficult to accurately distinguish the signal from each labeled peptide, leading to inaccuracies in quantifying the protein abundance across different samples. Barley grain proteins were isolated from the samples for identification and quantification. The isolated proteins were then digested into peptides using trypsin and labeled using TMT. These peptides were separated by liquid chromatography based on physicochemical properties before being analyzed in mass spectrometry. In this step, mass spectrometry occurs twice. This technique, in which both intact peptide masses (MS1) and the masses of their fragments after fragmentation (MS2) are measured in the mass spectrometer (see Figure 3.9 for the visualization of this method), is called tandem mass spectrometry (Blakeley-Ruiz & Kleiner, 2022).

Understanding the metaproteome of the cereal grains can lead to controlling that microbial community to improve malting quality and tailoring desired properties. Modifying the microbial community can enhance malt quality by improving grain germination, lautering performance, extract yield, and wort filtration. The majority of microbial investigations carried out on barley or malt samples have employed conventional culture-dependent techniques that compare the quantitative changes in microbial populations. These traditional techniques which are dependent on the microbial cultures are performed by selectively concentrating fast-growing microorganisms. However, they end up comprising just a small portion of the local microbial community (Kaur et al., 2015). Evaluation of microbial community dynamics and diversity are possible with

culture-independent techniques like metaproteomics where it's possible to analyze the entire set of proteins present in a sample from an environmental source without the need to isolate individual microbes in culture.

Metaproteomic investigations can improve malt properties through physical and chemical treatments, such as inoculating beneficial *LAB* or filamentous fungal species, inactivating microbial populations on barley grains, and selecting appropriate malting treatments (Li et al., 2018). Improving the characterization of barley and malt-associated microbial communities can be expected to open up various possibilities for improving the malt's properties and ensuring the safety of the brewing process and the final product.

Manually interpreting every spectrum for a complicated protein/peptide combination obtained from an LC-MS/MS run is not feasible. Therefore, using software to automate the identification of potential peptide sequences from MS/MS spectra is the key and one of the most significant contributions to the area of mass spectrometry-based proteomics. A bioinformatics method called "database search" is used to identify these proteins, matching peptide tandem mass spectra with theoretical spectra obtained from the protein database. These databases of proteomes are derived from publicly available references that can be accessed on NCBI RefSeq or UniProt (Blakeley-Ruiz & Kleiner, 2022).

The searchable database is constructed with the microbial community of barley seeds and the bacteria and fungi anticipated to be present in the malting regime.

For successful and routine testing, microbial detection/identification methods must be fast, specific, reliable, quantifiable, sensitive, and affordable. Numerous sectors, including medical, pharmaceutical, water purification, biological, virology, epidemiology, forensics, food and beverage, and agriculture can benefit from the use of these methods (Oldham & Held, 2023). The fact that the results are specific and quantifiable provides the clearest information about the microbe causing contamination and its amount, helping to handle it appropriately (Oldham & Held, 2023).

In barley-associated metaproteomics studies, the presence of false positives may result in the unnecessary treatment or destruction of products, while false negatives cause the neglections of microorganisms and prepare the ground for

their persistence, growth, and spread in the contaminating population (Oldham & Held, 2023). It also needs to be remembered that in studies based on mass spectrometry matching, it is not possible to ensure full spectrum matches with reference samples due to different equipment, operators, geographical locations, experimental setups, and similar reasons. And in case of encountering a new microbial strain where its mass fingerprint is not available in the reference database, without a positive control -ideally a well-characterized strain of the type of microbe being attempted to identify-, this isolate cannot be identified (Oldham & Held, 2023).

Protein databases, such as UniProt, serve as extensive repositories of protein sequence information. UniProt is the primary worldwide resource for knowledge on protein sequence and function; and a joint project of the Protein Knowledge Resource (PIR), the Swiss Institute of Bioinformatics (SIB), and the European Bioinformatics Institute (EBI) (Nudin et al., 2018). The database contains more than sixty million protein sequences, of which half have been carefully selected by specialists based on the available experimental information and by prediction (Nudin et al., 2018).

The algorithms strive to match the experimental data to the most probable protein sequences (Bahule et al., 2024). However, the complexity of biological systems often presents significant challenges. Enzymatic or chemical hydrolysis of polypeptide aliquots followed by peptide separation generates overlapping sets of smaller fragments, which, when analyzed from complex samples, often produce spectra with overlapping peptide signals (Bhagavan, 2002; Zhang et al., 2013).

This overlap might happen in case of a mass difference that is too small between the labeled and unlabeled peptides (Zhang et al., 2013). Once the peaks representing the peptides in the mass spectrum overlap, it makes it difficult to accurately measure the intensity of each peak, giving a high number of false positives, causing quantification errors as the software might miscalculate and incorrectly identify the abundance of each peptide (Zhang et al., 2013).

A statistical measure known as the adjusted p-value (APV) is used to correct for multiple hypothesis testing, to make sense of the generated ambiguous results employed by the algorithm. In proteomics, while thousands of proteins are

typically analyzed simultaneously, they increase the likelihood of false positives. Adjusting the p-value helps to control the false discovery rate (FDR), ensuring that identified protein changes are more reliable.

FDR (False Discovery Rate) is also a statistical measure used to control the number of false positives in peptide identification. While not directly related to isotopic peak overlap in MS1-based quantification, it is influenced by the overall accuracy of peptide identification. Lower FDR values indicate higher confidence in peptide assignments, reducing the likelihood of incorrect identifications due to factors such as spectral similarity or database errors (Blakeley-Ruiz & Kleiner, 2022; Dupree et al., 2020). Additionally, the existence of protein isoforms, post-translational modifications, and sequence variations cause further complications to the matching process. In instances where multiple proteins share highly similar peptide sequences, differentiating between these proteins becomes particularly challenging. Alongside incorrect protein identifications, it can also result in the underestimation of protein diversity within the sample.

Turvey et al., (2017), exemplifies the importance of false positives in their study; showing that the microbes that are listed under different spoilage categories (e.g. obligate or potential) for the end product might come from the same genus, supporting the need to provide a species-level identification of microorganisms.

3
MATERIALS AND METHODS

3.1 Sample History

The sample history is vital information for meta-omics of the malting barley and in this study, they were sampled through the accelerated malting regime (AMR). This work aimed to find the bacterial and fungal protein expressions for the AMR with increasing temperatures during germination or to define the difference between the temporal changes of these expressions. The barley samples used in this thesis are part of the projects conducted at KU Leuven Laboratory of Enzyme, Fermentation, and Brewing Technology.

In the following sections, the terms 'cultivar' and 'variety' have been used interchangeably to describe the barley types used in the accelerated malting regime. This decision can be explained by the historical and cultural context of barley cultivation in France and England, where the sampled grains initially came from. The farmers in these regions have cultivated barley for centuries, selecting and reproducing plants with desirable traits such as high yield, disease resistance, and specific malting characteristics. This long history of domestication and selection has led to the development of numerous barley populations with unique attributes like the ones utilized in this study. While some of these populations may have evolved naturally and may be termed 'varieties' in the strictest sense, many have been purposefully created and maintained by farmers, making them more correctly categorized as 'cultivars'.

Furthermore, the commercialization of barley for malting purposes has further complicated the terminology. Maltsters frequently refer to the barley they utilize as 'varieties', even though these are *cultivars* created and maintained by seed companies. This widespread usage reflects the malting industry's practical considerations, which prioritize the functional attributes of barley over its precise taxonomic categorization.

3.1.1 Barley Cultivars

For this study, the barley cultivars "Fandaga", "Fantex", "Planet", and "Faro" were used, with the first three being two-row spring barley cultivars and the last being a six-row winter barley cultivar. These were abbreviated in the following sections as FAN, FAR, FTX, and PLT, respectively. FAN, FAR, and FTX originate from France, and PLT is from England. This barley quality analysis is performed by Albert Maltings (Puur-Sint-Amands, Belgium) and the mechanical and physicochemical properties of four barley varieties used in the study can be found in Table A.1.

3.1.2 Pilot Scale Malting Process for Accelerated Malting Regime

The samples of barley employed in the study were taken from the four different barley cultivars that germinated in AMR. The subjected AMR was the pilot-scale malting plant at the Faculty of Engineering Technology, KU Leuven Technology Campus Ghent.

In the malting regime, the samples of raw barley (2,000 g for each of the four types) were put into cylindrical mesh stainless steel cans (with dimensions 20 cm diameter × 30 cm height) that tightened with a lid to hydrate the barley grains. The three biological replicates employed for each cultivar yielded twelve buckets that were poured into the cylindrical-conical steeping vessel with 700 L of water (pH 8.2). The grains were soaked for 5 hours and 30 minutes in the first wet steep with a temperature set to 22 °C.

An air rest for 18 hours at 21 °C was conducted after the first steep and the second wet steep was at 20 °C for 1 hour and 15 minutes. After steeping, the grains' moisture content was tested using a rapid moisture analyzer (HE73, METTER TOLEDO, Switzerland), and the moisture was adjusted by spraying water on the grains until a final moisture content of 45-47% was achieved.

The buckets were then moved to the germination/kilning vessel and allowed to germinate for 74 hours. The specified germination temperature was at 20 °C. During germination, the buckets were heavily aerated with humidified air (95% > relative humidity > 97%) and manually stirred twice a day to prevent the developing barley rootlets from clumping together.

The resulting % moisture content of green malts of the four barley cultivars is presented in Figure A.1, the experiment and analysis are collected by Albert Maltings (Puur-Sint-Amands, Belgium).

After germination, the samples were kilned by blowing warm air through the germination/kilning tank.

3.1.3 Sampling Points

Protein alterations happen gradually throughout the stages of steep and germination. This suggests a coordination between the important metabolic processes of malting. Therefore, samples from the steep and germination stages of malting were chosen so that metaproteomics could be used to track temporal changes in the barley microbiome during the accelerated regime.

The diagram which summarizes the AMR conditions and the sampling points for the study is given in Figure 3.1.

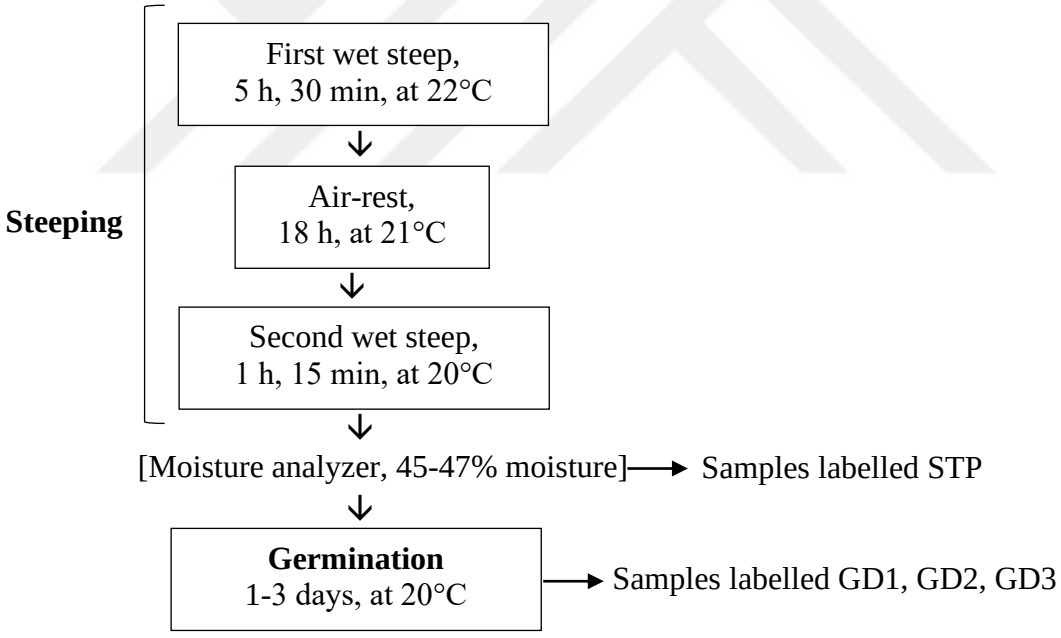


Figure 3.1 Accelerated malting regime steeping and germination information relevant to the sampling points

The sampled barley grains were; the grains taken before the onset of germination / at the end of the steeping process (labeled as STP), and the chitted germinating grains for the three germination days (labeled for the following days of

germination, as GD1, GD2, and GD3). This “chit” formation can be seen in Figure 3.2.



Figure 3.2 Seed morphologies of various malting stages used for proteome analysis for barley variety Faro, samples belonging to malting stages: end of steep, ends of germination day 1 (showing chitting), germination day 2 and germination day 3.

Each variety-specific time-point set had three duplicates, which were labeled and gathered into 5-milliliter Eppendorf tubes. Within the framework of the master's thesis, 48 samples—four distinct barley variety and their four time-stamp points (STP, GD1, GD2, and GD3) with triple repetitions each—were examined.

3.2 Sample Preparation for the Metaproteomic Analysis of Barley Microbiome

To prepare samples for metaproteomic analysis, protein and microorganism extraction was performed. The obtained microbial proteins were precipitated; the resulting pellet was purified and resolubilized. The protein sample was then ready to be quantified in BCA Assay to calculate the amount necessary for the protein digestion into peptides. The following sections of 3.2 give the details of this methodology. The experimental procedure for the barley metaproteomics analysis, showing protein precipitation, resolubilization of the protein pellet, protein quantification and digestion steps, briefly can be found in the Appendix as Figure A.2.

3.2.1 Microbial Protein Extraction

The barley grains of Steep, Germination Day 1, Germination Day 2, and Germination Day 3 for varieties of Fandaga, Fantex, Planet, and Faro that were stored in 5 mL Eppendorf tubes were thawed and followingly weighted. The average empty tube weight (derived from ten 5 mL Eppendorf tubes) was deducted from the weight of Eppendorf tubes containing the samples to find the net weight of the samples. Each sample weight is then approximated to 3 grams. The barley grains were suspended with 3 mL of saline solution (137 mM NaCl) to reduce the osmotic stress on the microbial cells. The samples were ultrasonicated with water bath extraction for 15 minutes at room temperature.

With the saline solution, the microbes on the grains were removed especially from the surface of the barley to the liquid. The liquid phase in the 5 mL Eppendorf tubes was transferred to corresponding labeled 50 mL Falcon tubes using syringes to avoid the accumulation of debris. Barley grains remaining in 5 mL Eppendorfs were then treated with approximately 3 mL 137 mM NaCl solution and ultrasonicated for 15 minutes again to aid in higher expression of microbial diversity for the samples.

The bottom of the Eppendorfs were punctured with a needle, creating a hole underneath to wash the liquid down. These sample containing punctured Eppendorfs were fitted on top of 5 mL Eppendorfs without caps that served as “collection” vials. The sets of two Eppendorfs stacked were secured together using parafilm to prevent the sample tube from popping out and liquid from spilling during the centrifugation process as shown in Figure 3.3.

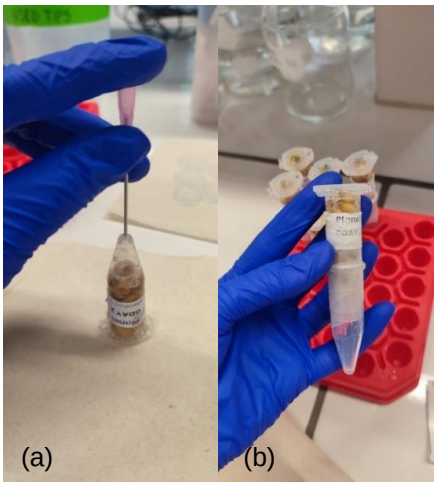


Figure 3.3 (a) Puncturing the bottom of 5 mL Eppendorfs with a needle; (b) fitting the Eppendorf on top of an empty vessel to collect the rinse solution containing microbial cells and debris.

These coupled Eppendorf tubes were then placed for centrifugation (Figure 3.4). This centrifugation was used to separate the microbial cells in the liquid phase from the grains and increase the collected sample amount (to fully transfer the liquid with microbial diversity) for further experiments. The samples were centrifuged at 2,000 xg (4°C) for 2 minutes in a fixed-angle centrifuge. The liquid phase rinsed down in collection tubes were transferred to the previously mentioned 50 mL Falcon tubes by pouring. The volumes were then recorded for the samples in 50 mL Falcon tubes.



Figure 3.4 Samples placed for centrifugation

3.2.2 Protein Precipitation

To extract and precipitate the proteins from isolated microbes and precipitate protein contained within the liquid, ice-cold (stored at -20°C) 20% TCA / 80% acetone solution was added to the samples with a ratio of 1:4 (volume of sample: volume of TCA-acetone solution). This step was carried out under a fume hood due to the need for ventilation for working with trichloroacetic acid. The samples were vortexed before being placed at -20°C for overnight incubation to ensure complete protein precipitation.

3.2.3 Treatment of the Protein Pellet

The samples for meta-proteomics analysis were maintained on ice as much as possible throughout the steps outlined below in section 3.2.3 until section 3.2.3.2. In protein precipitation, it's aided for the proteins to be accumulated as a pellet, for this a two-step centrifugation was followed. The first centrifugation step was used to concentrate proteins to the bottom of the tubes and carried out in a swing bucket centrifuge (axial) at 4,000 xg (-10°C) for 2 minutes to prevent the precipitate from smearing against the side of the tubes which would eventually cause difficulty in resolubilizing the pellet with a small volume of urea in the following steps of the experimental procedure. The second centrifugation is carried out in a fixed-angle centrifuge at 8960 xg (-10°C) for 18 minutes.

3.2.4 Purification and Resolubilization of the Protein Pellet

The supernatant was discarded after fixed-angle centrifugation, and 1 mL ice-cold (-20°C) acetone was added to the pellet to further remove excess pigments and wash away any leftover TCA that would interfere with pellet re-solubilization. The samples are incubated for 30 minutes at -20°C with intermittent vortexing every 10 minutes After discarding the acetone, the pellets were left to air-dry for 15-20 minutes inside the fume hood. The size of the protein pellet varied for steep and germination samples as shown in Figure 3.5.

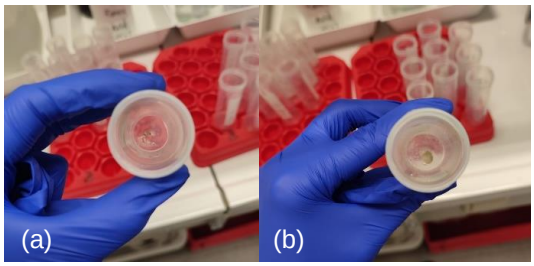


Figure 3.5 Size comparison of the air-dried protein pellets for (a) steep and (b) germination day 2.

Proteins can dissociate and unfold into their natural conformations when urea breaks non-covalent connections between, such as ionic bonds, hydrogen bonds, and hydrophobic interactions. This is necessary for trypsin digestion and later phases in the metaproteomic analysis process, such as allowing the proteins to be separated by electrophoresis and analyzed by mass spectrometry.

Once the acetone had evaporated fully, a gradient addition of 8 M urea (prepared in 50 mM Ammonium Bicarbonate (AmBic)) was carried out to resolubilize the protein pellets. Proportional to the pellet size, the amounts of 8 M urea administered to the time-points S, GD1, GD2, and GD3 were 300 µL, 400 µL, 450 µL and 500 µL, respectively. 8 M urea is essential for protein solubilization, and denaturation in metaproteomic analysis. It can also lead to excessive protein unfolding and loss of native structure. It is always favorable to keep the amount of urea in low volumes as adding this solvent in high volumes also dilutes the sample and causes a need for higher volumes of samples in further analyses. In this thesis, the sample preparation was carried out to enhance the microbial diversity, therefore the protein concentration of the samples was still obtained at a high rate regardless of the diluting urea amount.

Protein samples were incubated at room temperature for 40 minutes. Due to the pellet sizes, the 8 M urea volume was increased by 100 µL for each sample to aid pellet solubilization. The samples were ultrasonicated in a water bath for 5 minutes to aid the re-solubilization process. 8 M urea (without exceeding a total volume of 700 µL for each sample) was added to the samples that showed no sign of dissolving. Incubation for 20 minutes with intermittent vortexing every 10 minutes was performed. Samples were treated without exceeding 15 minutes. If necessary, the remaining 10 minutes of water bath ultrasonication was performed.

To avoid heat damage to proteins, the water inside the bath was replaced before each time interval, whether for 5 minutes or 10 minutes of use.

Finally, after completing their resolubilization, protein extracts were transferred in 1.5 mL Protein LoBind Eppendorf tubes and kept at -80°C until the next step of the experiments.

3.3 Protein Quantification using BCA Assay

The Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) was used to quantify the proteins dissolved in 8 M urea. BCA Assay utilizes the biuret reaction to quantify proteins in solution. The biuret reaction is a colorimetric assay that detects the presence of peptide bonds in a sample when copper ions (Cu^{+2}) in an alkaline solution bind to peptide bonds. In its free form, Cu^{+2} is pale blue and as a response to reacting with peptide bonds in the sample, it forms a purple complex. The intensity of the purple/mauve (lilac) color is directly proportional to the concentration of peptide bonds in the sample (Figure 3.6). The BCA kit does not contain biuret itself, but it does contain reagents that facilitate this reaction, such as bicinchoninic acid (BCA).

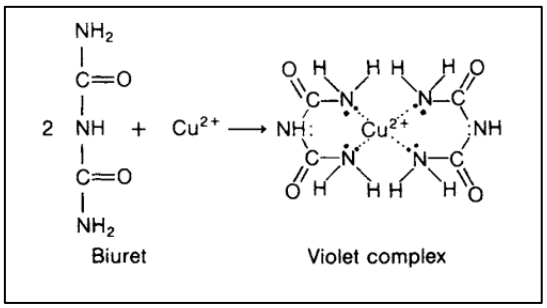


Figure 3.6 Formation of Cu^{+2} and peptide bond complex (together called "biuret reaction") giving a violet-colored complex (Bhagavan, 2002)

Bovine serum albumin is commonly used to calibrate the biuret method because it is a pure protein with a consistent nitrogen content and a known number of peptide bonds per molecule (Bianchi-Bosisio, 2004). Since peptide bonds are the target of the biuret reaction, it is a reliable standard for measuring the protein concentration. In addition to the peptide bonds, cysteine, tryptophan, and tyrosine amino acids can also reduce copper ions. However, peptide bonds are the most

abundant source of reducing groups in proteins, so the biuret reaction is primarily a measure of the peptide bond concentration.

For the BCA analysis, the samples stored at -80°C after 8 M urea resolubilization were thawed at room temperature and the standard is thawed at 37°C. BSA (Bovine serum albumin) standards and sample solutions were prepared according to the final concentrations given in Table 3.1.

Table 3.1 Final concentrations of BSA standards

Tube	Concentration [mg/mL]
A	2
B	1
C	0.5
D	0.25
E	0.125
Blank	0

BCA only works for urea concentrations below 3 M, a 1:3 dilution (with water or 1% SDS) was made. The diluent used for the samples and in preparation of standards must be chosen the same. 10 µLs of samples and standards and 200 µL of working solution were added to each microplate well for the type 96-well plate. The microplate was incubated at 37°C for 30 minutes. Absorbance at 562 nm was measured with a plate reader and sample protein concentration was calculated using the obtained standard curve.

3.4 Protein Digestion and Purification

3.4.1 Protein Digestion using Trypsin

The bottom-up proteomics approach involves several steps before protein digestion can occur. First, proteins are denatured to disrupt their tertiary structure, allowing proteases to access and break them down into peptides. Further, to allow for complete linearization of the amino acid sequence, cysteine bridges need to be reduced and alkylated to prevent their re-formation. Afterward, a specific protease

enzyme, trypsin, is used to cleave peptide bonds, particularly between lysine and arginine residues (Neagu et al., 2022). The proteins are rapidly fragmented into peptides with distinct masses, allowing for amino acid sequence identification and quantification.

The corresponding volumes of samples containing 50 µg protein were transferred to Protein LoBind Eppendorf tubes. Afterward, the volumes of all samples were equalized with 50 mM AmBic. A 1:20 dilution of the stock solution of TCEP/CAA (0.2/0.5 M) was added to the samples. Samples were incubated in the dark for 45 minutes due to CAA being light-sensitive. Benzonase was not added to degrade DNA due to the ultrasonication step applied for the pellet resolubilization and samples being treated with ultrasonication after thawing. In order to reduce the urea concentration of the samples from 8 M to 2 M, dilution was done with 50 mM AmBic. For each 0.05 mg of protein extract, 1 µg of Trypsin was added. Samples were then incubated at 24°C for 21 hours.

3.4.2 C-18 Peptide Clean-up of the samples

200 µL pipette tip (Eppendorf) was cut from the second mark of the bottom with a lancet. Using this new tool made out of a tip, Empore Solid Phase Extraction (SPE) C18 Extraction disk was punched out for the same sizes. This punching method is shown in Figure 3.7 (a). The punched plug was packed into 200 µL low protein binding pipette tips using an 18-gauge flat-top needle as shown in Figure 3.7 (b). The steps were repeated for each tip to contain three plugs.

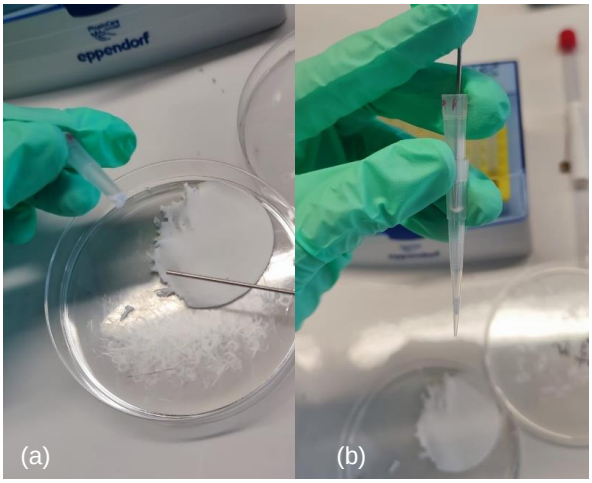


Figure 3.7 Preparation of the "C-18 peptide clean-up column" with the C-18 disk plugs

Tryptic digestion was quenched by the addition of 10% TFA to a final concentration of 1% (vol/vol) TFA.

The tips stacked with plugs were fitted onto 2 mL Eppendorf tube caps that were pierced with a lancet. The caps were placed onto capless 2 mL Eppendorf tubes for easy operation of the clean-up procedure, shown in Figure 3.8.



Figure 3.8 Centrifugation step in the "C-18 peptide clean-up column" with the C-18 disk plugs

- Activation of the C-18 Columns

For the C-18 peptide clean-up 100% (v/v) acetonitrile (ACN), 0.1% (v/v) trifluoroacetic acid (TFA) and 70% ACN / 0.1% TFA (v/v) were prepared. Assembled C-18 peptide clean-up columns were placed inside a bench-top centrifuge. C-18 columns were activated by pipetting 200 µL of 100% ACN on top of the C-18 tips and centrifuged at 500 xg for 4 minutes at room temperature, the flow-through in the collection vial was discarded. The plugs were then equilibrated with 200 µL of 0.1% TFA and centrifuged at 500 xg for 5 minutes, TFA flow-through was also discarded.

- Purification of the peptides

The tryptic digest samples were loaded onto the respectively labeled C-18 column tips and centrifuged at 500 xg for 3 minutes at room temperature. Columns were then washed by pipetting 200 µL of 0.1% TFA, centrifuged at 500 xg for 5 minutes, and flow-through was discarded. An additional 2 minutes of centrifuge time was given for the samples that didn't wash off easily as 0.1% TFA has a high

surface tension. The washing step removes excess urea, TFA, AmBic, and such while the peptides are retained by the resin.

The peptides were eluted into fresh 1.5 mL Protein LoBind Eppendorf tubes. using 50 µL of 70% ACN/0.1% TFA and centrifuged at 500 xg for 5-7 minutes. This step was repeated and the elutes were combined.

Caps with three holes punctured were fitted onto the 1.5 mL Protein LoBind collection vials. The samples were then snap-frozen using liquid nitrogen, freeze-dried for two hours, and stored at -80°C until further analysis.

3.5 Peptide Labelling with TMT Isobaric Mass Tags

The freeze-dried samples were centrifuged at 20,000 xg for 10 minutes before resuspending them to ensure peptides were at the bottom of their sample tubes.

The freeze-dried peptides were resuspended in 9.33 µL of 71.4 mM HEPES solution (pH 8.5), vortexed, spun down, and ultrasonicated in a water bath for 15 minutes. They were spun down again before the addition of the TMT reagent. The TMT Lot used for this thesis was YH 373067. The TMTpro Label Reagent aliquots were all set to the concentration of 13 mM with anhydrous ACN and sat at room temperature before use. The samples were spun down before use, and 4 µL of the respective TMT reagent was added to the corresponding peptide aliquot. In this step the samples were worked as sets of 24, details of TMT 18-plex label reagents, experiment numbers, and MS runs can be found in Appendix Table A.2.

After the addition of TMT 18-plex label reagents, samples were vortexed, spun down, and incubated for an hour at room temperature. 1.5 µL aliquots from each sample were transferred into a new 1.5 mL Protein LoBind Tube. To quench the TMT-labelling process of the 12 aliquots, 1.5 µL of 1% hydroxylamine solution (w/v) was added, bringing the final concentration to 0.5% (w/v). The rest of the samples were placed in the -80°C freezer. To stop the TMT labeling, the samples are vortexed, spun down, and incubated for the next 15 minutes.

For each MS-RUN, the sets of 12 samples each containing a total volume of 3 µL were transferred into a single 1.5 mL Protein LoBind Tube. This multiplexed sample of the respective MS-RUN was snap-frozen using liquid nitrogen and then freeze-dried overnight. The dried peptides were stored at -80°C.

Freeze-dried peptides were centrifuged at 500 xg for 5 minutes followed by centrifugation at 20,000 xg for 10 minutes both carried out at room temperature. The samples were then resuspended in 20 µL of freshly prepared 2% ACN / 0.1% formic acid (v/v), vortexed, spun down and ultrasonicated in a water bath for 15 minutes. The samples were transferred into 0.5 mL autosampler vials and sent to the VIB Proteomics Core (Ghent, Belgium) for LC-MS/MS analysis.

3.6 Raw Data Processing and Peptide Identification

An experimental scheme illustrating the step of peptide-labeling with TMT isobaric mass tags and methodology that follows according to the ionization and separation of peptides in a mass spectrometer is given in Figure 3.9.

Metaproteomics in this study is done by separating the proteins in the barley samples by mass spectrometry identifying the peptides that make up each protein and using the bottom-up method to identify the microorganism that contributes found protein to the malting environment. The identification of these peptides from a complex mixture of proteins is difficult, and followingly the accuracy of the identifications is affected by the quality of the reference sequence database used. The fragmentation of peptides during mass spectrometry and the homology between peptides from different organisms can contribute to false identifications due to similarities between fragments and sequences.

Using a “bigger than need” database can enhance the possibility of having “false positives” which are peptides that are not actually present in the sample but are identified by the search engine because of their similarity to the peptides in the database. Such misidentifications are fully relevant to the incomplete or inaccurate database. In order to employ a reference database for proteomic analysis, relevant search criteria should be used to reduce the number of false positives. Therefore, to reduce false positives, the protein databases should be constructed according to the expectations concerning the samples, peptide thresholds should be set to a reasonable tolerance, and relevant post-translational modifications (PTMs) must be considered (Dupree et al., 2020).

Allocation of experimental data to peptide sequences was performed by the “Comet” search engine. Data analysis is especially critical for metaproteomics,

where the microflora is initially unknown. Using a global database in metaproteomics analysis will produce meaningless and misleading results. It is not possible to unambiguously assign peptides to MS data by peptide sequence length or fragmentation patterns alone. In this case, the amount of peptides identified is a function of the reference sequence database used, the accuracy of the peptides identified, and the amount of peptides misidentified.

The score threshold, which is a cut-off value used to determine the validity of peptide identification in metaproteomics is based on the similarity between the experimental peptide spectrum and the database peptide sequences. A higher score indicates a stronger match, and a lower score indicates a weaker match. The choice of score threshold is crucial to balance true and false positives. A lower threshold will increase false positives, while a higher threshold will reduce true positives. The ideal threshold depends on the specific goals, sample complexity, and search engine specificity. Using large, inappropriate databases will result in overfitting and cause true peptide spectrum matches (PSMs) to be filtered out by score thresholds.

The dedicated reference database utilized in this study is specifically tailored by the previous studies of the Enzyme, Fermentation and Brewery Laboratory group, for the microbes that are expected to be present in the barley malting regime. The database was created to include the barley microbiome, the expected bacterial and fungal species, and the microorganisms expected to be seen during malt production. Constructing a comprehensive reference sequence database for metaproteomics analysis of barley required a two-step approach: first, metagenomic sequencing to identify the microbes present in the sample and then using this information to construct a database of their proteins. The reference sequence database was obtained by text-mining metagenomic barley studies published in PubMed.

The bioinformatics analyses of processing the raw data and the identification of peptides were done using R version 4.3.2. Using MSConverter, the raw MS data files were converted to the mzML format. All spectra for each barley cultivar were searched against the constructed database using Comet version 2023.01.

The searches were performed using a 20 ppm precursor mass tolerance with the fragment ion binning set to 0.02 and with 2 allowed missed cleavage sites. The parameters are summarized in Equation (3.1).

```
peptide_mass_tolerance = 20.00

fragment_bin_tol = 0.02

allowed_missed_cleavage = 2
```
(3.1)

Trypsin produces peptides with easily ionized amino acids like lysine or arginine. The TMT tags on lysine residues and peptide N termini and carbamidomethylation of cysteine residues were set as static modifications. The paramaeters are as in Equation (3.2).

```
add_Nterm_peptide = 304.2071
&
add_C_cysteine = 57.021464
add_K_lysine = 304.2071
```
(3.2)

The sample enzyme trypsin with cleavages at the carboxyl side of basic amino acids (lysine [K] and arginine [R]) and a variant of trypsin that cleaves after proline (P) residues were set alongside the enzymes given in the Comet parameter fragment below in Equation (3.3), 1s showing allows.

```
[COMET_ENZYME_INFO]
0.  Cut_everywhere          0       -           -
1.  Trypsin                 1       KR          P
2.  Trypsin/P               1       KR          -
3.  Lys_C                   1       K           P
4.  Lys_N                   0       K           -
5.  Arg_C                   1       R           P
6.  Asp_N                   0       D           -
7.  CNBr                    1       M           -
8.  Glu_C                   1       DE          P
9.  PepsinA                 1       FL          P
10. Chymotrypsin            1       FWYL        P
11. No_cut                  1       @
```
(3.3)

PeptideProphet (Trans-Proteomic Pipeline) was utilized for post-peptide validation. Based on the number of tryptic termini of the assigned peptides and the Comet expectation value (database search score), PeptideProphet provides a probability for each Comet peptide identification indicating the likelihood that it has been accurately recognized. For metaproteomics, control over FDR is necessary. FDR is estimated by matching the reversed sequence database to the

experimentally derived spectra. FDR is estimated by matching the reversed sequence database (mass spectrometry raw data) to the experimentally derived spectra (reference sequence database in Comet). The number of 19,158 unique peptides (5,517 from microbes with FDR = 5%) was calculated.

This means that only the peptides with minimum PeptideProphet probability ≤ false discovery rate of 0.05 were accepted and considered as a match. This was done to restrict the occurrence of false positives within the group of identified peptides to a level of 5%. The whole lists of each variety of barley's identified peptides were exported as Microsoft Excel (.xls) files. R software (version 4.3.2) was used to further process the peptide lists in order to produce the peptide sequences in a format that is readable by the Unipept software.

3.7 Microbial Taxonomic Analysis with Unipept

The combination of Uniprot pan proteomes derived from text mining, pre-study, plus barley and common contaminants consisted of 4,994 taxonomic identifiers (exclusive common contaminants), a total of 19,668,200 protein sequences. For the taxonomic analysis via Unipept, all peptides matched against UniProtKB (UniProt Knowledge Base) (1,304,333 taxonomic entries) to identify the lowest common ancestor organism.

Peptide quantification via an in-house developed R pipeline applying *limma* (Linear Models for Microarray Data), the empirical Bayes technique for two-group comparison in a proteomic study was used. The R programming language was used to create the pipeline, and when the script was executed, all necessary packages were automatically installed. Volcano-plot graphs of the temporal metaproteomics analysis of barley varieties obtained.

3.7.1 Lowest Common Ancestor (LCA)

Sequences retrieved from open databases like UniProt can only have their taxonomy assigned by lowest common ancestor (LCA) methods and similarity searches. Proteins that come from unbinned contigs with many genes are classified using the consensus taxonomy of all the genes in the contig. Species-specific taxonomy, however, cannot be established if available reference genomes

lack sequences that are at least 95% identical to the target protein (Blakeley-Ruiz & Kleiner, 2022).

Using Unipept's LCA technique, all detected peptide sequences (identified at 5% FDR from Trans Proteomics Pipeline) were submitted to taxonomy analysis. The analysis was conducted using Unipept online version 5.0.6, which permitted the use of an advanced missing cleavage handling option. In order to guarantee precise identification of the microorganisms on barley malt during germination, an analysis was conducted to determine the frequency of existence of each species of bacteria and fungi. The identification of species that occurred more than once was done at the species, genera, and family levels.

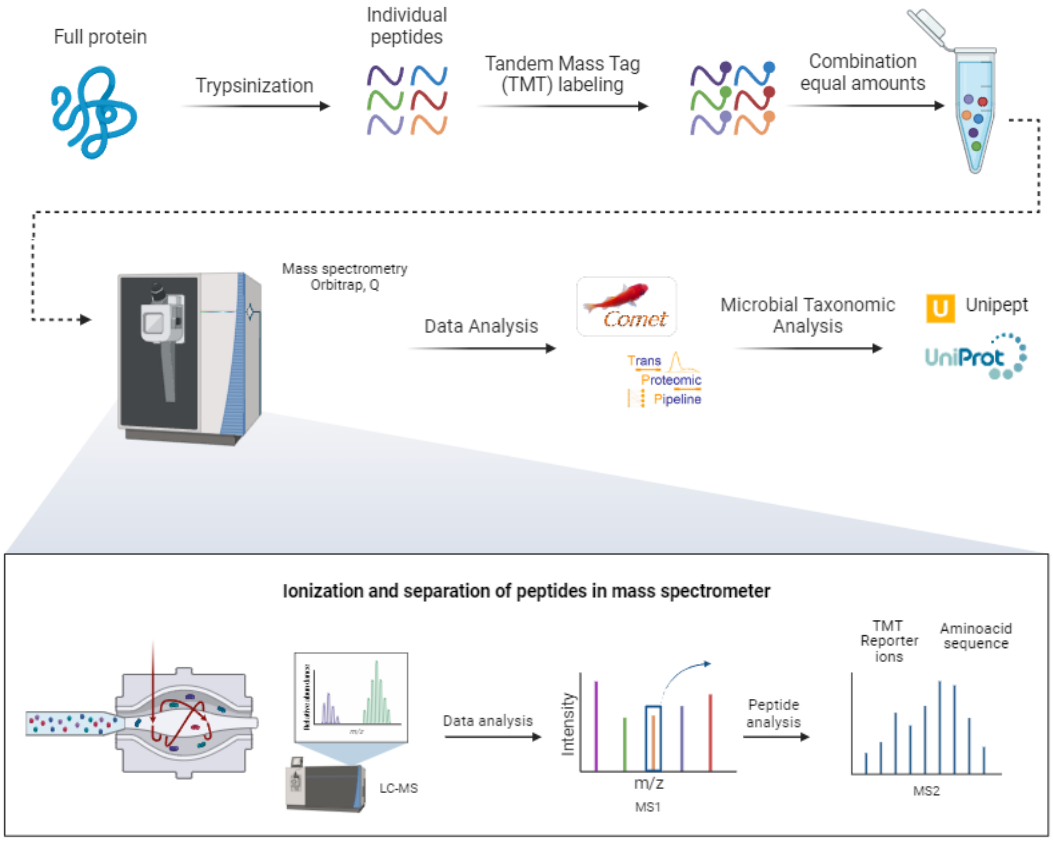


Figure 3.9 Experimental scheme illustrating the step of Peptide-labelling with TMT Isobaric Mass-Tags of the submitted thesis.
Constructed with BioRender.com for this study.

4
RESULTS AND DISCUSSION

4.1 Taxonomic Analysis with Identified Peptides via Unipept Search

The identified peptides were searched with Unipept using their lowest common ancestor (LCA) to link the peptides to microorganisms. The Upset plot of the distribution of identified LCAs between the barley cultivars can be found in Figure 4.1. The Upset-plot of AMR (present below) showed an interesting commonality between the different cultivars of malting barley. Although the set size of AMR is low with around 60, more than half of the identified LCA per cultivar is found to be shared by all four of the barley cultivars. Since the highest intersection size (indicating the unique LCAs) is only achieved by the intersection of all varieties.

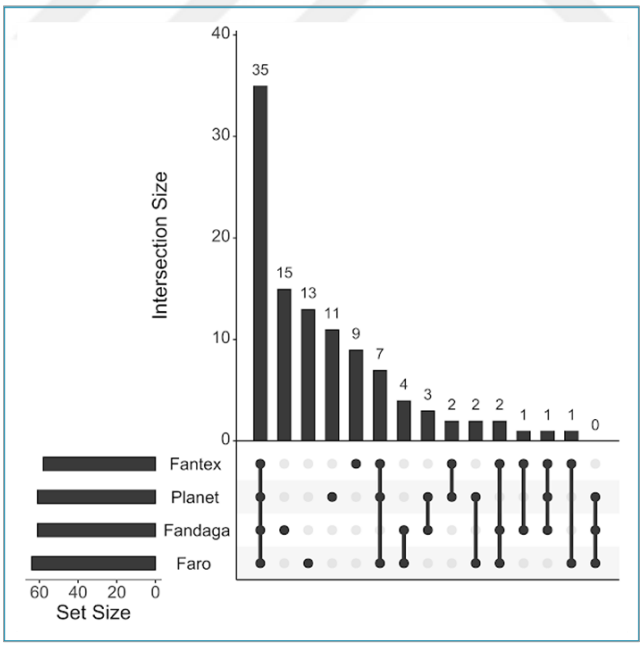


Figure 4.1 Upset-plot of the different LCA set intersections between the different barley cultivars. The set size indicates the number of unique LCA within each barley variety. Dots and lines mark the individual set comparisons.

Once the upset-plot investigation is replicated but to a contrast of the sample time-points, given in Figure 4.2, it shows the expected lowest commonality results for the contrasts of STP, GD1, and GD3 due to the distinction between the steps, which can be explained by the changing dynamic of microorganisms in the malting regime as the malting steps progress. The highest intersection for the unique lowest common ancestors was achieved by the intersection of all time points.

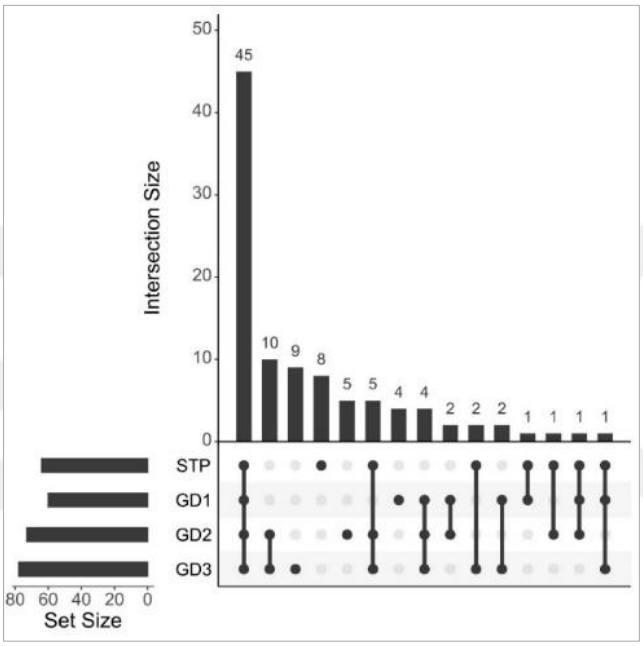


Figure 4.2 Upset-plot of the different LCA set intersections between the timepoints of research. Set size indicates the number of unique LCA within each step of malting. Dots and lines mark the individual set comparisons.

The intersection of the time-points GD2 and GD3 gives the second highest intersection size, indicating the finalization of germinating within the microbial community and showing a stabilization between the last two days of the germination step. Subsequently, though the number of unique lowest common ancestors is lower in comparison to the highest intersection, the intersection sizes of GD3 and STP followed each other. Both germination day 3 and steep results show a variety in terms of microorganisms and their low intersection with each other points out the microbial community's anticipated shift in dynamics.

From this, it can be concluded that there is a main static microbial population after steeping until germination day 3 (GD3) in AMR, with a stepwise addition of a

new enduring population after GD2, and the appearance of another population at GD3.

4.2 Quantitative Metaproteome Analysis of AMR

To investigate the quantitative metaproteome analysis, TMT report ion intensities were extracted from the mass spectrometry raw data files using R and assigned to their respective identified peptide found with Comet search. Peptide identifications having an FDR > 5% were eliminated from the data before it was converted, using variance stabilizing normalization. By combining protein identification with quantitative analysis and statistical significance assessment, it was possible to identify proteins that are differentially expressed between the sampling points. For this, the logFC (log fold change), which measures the change in protein abundance between two experimental conditions is given in Tables 4.1 to 4.5. A positive logFC value indicates an increase in protein abundance, while a negative logFC represents a decrease. The up/down regulation shows where the peptide is between the malting days, an upregulation is the positive logFC value.

In the case of the data labeled as "GD1_STP", a positive fold change indicates a higher peptide abundance in germination day 1, and a negative a higher abundance at steep-out. The probability of finding the peptide as differentially abundant increases as the value of APV approaches 0 and a cut-off value of 0.05 was chosen. This represented a false discovery rate of 5% and indicated that within the group of peptides with an APV $\leq$ 0.05, on average 5% of the peptides declared differentially abundant are false positives.

The most strongly regulated peptides are listed along with the number of proteins the peptide is associated with (N.O.P.), the log fold-change, the adjusted p-values, and the malting time point contrast statistically tested. Additionally, the LCA for the peptide is indicated together with the superkingdom it belongs to.

Tables 4.1 to 4.4 show the top three significantly up/down regulated unique peptides per variety for each tested contrast, e.g. GD1 vs. STP. Multiple significant differences were identified for all cultivars between the contrasts of germination day 1 and steeping or germination day 3 and steeping. However, Planet (given in Table 4.4) only showed regulated peptides for the contrasts

GD2_GD1 and GD3_GD2, while Fandaga (given in Table 4.1) showed a single regulated peptide on GD3_GD2 contrast with the lowest common ancestor being Bacteria, *Acinetobacter*. The details of bacteria and fungi distributions are given in volcano plot graphs in section 4.3. The majority of common LCA in all barley varieties are Bacteria genera *Pseudomonas* and *Acinetobacter*.

The presence of the mentioned (and seen in Table 4.1) single-regulated bacterial peptide for Fandaga during the late germination stage (GD3_GD2) is interpreted as the bacterial population being steady between those stages of germination. This stands in contrast to the more active microbial changes seen in other cultivars. The restricted bacterial response in Fandaga may be a sign of a unique microbial adaptation to the particular genetic background of this cultivar, or it might be a sign of possible technical difficulties in obtaining all of the bacterial protein spectrum

Table 4.1 Top three significantly up/down regulated peptides between stages for the barley variety Fandaga

Peptide (FANDAGA)	nProtein	logFC	APV	LCA	Group	
VDSVGYGETRPVADNATEEGR	32	2.97	3.9E-08	*Pseudomonas*	Bacteria	GD1_STP
EYDDACDPK	800	2.01	1.1E-07	*Pseudomonadota*	Bacteria	
QVLTQQYGVESTR	3	1.83	1.1E-06	*Pseudomonas putida*	Bacteria	
TYLMESDTK	5	-1.05	1.1E-04	*Dikarya*	Fungi	
NGNSTESPNGR	1	-1.40	1.4E-03	*Pantoea*	Bacteria	
STLKPEGQQALDQLYSQLSSMDPK	4	-1.44	3.4E-05	*Enterobacterales*	Bacteria	
DSGQQNSNNNNQQR	11	2.67	5.2E-05	*Acinetobacter*	Bacteria	GD2_GD1
LYNAGQVCTSSK	38	2.02	1.7E-06	*Lactobacillales*	Bacteria	
NNQQQQQQQNDNR	17	2.02	2.5E-03	*Acinetobacter calcoaceticus/baumannii complex*	Bacteria	
FGVMSIPTMIFFK	3	-0.67	3.9E-03	*Paenibacillaceae*	Bacteria	
FALPAVNCVGTDSINAALEAAAK	3	-0.72	5.9E-03	*Pantoea*	Bacteria	
QVAESACAPDDTMGHMPFPVTADDVYNAIVAANAIAQTYK	1	-0.94	1.0E-03	*Paenibacillus nuruki*	Bacteria	

Table 4.1 Top three significantly up/down regulated peptides between stages the barley variety Fandaga (continued)

DSGQQNSNNNNQQR	11	1.31	3.40E-02	*Acinetobacter*	Bacteria	GD3_GD2
GGCGTGGCGCSH	4	-0.41	4.57E-02	*Erwiniaceae*	Bacteria	
IGSEQYNQK	23	-0.42	4.76E-02	*Pseudomonadota*	Bacteria	
AIDMPFLLPIEDVFSISGR	8	-0.51	4.57E-02	*Gammaproteobacteria*	Bacteria	
NDSNAEVAGNR	1	-0.59	1.78E-02	*Erwinia*	Bacteria	
FQQNDTSSR	2	-0.69	4.57E-02	*Pantoea*	Bacteria	
DSGQQNSNNNNQQR	11	4.08	1.1E-07	*Acinetobacter*	Bacteria	GD3_STP
VDSVGYGETRPVADNATEEGR	32	3.13	1.8E-08	*Pseudomonas*	Bacteria	
NYQQAVAGQEGGVR	9	2.85	5.7E-08	*Acinetobacter*	Bacteria	
AIDMPFLLPIEDVFSISGR	8	-1.82	7.8E-08	*Gammaproteobacteria*	Bacteria	
STLKPEGQQALDQLYSQLSSMDPK	4	-2.14	8.3E-07	*Enterobacterales*	Bacteria	
NGNSTESPNGR	1	-2.23	2.8E-05	*Pantoea*	Bacteria	

The differentially abundant peptides for the Fantex cultivar are given in Table 4.2. For this cultivar, the distribution of bacteria and fungi was consistent. It is also noticed that the up regulation was mostly bacterial, and the fungi were only seen in down regulation for a total of 692 regulations.

The pronounced bacterial dominance in upregulated peptides for the Fantex cultivar, in contrast to the absence of fungal upregulation, is intriguing. Given the analysis focused on top significant regulated peptides, it's plausible that less abundant fungal peptides were overlooked. However, considering fungi's established role in plant-microbe interactions, this disparity warrants further investigation to elucidate potential implications for barley quality and malting processes.

Table 4.2 Top three significantly up/down regulated peptides between stages for the barley variety Fantex

Peptide (FANTEX)	nProtein	logFC	APV	LCA	Group	
VDSVGYGETRPVADNATEEGR	32	2.39	2.9E-04	*Pseudomonas*	Bacteria	GD1_STP
NNAGGHANHS	311	1.69	5.8E-03	*Bacteria*	Bacteria	
TWGDLGVTGDLANKPVQLFGR	291	1.66	9.7E-05	*Pseudomonas*	Bacteria	

Table 4.2 Top three significantly up/down regulated peptides between stages the barley variety Fantex (continued)

IALNEIVEQGFEALLK	1	-1.04	1.7E-03	*Paenibacillus nuruki*	Bacteria	
SGSTTGVFCGTIR	1	-1.36	1.2E-05	*Pleosporaceae*	Fungi	
AQVINTNSLSLITQNNINK	731	-1.57	2.3E-03	*Enterobacteriaceae*	Bacteria	
NNQQQQQQQNDNR	17	3.08	1.7E-02	*Acinetobacter calcoaceticus/baumannii complex*	Bacteria	GD2_GD1
NYQQAVAGQEGGVR	9	1.06	1.7E-03	*Acinetobacter*	Bacteria	GD2_GD1
GTLVTFGDVLFDFNK	72	1.01	4.1E-02	*Pseudomonas*	Bacteria	GD2_GD1
MVVTLIHPIAMDEGLR	12	-0.68	1.7E-02	*Gammaproteobacteria*	Bacteria	GD2_GD1
MGGQASHGGGR	6	-1.11	1.9E-02	*Bacteroidota*	Bacteria	GD2_GD1
AHAEDPAEG	2	-1.28	1.7E-03	*Micrococcales*	Bacteria	GD2_GD1
DSGQQNSNNNNQQR	11	2.11	4.8E-02	*Acinetobacter*	Bacteria	GD3_GD2
APGQHGQAR	27	1.74	2.1E-03	*Acinetobacter*	Bacteria	GD3_GD2
GFGFIQQDNGPDVFAHFK	6	1.36	6.0E-04	*Acinetobacter*	Bacteria	GD3_GD2
VPVPTGSITDLTLETK	2	-0.51	2.1E-02	*Bacteria*	Bacteria	GD3_GD2
EQIIEGVAALSVMEIVELISAMEEK	6	-0.64	1.0E-02	*Enterobacterales*	Bacteria	GD3_GD2
SGSTTGVFCGTIR	1	-0.66	6.2E-03	*Pleosporaceae*	Fungi	GD3_GD2
VDSVGYGETRPVADNATEEGR	32	3.48	3.7E-06	*Pseudomonas*	Bacteria	GD3_STP
APGQHGQAR	27	3.48	6.9E-07	*Acinetobacter*	Bacteria	GD3_STP
NNAGGHANHS	311	2.90	6.3E-05	*Bacteria*	Bacteria	GD3_STP
LDFDPLCLQDSPLGIR	1	-1.60	7.4E-07	*Bipolaris sorokiniana*	Fungi	GD3_STP
QYDINEAVALLK	33	-1.74	3.7E-05	*Enterobacterales*	Bacteria	GD3_STP
SGSTTGVFCGTIR	1	-2.23	2.9E-08	*Pleosporaceae*	Fungi	GD3_STP

The results for FARO (given in Table 4.3) exhibited a microbial profile characterized by downregulated fungi and upregulated bacteria as similar to the other varieties. The LCAs were mainly of Bacteria genera *Pseudomonas* and *Acinetobacter* for the variety's top significant peptides in upregulation. Notably, for the top-regulated peptides, the identified fungal genus *Pleosporaceae* was singular and significant. The abundance of these fungi decreasing over the malting process is seen in GD1_STP comparison data. The presence of this genus in steeping is also further supported by the comparison of GD3_STP, where it is

again seen in downregulation, suggesting a potential role for *Pleosporaceae* in the early stages of malt development.

Table 4.3 Top three significantly up/down regulated peptides between stages for the barley variety Faro

Peptide (FARO)	nProtein	logFC	APV	LCA	Group	
VDSVGYGETRPVADNATEEGR	32	3.34	8.1E-06	*Pseudomonas*	Bacteria	GD1_STP
GAGESSADQDQAR	4	2.39	2.5E-05	*Pseudomonas*	Bacteria	
DTDKPLLLPVEDVFSITGR	90	1.69	2.2E-04	*Bacteria*	Bacteria	
SGSTTGVFCGTIR	1	-0.96	3.7E-04	*Pleosporaceae*	Fungi	
GPVPGNAFTVTFR	1	-0.99	3.8E-04	*Acidovorax*	Bacteria	
ETDAAATAEDR	5	-1.22	6.8E-03	*Bacteria*	Bacteria	
DSGQQNSNNNNQQR	11	3.29	3.9E-07	*Acinetobacter*	Bacteria	GD2_GD1
NYQQAVAGQEGGVR	9	1.83	1.5E-04	*Acinetobacter*	Bacteria	
DTDKPLLLPVEDVFSITGR	90	1.44	7.0E-03	*Bacteria*	Bacteria	
NWLIGDAIYTPGTTGDALR	1	-0.48	1.4E-02	*Paenibacillus nuruki*	Bacteria	
YYFTNNLYAR	84	-0.59	1.7E-02	*Pseudomonas*	Bacteria	
NNQQQQQQQNDNR	17	2.77	7.8E-04	*Acinetobacter calcoaceticus/baumannii complex*	Bacteria	GD3_GD2
DSGQQNSNNNNQQR	11	2.43	1.2E-05	*Acinetobacter*	Bacteria	
NYQQAVAGQEGGVR	9	0.92	3.8E-02	*Acinetobacter*	Bacteria	
AAVQEGIVPGGGTALLR	16	-0.42	4.9E-02	*Alphaproteobacteria*	Bacteria	
NWLIGDAIYTPGTTGDALR	1	-0.44	4.7E-02	*Paenibacillus nuruki*	Bacteria	
YVAEALGVTPANILFDK	1	-0.53	4.9E-02	*Paenibacillus nuruki*	Bacteria	
DSGQQNSNNNNQQR	11	5.06	1.0E-08	*Acinetobacter*	Bacteria	GD3_STP
VDSVGYGETRPVADNATEEGR	32	3.99	2.9E-07	*Pseudomonas*	Bacteria	
NYQQAVAGQEGGVR	9	3.45	5.9E-08	*Acinetobacter*	Bacteria	
NDSNAEVAGNR	1	-1.66	1.2E-02	*Erwinia*	Bacteria	
YDANNVYLAAFYGEAR	1	-1.71	4.2E-04	*Erwinia*	Bacteria	
SGSTTGVFCGTIR	1	-1.83	8.8E-07	*Pleosporaceae*	Fungi	

PLT variety exhibited only upregulated peptides identified for GD2_GD1 and GD3_GD2 contrasts, suggesting a potential increase in microbial abundance without significant losses during the malting process. And similar to FARO, PLT displayed a microbial profile dominated by downregulated fungi *Pleosporaceae*

during steeping and upregulated bacteria, primarily *Pseudomonas* and *Acinetobacter*.

Table 4.4 Top three significantly up/down regulated peptides between stages for the barley variety Planet

Peptide (PLANET)	nProtein	logFC	APV	LCA	Group	
VDSVGYGETRPVADNATEEGR	32	2.81	2.3E-06	*Pseudomonas*	Bacteria	GD1_STP
EKPTWLEVDAGK	65	1.67	3.9E-05	*Bacteria*	Bacteria	
NGSVSGENDTGR	19	1.67	1.7E-04	*Enterobacterales*	Bacteria	
NSIDGNGLALK	1	-1.13	1.6E-04	*Paenibacillus nuruki*	Bacteria	
SGSTTGVFCGTIR	1	-1.14	6.3E-04	*Pleosporaceae*	Fungi	
NGNSTESPNGR	1	-1.37	2.9E-04	*Pantoea*	Bacteria	
GTDAYNQK	182	0.56	4.3E-02	*Bacteria*	Bacteria	GD2_GD1
DSQEYVSK	96	0.56	1.4E-02	*Bacteria*	Bacteria	
IGFGDNDNTYGVR	8	0.37	4.7E-02	*Acinetobacter*	Bacteria	
DSGQQNSNNNNQQR	11	1.27	2.7E-03	*Acinetobacter*	Bacteria	GD3_GD2
DYNVMDGAALYQK	2	0.55	1.3E-02	*Rhodococcus*	Bacteria	
VDSVGYGETRPVADNATEEGR	32	2.69	1.9E-06	*Pseudomonas*	Bacteria	GD3_STP
ITEQDAQEMIDHLVMK	95	2.32	1.2E-06	*Enterobacterales*	Bacteria	
SALVNEYNVDASR	69	2.28	1.2E-06	*Acinetobacter*	Bacteria	
NSIDGNGLALK	1	-1.65	4.1E-06	*Paenibacillus nuruki*	Bacteria	
NGNSTESPNGR	1	-1.99	7.4E-06	*Pantoea*	Bacteria	
SGSTTGVFCGTIR	1	-2.17	2.8E-06	*Pleosporaceae*	Fungi	

Quantitative metaproteome analysis revealed substantial peptide abundance differences between bacterial species across barley varieties. Table 4.5, focusing on Germination Day 3, supports the hypothesis that the absence of fungi in specific malting steps indicates a shift in microbial community composition.

Interestingly, in the below data, the genus *Pseudomonas* was expressed fairly less, this could be attributed to the genus' high abundancy, potentially exhibiting stable levels across cultivars and malting stages. Consequently, a highly abundant taxon might not show substantial fluctuations in relative abundance, even if its absolute abundance is high, making it challenging to detect changes using fold change analysis. Therefore, to accurately assess the dynamics of such dominant species,

absolute abundance measurements are crucial (and are provided in section 4.4 of this thesis).

Table 4.5 Top three significantly up/down regulated peptides of the comparisons made between the barley varieties for the time point Day 3 in malting step Germination.

Peptide (TIME_POINTS)	nProtein	logFC	APV	LCA	Group	
QQCLTDEK	1	3.53	6.5E-05	*Desulfonema magnum*	Bacteria	FAN_FARO
GGSGNFAENR	23	2.45	6.7E-03	*Pseudomonadota*	Bacteria	
KGGQNSHGGGR	141	1.98	5.3E-04	*Bacteria*	Bacteria	
STLKPEGQQALDQLYSQLS SMDPK	4	-2.11	2.1E-06	*Enterobacterales*	Bacteria	
TGAGSNDAR	6	-2.17	2.8E-04	*Micrococcaceae*	Bacteria	
QEHGGEGEQK	2	-4.82	2.9E-05	*Pseudacidovorax intermedius*	Bacteria	
QQCLTDEK	1	3.49	1.3E-04	*Desulfonema magnum*	Bacteria	FAN_FTX
GGSGNFAENR	23	2.12	1.8E-02	*Pseudomonadota*	Bacteria	
KGGQNSHGGGR	141	1.65	2.4E-03	*Bacteria*	Bacteria	
TGAGSNDAR	6	-1.83	1.2E-03	*Micrococcaceae*	Bacteria	
STLKPEGQQALDQLYSQLS SMDPK	4	-1.84	8.8E-06	*Enterobacterales*	Bacteria	
AQTSGQAEK	6	-1.85	5.3E-03	*Erwiniaceae*	Bacteria	
QQCLTDEK	1	3.22	1.4E-04	*Desulfonema magnum*	Bacteria	FAN_PLT
DQQQQNNQQQQQNDNR	16	2.61	4.2E-05	*Acinetobacter calcoaceticus*	Bacteria	
KGGQNSHGGGR	141	2.42	1.8E-04	*Bacteria*	Bacteria	
AQSEMDNR	7	-1.41	2.3E-05	*Actinomycetes*	Bacteria	
MAAADGVR	2	-1.50	7.0E-06	*Bacteria*	Bacteria	
STLKPEGQQALDQLYSQLS SMDPK	4	-1.80	7.0E-06	*Enterobacterales*	Bacteria	
QEHGGEGEQK	2	4.43	5.4E-04	*Pseudacidovorax intermedius*	Bacteria	FAR_PLT
NNQQQQQQQNDNR	17	1.85	3.3E-03	*Acinetobacter calcoaceticus/baumannii complex*	Bacteria	
DSGQQNSNNNNQQR	11	1.80	1.8E-03	*Acinetobacter*	Bacteria	
FAVDQEKLEK	80	-0.91	5.0E-03	*Bacteria*	Bacteria	
ADSTQQVVGGNR	1	-1.04	5.0E-03	*Erwiniaceae*	Bacteria	
NGSVSGENDTGR	19	-1.07	3.2E-02	*Enterobacterales*	Bacteria	

Table 4.5 Top three significantly up/down regulated peptides of the comparisons made between the barley varieties for the time point Day 3 in malting step Germination (continued)

ADSTQQVVGGNR	1	1.05	4.5E-03	*Erwiniaceae*	Bacteria	FTX_FAR
DTGVNNDGSR	1	1.00	8.9E-03	*Erwinia persicina*	Bacteria	
NNAGGHANHS	311	0.94	3.0E-02	*Bacteria*	Bacteria	
SQTGDFTR	483	-1.16	5.8E-04	*Bacteria*	Bacteria	
NSDAATGYR	10	-1.67	5.6E-05	*Pseudomonas*	Bacteria	
QEHGGEGEQK	2	-4.96	1.1E-04	*Pseudacidovorax intermedius*	Bacteria	
NNQQQQQQQNDNR	17	2.22	8.6E-04	*Acinetobacter calcoaceticus/baumannii complex*	Bacteria	FTX_PLT
DSGQQNSNNNNQQR	11	2.07	5.9E-04	*Acinetobacter*	Bacteria	
NNAGGHANHS	311	1.79	1.1E-03	*Bacteria*	Bacteria	
TDWVFSMPTR	32	-0.85	1.8E-03	*Bacteria*	Bacteria	
TDTELGALR	70	-0.89	3.1E-03	*Rhizobiaceae*	Bacteria	
SQTGDFTR	483	-0.97	1.3E-03	*Bacteria*	Bacteria	

It must be underlined that the presented tables offer a preliminary overview of the **top three** up- and downregulated peptides for each cultivar; representing a subset of the comprehensive quantitative metaproteomic analysis. The more extensive data set, encompassing additional comparisons between varieties and time points, is available upon request to provide a deeper understanding of the complex microbial dynamics at play.

4.3 Microbial Metabolism and Its Role in Accelerated Malting Process

When examined for all contrasts, the GD2 vs GD1 and GD3 vs GD2 volcano plots of all barley species showed low microbial differences. The time points with the strongest changes are seen at the beginning of germination and the end of germination. In Figures 4.3, 4.4, 4.5, and 4.6 the GD1 vs. STP and GD3 vs. STP volcano plots are presented.

When these graphs are examined, the results also show here overwhelming changes in peptide abundance in bacterial species in contrast to fungal species.

However, as can be shown in section 4.2, the few fungal species peptides generally appear to be downregulated in GD3, which is evident in the Fantex, Faro, and especially the Planet volcano plot. The primary bacteria whose peptides show an increase in abundance was observed for all barley varieties belong to *Pseudomonas* & *Pseudomonadota*, with 48.8% of peptides belonging to Planet, 43.7% to Faro, 42.3% to Fandaga and 34.3% to Fantex.

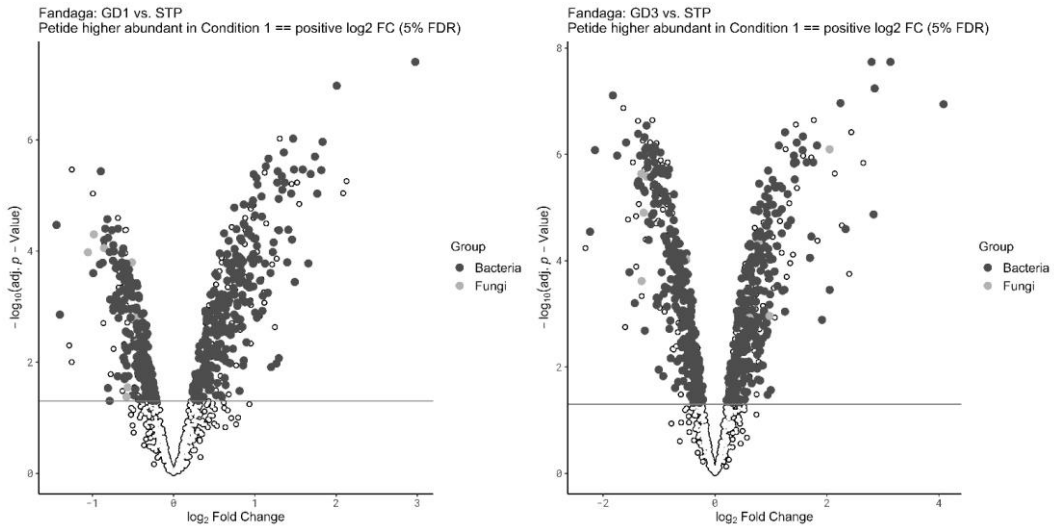


Figure 4.3 Volcano plots for Fandaga GD1 vs. STP and GD3 vs. STP comparisons

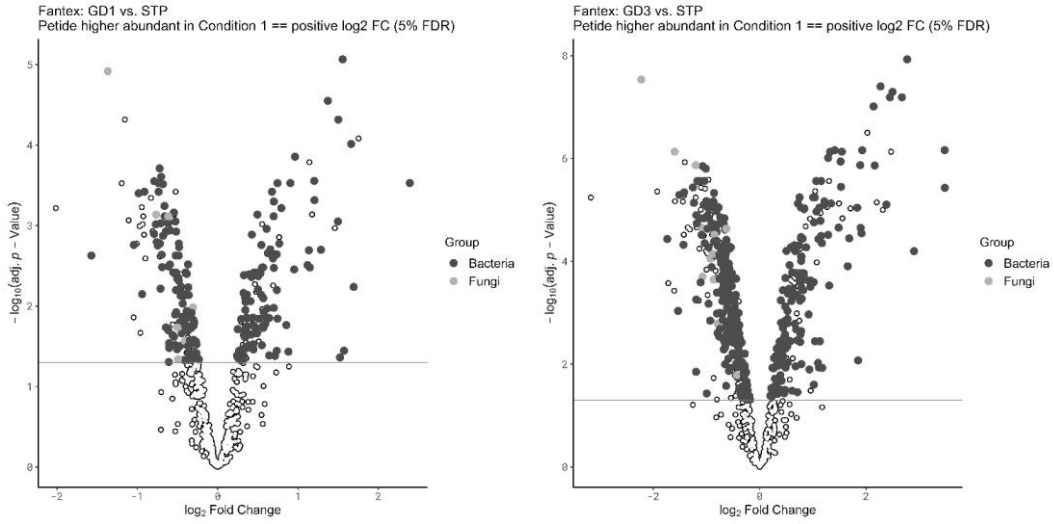


Figure 4.4 Volcano plots for Fantex GD1 vs. STP and GD3 vs. STP comparisons

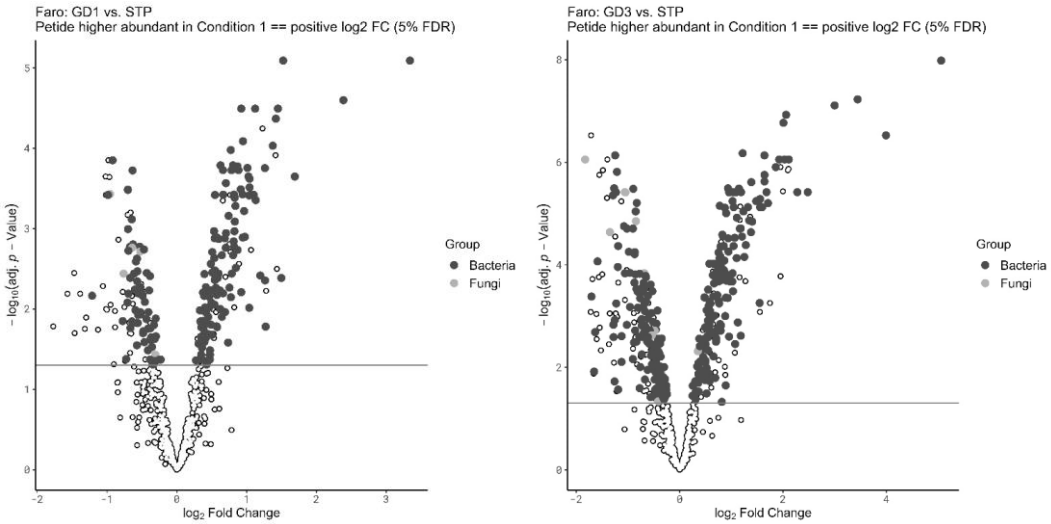


Figure 4.5 Volcano plots for Faro GD1 vs. STP and GD3 vs. STP comparisons

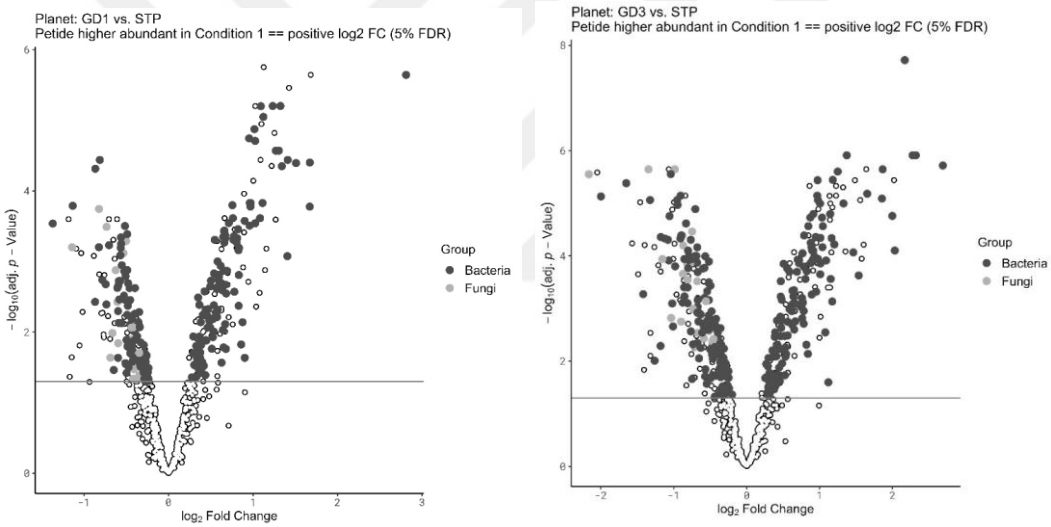


Figure 4.6 Volcano plots for Planet GD1 vs. STP and GD3 vs. STP comparisons

With Figure 4.7 the volcano plots of all barley varieties for the time-point contrasts are given. This finding was similar to the ones examined separately for per barley cultivar, the contrasts GD2 vs GD1 and GD3 vs GD2 showed lower difference and a higher threshold value, meaning the statistically insignificant change in differential peptide expressions for the days between the start and end of germination to be higher.

The volcano plots showing a single barley variety in terms of time points were interpreted as overwhelming changes in peptide abundance in bacterial species as in contrast to fungal species. Interestingly, the volcano plots given in Figure 4.7

for the overlap of all barley varieties in terms of time points, show the fungi expression to be more visible and equally frequent to bacteria expression compared to that of separate volcano plots presented for barley varieties.

However, this difference can not be taken into account as an indication of fungi expression in contrast to stated suppression, due to the primary bacteria whose peptide shows an increase in abundance observed for all varieties belonging to the genus *Pseudomonas* & phylum *Pseudomonadota*. As the same bacteria overlap in volcano plots of Figure 4.7. is expected, the fungi species for different barley varieties are anticipated to also differ.

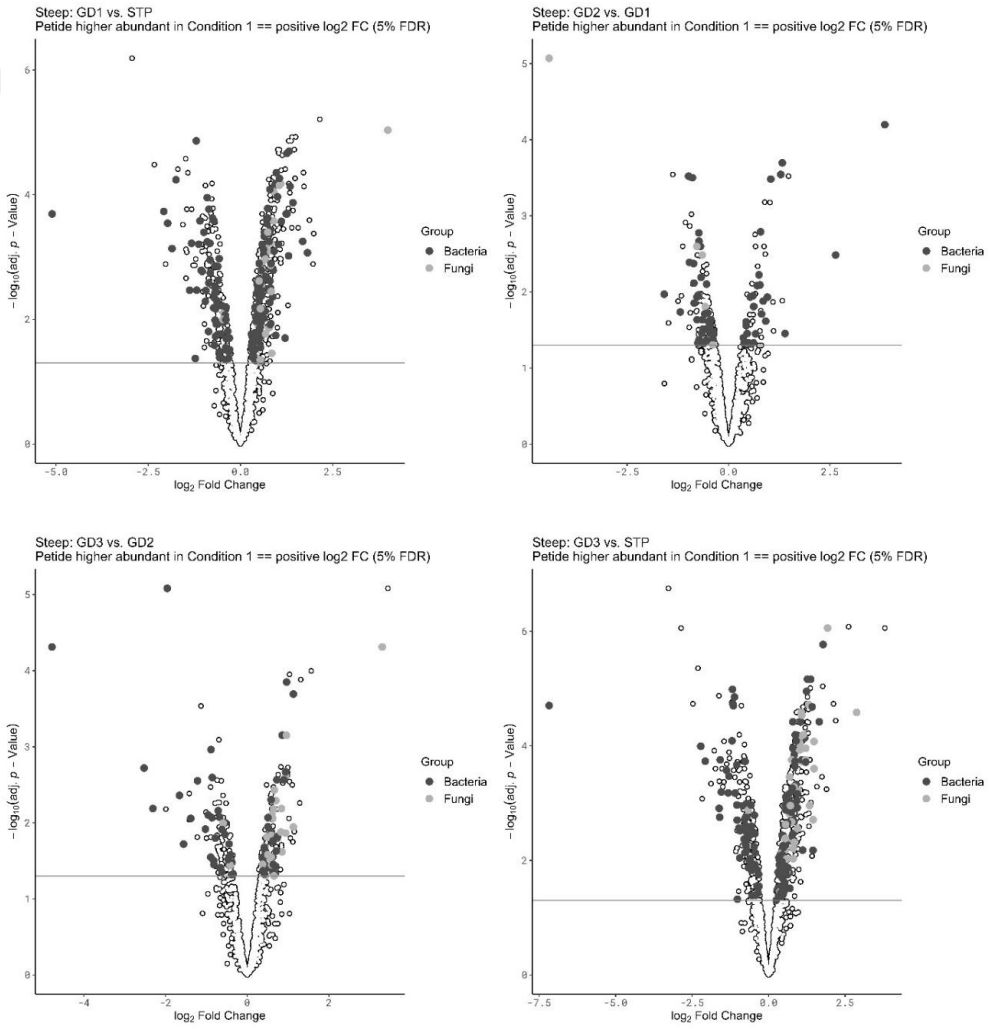


Figure 4.7 Volcano plots of all barley varieties for the time-point contrasts GD1 vs. STP, GD2 vs. GD1, GD3 vs. GD2 and GD3 vs. STP.

4.4 Uniquely Identified Microorganisms for AMR

Understanding the microbial composition of barley varieties is crucial as it provides insights into the potential sources of contamination during cultivation, harvest, or storage. The presence of specific microorganisms can influence the quality and safety of barley-derived products. By identifying the microorganisms present at the genus level, it is possible to understand the potential risks and develop strategies to reduce them.

The identified species for the barley cultivars are given in Figures 4.8, 4.9, 4.10, and 4.11. Through this, it can easily be seen that there is inconsistency in the number of identified genera between the barley varieties. This is most easily observed through a comparison of FAN which has 16 uniquely identified species names, and PLT with only 7. FAN's higher number might indicate greater microbial diversity**,** or a more complex community ecology compared to PLT. This finding is a result of distinct microbial community structures of the barley varieties, however, as discussed in section 4.1, the cultivars show a commonality in terms of their lowest common ancestors, hence, not enough of a significant distinction is caught here.

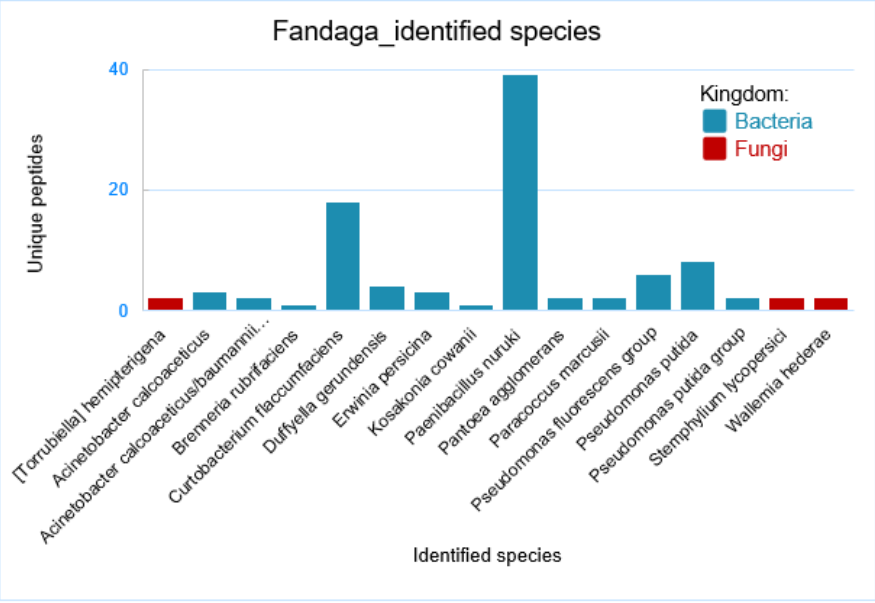


Figure 4.8 Identified species for the barley cultivar Fandaga, showing the dominating Kingdom of the microorganisms

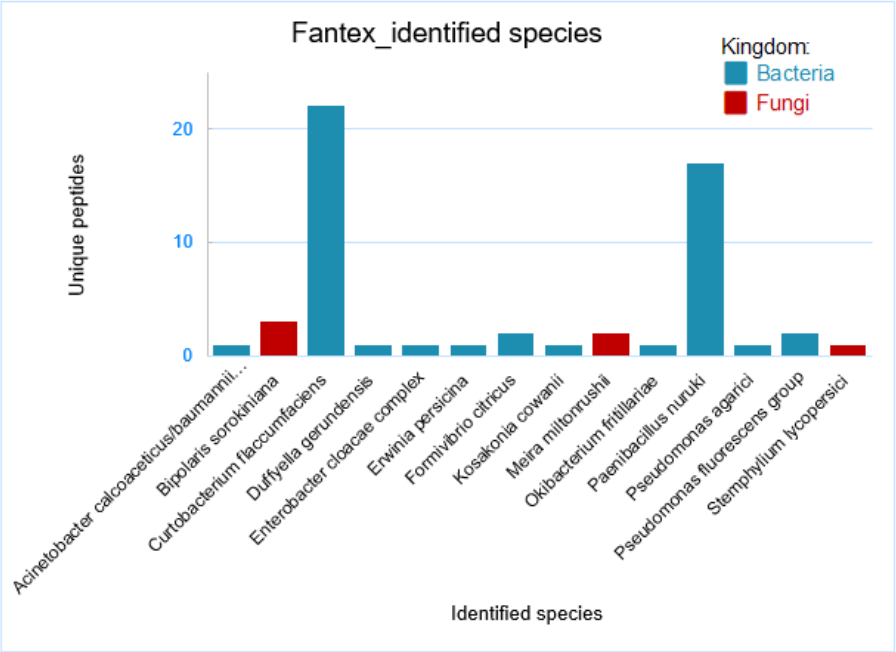


Figure 4.9 Identified species for the barley cultivar Fantex, showing the dominating Kingdom of the microorganisms

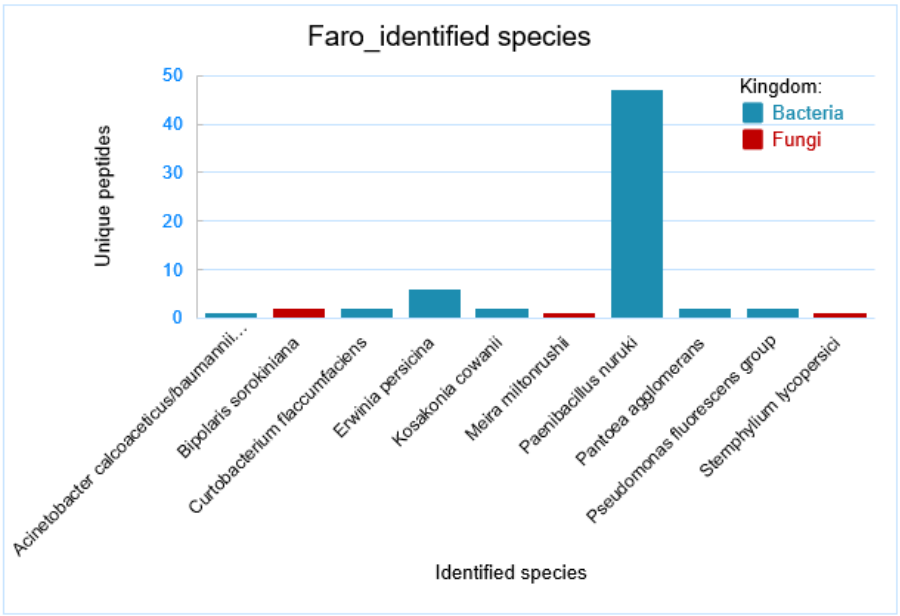


Figure 4.10 Identified species for the barley cultivar Faro, showing the dominating Kingdom of the microorganisms

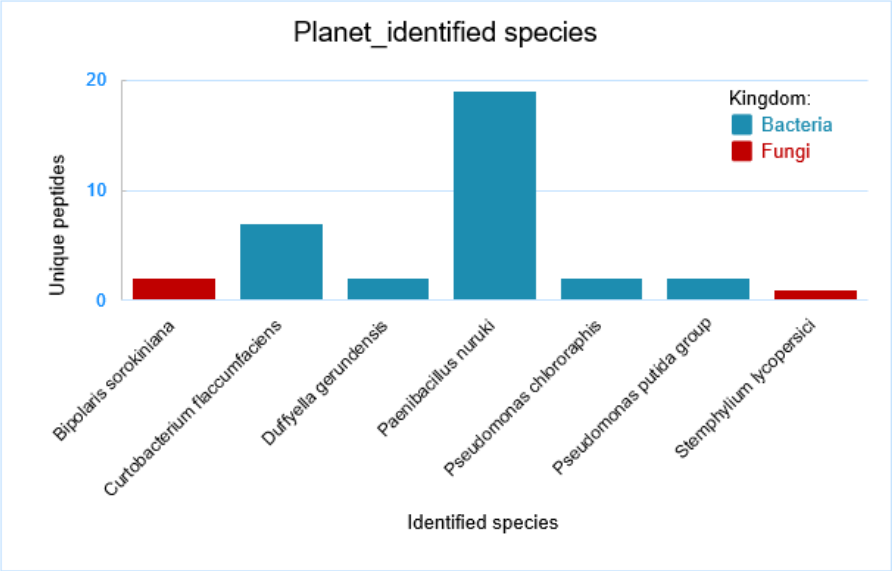


Figure 4.11 Identified species for the barley cultivar Planet, showing the dominating Kingdom of the microorganisms

It is also observed in Figures 4.8 to 4.11, there's a consistent dominance of the *Paenibacillus nuruki* identified across all cultivars, this implies a significant role for these bacteria in barley microbiomes.

The identified genera for the barley cultivars are given in Figures 4.12, 4.13, 4.14, and 4.15 to summarize the discussions made in sections 4.2 and 4.3. The presented figures absolutely underscore the predominance of the *Pseudomonas* genus within the microbial community. The finding aligns with the previous observations regarding the genus' association with upregulated peptides and its prominent representation in the volcano plot analysis. However, the comparative nature of these figures serves to further emphasize the substantial abundance of *Pseudomonas* relative to its microbial counterparts, both bacteria and fungi genera, thereby solidifying its status as a key player in the observed ecosystem.

The dominant species (*Paenibacillus nuruki*) is of the genus *Paenibacillus*; and not from the genus *Pseudomonas*, the highest abundant genus identified for all cultivars. This case creates an intriguing contrast to the earlier discussed findings and highlights the complexity of microbial community analysis. It's possible to give the potential explanations for this observation (as also mentioned in section 2.7):

- The level of taxonomic resolution achieved in the study might influence the results; while species-level identification provides more precise information, it might be limited by database availability and the complexity of the samples.
- *Paenibacillus* might have a higher protein abundance compared to other genera, leading to a greater number of identified peptides and its subsequent dominance at the **species level**.
- *Pseudomonas* genus, on the other hand, might encompass multiple species with varying protein abundances, resulting in a high **genus-level** representation but a lower abundance at the species level.

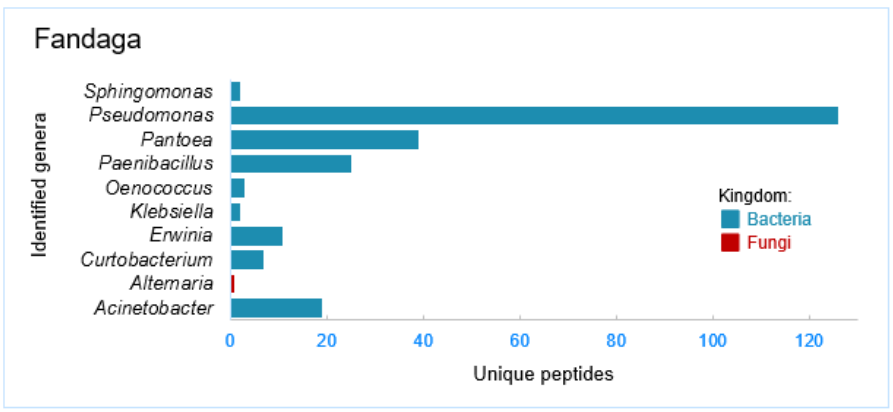


Figure 4.12 Identified genera for the barley cultivar Fandaga, showing the dominating Kingdom of the microorganisms

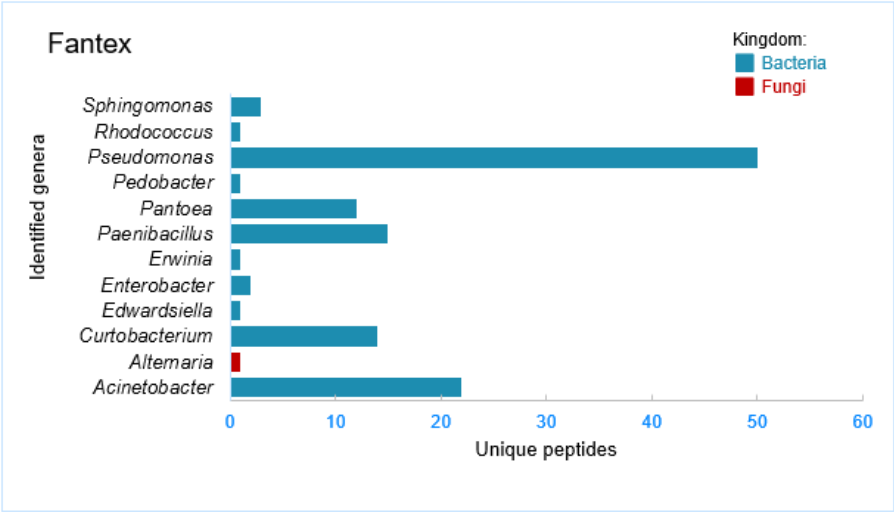


Figure 4.13 Identified genera for the barley cultivar Fantex, showing the dominating Kingdom of the microorganisms

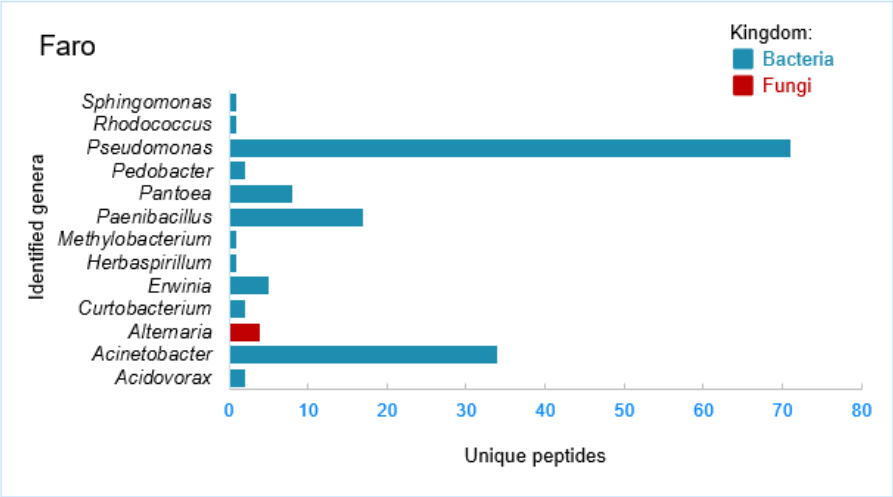


Figure 4.14 Identified genera for the barley cultivar Faro, showing the dominating Kingdom of the microorganisms

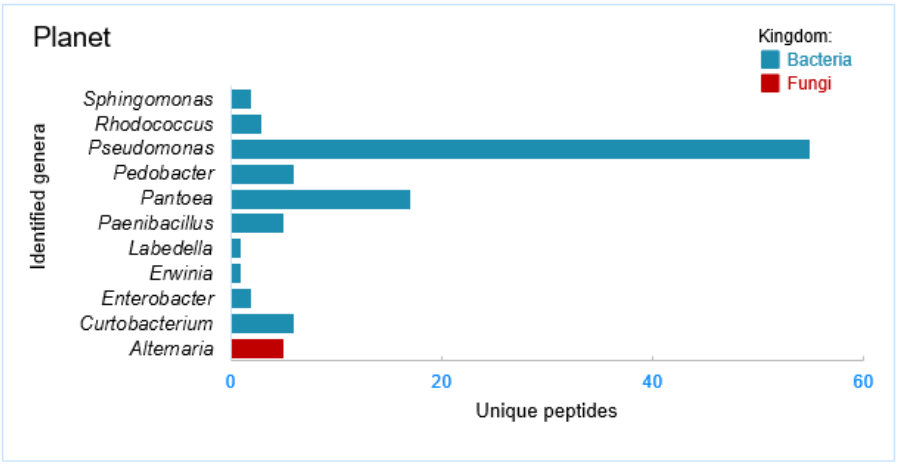


Figure 4.15 Identified genera for the barley cultivar Planet, showing the dominating Kingdom of the microorganisms

When investigated for the identified common species for Steeping and Germination days, the fungi species for different barley varieties differ profoundly with only two species of Fungi being common, given in Table 4.6.

Table 4.6 Identified common species for the malting steps Steeping and Germination for AMR

Common Species for Steeping and Germination	Kingdom
Burkholderia cepacia complex	Bacteria
Chytriomyces confervae	Fungi
Curtobacterium flaccumfaciens	Bacteria
Duffyella gerundensis	Bacteria
Enterobacter roggenkampii	Bacteria
Erwinia persicina	Bacteria
Paenibacillus nuruki	Bacteria
Pseudacidovorax intermedius	Bacteria
Pseudomonas fluorescens group	Bacteria
Stemphylium lycopersici	Fungi

A detailed list of uniquely identified species for each germination day is provided in Appendix B. While these data offer valuable insights, the primary focus of this section is on overarching trends and patterns. The unique species identified from the germination days of all varieties can be found in Appendix Table B.1. The results showed the highest number of peptides identified for the species *Curtobacterium flaccumfaciens, Paenibacillus nuruki, Erwinia persicina,* and *Duffyella gerundensis*. It is essential here to recognize that the species listed may not perfectly align with those presented in Figures 4.8 to 4.11 due to the inherent variability of peptide selection in mass spectrometry, the semi-random selection of peptides by the software, slight variations in sample analysis can lead to differences in identified species, even between identical starting samples.

The findings of the unique microorganisms for the STP step for all barley varieties showed equal numbers of listed Bacteria and Fungi species, the list for this can be found in Appendix Table B.2. Followed by the identified unique microorganisms of the time-points of Germination days showing the overwhelming Bacteria amount once more as the kingdom is seen dominating in the Appendix Tables B.3, B.4 & B.5.

4.5 AMR Samples Malt Quality Properties

Malt quality assessment of accelerated malting regime (AMR) per cultivar is given in Table 4.7, the results are presented as mean values (n=3) ± standard deviation and the malt quality analyses done by Albert Maltings (Puur-Sint-Amands, Belgium). Each barley cultivar is presented in terms of friability, viscosity, PUG, WUG, fine extract, diastatic power (DP), FAN, and Kolbach Index. The malt quality results showed very little difference from the industrial malting regime ones. The most notable ones are the drop in diastatic power and friability values of the produced malt. The friability (mentioned in section 2.4 in detail) might cause inefficient starch conversion, which is unfavorable due to low extract yields.

Table 4.7 Malt quality properties per cultivar

	Cultivar	Friability (%)	Viscosity (cPa)	PUG	WUG	Fine extract (%)	DP (°WK units)	FAN (mg/L)	Kolbach index (%)
Accelerated Malting Regime	Fandaga	98.2 ± 0.68	1.51 ± 0.03	0.2 ± 0.1	0.0 ± 0.0	81.15 ± 0.30	264 ± 1	239 ± 4	57 ± 1
	Fantex	93.6 ± 0.22	1.47 ± 0.01	0.9 ± 0.4	0.3 ± 0.1	82.04 ± 0.06	260 ± 1	162 ± 2	44 ± 1
	Faro	89.3 ± 0.61	1.49 ± 0.01	0.9 ± 0.2	0.2 ± 0.1	78.26 ± 0.23	273 ± 2	153 ± 2	43 ± 1
	Planet	87.8 ± 1.19	1.57 ± 0.01	4.1 ± 1.0	1.8 ± 0.4	82.18 ± 0.08	236 ± 1	148 ± 4	45 ± 2

4.6 Malt Quality and Correlation with Microbiome Activity

The AMR results regarding filtration and friability in comparison to the IMR are given in Figures 4.16 and 4.17, the results are expressed as mean values (n=3), error bars = standard deviation. Statistical comparisons between the two malting regimes for each barley cultivar were performed using a Student's t-test. Statistically significant different groups ($p \leq 0.05$) and ns = not significant. The malt analysis is performed by Albert Maltings (Puur-Sint-Amands, Belgium).

The difference in filtration between the malting regimes showed AMR expressing undesirable filtration results. Most of the time, this insufficient decline is attributed to stemming from high molecular weight beta-glucan or arabinoxylans. Comparison of the malt filtration quality of the accelerated malting regime (AMR) with the industrial malting regime (IMR).

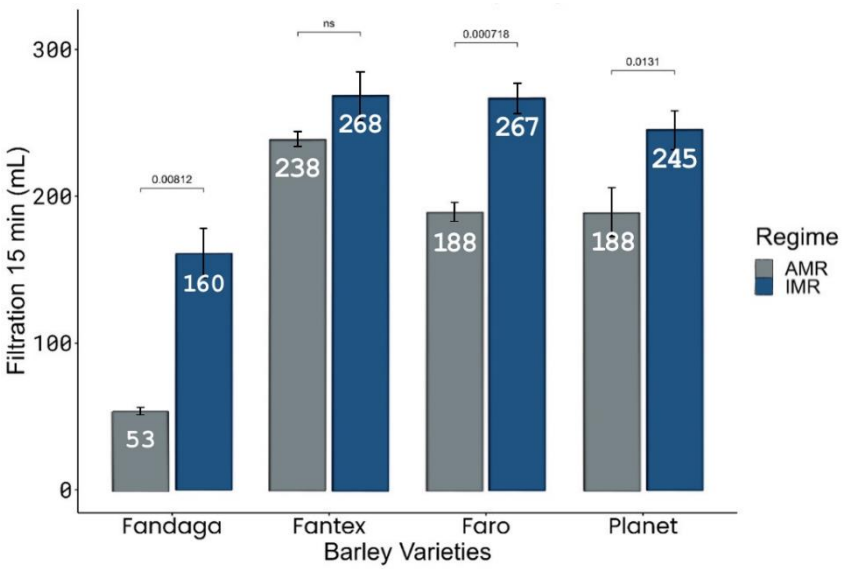


Figure 4.16 Comparison of the malt filtration quality of the accelerated malting regime (AMR) with the industrial malting regime (IMR)

When the β-glucan activity difference between IMR and AMR is investigated, the faster breakdown of β-glucan is seen with AMR & after kilning, equivalent amounts are obtained, except for the barley variety Faro.

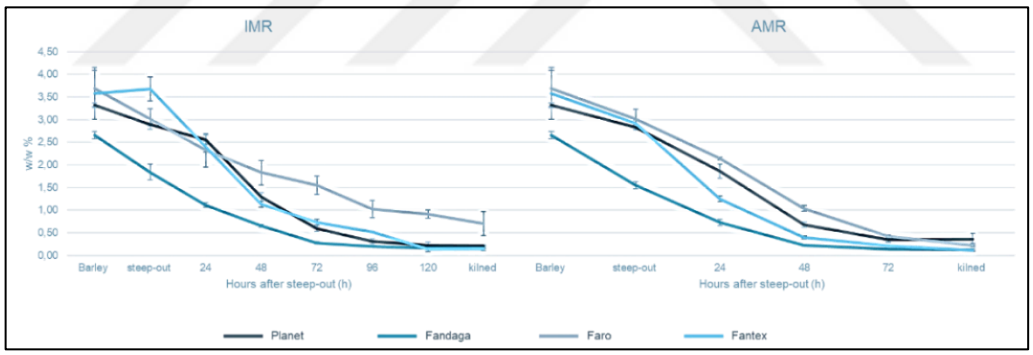


Figure 4.17 β-Glucan concentration

The results in Figure 4.17 showed the reason behind the inferiority of filtration to be not related to the β-glucan content of the malt. Followingly, the friability of the malt was investigated. A high degree of friability is often used to indicate well-modified malt because it relates to the extent of enzymatic endosperm cell wall breakdown during malting. Beta-glucanase activity can help increase friability by breaking down beta-glucans in cell walls. However, since the friability results for IMR and AMR were very close (given in Figure 4.18 and the AMR values were not abnormal when examined for beta-glucan concentration, it was seen that the problem in filtration did not arise due to beta-glucan).

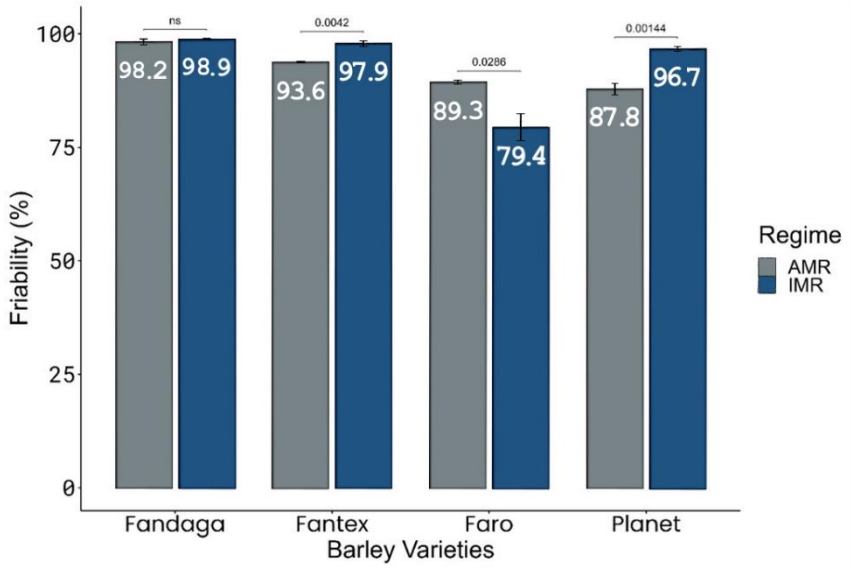


Figure 4.18 Comparison of the malt friabilimeter value (%) of the accelerated malting regime (AMR) with the industrial malting regime

A further potential source of filtration problems can be due to arabinoxylans. As shown in Figure 4.19, the endo-xylanase activity for IMR tends to increase between the 72nd hour after steep-out and kilning while AMR does not show such activity, and the swing moderately occur between 25 – 50 mU/g d.m throughout the analysis time for all four varieties.

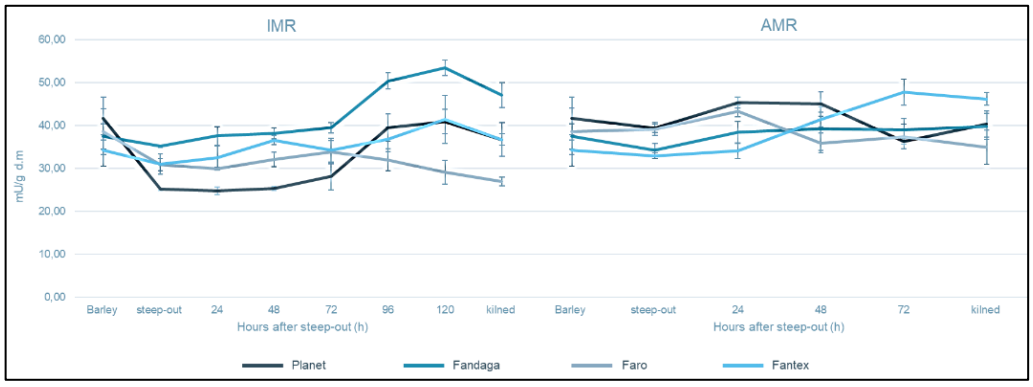


Figure 4.19 Endo-Xylanase activity

Interestingly, only sparse numbers of fungi were detected in the microbiome associated with AMR. The increase found in the genus *Pseudomonas* and phylum *Pseudomonadota* may explain this suppression of fungi observed, with an associated lack of fungal contribution to xylanase activity, since, for AMR, mostly constant xylanase activity was present.

5
CONCLUSION

This thesis aimed to characterize the temporal changes in the barley microbiome with increasing temperatures applied during steeping and germination under adjusted malting conditions, the interpretation of this goal is enabled by the quantitative metaproteome analysis.

The upset-plot showed that AMR resulted in a more homogenized microbiome, with more overlap in the types of bacteria and fungi present across different barley varieties. In line with this commonality, the identification of up/down-regulated peptides also showed the majority of LCA across all barley cultivars to be from the Bacteria genus *Pseudomonas* & phylum *Pseudomonadota*. With the volcano-plot analysis coupled with up/down regulation analysis, the findings were of that the down-regulation of fungal species' peptides is particularly evident in GD3, in the final stage of the malting process. Subsequently, the results of variety Fantex and its expression of fungi only in down-regulation suggested that the environment at the end of malting is particularly unfavorable for fungal growth.

The data of up/down regulation comparisons between malting regime time-points, microbial profile dynamics of different barley varieties in contrast to each other, and the taxonomic analyses with identified peptides were presented and found to be supporting the fungi suppression pointed out throughout the results of the thesis study.

Upset-plot of the distribution of LCAs detected between sampled time-points showed the lowest expected commonality results for the intersection of STP, GD1, and GD3 contrasts and supported the distinct dynamics between the stages of malting. From the taxonomic analysis results, it could be concluded that in AMR, there is a main static microbial population from steeping to GD3, a new emerging and permanent population rising after the time point GD2, and a newly emerged population obtained at GD3.

The volcano plots for the overlap of all barley varieties in terms of time-points, showed the fungi expression to be more visible and equally frequent to bacteria expression compared to that of separate volcano plots presented for barley varieties. However, this indication of fungi expression was not enough to oppose the suggested fungi suppression concluded in the thesis, due to the expectation of the same bacteria overlap in volcano plots and the fungi species for different barley varieties to differ.

Followingly and in support of the fungi suppression, the findings of uniquely identified microorganisms for AMR once again pointed out the overwhelming number of Bacteria, with the kingdom dominating the tables of identified unique microorganisms of the time-points of Germination days.

The thesis study's findings of up- and down-regulated peptides for the malting stages of a single barley variety concerning bacterial over-expression were supported with the comparisons between the varities in contrast to each other, and the absence of fungi in specific malting steps (focused on Germination Day 3) indicated a shift in microbial community composition.

This suppression of fungi is found to be significant since, compared to the industrial malt, malt produced by AMR also exhibited lesser friability and slower filtering. Following this comparison, the malt analysis results can further suggest (as investigated with ß-glucan) that the decrease in filtration speed to be not originating from the presence of ß-glucans and could be attributed to arabinoxylans.

It was fascinating to hypothesize that this results from the absence of fungi during germination, connecting that the fungi contribute to the production of xylanases to degrade arabinoxylans. It is also suggested in the literature to investigate the presence of microorganisms producing hydrolytic enzymes such as xylanases during malting to improve the filtration by enriching such microorganisms that degrade arabinoxylans (Malfliet et al., 2013).

For this thesis, further interpretation predicts for the arabinoxylan concentration to be higher in AMR. Despite the difference in endo-xylanase enzymatic activity seen in AMR is low, this hypothesis is still testable via arabinoxylan concentrations. At the time of the study conducted, no arabinoxylan data was

available. Though, another potential explanation for the low filtration speed could be attributed to the dominance of *Pseudomonas* & *Pseudomonadota*, as they are known biofilm-formers. These biofilms consist mainly of polysaccharides (Mann & Wozniak, 2012) and might thus impair the filtration of AMR malt.

Therefore, finalizing this thesis study, it was possible to propose the improvement of AMRs with the future steps: application of fungi starter culture to improve on xylanase activity, potentially resolving the filtratability of wort made from AMR malt or to use starter cultures to suppress *Pseudomonas* & *Pseudomonadota* growth during AMR.

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A
APPENDIX MATERIAL AND METHODS

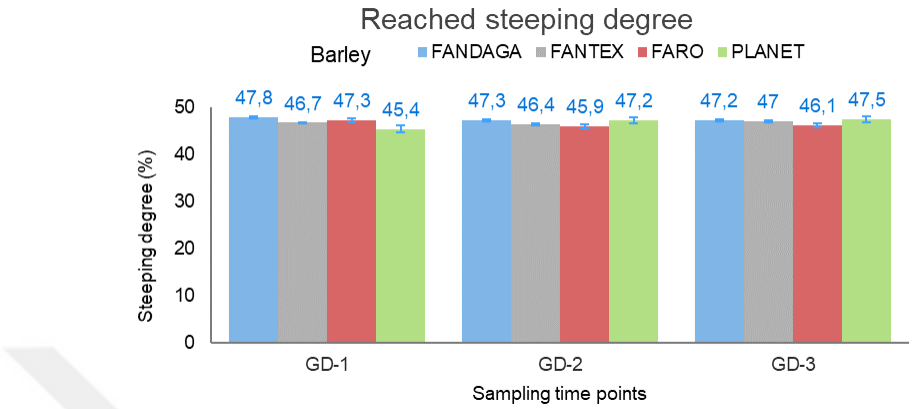


Figure A.1 Resulting % moisture content of green malts of the four barley cultivars

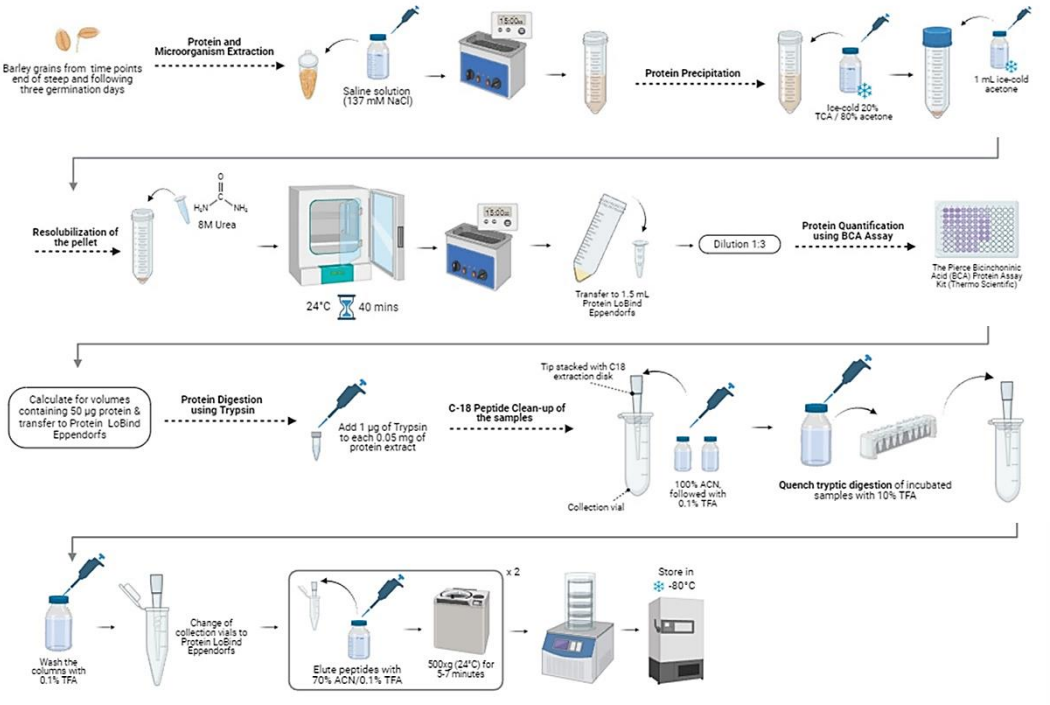


Figure A.2 Experimental procedure for the barley metaproteomics analysis, showing protein precipitation, resolubilization of the protein pellet, protein quantification and digestion steps, briefly. Constructed with BioRender.com for this study.

Table A.1 Mechanical and Physicochemical properties of four barley varieties used in the study

	Cultivar	Moisture content (%)	Total protein (%)	Soluble protein (%)	FinalAtte (%)	Color (EBC)	Homogenity (%)
Accelerated Malting Regime	**Fandaga**	4.17	10	5.7	83.4	7.17	99.8
	Fantex	4.04	9.8	4.3	82.5	3.17	99.1
	Faro	4.01	10.51	4.5	84	3.33	99.1
	Planet	4.36	9.2	4.1	82.1	4.67	95.9

Table A.2 The accelerated malting regime barley samples for the variety Fantex (FTX) given as an example of MS-RUN TMT-labelling scheme

Barley Metaproteomics: Accelerated Malting Regime **TMT-Metaprotemics-E06**

Fantex (FTX): MS-RUN-03

SN	Sample ID	Description	Resuspension solution 71.4 mM HEPES	TMT 18-plex label reagent	Volume of 13 mM TMT label (μL)	Total volume
1	**X01**	AMR-FTX-STP-R1	9.33	126	4	13.33
2	**X02**	AMR-FTX-STP-R2	9.33	127N	4	13.33
3	**X03**	AMR-FTX-STP-R3	9.33	127C	4	13.33
4	**X11**	AMR-FTX-GD1-R1	9.33	128N	4	13.33
5	**X12**	AMR-FTX-GD1-R2	9.33	128C	4	13.33
6	**X13**	AMR-FTX-GD1-R3	9.33	129N	4	13.33
7	**X21**	AMR-FTX-GD2-R1	9.33	129C	4	13.33
8	**X22**	AMR-FTX-GD2-R2	9.33	130N	4	13.33
9	**X23**	AMR-FTX-GD2-R3	9.33	130C	4	13.33
10	**X31**	AMR-FTX-GD3-R1	9.33	131N	4	13.33
11	**X32**	AMR-FTX-GD3-R2	9.33	131C	4	13.33
12	**X33**	AMR-FTX-GD3-R3	9.33	132N	4	13.33

B
APPENDIX RESULTS AND DISCUSSION

Table B.1 Identified unique species for the germination days of AMR

Unique Species Identified for All Germination Days	No. peptides
Acinetobacter calcoaceticus	3
Acinetobacter calcoaceticus/baumannii complex	1
Acinetobacter pittii	1
Bacteroides zoogleoformans	1
Burkholderia cepacia complex	1
Candidatus Aquirickettsiella gammari	1
Candidatus Baumannia cicadellinicola	1
Candidatus Portiera aleyrodidarum	1
Chytriomyces confervae	1
Curtobacterium flaccumfaciens	17
Desulfonema magnum	1
Dokdonella immobilis	1
Duffyella gerundensis	8
Enterobacter roggenkampii	1
Erwinia persicina	10
Escherichia coli	2
Kitasatospora griseola	1
Kosakonia cowanii JCM 10956 = DSM 18146	1
Litoreibacter ponti	1
Melghirimyces thermohalophilus	1
Microbotryum intermedium	1
Okibacterium fritillariae	1
Paenibacillus nuruki	33
Pantoea agglomerans	3
Paraburkholderia guartelaensis	1
Paraburkholderia phenoliruptrix	1
Parasutterella excrementihominis	1
Pseudacidovorax intermedius	1
Pseudomonas chlororaphis	1
Pseudomonas fulva	1
Pseudomonas poae	1
Pseudomonas putida	6

Table B.1 Identified unique species for the germination days of AMR (continued)

Saccharibacillus brassicae	1
Solimicrobium silvestre	1
Sphingomonas faeni	2
Stemphylium lycopersici	1
Zymoseptoria tritici IPO323	1

Table B.2 Identified unique microorganisms of the malting step "Steeping" for AMR

Unique Microorganisms found for Steep	Kingdom
Aureobasidium	Fungi
Candidatus Aquirickettsiella gammari	Bacteria
Dokdonella immobilis	Bacteria
Kitasatospora griseola	Bacteria
Melghirimyces thermohalophilus	Bacteria
Mucorales	Fungi
Pleosporaceae	Fungi
Zymoseptoria tritici IPO323	Fungi

Table B.3 Identified unique microorganisms of the time-point Germination Day 1 for AMR

Unique Microorganisms found for Germination Day 1	Kingdom
Candidatus Baumannia cicadellinicola	Bacteria
Paraburkholderia phenoliruptrix	Bacteria
Pseudomonas putida group	Bacteria
Serratia	Bacteria

Table B.4 Identified unique microorganisms of the time-point Germination Day 2 for AMR

Unique Microorganisms found for Germination Day 2	Kingdom
Duganella	Bacteria
Microbotryum intermedium	Fungi
Paraburkholderia guartelaensis	Bacteria
Pseudomonas poae	Bacteria
Staphylococcaceae	Bacteria

Table B.5 Identified unique microorganisms of the time-point Germination Day 3 for AMR

Unique Microorganisms found for Germination Day 3	Kingdom
Candidatus Portiera aleyrodidarum	Bacteria
Desulfonema magnum	Bacteria
Klebsiella	Bacteria
Litoreibacter ponti	Bacteria
Micrococcaceae	Bacteria
Mycobacteriales	Bacteria
Plantibacter	Bacteria
Pseudomonas fulva	Bacteria
Vibrio	Bacteria

PUBLICATIONS FROM THE THESIS

Conference Papers

1. Kızılyar, E. G., Weiland, F., & Balkanlı, D. (2024). Temporal metaproteomics of a barley germination regime with applied increased temperatures during steeping and germination. In M. Faisal (Ed.), *Academy of Scientific and Educational Studies (ASES) Congress book of VIII. International Health, Engineering and Sciences Conference 2024* (pp. 109–110). ASES Publications. https://asescongress.com/wp-content/uploads/2024/05/8.-INTERNATIONAL-HEALTH-ENGINEERING-AND-SCIENCES-CONFERENCE-BOOK.pdf