Studies on polyamines in barley grains: Relationship with protein, distribution within the kernel and enrichment in milling fractions

By

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Dedication

This thesis is dedicated to Sĩ Nhật (Brian) when he was 25 years old.

Thank you for your courage, curiosity and perseverance that have helped us go this far.

We both know that there are *still* places that you want to go, people to meet and things to do.

"And more, much more than this,

I did it my way".

Paul Anka (Canadian-born singer and songwriter)

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Thesis Abstract

Barley (*Hordeum vulgare* L.) is one of the important and climate-resilient crops that serves as a rich source of health-promoting bioactive compounds. While recent research has focused on β-glucans and phenolic compounds in barley grains, this thesis investigated the occurrence, distribution and enrichment of polyamines. Polyamines (e.g., putrescine, spermidine and spermine) are aliphatic nitrogenous compounds recognized for their antioxidant and anti-inflammatory properties. The study first examined a polyamine extraction method, reporting that 1 M hydrochloric acid was more effective than 5% (w/v) trichloroacetic acid for recovering free polyamines from barley flour. Analysis of thirteen Canadian varieties revealed significant genotypic variations. The total polyamine content ranged from 5.48 to 11.23 mg/100 g dry basis (db), with hulless food-grade varieties generally exhibiting higher levels (average 9.73 mg/100 g db) than malting (average 7.00 mg/100 g db) or feed (average 7.81 mg/100 g db) cultivars. Mechanical fractionation (pearling, dehulling and milling) and hand-dissection techniques demonstrated gradient distribution within the kernel. Polyamines were highly concentrated in metabolically active tissues, particularly the germ (52.0–65.5 mg/100 g db) and the aleurone layer, whereas the starchy endosperm was relatively depleted. A significant and persistent positive correlation was established between polyamine levels and protein content ($r = 0.70–0.84$, $p < 0.001$) across both whole kernels and processed fractions. Furthermore, polyamine concentrations correlated with other markers of whole-grain status, such as ash, germ agglutinin, and total phenolic content. These results provide a scientific basis for using dry fractionation to create value-added and polyamine-enriched barley ingredients for functional food applications.

Chapter 1 — Overview

1.1. General Introduction

During the first two decades of the 21st century, challenges in agriculture and population health that originated in the previous century continued to affect the world. The Green Revolution, once considered the solution to feeding an ever-growing population, has gradually faded from collective memory, giving way to the urgent issue of climate change, which now commands global attention. On one hand, average human life expectancy has increased significantly due to improved access to food and better control of infectious diseases (Barkat, Alsamara, Mimouni, et al., 2024). It should be noted that undernourishment can weaken the immune system and consequently increase the susceptibility to infections (Fan, Yao, Liu, et al., 2022). On the other hand, the burden has shifted to non-communicable diseases, particularly type II diabetes, cardiovascular conditions and certain cancers, which are closely linked to lifestyle and dietary habits (Alamnia, Sargent, & Kelly, 2023; Uddin, Lee, Khan, et al., 2020). Elevated consumption of processed, energy-dense foods combined with reduced intake of fruits, vegetables and dietary diversity has been reported to result in insufficient intake of essential nutrients (e.g., protein, vitamins and minerals). This, in turn, may contribute to increased metabolic risk factors such as obesity, dyslipidemia and hypertension (Alamnia, Sargent, & Kelly, 2023). Although these concerns vary between developing and developed countries, along the farm-to-fork supply chain, producers question what crops to grow to ensure profitability and resilience to evolving weather conditions, while consumers wonder what foods to include in their diet to maintain optimal health. Addressing these questions requires collaboration among crop breeders, food scientists, clinical researchers and governmental agencies.

In response to the first question about sustainable agriculture, there has been an ongoing quest for new crops and varieties that are resilient to climate change (Kopeć, 2024). Climate change, driven by human activities, is characterized globally by rising average temperatures, elevated atmospheric carbon dioxide levels, altered precipitation patterns and more frequent extreme weather events (Kopeć, 2024). These factors collectively threaten agricultural output and food security. Moreover, the manifestations of climate change vary by geographical location, adding complexity to weather prediction and posing region-specific challenges for crop production (Xie, Deser, Vecchi, et al., 2015). Projected global yield losses for major staple cereal crops, including wheat, corn, rice, millet and sorghum, range from 7 to 23% under the most extreme climate change scenarios without adaptation measures (Mirzabaev, Bezner Kerr, Hasegawa, et al., 2023). This underscores the urgent need to identify and improve crops capable of withstanding such environmental changes. During the Green Revolution of the 20th century, significant progress in crop breeding was achieved through the introduction of dwarfing alleles, which substantially increased yields in cereals, particularly wheat and rice (Gaur, Channappa, Chakraborti, et al., 2020; Pearce, 2021). However, these high-yielding varieties performed poorly under dry conditions due to reduced coleoptile length and their dependence on controlled irrigation and synthetic fertilizers (John & Babu, 2021). Rather than continuing to cultivate varieties optimized for ideal conditions and relying on monoculture farming of wheat or rice, it is crucial to consider alternative crops that can thrive in more challenging environments under current climate change. In this regard, barley has emerged as a promising candidate, thanks to its resilience, adaptability and appreciable nutritional value.

Barley (*Hordeum vulgare* L.) is a diploid crop (2*n* = 14) belonging to the *Gramineae* family. It was domesticated approximately 10,000 years ago and became one of the founder crops of Neolithic agriculture (Sato, 2020). Today, barley ranks as the fourth-largest grain crop in the world, playing a significant role in the agricultural supply chain. Worldwide, about 65% of barley production is used for general purposes (i.e., animal feed and forage), followed by malt production (33%) and human consumption (2%) (Idehen, Tang, & Sang, 2017). The inherent diversity of barley is considered conducive to its wide range of uses throughout human history (Newton, Flavell, George, et al., 2011). Barley varieties are generally classified as hulled (covered) and hulless (naked), depending on whether the hull layer remains attached to the caryopsis. Hulled varieties are primarily used for malting and general purposes, whereas hulless varieties are important ingredients in barley-based food products (Izydorczyk & Edney, 2017). Although barley makes only a modest contribution to global human diets, it serves as a staple food for populations living in marginal environments due to its remarkable adaptability. Barley can thrive in arid areas such as Northern Africa and the Middle East, as well as in high-altitude areas like the Ethiopian Highlands and the Tibetan Plateau (Newton, Flavell, George, et al., 2011). Furthermore, barley landraces have demonstrated tolerance to poor-quality soils with micronutrient deficiencies (Schmidt, George, Brown, et al., 2019). In terms of nutritional composition, hulled and hulless barley grains contain starch (57.7 and 60.7% dry basis (db), respectively), protein (12.2 and 15.1% db), lipid (2.5 and 2.7%), mineral (2.1 and 1.6%) and dietary fibre (20.6 and 16.6%) (Lukinac & Jukić, 2022). Another advantage of barley is its relatively shorter growth period compared to wheat and corn, enabling completion of its life cycle in regions with short growing seasons (Perera, Abdollahi, Zaefarian, et al., 2022).

In western Canada, barley is grown in the Prairie regions, with Alberta leading production, followed by Saskatchewan and Manitoba. According to a recent report from the Canadian Grain Commission, approximately half of all barley planting areas are occupied by the hulled cultivar AAC Synergy (Izydorczyk & McMillan, 2024). Canadian barley breeders have made significant strides in developing and commercializing elite cultivars, including AC Ranger and Vivar for general purposes (Helm, Cortez, Salmon, et al., 2003; Therrien, 2002), AAC Synergy and AC Metcalfe for malting (Legge, Metcalfe, Haber, et al., 2003; Legge, Tucker, Fetch, et al., 2014) and Roseland and AAC Beckett for food (Badea, Therrien, & Lukow, 2017; Badea & Tucker, 2025). These achievements underscore the expertise and innovation of Canadian barley breeding programs. The genetic diversity of barley germplasm, largely derived from the natural pool of alleles, offers opportunities for breeding programs to select traits that enhance resilience, nutritional quality and yield stability. Related species of cultivated barley (*H. vulgare* L. ssp. *vulgare*), such as wild barley (*H. vulgare* L. ssp. *spontaneum*), serve as valuable genetic resources for varietal improvement due to their demonstrated phenotypic plasticity (Hosseini, Mojab, Yassaie, et al., 2025). In addition to wild barley as the progenitor of cultivated forms, Sato (2020) reported that the *Hordeum* genus comprised 45 species, with more than 485,000 *Hordeum* accessions conserved across over 200 institutions worldwide. Collectively, these resources contribute to the germplasm diversity available for developing new barley varieties. To support breeding programs, the genetic map of barley has been continuously developed using both classical and modern approaches, including linkage analysis, high-density molecular markers and whole-genome sequencing (Sato, 2020). As a result, barley has gained attention in the pursuit of sustainable and climate-resilient food systems, thanks to its agronomic potential, nutritional relevance and the wealth of genetic resources and data available.

The chemical components of barley grains suggest avenues for improving public health if they are incorporated into the diet. It has been well established that dietary choices can contribute to the prevention of non-communicable diseases (Alamnia, Sargent, & Kelly, 2023; Ribeiro, Andrade, Hermsdorff, et al., 2019). Non-nutritive components, particularly dietary fibres and plant secondary metabolites, have been reported to reduce fat and glucose absorption and modulate metabolic pathways involved in the pathogenesis of certain chronic diseases, owing to their antioxidant and anti-inflammatory activities (Nguyen & Beta, 2024; Ribeiro, Andrade, Hermsdorff, et al., 2019). These health-promoting properties have fueled growing interest in identifying food-based strategies that can be integrated into everyday diets. Whole grain barley is a rich source of various bioactive compounds, including β-glucan and numerous small molecules with diverse bioactivities (Idehen, Tang, & Sang, 2017). In recent years, research efforts have focused on understanding the beneficial effects of these compounds, particularly β-glucan. However, given the multiple uses of barley, increasing the content of one compound for functional food formulation may not be suitable for other applications; for example, high β-glucan levels are undesired in malting (Vis & Lorenz, 1998). Furthermore, prioritizing dietary fibres in barley grains may lead to neglect of other small bioactive components, such as nitrogenous (folates, polyamines) and non-nitrogenous metabolites (phenolic acids, flavonoids, tocols, lignans and phytosterols) (Idehen, Tang, & Sang, 2017). These substances play important roles in plant physiology; for instance, folates are involved in signalling cascades and resistance to abiotic stress (Gorelova, Ambach, Rébeillé, et al., 2017). Understanding these metabolic networks is therefore essential for a holistic evaluation of the nutritional potential of barley grains, as many of these compounds may act synergistically to enhance health outcomes.

The growing awareness of the wide variety of secondary metabolites in barley grains has prompted closer attention to polyamines, a group of nitrogenous compounds gaining recognition for their potential nutraceutical significance. Polyamines have been reported to play roles in regulating flower development, fruit maturation and plant responses to biotic and abiotic stresses (Chen, Shao, Yin, et al., 2019). They are low-molecular-weight, aliphatic nitrogenous bases containing two or more amino groups and occur naturally in both free and conjugated forms. Their production is tightly regulated through interconnected metabolic pathways, underscoring their importance in plant growth and adaptation. Previous studies have identified spermidine derivatives in foxtail millet (Xiang, Zhang, Apea-Bah, et al., 2019), as well as the occurrence of spermidine in barley seedlings (Smith & Best, 1977). More recently, Drawbridge, Apea-Bah, Hornung, et al. (2021) detected noticeable concentrations of a conjugate between spermidine and caffeic acid in methanolic extracts from whole grains of Canadian hulless barley varieties. Regarding the bioactivities and human health impacts, polyamines were proven to have antioxidant and anti-inflammatory properties (Hussain, Tan, Ren, et al., 2017) and the consumption of polyamine-enriched diets has been associated with reduced mortality and improved longevity in animal models and epidemiological studies (Kim, Baek, & Lee, 2024). Consequently, dietary polyamines have attracted attention in the development of functional food aimed at preventing non-communicable diseases. Despite these promising observations, variability in polyamine content across cereal species and cultivars remains underexplored. Given the discovery of polyamine–phenolic acid conjugate in barley grains and the demonstrated health-promoting effects of polyamines, this class of phytochemicals warrants further investigation for its nutraceutical potential, research that could inform breeding strategies and foster innovation in barley-based functional foods.

1.2. Hypotheses and Rationale

The hypotheses included in this series of studies and their rationale are as follows:

- **Hypothesis H_1:** "Extraction efficiency of polyamines depends on the extraction solvent."

Rationale: Multiple solvents have been documented in the literature for extracting polyamines from plant matrices, including hydrochloric (HCl), trichloroacetic (CCl_3COOH) and perchloric ($HClO_4$) acid. Solvent properties can affect matrix breakdown and analyte solubility, leading to potential fluctuations in extracted polyamine levels. Due to its explosiveness, perchloric acid may be unsuitable for large-scale extraction. Therefore, the extraction yields of polyamines from barley grain using hydrochloric and trichloroacetic acid will be compared.

- **Hypothesis H_2:** "Polyamine contents are appreciable in barley grains."

Rationale: Polyamines can conjugate with phenolic acids to form phenolamides in cereal grains. Based on the reported high concentration of a phenolamide (*N*1, *N*8-dicaffeoylspermidine) in Canadian hulless barley grains, it is anticipated that barley grains also contain a sizable quantity of free polyamines, defined as those extractable with HCl (1 M). Additionally, since polyamines are essential for cellular vitality, cereal crops may accumulate them in their grains in preparation for germination. Other cereals, such as wheat, corn and sorghum, have also been reported to contain observable levels of polyamines (Mohajeri, Ayatollahi, Kobarfard, et al., 2023; Paiva, Netto, Queiroz, et al., 2022).

- **Hypothesis H_3:** "Grain polyamine contents differ among barley varieties."

Rationale: Barley varieties exhibit considerable phenotypic and genetic variation due to their multiple uses. In addition to being hulled or hulless, barley grains differ in quantitative traits, including proximate composition (i.e., content of moisture, protein, carbohydrate, lipid, and ash) as well as metabolite profiles. These differences may arise from variations in pathways involved

in nitrogen assimilation, protein biosynthesis, and polyamine metabolism; thus, it is plausible that polyamine profiles vary among varieties.

- **Hypothesis H_4:** "Polyamine contents of barley grains correlate with the protein levels."

Rationale: The anabolism of polyamines and proteins in plants is closely interconnected through their shared reliance on nitrogen assimilation and amino acid metabolism. Therefore, polyamine synthesis depends on the availability of the nitrogen pool, most of which is directed toward protein synthesis. Grains with higher protein content may exhibit enhanced nitrogen uptake and metabolic capacity due to increased enzymic activity, conditions that can facilitate polyamine synthesis. Hence, a correlation between polyamine concentrations and protein levels is expected.

- **Hypothesis H_5:** "Polyamines have an uneven distribution pattern within the barley grain."

Rationale: The barley kernel comprises various anatomical regions (germ, pericarp, testa, aleurone and endosperm), each distinguished by biological function and chemical composition. Gradient spatial distribution patterns within the cereal kernel have been reported for macronutrients (starch and protein) (Shewry, Wan, Hawkesford, et al., 2020), micronutrients (minerals and vitamins) (Brier, Gomand, Donner, et al., 2015; Giordano, Reyneri, & Blandino, 2016) and phytochemicals (phenolic compounds and carotenoids) (Landberg, Kamal-Eldin, Salmenkallio-Marttila, et al., 2008; Ndolo & Beta, 2013). As polyamines are essential for biological activities, their synthesis and deposition are likely regulated and allocated to tissues according to their functional needs.

- **Hypothesis H_6:** "Pearling of barley grains as a mechanical fractionation method results in a product differing in polyamine content."

Rationale: Pearling, which uses abrasive force, gradually removes outer layers from barley grains before reaching the endosperm. Sequential pearling produces fractions that are

histologically distinct. If polyamines are unevenly distributed within the kernel, pearling fractions will exhibit varying polyamine concentrations depending on the tissues removed or retained.

- **Hypothesis H_7:** "Polyamine concentrations correlate with protein content at sub-kernel level."

Rationale: Variation in protein content across different cereal grain tissues is well documented. If polyamines also show a gradient distribution pattern within the barley kernel, their relationship with protein may also vary topologically. It can be speculated that polyamines and protein co-localize to facilitate biological processes during germination. Evaluating this relationship at a finer scale can help determine whether correlations observed at the whole-kernel level are consistent across tissues and provide insight into barley grain physiology.

- **Hypothesis H_8**: "Sieving whole barley flour yields fractions enriched in polyamines."

Rationale: Milling is one of the most common cereal processing methods. Each anatomical portion of the kernel exhibits different milling behaviour, producing distinct particle size distributions in the resulting flour. If polyamines are heterogeneously distributed within the kernel, sieving can isolate fractions enriched in tissues with high polyamine levels.

- **Hypothesis H_9:** "Polyamines correlate with markers of whole-grain status."

Rationale: Ross, van der Kamp, King, et al. (2017) suggested that the whole-grain status of a cereal product could be characterized by the proportion of materials that are absent in refined-grain ingredients, i.e. germ and bran. Several chemical components have been proposed as markers of whole-grain status due to their specific localization in the kernel, such as germ agglutinin in the embryonic axis or select phenolic compounds in the aleurone layer. Increased ash content is conventionally an indicator of the presence of non-endosperm components in the milling products. If polyamines are similarly concentrated in certain anatomical regions, their

contents may align with whole-grain markers. Correlation analysis will be based on polyamine profiles and the chemical composition of whole barley flour and its milling fractions.

1.3. Objectives

This body of work aims to address the following objectives:

- **Objective O_1:** Adapt a method for extracting polyamines and determining their contents in Canadian barley grains.
- **Objective O_2:** Examine the potential of various barley varieties as sources of polyamines.
- **Objective O_3:** Establish the relationship between polyamine contents and protein levels in barley grains at both the kernel and sub-kernel levels.
- **Objective O_4:** Characterize the distribution of polyamines within the barley kernel using mechanical fractionation and hand-dissection approaches.
- **Objective O_5:** Analyze the effectiveness of mechanical fractionation methods in producing polyamine-enriched fractions from barley grains.
- **Objective O_6:** Determine the relationship between polyamines in barley grains and other markers of whole-grain status.

1.4. Organization of Experimental Sections

This thesis is prepared in manuscript format and contains three experimental sections designed to evaluate the validity of the formulated hypotheses and address the proposed objectives:

- **Chapter 3:** Tests hypotheses H_1, H_2 and H_3 and addresses objectives O_1, O_2 and part of O_3. Thirteen barley varieties grown in Manitoba, Canada, representing different end uses, were examined for their polyamine profiles. Published as: Si Nhat Nguyen and Trust Beta.

"Polyamines in Canadian barley grains: Determination of their content and relationship with protein level". *Journal of Cereal Science* 114 (2023): 103781. The contributions of each author are as follows: **Si Nhat Nguyen:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing; **Trust Beta:** Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition.

- **Chapter 4:** Tests hypotheses H_4, H_5 and H_6 and contributes to objectives O_3, O_4 and O_5. Two hulled malting varieties and two hulless food varieties were used. Both dehulling and pearling techniques were applied to study polyamine distribution. Published as: Si Nhat Nguyen, Matthew G. Bakker and Trust Beta. **"Distribution of polyamines in kernel tissues of Canadian barley cultivars and their association with protein content".** *Journal of Cereal Science* (2025): 104225. The contributions of each author are as follows: **Si Nhat Nguyen:** Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – Original Draft, Writing – Review & Editing; **Matthew Bakker:** Investigation (Collection of scanning electron micrographs), Writing – Review & Editing; **Trust Beta:** Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition.
- **Chapter 5:** Tests hypotheses H_8 and H_9 and addresses part of objective O_5 and all of O_6. Five hulled and one hulless barley cultivars were milled at a laboratory scale and sieved to obtain different fractions. Manuscript submitted to the *Applied Food Research* and currently under revision as of June 16th, 2026: Si Nhat Nguyen, Marie-Françoise Samson, Joëlle Bonicel, Hamza Mameri and Trust Beta. **"Enrichment of polyamines across barley milling fractions and their correlation with markers of whole-grain content"**. The contributions of each author are as follows: **Si Nhat Nguyen:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Visualization; **Marie-Françoise Samson:** Methodology, Formal analysis, Investigation

(Determination of barley germ agglutinin content), Writing – Review & Editing; **Joëlle Bonicel:** Validation, Investigation (Determination of moisture content), Writing – Review & Editing; **Hamza Mameri:** Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration, Funding acquisition; **Trust Beta:** Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Chapter 2 — Literature Review

2.1. Polyamines: Definition, occurrence in nature and cellular uptake

2.1.1. Definition of biogenic amines and polyamines

Biogenic amines are nitrogenous, low-molecular-weight compounds produced by living organisms, primarily through the decarboxylation of amino acids and, to a lesser extent, through the transamination of carbonyl compounds (Saha Turna, Chung, & McIntyre, 2024). They are commonly classified into aromatic, heterocyclic and aliphatic groups based on chemical structure (Givanoudi, Heyndrickx, Depuydt, et al., 2023). Heterocyclic biogenic amines, such as histamine and tryptamine, contain the imidazole or indole moieties derived from histidine and tryptophan, respectively. Aromatic biogenic amines, including serotonin and catecholamines, are characterized by a benzene ring and are biosynthesized primarily from the aromatic amino acid tyrosine. Heterocyclic and aromatic biogenic amines play essential physiological roles as neurotransmitters and signalling molecules in both vertebrates and invertebrates (Breed & Moore, 2016). Aliphatic biogenic amines are synthesized from aliphatic amino acids via decarboxylation and typically contain straight or branched carbon chains without aromatic rings. This group includes monoamines and polyamines, which contain two or more amino groups. Polyamines are synthesized by decarboxylation of diamino acids (cadaverine from lysine and putrescine from ornithine or arginine), followed by aminopropyl transfer reactions (spermidine from putrescine and spermine from spermidine). They are involved in fundamental cellular processes, including cell growth, gene regulation and nucleic acid stabilization (Soda, 2022). Polyamines differ from other biogenic amines in both the number of amino groups, possessing multiple amine functionalities rather than a single amine and in their aliphatic carbon skeleton, which lacks aromatic or heterocyclic ring structures (Figure 2.1).

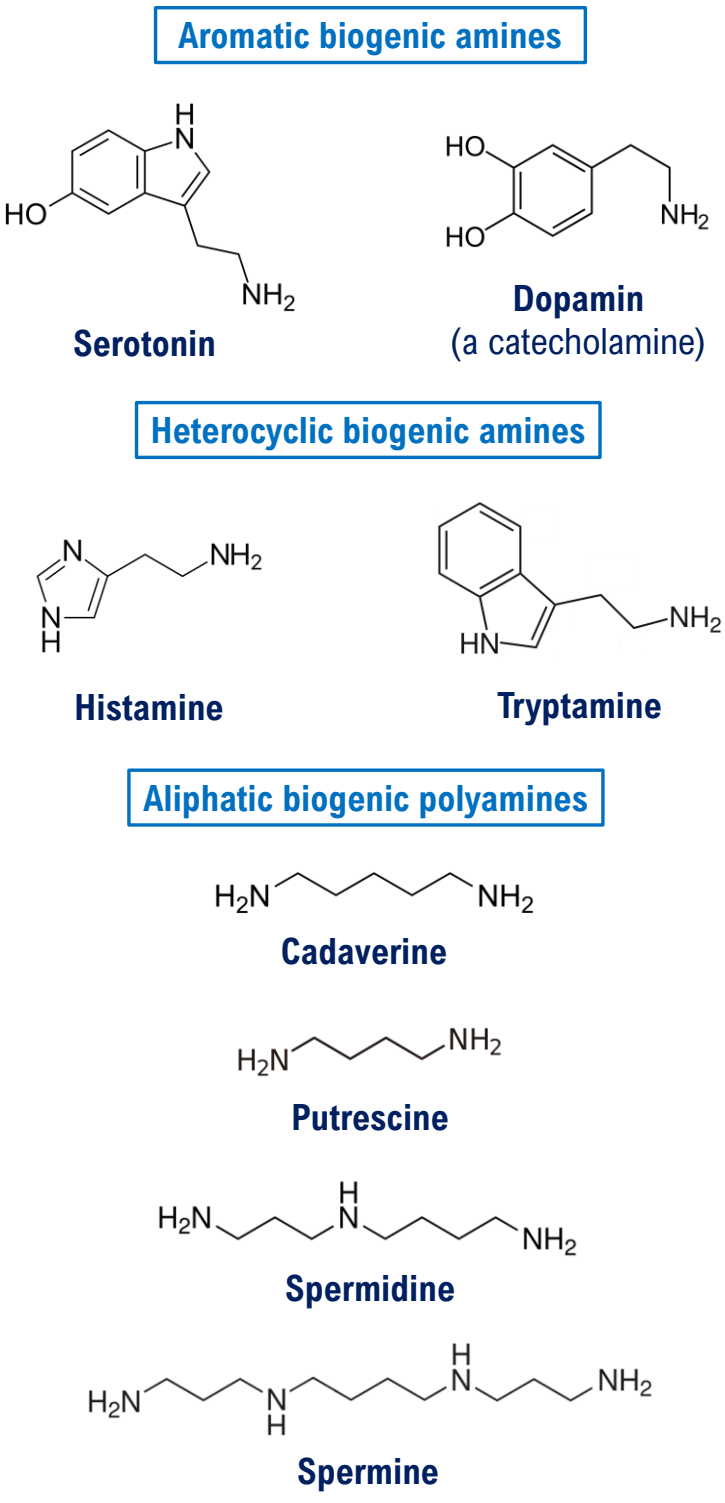

Figure 2.1. Chemical structures of some biogenic amines

2.1.2. Polyamine occurrence in nature

Polyamines are ubiquitous across all three domains of life (Archaea, Bacteria and Eukarya), highlighting their fundamental physiological importance (Michael, 2016). Phylogenetic analyses based on sequence conservation suggest that spermidine synthase was likely encoded in the last universal common ancestor (LUCA) genome, indicating that polyamine metabolism emerged very early in evolution (Li, Liang, Baniasadi, et al., 2025). Despite their universal occurrence, polyamine biosynthesis and diversity vary among domains of life. Putrescine serves as the central precursor of higher polyamines and can be produced via multiple enzymatic pathways. In bacteria and eukaryotes, putrescine is primarily generated via the decarboxylation of ornithine catalyzed by ornithine decarboxylase (ODC) (Li, Liang, Baniasadi, et al., 2023). An alternative pathway, frequently observed in bacteria and plants, involves arginine decarboxylation to agmatine, which is then converted to putrescine through agmatinase or related enzymes (Li, Liang, Baniasadi, et al., 2023). These parallel pathways provide metabolic flexibility, allowing organisms to adapt polyamine biosynthesis to nutrient availability and environmental conditions. After putrescine formation, spermidine is synthesized by adding an aminopropyl group, typically derived from decarboxylated S-adenosylmethionine, in a reaction catalyzed by spermidine synthase (Li, Liang, Baniasadi, et al., 2023). Some fungi and bacteria lack this enzyme and therefore do not produce spermine (Pegg & Casero, 2011). Thermophilic bacteria and Archaea exhibit a great diversity in polyamine metabolism, often synthesizing uncommon or long-chain polyamines (e.g. thermospermine, caldopentamine) or branched polyamines (e.g., tetrakis(3-aminopropyl)ammonium) (Pegg & Casero, 2011). These specialized compounds are believed to help maintain nucleic acid stability and participate in protein synthesis even under extreme environmental conditions like high temperatures (Pegg & Casero, 2011).

2.1.3. Cellular uptake of polyamines

a) Polyamine uptake of microbial cells

Since polyamines are positively charged under normal physiological conditions, the extracellular uptake of these compounds requires membrane proteins and the consumption of adenosine triphosphate (ATP) for active transport. The uptake of polyamines has been studied in both eukaryotic and prokaryotic cells, and it has been suggested that there may be convergent evolution in polyamine recognition and the binding modes of transport proteins (Sim, von Bülow, Hummer, et al., 2021). In *Escherichia coli*, two polyamine-transporting systems have been reported: one is putrescine-specific, and the other is spermidine-preferential, meaning that spermine uptake is the lowest rate (Igarashi & Kashiwagi, 1999). They are composed of a periplasmic substrate-binding protein that recognizes the polyamines through interactions with the amino and methylene groups of the molecules, two transmembrane proteins that form a channel for polyamine passage and a membrane-associated ATPase that supplies energy via ATP hydrolysis (Igarashi & Kashiwagi, 1999). Given the importance of polyamines for cellular vitality, the polyamine-transporting systems play a crucial role in mediating the virulence of bacterial pathogens infecting the respiratory and gastrointestinal tracts (Banerji, Kanojiya, Patil, et al., 2021; Jelsbak, Thomsen, Wallrodt, et al., 2012). For example, Jelsbak, Thomsen, Wallrodt, et al. (2012) found that the expression of virulence genes involved in epithelial invasion in *Salmonella enterica* serovar Typhimurium was responsive to exogenous polyamines. Contrarily, the authors observed that a polyamine-deficient mutant of the same strain was impaired in its ability to invade. In this context, Di Martino, Campilongo, Casalino, et al. (2013) highlighted that human bacterial pathogens have developed strategies to either utilize the polyamine reservoir or modulate polyamine-metabolizing processes to promote their proliferation within the host.

b) Polyamine uptake of plant cells

Early research by Pistocchi, Bagni, and Creus (1987) examined the kinetics of polyamine uptake and the distribution of absorbed polyamines within carrot cells (*Daucus carota*) from a callus culture. Based on kinetic characteristics, the authors proposed a biphasic model for polyamine uptake by carrot cells, with unequal affinities at low and high external polyamine concentrations. Polyamines transported into the intercellular environment were partitioned differently, with putrescine predominantly in the cytosol and spermidine localized in the cell wall. The authors also found that polyamine absorption was influenced by the electrical gradient in the presence of calcium ions, suggesting that an antiport mechanism may be involved. Later studies identified several polyamine transporters in diverse plant models, including *Arabidopsis thaliana* and rice (*Oryza sativa*) (Mulangi, Phuntumart, Aouida, et al., 2012). These transporters belong to the *L*-type amino acid transporter (LAT) family, also known as polyamine uptake transporters (PUT). Polyamine transporters are located at the plasma membrane of plant cells for extracellular uptake and on the membranes of several organelles to facilitate intercellular trafficking of polyamines (Pottosin, Olivas-Aguirre, Dobrovinskaya, et al., 2021). The expression of genes encoding specific polyamine transporters can be tissue-specific; for example, the gene encoding OsPUT1 in rice is not expressed in the root or grain (Mulangi, Phuntumart, Aouida, et al., 2012). Furthermore, LAT/PUT transporters are not strictly specific to polyamines but also facilitate the movement of structurally similar compounds, such as the herbicide paraquat, certain amino acids and vitamin B1 (Flores-Hernández, Gonzalez, Alvarado-Guitron, et al., 2025). These findings suggest that polyamine transporters serve as key players in plant metabolic and stress-response networks and that their modulation could have broad effects on herbicide sensitivity, nutrient uptake and resilience to environmental stresses.

Understanding the regulation and substrate specificity of amino acid and polyamine transporters may therefore provide valuable strategies for crop improvement and stress management. Begam, D'Entremont, and Good (2020) reported that an Arabidopsis knockout mutant lacking LAT-5 exhibited reduced shoot and leaf growth, accompanied by a modest increase (3%) in seed nitrogen content. This increase was associated with elevated concentrations of the nitrogen-transporting amino acids asparagine and glutamine in seed pods, suggesting altered nitrogen partitioning within the seed tissues. In the context of stress responses, Peña-Lucio, Pieckenstain, Gonzalez, et al. (2025) demonstrated that *put-1* and *put-5* Arabidopsis mutants, which lack the polyamine transporters PUT-1 and/or PUT-5, failed to mount polyamine-mediated oxidative stress responses upon infection with *Botrytis cinerea*. This response is a key component of plant defence against fungal pathogens. In contrast, *PUT-1* and *PUT-5* were strongly induced in wild-type plants during infection, underscoring their role in stress signalling. Similarly, a metabolome-based and genome-wide association study conducted by Yang, Zhang, Li, et al. (2024) in wild and cultivated tomatoes (*Solanum pimpinellifolium*, *S. lycopersicum* var. cerasiforme, *S. lycopersicum*) identified a domestication-selected gene cluster regulating polyamine metabolism and transport. This cluster includes genes involved in polyamine uptake (*SlPUT3*) and acylation (*SlPPOE*, *SlPPOF*, *SlAT4*, *SlAT5* and *Sl4CL6*). Overexpression of *SlPPOE*, *SlPPOF* and *SlAT5* enhanced phenolamide accumulation and improved salt tolerance, while *SlPUT3*-mediated polyamine transport was essential for salt stress resistance. Exogenous spermine improved salt tolerance in *SlPUT3*-overexpressing plants but reduced tolerance in *slput3* mutants, confirming the protective role of polyamines and phenolamides. Collectively, these studies highlight polyamine transporters as central regulators of nutrient allocation and stress responses, making them promising targets for developing stress-resilient crops.

2.2. Polyamine production by fermentation

2.2.1. Biochemical and microbiological aspects

Fermentation can significantly contribute to the polyamine profile of fermented foods and beverages thanks to the microbial decarboxylase activities, which can diversify the polyamine profile and/or enhance the polyamine contents (Barbosa, Martins Dala Paula, & Souza, 2024). In addition to the decarboxylation pathway, certain strains have been reported to deaminate agmatine to yield putrescine, including a strain of *Lactobacillus hilgardii* isolated from wine (Landete, Arena, Pardo, et al., 2008). Some traditional fermented foods known to be sources of polyamines include natto (Japanese fermented soybeans), miso (Japanese fermented bean paste), tempeh (Indonesian fermented soybean), sauerkraut (fermented cabbage), cheese and fermented sausage. Within the same category of fermented products, variations in polyamine levels are expected due to differences in the starting material, the type of starter culture and fermentation conditions. Lactic acid bacteria (LAB) have been reported to be key players in the conversion of various amino acids into biogenic amines, including aliphatic polyamines (Barbosa, Martins Dala Paula, & Souza, 2024). Certain LAB cultures used in wine and cider production were found to have polyamine-forming activity (Coton, Romano, Spano, et al., 2010). In this context, the term “metabiotics” has been used to designate a range of health-promoting bioactive compounds of low molecular weight produced by probiotic bacteria, such as short-chain fatty acids, polyamines, vitamins and bioactive peptides (Biswas & Das Mohapatra, 2023). Synergy in polyamine metabolism was also observed in the gut microbiota. Kitada, Muramatsu, Toju, et al. (2018) reported that putrescine levels were increased following arginine administration with probiotic *Bifidobacterium* spp. through an acid-triggered (pH < 6.5) and cooperative pathway involving *Escherichia coli*, *Enterococcus faecalis* and *Bifidobacterium* spp.

2.2.1. Food safety aspects

Despite their high polyamine content, a major food safety concern with fermented foods is the formation of toxic biogenic amines. Histamine and tyramine are commonly produced during microbial fermentation of plant- and animal-derived raw materials, and both biogenic amines have been reported to exhibit in vitro cytotoxicity at levels commonly found in some products (Linares, del Rio, Redruello, et al., 2016). Specifically, tyramine can induce intestinal cell death, whereas histamine can trigger apoptosis (Linares, del Rio, Redruello, et al., 2016). However, establishing the toxicity limits for biogenic amines can be challenging due to individual differences in detoxification mechanisms and their efficiency (Saha Turna, Chung, & McIntyre, 2024). Furthermore, the simultaneous presence of multiple biogenic amines can modulate their toxicity. Putrescine and cadaverine can increase histamine toxicity by competing for enzymes involved in histamine catabolism, leading to histamine accumulation and more severe intolerance reactions (Dala-Paula, Custódio, & Gloria, 2023). The production of toxic biogenic amines can be mitigated by the robust selection of starter culture and raw materials to minimize the initial presence of spoilage bacteria, as well as the application of certain plant extracts to inhibit their growth (Saha Turna, Chung, & McIntyre, 2024; Świder, Roszko, & Wójcicki, 2024). Strains of amine-oxidizing LAB have also been proposed as a method to reduce levels of harmful biogenic amines. Based on a collection of LAB strains lacking decarboxylase activity, Guarcello, De Angelis, Settanni, et al. (2016) conducted the screening of those having amine oxidase activities for the manufacture of fermented dairy products with low biogenic amine levels. However, this approach may compromise the levels of beneficial polyamines and the organoleptic profile of the fermented products due to the release of aldehydes and hydrogen peroxide as degradation substances in the medium (García-García, Rocha-Martin, Fernandez-Lorente, et al., 2018).

2.3. Impact of food processing on polyamines in foods

The polyamine content of food products can be affected by various food processing techniques. In the context of thermal treatment, Lima, Costa, Monaco, et al. (2017) observed an increase in polyamine content in organic green beans (*Phaseolus vulgaris*) following a 10 min boil. The author attributed the augmentation to a softened food matrix, which facilitated polyamine extraction. However, Reis, Dala-Paula, Tavano, et al. (2020) revealed that the spermidine content of mushrooms (*Agaricus bisporus*) decreased by 14% after sterilization at 97 °C for 20 min. Similarly, Muñoz-Esparza, Costa-Catala, Comas-Basté, et al. (2021) reported that boiling for 15 min or grilling for 7 min reduced the total polyamine content of diverse foodstuffs by up to 64%, possibly due to the extraction of polyamines into the cooking water or to their participation in Maillard reactions at high temperatures. Pyrolysis degradation was believed to be responsible for the reduction in polyamine levels during coffee roasting (Oliveira, Franca, Glória, et al., 2005). Meanwhile, microwave treatment for 5 min or sous vide cooking for 25 min did not significantly alter the polyamine levels of the examined commodities (Muñoz-Esparza, Costa-Catala, Comas-Basté, et al., 2021). With respect to non-thermal treatments, submerging pork loin in 20% w/w brine was reported to induce a 15% loss of spermidine in the final product relative to the non-cured sample (Paulsen & Bauer, 2007). In view of biochemical processes, Palavan-Ünsal, Damla Arisan, and Terzioglu (2007) examined changes in polyamine levels during the enzymatic browning of tea leaves. The spermidine content increased during the withering and rolling stages, likely as a defence mechanism against abiotic stress. However, this value decreased after tea fermentation, coinciding with a decrease in ODC transcript levels. In this case, the external stress caused by tea processing disrupted the balance between polyamine synthesis and degradation in tea leaves, leading to a decrease in spermine level.

2.4. Extraction and quantification of polyamines

The extraction and quantification procedures for polyamines have been developed for a wide range of samples, including solid (food matrices, microbial biomass and human, plant and animal tissues) and liquid (beverages and biological fluids) (Dai, Wu, Wang, et al., 2014; Latorre-Moratalla, Bosch-Fusté, Lavizzari, et al., 2009; Lavizzari, Teresa Veciana-Nogués, Bover-Cid, et al., 2006; Magnes, Fauland, Gander, et al., 2014). Polyamines are commonly extracted from biological samples using strong acids (e.g., perchloric, hydrochloric, or trichloroacetic acid) and at cold temperatures (Magnes, Fauland, Gander, et al., 2014). Under acidic conditions, the protonation of negatively charged cellular components takes place, which can offset their interactions with polyamines and thus facilitates extraction (Mateos, Reyes, Vicente, et al., 2000). The low temperature (typically 0–4 ºC) can also limit undesirable biochemical reactions, notably the oxidation of polyamines catalyzed by polyamine oxidases (Palavan-Ünsal, Damla Arisan, & Terzioglu, 2007). In the case of animal tissues, protein precipitation and fat removal are recommended (Inoue & Mizutani, 1973). Following extraction, polyamines are typically quantified using chromatographic and/or spectrometric techniques. Because aliphatic polyamines lack intrinsic chromophores or fluorophores, derivatization is generally required for detection. In conventional chromatographic approaches, polyamines are derivatized with reagents such as dansyl chloride, *o*-phthalaldehyde, or isobutyl chloroformate, which react with amino groups to introduce chromophoric or fluorogenic moieties, thereby allowing detection by ultraviolet (UV) or fluorescence detectors (Al-Hadithi & Saad, 2011; Dai, Wu, Wang, et al., 2014). High-performance liquid chromatography (HPLC), particularly reversed-phase HPLC, is the most widely used approach. The polarity and retention of polyamine are modified after derivatization, resulting in lower limits of detection and quantification, as well as good recovery and

reproducibility in complex matrices (Jiang, Ling, Yi, et al., 2024). On the other hand, hydrophilic interaction liquid chromatography (HILIC) can sometimes be performed without polyamine derivatization. However, it may show more pronounced matrix effects and might not be suitable for samples having low analyte concentrations and relatively complex matrices (Nalazek-Rudnicka, Kubica, & Wasik, 2020).

2.5. Biological activities and health-promoting effects of polyamines

2.5.1. In vitro *and* in vivo *studies on bioactivities of polyamines*

*In vitr*o and *in vivo* research have demonstrated that polyamines function as antioxidants and anti-inflammatory agents. Their antioxidant actions occur through two mechanisms: i) anion binding at higher degrees of protonation, which localizes to oxidation-sensitive cellular structures such as membranes and DNA and ii) cation binding at lower degrees of protonation, which sequesters transition metals such as the ferrous (Fe^{2+}) and ferric ion (Fe^{3+}) (Løvaas, 1996). Løvaas and Carlin (1991) reported that spermine effectively suppressed the Fe^{3+}-catalyzed oxidation of brain phospholipid liposomes in the presence of xanthine oxidase; however, this antioxidant effect diminished at higher Fe^{3+} concentrations. Toro-Funes, Bosch-Fusté, Veciana-Nogués, et al. (2013) evaluated the in vitro antioxidant activity of polyamines in both water- and fat-soluble systems. In soybean oil, spermidine and spermine perform particularly well at concentrations of 30–1250 μg/mL and help delay oxidation at multiple stages, including the formation of peroxides and carbonylic products. The authors concluded that their protective effects were comparable to those of the food preservative octyl gallate under the test conditions. *In vivo* research conducted in rats (Wu, Cao, Jia, et al., 2017) and piglets (Cao, Xu, Jia, et al., 2018) demonstrated that dietary supplementation of spermine lowered the levels of carbonyls (e.g., malondialdehyde) and carbonyl adducts (e.g., protein carbonyl) as markers of oxidative

damage in the liver and spleen of the animals. These studies reported that polyamine-enriched diets improved the intrinsic antioxidant arsenal, namely catalase, superoxide dismutase, glutathione peroxidase and glutathione levels. Mechanistically, these effects are linked to the activation of the Nrf2 signalling pathway, which leads to increased antioxidant defences and enhanced antioxidant-related gene expression (Cao, Xu, Jia, et al., 2018).

2.5.2. Preclinical and clinical studies on health-promoting effects of polyamines

Beyond studies demonstrating the antioxidant and anti-inflammatory properties of polyamines, growing preclinical and clinical evidence have indicated that polyamines function as cellular protectors and regulators that may support healthy aging. Early studies revealed that exogenous spermidine can delay aging and extend the lifespan of yeast and invertebrates by inhibiting histone deacetylases and activating autophagy (Eisenberg, Knauer, Schauer, et al., 2009; Morselli, Galluzzi, Kepp, et al., 2009), offering potential translational benefits. Preclinical studies in mice showed that oral administration of natural spermidine significantly extended lifespan, primarily through cardioprotective effects that included enhanced autophagy and mitophagy, improved mitochondrial respiration and preservation of cardiomyocyte function (Eisenberg, Abdellatif, Schroeder, et al., 2016). The molecular mechanisms of cardioprotection by spermidine in rats were elucidated by Yan, Yan, Wang, et al. (2019), which involved activation of the AMPK (adenosine monophosphate-activated protein kinase)/mTOR (mammalian target of rapamycin) pathway and facilitation of autophagic flux. The authors noted that chloroquine-induced inhibition of autophagy abolished the protective effects. In humans, a large population-based study reported a strong inverse association between dietary spermidine intake and all-cause mortality, suggesting a beneficial role in longevity (Kiechl, Pechlaner, Willeit, et al., 2018). Supporting this observation, a dietary intervention trial found that consuming polyamine-rich

natto increased circulating spermidine and reduced lymphocyte function-associated antigen-1 (LFA-1, a marker of immunosenescence and chronic inflammation) in healthy male participants (Soda, Uemura, Sanayama, et al., 2021). Collectively, dietary polyamines, particularly spermidine, are recognized as bioactive compounds that modulate inflammation, oxidative stress and age-related pathogenesis (Hofer, Daskalaki, Bergmann, et al., 2024).

2.6. Bioaccessibility and absorption of polyamines in the human body

Bioaccessibility refers to the fraction of a consumed compound of interest that is released from the food matrix during digestion, whereas bioavailability designates the proportion of an ingested compound that is released and absorbed in the gastrointestinal tract, subsequently becoming available for utilization or storage in destined tissues (Oduro-Obeng, Chaudhry, Zogona, et al., 2024). Dietary intake is a major source contributing to the human polyamine pool, along with de novo synthesis and uptake from the colonic microbiota (Vargas, Wertheim, Gerner, et al., 2012). The bioaccessibility of polyamines has been measured in selected food matrices using simulated gastrointestinal digestion models. Reis, Dala-Paula, Tavano, et al. (2020) reported that spermidine in fresh mushrooms (*A. bisporus*) was completely released during the gastric phase and remained stable throughout the intestinal phase. Dala-Paula, Deus, Tavano, et al. (2021) investigated the bioaccessibility of bioactive amines in 70% dark chocolate and observed increases in the concentrations of spermidine and spermine during gastric digestion. However, a slight reduction in polyamine levels was observed after the intestinal phase, likely due to interactions or conjugation with other food components present in the digesta (e.g., polyphenols or peptides). Following digestion, polyamines reach the gut lumen at millimolar concentrations and are quickly absorbed in the duodenum and jejunum (Milovic, Faust, Turchanowa, et al., 2001). Despite rapid absorption, enterocyte-mediated polyamine metabolism

can limit postprandial blood polyamine concentrations. Human intestinal epithelium requires high levels of polyamines due to its frequent renewal (Seiler & Raul, 2007). Therefore, postprandial increases in blood polyamine levels are modest and typically below 20 μM (Milovic, 2001). However, long-term consumption of polyamine-rich foods has been reported to increase steady-state blood polyamine levels in both mice and humans (Soda, Dobashi, Kano, et al., 2009).

2.7. Dietary intake and homeostasis of polyamines

Polyamines are present in all living organisms and are widely found in both plant and animal foods, with variations in the content and relative abundance of individual polyamines (Muñoz-Esparza, Latorre-Moratalla, Comas-Basté, et al., 2019). Ali, Poortvliet, Strömberg, et al. (2011) estimated that adolescents consumed an average of 316 μmol/day of polyamines (337 μmol/day in males and 303 μmol/day in females), derived from mean daily intakes of putrescine (215.5 μmol), spermidine (66 μmol) and spermine (34.5 μmol). Although there is no official recommended dietary allowance for polyamines, the authors suggest a dietary intake of 540 μmol/day, based on the Swedish Nutrition Recommendations and the available database on food polyamines (Ali, Poortvliet, Strömberg, et al., 2011). Dietary habits and cultural patterns lead to differences in polyamine intake levels (Cantabrana, Peña-Iglesias, Castro-Estrada, et al., 2025). The mean daily dietary intake of spermidine ranges from approximately 7 to over 25 mg (48 to 172 μmol), with the Mediterranean diet providing the highest amounts and being widely associated with improved human health (Madeo, Eisenberg, Pietrocola, et al., 2018). Dietary polyamines complement endogenous synthesis in the human body, which declines with aging, and growing evidence suggests that reduced polyamine biosynthesis contributes to age-associated cellular dysfunction (Li, Brazill, Liu, et al., 2017; Soda, 2022). The human gut microbiota is

essential for regulating host polyamine levels through its metabolic functions. Microbes in the gut produce polyamines in the intestinal lumen, which directly impact epithelial and immune cells and help maintain intracellular polyamine balance through specialized uptake mechanisms (Chen & Fang, 2025). On the other hand, dysregulation of polyamine metabolism and associated pathways, such as the activity of transcription factor eIF5A2, has been implicated in cancer development and neoplastic progression (Murray-Stewart, Dunworth, Foley, et al., 2018).

2.8. Polyamines in the physiology of plants and cereal grains

2.8.1. Relationship between polyamines and nitrogen metabolism

As nitrogenous compounds, polyamines contribute to the plant nitrogen pool, and increased nitrogen availability through fertilization or cell culture supplementation generally elevates total polyamine levels in plants (Gény, Broquedis, Soyer, et al., 1996; Serapiglia, Minocha, & Minocha, 2008). Owing to their high intracellular concentrations, free polyamines (putrescine, spermidine and spermine) and their conjugates, particularly those accumulated in seeds, can serve as nitrogen storage forms to support germination (Moschou, Wu, Cona, et al., 2012). However, because polyamines are involved in numerous physiological processes, they are readily mobilized during plant growth and in response to stress. During pathogen interactions, polyamines are oxidized by diamine oxidase (DAO) or polyamine oxidase (PAO), generating hydrogen peroxide and ammonia (Gerlin, Baroukh, & Genin, 2021). The resulting hydrogen peroxide promotes lignification and strengthens the cell wall, enhancing resistance to pathogen invasion, whereas excess ammonia is detoxified via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway and recycled into amino acid synthesis (Gerlin, Baroukh, & Genin, 2021; Sánchez-Rodríguez, Rubio-Wilhelmi, Ríos, et al., 2011). Consequently, polyamines and their catabolic products play a central role in nitrogen turnover in plants (Moschou, Wu, Cona, et

al., 2012). Beyond biotic stress, plants are increasingly exposed to abiotic stresses associated with climate change, including salinity, extreme temperatures, drought, flooding and enhanced UV radiation (Pereira, 2016). Given the ability of polyamines to enhance the tolerance of plants to stress, understanding the regulation of polyamine metabolism and nitrogen reutilization is crucial for addressing emerging agronomic challenges under changing environmental conditions (He, He, & Ding, 2018).

2.8.2. Roles of polyamines in plant-fungi interactions

As ubiquitous compounds in living organisms, polyamines are also present in fungi, where they are essential for cellular growth and development (Rocha & Wilson, 2019). In addition to their regulatory roles, polyamines can serve as nitrogen and carbon sources for filamentous fungi (Majumdar, Minocha, Lebar, et al., 2019). Despite these generally supportive functions, the effects of exogenously applied polyamines on fungal pathogenicity could depend on the context. For example, treatment of *Magnaporthe grisea* with putrescine, spermidine, or spermine inhibited appressorium formation, a critical structure for host penetration (Choi, Kang, Lee, et al., 1998). In contrast, polyamines have been shown to enhance mycotoxin production in certain pathogenic fungi. Supplementation of putrescine in *Fusarium graminearum* cultures induced the synthesis of the trichothecene mycotoxin deoxynivalenol (DON), whereas polyamine inhibitors suppressed this response (Crespo-Sempere, Estiarte, Marín, et al., 2015). This induction was associated with increased expression of *TRI5* and *TRI6*, which encode a terpene synthase and a transcription factor essential for trichothecene biosynthesis (Crespo-Sempere, Estiarte, Marín, et al., 2015; Kolawole, Meneely, Petchkongkaew, et al., 2021). Similar activation of trichothecene production following polyamine supplementation has also been reported in axenic cultures of *F. graminearum* (Gardiner, Kazan, Praud, et al., 2010). Conversely, polyamines

are integral components of plant defence responses against fungal pathogens (Gerlin, Baroukh, & Genin, 2021) and endogenous polyamines in maize have been shown to contribute to resistance against *Aspergillus flavus* infection (Majumdar, Minocha, Lebar, et al., 2019). However, once fungal invasion is established, characterizing the fungal secretome, including hydrolytic enzymes and transporters, may provide valuable insights into the mechanisms underlying polyamine acquisition from plant tissues (Vincent, Rafiqi, & Job, 2019).

2.8.3. Roles of polyamines in cereal grain physiology

Botanically, cereal grains are dry fruits (caryopses) in which the ovary wall (pericarp) is fused directly to the seed coat, forming a single structure. In general, the development of cereal grains after pollination can be divided into three major phases: early grain development (involving cell division and differentiation), grain filling (the longest phase, during which starch and protein accumulate) and grain maturation (characterized by a reduction in moisture content and cessation of physiological activity) (Shewry, Mantila, & Serna-Saldivar, 2023). Several studies have demonstrated the importance of polyamines during the grain-filling period, when biochemical processes are most active. Xu, Jian, Li, et al. (2021) investigated the relationship between polyamines and amino acids in filling grains of six different rice cultivars. The authors observed increased amino acid contents in cultivars with higher free polyamine concentrations. Mechanistically, there were positive correlations between free polyamine concentrations and the activities of enzymes involved in the nitrogen assimilation and synthesis of amino acids, including glutamine synthetase, glutamate synthetase, aspartate transaminase, aspartate kinase and alanine transaminase. On the other hand, the application of the polyamine synthesis inhibitor methylglyoxalbis(guanylhydrazone) (MGBG) at the beginning of the grain-filling phase reduced the activities of enzymes required for nitrogen uptake and amino acid metabolism (Xu, Jian, Li,

et al., 2021). This corroborated the roles of polyamines, particularly spermidine and spermine, in amino acid acquisition during the grain-filling period in rice. Apart from the rapport with amino acid-metabolizing enzymes, Liu, Gu, Wu, et al. (2013) analyzed the effect of applying exogenous polyamines on the production of growth hormone in wheat during the grain-filling phase. The researchers observed that external applications of spermidine and spermine increased the grain-filling rate and grain weight, whereas exogenous putrescine had no significant effects.

2.9. Phenolamides: Structure, biological importance, distribution and bioactivities

One important class of biogenic amine derivatives is phenolamides (also referred to as hydroxycinnamic acid amides), which are formed by conjugation of hydroxycinnamic acids (e.g., ferulic, caffeic, or *p*-coumaric acids) with biogenic amines or polyamines via amide bonds (Li, Zhao, Zhao, et al., 2018). With polyamines, conjugation often occurs at multiple amino groups, allowing the attachment of several hydroxycinnamoyl moieties at different positions and resulting in a broad structural diversity. Based on plausible biosynthetic reactions, Li, Zhao, Zhao, et al. (2018) annotated a total of 846 phenolamides derived from the most common phenolic acid and polyamine or aromatic monoamine substrates. The occurrence of phenolamides has been reported in grains of hulless barley (Drawbridge, Apea-Bah, Hornung, et al., 2021), proso millet (Yuan, Xiang, Zheng, et al., 2022) and foxtail millet (Xiang, Zhang, Apea-Bah, et al., 2019). As part of the reproductive organ of angiosperms, floral pollens were reported to contain a variety of phenolamides, which account for approximately 1% of their total weight (Qiao, Feng, Zhang, et al., 2023). The biosynthesis of phenolamides is catalyzed by hydroxycinnamoyl transferases, which belong to the coenzyme A (CoA)-dependent acyltransferase family (Xu, Wang, Zhuang, et al., 2023). These enzymes employ activated hydroxycinnamoyl-CoA as acyl donors and biogenic amines as acyl acceptors (Xu, Wang, Zhuang, et al., 2023). The presence of one or more aromatic

benzene rings confers phenolamides an enhanced capacity to absorb UV radiation, as well as greater hydrophobicity or increased antimicrobial activity (Menezes & Campos, 2024). These physicochemical properties are biologically relevant for the protection of cereal grains. Phenolamides contribute to defence mechanisms by protecting tissues from abiotic (e.g., UV-induced) and biotic stresses (e.g., pathogen invasion), thereby helping maintain seed viability (Liu, Jiang, Ma, et al., 2022).

Phenolamides in cereal grains have been characterized with respect to their histological localization. The distribution of hordatines, dimers of hydroxycinnamic acid agmatines typically found in barley, was determined by imaging mass spectroscopy (Gorzolka, Bednarz, & Niehaus, 2014). The findings revealed that most hordatines and their precursors were found in barley germ, consistent with their roles in stress responses and seed germination. However, the β-glycosidic derivative of hordatine A, which contributes to the astringency of beer aftertaste, was localized in the aleurone layer of ungerminated barley (Kohyama & Ono, 2013). Another group of species-specific phenolamides is the avenanthramides (i.e., amides of anthranilic acid), which are naturally occurring in oats. The pearling study of Liu and Wise (2021) indicated that the majority of avenanthramides was retained in the 4% pearling fraction and declined dramatically as the abrasion degree was increased to 13.5% of grain weight. In terms of nutraceutical potential, phenolamides have also been reported to exhibit potent antioxidant and anti-inflammatory properties (Roumani, Duval, Ropars, et al., 2020). Wang, Zhu, and Sang (2023) reported that *p*-coumaroylagmatine and feruloylagmatine in barley could neutralize methylglyoxal, a toxic and reactive dicarbonyl implicated in the onset of diabetic conditions. Their animal study demonstrated that adducts between methylglyoxal and phenolamide can be excreted in urine and feces (Wang, Zhu, & Sang, 2023). In a clinical study focusing on avenanthramides (AVA), the cohort consuming AVA-supplemented oat cookies daily showed a

global improvement in eccentric exercise-induced inflammation, including lower levels of plasma cytokines and IL-6 after 8 weeks, compared with those of the control group receiving oat products void of AVA (Zhang, Zhao, Zhang, et al., 2020). The mechanism behind the anti-inflammatory property of AVA-C was later elaborated in the context of atherosclerosis pathogenesis, in which AVA-C was found to downregulate the nuclear factor (NF)-κB signalling pathway (Park, Choi, Abekura, et al., 2021).

2.10. Barley grain structure

From a structural and histological perspective, the barley grain is a complex, multilayered caryopsis with distinct tissues arranged to support protection, storage and germination. The outermost layer of barley is the husk, formed by the lemma and palea, which serve to protect the grain (Grant, Brennan, & Hoad, 2020). Unlike most other cereals, many barley varieties retain their hulls attached to the caryopsis at maturity and are commonly referred to as covered or hulled barley, whereas some barley varieties are free-threshing and are known as naked or hulless barley. In covered barley, adhesion occurs when the lipid-coated surface of the caryopsis contacts the inner hull surface, and the distinction between covered and naked caryopsis phenotypes is controlled by a transcription factor gene that is part of the ethylene response factor (ERF) family (Taketa, Amano, Tsujino, et al., 2008). Beneath the hull, the bran is composed of the pericarp, the seed coat (testa) and the aleurone layer. In barley, the aleurone is a living tissue typically comprising multiple cell layers with thick cell walls and is rich in protein bodies and lipid droplets (Antonini, Zara, Valentini, et al., 2018). This layer also contains a substantial quantity of proteins, minerals, vitamins and phenolic compounds in comparison with other anatomical parts of the barley grain (Sullivan, Arendt, & Gallagher, 2013). Structurally, the

aleurone forms a continuous sheath that surrounds the endosperm. The endosperm is the largest tissue component and consists of large, thin-walled and inactive storage cells (Enari & Sopanen, 1986). This anatomical part is packed with starch granules embedded in a protein matrix and serves as a source of carbon and nitrogen during germination (Jääskeläinen, Holopainen-Mantila, Tamminen, et al., 2013). At the basal end of the barley grain, the embryo includes the scutellum and embryonic axis, which houses the plumule and radicle and is specially adapted for nutrient absorption and quick cell growth during germination.

2.11. Techniques to study the distribution of phytochemicals in whole cereal grains

2.11.1. Grain fractionation techniques

a) Hand dissection

Several authors have employed a hand-dissection approach to separate the botanical fractions of the kernel manually. In the typical procedure, husked cereal grains are initially surface-sterilized by soaking in disinfecting reagents, commonly a dilute sodium hypochlorite solution (Daneri-Castro & Roberts, 2016; Hall & Laidman, 1968; Ndolo & Beta, 2013). A lengthwise cut is made to expose the anatomical parts of the grain, followed by the removal of the germ. The half-grains obtained by the longitudinal cut are then imbibed in water for a prolonged period (15–72 h) (Hall & Laidman, 1968; Ndolo & Beta, 2013). Moisturization is necessary to soften the endosperm and make the tissues more readily separable from each other. It can be combined with disinfection by soaking in 50% ethanol if initial surface sterilization is skipped (Barron, Surget, & Rouau, 2007). The starchy endosperm can be isolated from the aleurone layer by scraping with a scalpel (Daneri-Castro & Roberts, 2016; Hall & Laidman, 1968). Gartaula, Dhital, Pleming, et al. (2017) reported a popping method to obtain the endosperm fraction from grains that did not undergo longitudinal cutting, in which softened

endosperm was extracted from the outer envelope after removing the two distal ends. Finally, the outer pericarp can be peeled off from the aleurone layer (Barron, Surget, & Rouau, 2007; Betts, Berkowitz, Liu, et al., 2017). The chief advantage of manually dissecting grains is the improved purity of the resulting fractions; however, it can be time-consuming. To reduce the labour involved in this technique, the embryo of the whole grain can be initially separated manually, and the degermed kernel can then be milled to produce fractions rich in endosperm or bran (Posner & Li, 1991; Shao, Xu, Sun, et al., 2014). In this procedure, the purity of the fractions would be compromised.

b) Pearling

Pearling grains is the process of removing the outer layers of the grains by abrasion to remove the indigestible hull (if present) and reduce cooking times of the remaining grain, because the outer layers contain most of the fibre of the grain. Pearling of other cereal grains can also be done as a useful technique to determine the distribution of phytochemicals and other constituents within the grains. Previous studies on pearled barley and wheat involved sequentially pearling grains, extracting fractions with each pass that accounted for 5% of the kernel weight, removing up to 20 or 30% of the kernel in total (Beta, Nam, Dexter, et al., 2005; Giordano, Reyneri, & Blandino, 2016; Madhujith & Shahidi, 2006). The 5% increments showed differences in composition among fractions with respect to total phenolic content and antioxidant activity (Beta, Nam, Dexter, et al., 2005; Madhujith & Shahidi, 2006) and folate (Giordano, Reyneri, & Blandino, 2016). The advantage of pearling is that after the preliminary testing and instrument set-up to achieve the desired fractions, the pearling process is faster and less tedious than hand fractionation of the grains. Scarification and polishing are similar techniques to pearling for removing the outer layers of the kernel without significantly compromising the structural

integrity of the endosperm. While pearling involves the friction between the surface of kernels and/or abrasion of kernels against abrasive stone, scarification relies on the forced contact between kernels and abrasive surface to produce a greater quantity of abraded fractions (De Brier, Lemmens, Gomand, et al., 2021; Flores, Hicks, & Wilson, 2007). Polishing is commonly employed in rice processing to remove the bran layer from brown rice, yielding white polished rice as the finished product. Rice polishing can be performed using either an abrasive or a frictional rice polisher, in which the kernels interact with one another and with the machine surfaces as the rotating drum or stirrer is in motion (Li, Han, Jia, et al., 2021).

b) Milling

Milling is the most industrially relevant and most extensively used technique for separating wheat kernels into fractions; however, milling of minor cereal crops such as barley and oats is less common on a commercial scale (Izydorczyk, Lagassé, Hatcher, et al., 2005; Izydorczyk, McMillan, Bazin, et al., 2014). Milling involves tempering and grinding cereal grains to flour, then sifting the flour to separate the bran, germ and endosperm. After milling, mill streams can be combined to achieve flour with specific physicochemical, functional, or nutritional properties. The milling technique can be modified to obtain distinct fractions of varying particle size. The fractions obtained from milling can be analyzed for their starch, protein, ash, fibre, and phytochemical content, as well as other characteristics such as particle size. Milling does not separate botanical tissues, such as the germ and aleurone, as cleanly as hand dissection; however, examination of milled fractions can provide information on phytochemical distribution in the grain. Furthermore, the presence and composition of certain nutrients and phytochemicals may serve as biomarkers for the botanical tissues of the grain within milled fractions. The presence of bound phenolic acids in the milled fraction indicates that

the fraction comprises bran layers since bound phenolic acids are predominantly found in the cell walls of the bran (Barron, Samson, Lullien-Pellerin, et al., 2011). The bran mill stream may be further processed and purified to obtain additional fractions with unique characteristics. Milling cereal grains generates several streams of by-products containing unequal proportions of the grain outer layers. According to the Association of American Feed Control, the by-products of wheat milling can be classified based on their crude fibre content as bran (> 95 g/kg), middlings (70–95 g/kg), shorts (40–70 g/kg), red dog (15–40 g/kg) and feed flour (< 15 g/kg) (Huang, Shi, Su, et al., 2014). These terms are sometimes used in milling studies.

2.11.2. Imaging techniques

a) Mass spectrometry imaging

Mass spectrometry imaging (MSI) is a technique that has gained popularity in food science research for visualizing the distribution of components within samples. In brief, the principle of this technique is based on ion signals produced and detected when a thin layer of the sample is exposed to an ion beam (Yoshimura & Zaima, 2020). The major types of ionization in MSI include secondary ion mass spectrometry (SIMS), matrix-assisted laser desorption/ionization (MALDI) and desorption electrospray ionization (DESI). Given its ability to analyze a plethora of molecules simultaneously, MSI has been used to study temporal changes in the distribution of various substances in complex biological processes, such as barley germination (Gorzolka, Kölling, Nattkemper, et al., 2016). The localization of a compound can be monitored by measuring the intensity of its characteristic ion within the sectioned sample layer. For instance, a flavonoid with an *m*/*z* value of 365.102 was identified during the germination of barley grains under saline stress conditions (Gupta, Rupasinghe, Callahan, et al., 2019). Sagara, Bhandari, Spengler, et al. (2020) employed MALDI coupled with a Fourier-transform orbital

trapping mass spectrometer to detect individual polyamines in soybean seeds histologically. The topographical mapping of polyamines showed that spermidine and spermine were more concentrated in the germ than in the cotyledons of soybean seed, with the root and shoot meristems showing the highest concentrations (Sagara, Bhandari, Spengler, et al., 2020). These results were later confirmed by experiments using mechanical fractionation. Despite the expense of mass spectrometry imaging equipment, this technique offers powerful analytical capabilities and avoids the need to physically separate grain tissues. Nevertheless, sample preparation and fixation are necessary prior to each analysis (Yoshimura & Zaima, 2020).

b) Fluorescence imaging

Fluorescence imaging relies on the emission of visible light (400–700 nm) from fluorescent compounds upon excitation with UV to red light, and the resulting fluorescence patterns can be observed using microscopy techniques (Khatib, Pouzet, Lafitte, et al., 2021). While phenolic acids and flavonoids exhibit autofluorescence, imaging of arabinoxylans and beta-glucans requires fluorescent dyes or stains, and it is important to select excitation and emission wavelengths so that the dye appears brighter than the autofluorescence (DeVree, Steiner, Głazowska, et al., 2021; Fulcher, 1982; Khatib, Pouzet, Lafitte, et al., 2021). Izydorczyk, McMillan, Bazin, et al. (2014) used fluorescence microscopy to show the localization of beta-glucan and arabinoxylans in transverse sections of whole grain oats and barley by staining with periodic acid-Schiff reagent for carbohydrates and counterstaining with Calcofluor for cell walls containing mixed linkage beta-glucans. In this study, phenolic acids esterified to arabinoxylans were observed in the outer pericarp and aleurone of both oats and barley as pale blue autofluorescence. In another research, Ndolo, Beta, and Fulcher (2013) examined the location and distribution of ferulic acid in various pigmented and non-pigmented cereal grains, including

varieties of barley, wheat and corn, using fluorescence microscopy. In this study, grain sections viewed under a fluorescence microscope were 10 µm thick. A UV/49-DAPI filter was used, with an excitation wavelength of 365 nm and an emission wavelength cutoff of >420 nm. To obtain quantitative results and identify minor variations in ferulic acid concentration within the grains, the authors used fluorescence intensity maps to visualize distinct fluorescence profiles. The fluorescence intensity mapping technique revealed differences in ferulic acid concentration within cell walls and aleurone cells that would otherwise be missed by traditional analytical methods (Ndolo, Beta, & Fulcher, 2013).

2.12. Valorization of the literature review

Parts of the literature review have been incorporated into peer-reviewed publications, with Si Nhat Nguyen as the lead author:

- Section 2.9 appears in: Si Nhat Nguyen, Trust Beta. **"Cereal-derived polyphenols and their bioactive properties".** *Current Opinion in Food Science* 56(2024): 101136.
- Sections 2.9 and 2.11 appear in: Si Nhat Nguyen, Pamela Drawbridge, Trust Beta. **"Distribution of cereal phytochemicals and micronutrients in whole grains: A review of nutraceutical, industrial and agricultural implications".** *Cereal Chemistry* 101(2024): 903-925.

Chapter 3 — Experimental Section 1 (Polyamines in Canadian barley grains: Determination of their content and relationship with protein level)

3.1. Chapter Abstract

Barley is one of the important crops with diverse end-use purposes. In every use of barley grains, the protein fraction plays an important function that impacts the quality of final products. Polyamines, having multiple nitrogen atoms in their chemical structure (e.g., putrescine, spermidine and spermine), may be linked to the levels of protein and nitrogen reserves. In this study, thirteen Canadian barley varieties were characterized for their total polyamines and protein content. The effects of extractants on the extractability of polyamines were considered, and HCl (1 M) was found to be more effective than trichloroacetic acid (5% w/v) in extracting free polyamines from whole barley flour. The total polyamine content of barley grains varied from 6.46 to 11.47 mg/100 g dry basis, and spermidine was the most abundant. Polyamine represented 0.045–0.071% w/w grain protein. Significant and positive correlations were found between protein content and levels of total polyamines ($r = 0.798$, $p = 0.001$), putrescine ($r = 0.771$, $p = 0.002$) and spermine ($r = 0.668$, $p = 0.009$). Principal Component Analysis and K-Means Cluster Analysis divided the barley samples into one cluster (hulless barley for food) differentiated from the other (hulled barley for malting and feeding) by their superiority in polyamine and protein content.

Keywords: cereal grain; barley protein; polyamine; spermidine

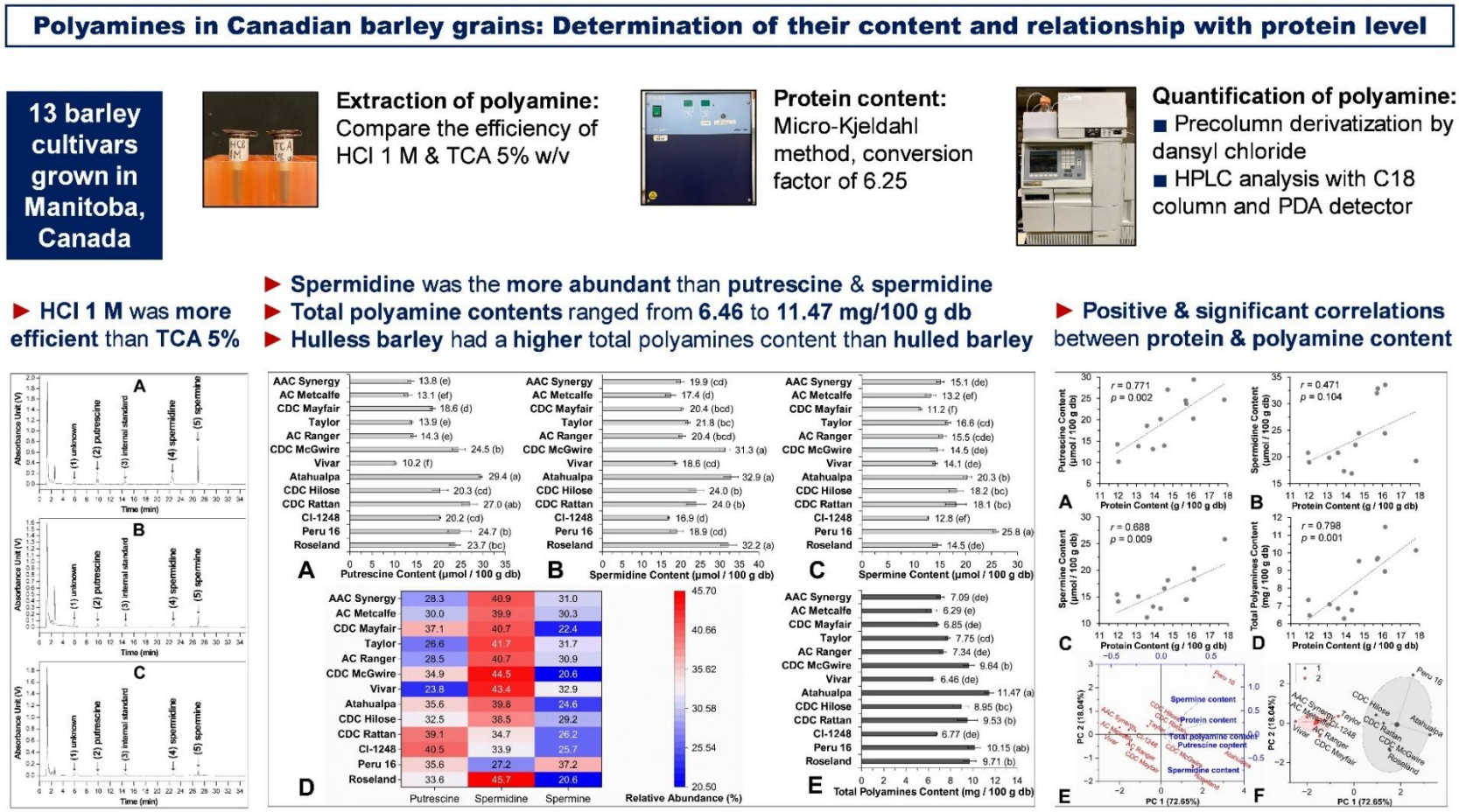

Figure 3.1. Graphical abstract of experimental section 1

3.2. Introduction

Barley is one of the major crops with global production volume positioning it at the fifth place after maize, wheat, rice and soybean (Meng, Rasmussen, Christensen, et al., 2023). Given the challenges of climate change, future projections show that barley yield in the Mediterranean Basin would experience a net decline of 9% because of heat and drought stress (Cammarano, Ceccarelli, Grando, et al., 2019), while the yield of this crop in the Canadian Prairies is expected to rise by 3.5% as a result of increased temperature and precipitation (Lychuk, Moulin, Izaurralde, et al., 2017). On the other hand, the elevated atmospheric CO_2 level would enhance the biomass accumulation of barley seeds; nevertheless, reducing their protein content (Rezaei, Rojas, Zhu, et al., 2022). The decrease in grain protein content of barley due to climate change may be problematic and could result in malnutrition among the populations in marginal areas relying on this crop as a staple food (Bista, Heckathorn, Jayawardena, et al., 2020). Moreover, since the protein fraction plays an important role in the end-use purposes of barley grains, the changes in protein level would compromise the quality of the final products. In animal feeding, a high protein content of barley grains is desirable to maintain an optimal performance of cattle (Miller, Beasley, Drury, et al., 2015). In beer making, the grain protein content of barley needs to conform to the requirements of producers to avoid certain technical complications, such as haze formation and low foam stability (Briggs, 2004).

The mechanisms by which a rise in atmospheric CO_2 concentration lowers the protein content of barley grains are still unclear (Rezaei, Rojas, Zhu, et al., 2022). Bista, Heckathorn, Jayawardena, et al. (2020) observed no statistical differences in the quantity of nitrogen-uptake proteins in barley roots under normal and increased ambient CO_2 levels. Delving into the nitrogen metabolism may help elucidate the alternation in protein content of barley grains in

response to different environmental circumstances. Below the macro-molecular level, the nitrogenous compounds in plants can be found as amino acids and polyamines, whereas NH_3 and NH_4^+ are not stored in the cells (Moschou, Wu, Cona, et al., 2012). Polyamines are aliphatic compounds of low molecular weight containing two or more amino groups. Plants can absorb the inorganic nitrogen source in the form of NO_3^- or NH_4^+ ions prior to converting them into NH_3 for the synthesis of amino acids via the glutamine synthetase/glutamate synthase cycle (Paschalidis, Tsaniklidis, Wang, et al., 2019). The first amino acid to be synthesized is usually glutamate, followed by the generation of other amino acids, including arginine and ornithine, the precursors of putrescine (Moschou, Wu, Cona, et al., 2012). Putrescine ($C_4H_{12}N_2$) is the simplest polyamine formed by decarboxylation of the diamino acids, while spermidine ($C_7H_{19}N_3$) and spermine ($C_{10}H_{26}N_4$) are produced from putrescine by prolonging the aliphatic chain with one or two *n*-propylamine units (Moschou, Wu, Cona, et al., 2012). In terms of physiological importance, polyamines participate in the response to stress as well as in other vegetative and reproductive processes of plants (Paschalidis, Tsaniklidis, Wang, et al., 2019).

Given the involvement of polyamines in the metabolism of amino acids, this class of nitrogenous compounds may hold an association with the changes in barley protein content. The amount of proteins in barley grains grown under normal and unfavourable conditions has been linked to the content of phytic acid, Zn and Fe (Dai, Wang, Zhang, et al., 2007; Gao, Persson, Vincze, et al., 2022). However, there is hardly any literature considering the relationship between polyamine levels and protein content of barley. Therefore, this study was aimed to determine the profile of polyamines among various Canadian barley cultivars after examining their extractability with two different solvents, i.e., HCl (1 M) and trichloroacetic acid (TCA) (5% w/v). Henceforth, the correlations between the level of individual and total polyamines of barley grains and their protein content were evaluated.

3.3. Materials and methods

3.3.1. Materials

a) Barley cultivars

A set of thirteen barley (*Hordeum vulgare*) cultivars of various end uses (for malting, animal feed and food) was provided by Agriculture Agri-Food Canada (AAFC). These barley cultivars were grown in Manitoba (MB), Canada, with harvest years ranging from 2009 to 2022. The botanical traits, end use and quality characteristics of each cultivar are described in Table 3.1. Following receipt, whole barley grains were ground using a laboratory centrifugal mill operating at 10,000 rpm (Model ZM200, Retsch Co., Haan, Germany) and passed through a 0.5 mm sieve. In this study, the term "whole barley grains" designates the caryopses with and without the adhering hulls for hulled and hulless barley, respectively. Additionally, barley caryopses did not undergo dehulling. The resultant whole grain barley flours were kept in air-tight polyethylene bags (Whirl-Pak Co., Madison, WI, USA) at -18 ^{o}C until further analysis. They were brought to room temperature prior to the commencement of each analysis.

b) Chemicals

Dansyl chloride (5-(dimethylamino) naphthalene-1-sulfonyl chloride, ≥ 99%) and polyamine external standards in the form of chloride salt (≥ 99%) were acquired from Millipore Sigma Corp. (Burlington, MA, USA). The latter comprised of putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrachloride. Internal standard (1,7-diaminoheptane, 98%) was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Acetonitrile, acetone and ethyl acetate were of HPLC grade. The trio were obtained from Fisher Scientific Inc. (Pittsburgh, PA, USA). Other chemicals were purchased from either Millipore Sigma Corp. or Fisher Scientific Inc. They were at least reagent grade. Water purified from the Milli-Q Direct system

(Millipore Sigma Corp.) was used in all the experiments, including solution preparation and composition of solvent gradient in HPLC.

3.3.2. Methods

a) Analyses of barley grain quality characteristics

Quality characteristics of barley grains in terms of thousand kernel weight and proximate composition were analyzed. One thousand-kernel weight (1000KW) was measured manually after eliminating broken kernels. Moisture content was determined by drying flour samples at 130 ^{o}C to a constant weight in a hot-air oven (Heratherm, Thermo Fisher Scientific Inc.) (AACC International Method 44-15.02). Total ash content was quantified by incineration at 550 ^{o}C for 18 h in a muffle furnace (Lindberg Blue M, Thermo Fisher Scientific Inc.) (AACC International Method 08-01.01). Soxhlet extraction using hexane was used to measure total lipid content (AOAC Official Method 2003.06). Total protein content was determined by Kjeldahl method (AOAC Official Method 992.23) with three steps: i) digestion of flour samples by concentrated H_2SO_4 with copper catalyst in a block heating unit (DK 20, VELP Scientifica Co., Usmate Velate, Italy); ii) distillation of NH_3 with steam and NaOH (40% w/v) prior to entrapment with H_3BO_3 in a distillation unit (KT 200 Kjeltec, FOSS Co., Hilleroed, Denmark); and iii) titration with standardized 0.05 N HCl solution using a bottle-top digital burette (Tirette, BrandTech Scientific Co., Essex, CT, USA). A nitrogen-to-protein conversion factor of 6.25 was applied. The total carbohydrate content was calculated by difference using the formula below:

Carbohydrate (g/100 g dry basis, db) = 100 – Moisture – Ash – Lipid – Protein

b) Extraction of free polyamines

Free polyamines are extracted from whole barley flours using two different solvents, namely HCl (1 M) and TCA (5% w/v). The extraction with each solvent was carried out

separately following the procedure of Gutierrez, Johansson, Centellas Quezada, et al. (2021) with minor modifications. Approximately 100 mg of whole grain barley flour was weighed into a 2-mL Eppendorf tube. Twenty µL of 1,7-diaminoheptane amine (internal standard) was spiked, followed by adding 1000 µL of extraction solvent. The mixture was vortexed for 30 s and sonicated for 15 minutes (B5510-MTH, Branson Ultrasonics Corp., Danburry, CT, USA) before incubating at 4 ºC for 60 min. The content was centrifuged (Sorvall Legend Micro 21R, Thermo Scientific Inc.) at 13,000 g at 4 ºC for 10 min. A portion of the supernatant (500 µL) was collected into a 15 mL centrifuge tube, and the pre-column derivatization was conducted shortly.

c) Derivatization of polyamines

Dansyl derivatives of free polyamines were obtained by performing a reaction with dansyl chloride using the method described by Yang, Mu, Zhang, et al. (2014). The dansylation reaction necessitates an alkaline pH of 9.5–11 (Mengerink, 2005). Therefore, 300 µL of NaOH (2 M) and 350 µL of saturated $NaHCO_3$ (solubility of about 9 % w/v at room temperature) were consecutively added to 500 µL of HCl (1 M) acid extracts. Meanwhile, a consecutive addition of the same reagents was done to 500 µL of TCA (5% w/v) extracts. The alkalized extracts in approximately 0.37 M carbonate buffer had a pH of 10 (verified by Whatman Panpeha pH strip, Cytiva Corp., Marlborough, MA, USA). Dansyl chloride solution in acetone (0.01 g/mL) was freshly prepared before each run, then 500 µL of this solution was added to the alkaline extracts. The mixture was vortexed and incubated at 60 ºC in a water bath (Model 10128-128, VWR International Co., Batavia, IL, USA). During incubation, the content was gently inverted and vortexed every 15 min. After 45 min, 50 µL of 25% NH_4OH solution was added to stop the reaction. The mixture was then kept at room temperature for 5 min to equilibrate prior to extracting derivatized polyamines. Liquid-liquid extraction of dansyl derivatives was done by

adding 1500 µL of ethyl acetate and then vortexing for 30 s prior to centrifugation at 5000 g at 15 ^{o}C for 10 min (Sorvall RC6 Plus centrifuge, Thermo Scientific Inc. with Fibrelite F14-6x250y rotor). After centrifuging, the organic phase was collected into a 5-mL amber Eppendorf tube and the extraction was repeated once more. The resultant extract was blown under a gentle nitrogen stream at ambient temperature until dryness. The dried extract was reconstituted in 300 µL acetonitrile and passed through 0.22 µm PVDF syringe filter (Milex-GV, Milipore Co.) before HPLC analysis.

d) HPLC analysis

Reconstituted and filtered extracts of derivatized polyamines in amber vial were subjected to the HPLC unit, comprising a separation module (Model 2695, Waters Co.), a guard column (ZORBAX Extend-C18, Agilent Technologies Co., packing material size of 5 µm, column dimensions of 4.6 × 12.5 mm), a reverse phase analytical column (ZORBAX Extend-C18, Agilent Technologies Co., 5 µm, 4.6 × 150 mm), a photodiode array detector (Model 2996, Waters Co.) and a data acquisition software (Empower Pro Build 1154, Waters Co.). A wavelength of 254 nm was selected for the detection. The column and sample temperatures were set at 30 and 15 ^{o}C, respectively. The injection volume was 20 µL. The gradient elution was composed of water (solvent A) and acetonitrile (solvent B). A gradient elution program reported by Yang, Mu, Zhang, et al. (2014) was employed: 45% A and 55% B (0–7 min), 35% A and 65% B (7–14 min), 30% A and 70% B (14–23.5 min), 10% A and 90% B (23.5–25 min) and finally 100% B (25–35 min) to equilibrate the column for the next run.

e) Preparation and treatment of polyamine standards

A stock solution containing a mixture of 500 µM of the three external standards in 0.1 M HCl was diluted into a series of working solutions at concentrations of 5, 10, 15, 20, 30, 40 and

50 µM. An internal standard solution (1000 µM of 1,7-diaminoheptane in 0.1 M HCl) was prepared separately from that of external standards. The derivatization was done in a reaction mixture consisting of 500 µL working solution of mixed external standards, 10 µL of internal standard solution, 100 µL of NaOH (1 M), 350 µL of saturated $NaHCO_3$ and 500 µL of dansyl chloride (0.01 g/mL). Calibration curves were constructed by plotting the concentration against the peak area ratio of the targeted compound (putrescine, spermidine, or spermine) to the internal standard. After sample weight normalization, individual polyamine contents were expressed as µmol per 100 g db. Total polyamine content is the sum of the concentrations of individual polyamines on a weight basis and was expressed as mg per 100 g db.

f) Statistical analyses

All the experiments were conducted in triplicate. Data are presented as mean ± standard deviation. Paired-sample *t*-test, Pearson correlation and one-way ANOVA followed by Tukey comparison at a significant level of 0.05 were performed on Origin V10.0 (OriginLab Co., Northampton, MA, USA). Data visualization by heatmap, Principal Component Analysis (PCA) and K-Means Cluster Analysis (KCA) were done using the same software.

3.4. Results and discussion

3.4.1. Quality characteristics of barley grains

Table 3.1 presents the quality characteristics of the barley samples set in terms of 1000KW and proximate composition. The 1000KW of the barley varieties varied between 35.4 and 59.6 g, in which Atahualpa was remarked for producing heavier seeds than the other cultivars. The barley grains used in this study had ash, lipid and carbohydrate contents of 1.78–2.94, 2.2–4.5 and 66.5–73.5 g/100 g db, respectively. The elimination of the hull layer could give

rise to higher lipid and lower ash content of hulless barley grains compared to hulled ones. The protein level of Canadian barley grains was from 12.0 to 17.8 g/100 g db, falling in the upper interval of the average range (8–18 g/100 g db) reported in the literature (Gubatz & Weschke, 2014). It should be noted that the grain protein content of barley may vary greatly depending on the genotype and cultivating conditions (Kong, Choo, Narasimhalu, et al., 1995). In Canada, the Prairies region was demonstrated to produce barley grains with higher protein content compared to the Eastern and Maritime provinces (Kong, Choo, Narasimhalu, et al., 1995). On the other side, milling of hull-less barley grains resulted in flours with a higher protein level than those of hulled varieties, likely due to the absence of the hull layer.

Protein content is an essential parameter of barley grains for the marketing of their end-use purposes. A high protein content is generally desirable for both human and animal nutrition when consuming or feeding barley-based diets (Bista, Heckathorn, Jayawardena, et al., 2020; Miller, Beasley, Drury, et al., 2015). This value is more strictly controlled in malting barley, of which variations in protein content would exert an adverse impact on beer quality. For instance, too high protein concentration in malts would make the beer more susceptible to haze formation (i.e., coagulation of protein in the presence of polyphenol) during cold storage (Briggs, 2004). However, removal of coagulable proteins may undermine the foaming properties of the final product (Briggs, 2004). In food applications, the protein quantity and quality of a material are of importance from nutritional and technological perspectives. Alu'datt, Rababah, Ereifej, et al. (2012) partially substituted wheat flour with barley flour to compensate for the deficit of a few essential amino acids in wheat-based products. Barley protein isolate also demonstrated its potential as a new food ingredient due to its good emulsifying and foaming properties (Alu'datt, Rababah, Ereifej, et al., 2012).

Table 3.1. Characteristics of the barley sample set

Barley cultivar	Utility	Botanical traits			Growing location	Harvest time	Thousand-grain weight (g)	Moisture content (g/100 g, wb)	Ash content (g/100 g, db)	Lipid content (g/100 g, db)	Protein content (g/100 g, db)	Carbohydrate content (g/100 g, db)
		Seed arrangement	Hull adherence	Seed colour								
AAC Synergy	Malting	Two-row	Hulled	Yellow	Brandon, MB	2021	44.4	9.4 ± 0.5 (cd)	2.36 ± 0.03 (c)	2.4 ± 0.1 (def)	13.1 ± 0.3 (f)	72.7
AC Metcalfe	Malting	Two-row	Hulled	Yellow	Brandon, MB	2021	40.2	9.3 ± 0.3 (cd)	2.36 ± 0.04 (c)	2.9 ± 0.1 (bc)	13.9 ± 0.5 (def)	71.5
CDC Mayfair	Malting	Six-row	Hulled	Yellow	Brandon, MB	2009	43.4	10.4 ± 0.3 (b)	2.33 ± 0.04 (c)	2.2 ± 0.2 (f)	13.6 ± 0.1 (ef)	71.4
Taylor	Malting	Two-row	Hulless	Yellow	Brandon, MB	2021	40.9	8.8 ± 0.5 (de)	1.87 ± 0.03 (g)	2.8 ± 0.2 (bcd)	14.5 ± 0.2 (cd)	72.0
AC Ranger	Feed	Six-row	Hulled	Yellow	Brandon, MB	2016	40.4	9.3 ± 0.3 (cd)	2.94 ± 0.03 (a)	2.3 ± 0.2 (ef)	12.0 ± 0.1 (g)	73.5
CDC McGwire	Feed	Two-row	Hulless	Yellow	Hamiota, MB	2022	41.2	7.1 ± 0.2 (f)	2.02 ± 0.05 (f)	2.7 ± 0.2 (cde)	15.7 ± 0.2 (b)	72.5
Vivar	Feed	Six-row	Hulled	Yellow	Brandon, MB	2016	39.5	10 ± 0.1 (bc)	2.72 ± 0.04 (b)	2.8 ± 0.05 (bcde)	12.0 ± 0.2 (g)	72.5
Atahualpa	Food	Two-row	Hulless	Yellow	Brandon, MB	2017	59.6	11.9 ± 0.3 (a)	2.13 ± 0.02 (de)	3.0 ± 0.1 (bc)	16.1 ± 0.1 (b)	66.8
CDC Hilose	Food	Two-row	Hulless	Yellow	Brandon, MB	2021	35.4	9.2 ± 0.1 (cd)	1.78 ± 0.04 (g)	4.5 ± 0.04 (a)	16.1 ± 0.4 (b)	68.4
CDC Rattan	Food	Two-row	Hulless	Yellow	Brandon, MB	2022	39.2	7.2 ± 0.4 (f)	2.08 ± 0.05 (ef)	3.0 ± 0.1 (bc)	14.7 ± 0.1 (c)	73.0
CI-1248	Food	Six-row	Hulless	Purple	Brandon, MB	2008	35.5	10.1 ± 0.3 (bc)	2.22 ± 0.01 (d)	3.1 ± 0.1 (bc)	14.3 ± 0.2 (cde)	70.4
Peru 16	Food	Six-row	Hulled	Black	Brandon, MB	2012	42.0	10.1 ± 0.4 (bc)	2.98 ± 0.01 (a)	2.6 ± 0.2 (cdef)	17.8 ± 0.2 (a)	66.5
Roseland	Food	Two-row	Hulless	Yellow	Brandon, MB	2020	41.2	7.9 ± 0.1 (ef)	1.86 ± 0.02 (g)	3.2 ± 0.04 (b)	15.7 ± 0.5 (b)	71.4

Data are presented as mean ± standard deviation (n = 3), except for thousand-grain weight and carbohydrate content (n = 1). Values that do not share a lowercase letter within a column are significantly different (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: wb: wet basis; db: dry basis.

3.4.2. Chromatogram, linearity and sensitivity of free polyamine quantification method

Figure 3.2 shows the peaks observed in the chromatographic runs of dansylated polyamines. Dansyl chloride can form stable derivative products with both primary and secondary amino groups and provide a chromophore for the detection under UV wavelength (Muñoz-Esparza, Latorre-Moratalla, Comas-Basté, et al., 2019). No major differences were found among the chromatograms of the standard mix, HCl extract and TCA extract of polyamines. Putrescine, spermidine and spermine in the acid extracts from whole barley flour were identified by referring to the commercially available standards after derivatization. The unknown peak eluted at 5.9 min (peak 1) that appeared in every chromatogram could be either the hydrolysis product of dansyl chloride (dansylic acid) or the reaction product with ammonia (dansyl amide). Residual dansyl chloride, if present, was eluted at 11.7 min (data not shown). The linearity and sensitivity of the polyamine quantification method are shown in Table 3.2. The method employed in this work demonstrated high linearity ($R2 > 0.9995$) and sensitivity for the polyamines considered (LOD < 1.5 μM, LOQ < 4.0 μM). However, dansylated spermine showed a slightly lower sensitivity than the derivatives of putrescine and spermidine, corresponding to the presence of minor background peaks before and after the elution of peak 5 (26.7 min). This phenomenon could be attributed to the formation of spermine derivatives with different degrees of derivatization, as well as the degradation of the dansylated compound (Mengerink, 2005).

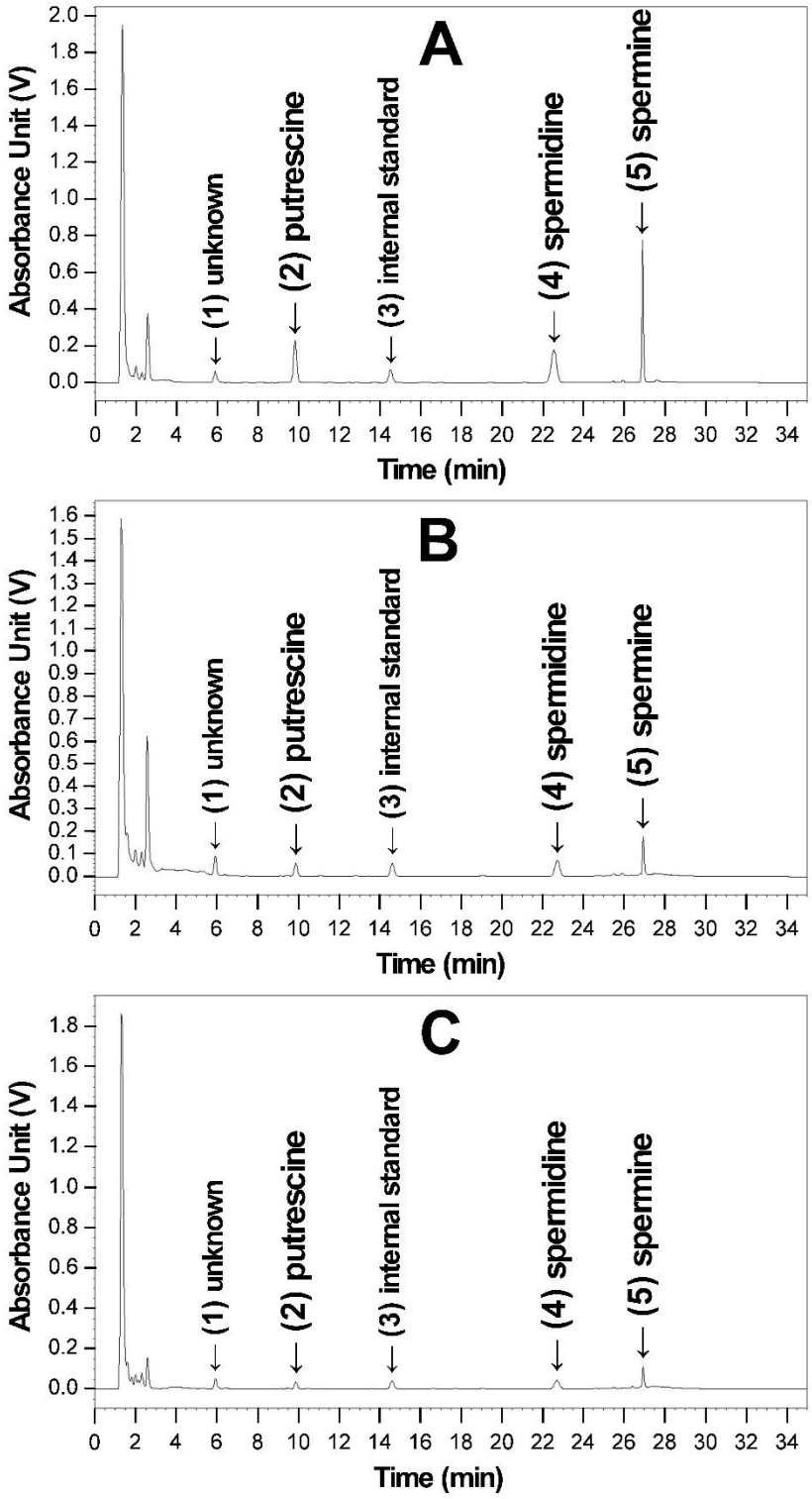

Figure 3.2. Chromatograms of dansylated polyamines from standard mix (A), HCl 1 M extract (B) and trichloroacetic acid 5% w/v (C)

For detectors of Waters™ systems, 1 volt (V) equals 1 absorbance unit (AU).

Table 3.2. Linearity and sensitivity of dansylated polyamines determined by the chromatographic method.

Compound	**Putrescine**	**Spermidine**	**Spermine**	**1,7-Diaminoheptane (Internal standard)**
Chemical formula (prior to derivatization)	$NH_2(CH_2)_4NH_2$	$NH_2(CH_2)_3NH(CH_2)_4NH_2$	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	$NH_2(CH_2)_7NH_2$
Retention time (min)	9.84	22.58	26.67	14.54
Slope of calibration curve	0.0526	0.0691	0.0901	NA
Intercept of calibration curve	0.0187	0.0131	0.0269	NA
R-squared	0.9997	0.9997	0.9996	NA
Limit of detection (μM)	1.03	1.03	1.21	NA
Limit of quantification (μM)	3.13	3.11	3.69	NA

NA: not applicable

3.4.3. Effects of different acids on the extraction yield of free polyamines

Table 3.3 depicts the effects of HCl (1 M) and TCA (5%) on the extraction yield of free polyamines from whole barley flour. Extraction is a prerequisite for the quantification of polyamines and other biogenic amines in biological samples, and acid solutions are the most common extraction solvents (Paiva, Evangelista, Queiroz, et al., 2015). Evaluation of chromatographic area was used to optimize the derivatization conditions of biogenic amines in wine samples using dansyl chloride (Jiang, Ying, Zhou, et al., 2011). In this work, eight out of thirteen barley cultivars demonstrated a significant difference ($p < 0.05$) in the peak area of at least one derivatized polyamine as the two different acids were employed. Use of 1M HCl resulted in higher peak area of putrescine (30–88%), spermidine (27–71%) and spermine (19–58%) compared to that of TCA 5% w/v. Moreover, the internal standard spiked in whole barley flour appeared to behave in an akin fashion to other polyamines, showing a higher peak area (20–57%) in HCl extracts as observed among ten out of thirteen cultivars. The disparity in chromatographic areas was noted for both hulled and hulless varieties. This implied that the presence of the hull layer materials in hulled barley flour was less likely to have a diminutive effect on the extraction yield of polyamines. In this case, the solvent property might play a significant role in determining the extractability of polyamines from whole barley flour.

It has been known that TCA can induce the precipitation of proteins owing to the sequestration of the surrounding water layer formed by hydrogen bonds (Koontz, 2014). In the extractions of polyamines from whole barley flour, TCA was speculated to perturb the hydration layer of the analytes and, in turn, reduce their solubility in the aqueous phase. This speculation was corroborated by the decreased number of barley samples showing a disparity in the spermine peak areas between the two extraction methods. Under acidic conditions, spermine remains in the

tetracationic form due to protonation, which may compete more effectively with TCA for water and facilitate the formation of the hydration layer. However, the dicationic and tricationic forms of putrescine and spermidine, respectively, might exhibit a lower affinity for water. In addition, the number of samples showing chromatographic area loss when changing from HCl to TCA was highest for putrescine, followed by spermidine and spermine. Similarly, the observed extent of chromatographic area loss occurred in the same sequence. On the other hand, Paiva, Evangelista, Queiroz, et al. (2015) concluded that the use of TCA (5% w/v) resulted in better recovery rates of polyamines in ground sorghum meal in comparison with that of HCl (1 M). The application of TCA in the extraction of polyamines from sorghum likely assisted the removal of certain interferents from the sample matrix (e.g., tannins) and eventually improved the extractability of the analytes. With regards to the peak area ratios of targeted polyamines to internal standard, only three pairs of values were shown to have a statistically significant difference ($p < 0.05$) when the two acids were applied. This suggested that the determination of polyamine content would give the same results regardless of the type of extractants used. A partial explanation to this observation could be attributed to similar behaviour between the targeted polyamines and the internal standard in the acidic medium. However, since some compounds might be co-extracted and interfere with the analysis (e.g., increased viscosity caused by β-glucan), the matrix effect of whole barley flour should be addressed in another study with more emphasis on analytical aspects.

Table 3.3. Peak areas and peak area ratios of derivatized polyamines using HCl (1 M) and trichloroacetic acid (5% w/v) as extraction solvents

Barley cultivar	Peak area (V × s)								Peak area ratio					
	Putrescine		Spermidine		Spermine		IS		Putrescine / IS		Spermidine / IS		Spermine / IS	
	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)	Extraction with HCl (1 M)	Extraction with TCA (5% w/v)
AAC Synergy	0.61 ± 0.06	0.44 ± 0.06	1.14 ± 0.11	0.85 ± 0.09	1.08 ± 0.12	0.84 ± 0.07	0.78 ± 0.03 (*)	0.58 ± 0.03	0.78 ± 0.06	0.75 ± 0.07	1.46 ± 0.11	1.46 ± 0.07	1.39 ± 0.14	1.46 ± 0.10
AC Metcalfe	0.58 ± 0.06	0.39 ± 0.08	1.00 ± 0.09	0.68 ± 0.14	1.01 ± 0.09	0.74 ± 0.15	0.80 ± 0.06	0.54 ± 0.15	0.73 ± 0.02	0.73 ± 0.06	1.24 ± 0.03	1.28 ± 0.09	1.26 ± 0.05	1.41 ± 0.13
CDC Mayfair	0.98 ± 0.04 (*)	0.52 ± 0.07	1.40 ± 0.05 (*)	0.82 ± 0.14	1.02 ± 0.05	0.71 ± 0.17	0.88 ± 0.04 (*)	0.57 ± 0.04	1.12 ± 0.01	0.91 ± 0.06	1.60 ± 0.06 (*)	1.43 ± 0.13	1.16 ± 0.04 (*)	1.23 ± 0.20
Taylor	0.63 ± 0.05 (*)	0.35 ± 0.04	1.28 ± 0.12 (*)	0.78 ± 0.03	1.06 ± 0.14	0.78 ± 0.06	0.80 ± 0.04 (*)	0.51 ± 0.09	0.79 ± 0.02	0.70 ± 0.17	1.60 ± 0.06	1.55 ± 0.29	1.32 ± 0.11	1.56 ± 0.20
AC Ranger	0.64 ± 0.04 (**)	0.46 ± 0.05	1.18 ± 0.08 (**)	0.89 ± 0.08	1.10 ± 0.07	0.89 ± 0.11	0.83 ± 0.02 (**)	0.61 ± 0.02	0.77 ± 0.05	0.77 ± 0.07	1.42 ± 0.09	1.47 ± 0.10	1.32 ± 0.09	1.47 ± 0.20
CDC McGwire	1.10 ± 0.05 (**)	0.69 ± 0.04	1.83 ± 0.05 (**)	1.20 ± 0.08	1.12 ± 0.05 (*)	0.71 ± 0.1	0.83 ± 0.02 (**)	0.55 ± 0.03	1.33 ± 0.03 (**)	1.26 ± 0.02	2.21 ± 0.06	2.17 ± 0.07	1.35 ± 0.08	1.28 ± 0.15
Vivar	0.47 ± 0.03 (*)	0.31 ± 0.01	1.10 ± 0.09 (*)	0.72 ± 0.04	1.06 ± 0.07 (*)	0.71 ± 0.09	0.86 ± 0.11	0.54 ± 0.05	0.55 ± 0.04	0.57 ± 0.04	1.29 ± 0.08	1.34 ± 0.05	1.25 ± 0.14	1.30 ± 0.06
Atahualpa	1.26 ± 0.04 (*)	0.77 ± 0.14	0.83 ± 0.01 (*)	0.56 ± 0.06	1.85 ± 0.19	1.27 ± 0.21	1.50 ± 0.13	1.03 ± 0.14	1.53 ± 0.03	1.37 ± 0.12	2.24 ± 0.21	2.26 ± 0.16	1.82 ± 0.14	1.83 ± 0.10
CDC Hilose	0.94 ± 0.16	0.61 ± 0.04	1.44 ± 0.24	0.95 ± 0.06	1.35 ± 0.24	0.96 ± 0.05	0.83 ± 0.06 (*)	0.53 ± 0.04	1.12 ± 0.12	1.14 ± 0.04	1.72 ± 0.17	1.79 ± 0.06	1.61 ± 0.18	1.81 ± 0.12
CDC Rattan	1.10 ± 0.06	0.88 ± 0.10	1.28 ± 0.07	1.09 ± 0.12	1.18 ± 0.01 (*)	0.99 ± 0.05	0.73 ± 0.04 (*)	0.61 ± 0.03	1.51 ± 0.15	1.43 ± 0.11	1.75 ± 0.20	1.79 ± 0.12	1.62 ± 0.09	1.63 ± 0.14
CI-1248	0.93 ± 0.07 (*)	0.59 ± 0.02	1.02 ± 0.07 (*)	0.66 ± 0.01	1.22 ± 0.09 (*)	0.88 ± 0.03	0.82 ± 0.03 (**)	0.54 ± 0.04	1.13 ± 0.11	1.11 ± 0.06	1.24 ± 0.12	1.24 ± 0.08	1.49 ± 0.16	1.65 ± 0.07
Peru 16	1.00 ± 0.08	0.77 ± 0.11	1.00 ± 0.08	0.68 ± 0.16	1.79 ± 0.09	1.41 ± 0.13	0.78 ± 0.01 (*)	0.57 ± 0.07	1.28 ± 0.12	1.34 ± 0.04	1.28 ± 0.11	1.38 ± 0.04	2.29 ± 0.1	2.47 ± 0.08
Roseland	1.00 ± 0.10 (*)	0.77 ± 0.06	1.77 ± 0.19 (*)	1.39 ± 0.13	1.06 ± 0.10	0.87 ± 0.02	0.80 ± 0.02 (*)	0.58 ± 0.03	1.25 ± 0.11	1.32 ± 0.08	2.21 ± 0.22 (**)	2.40 ± 0.20	1.32 ± 0.11	1.51 ± 0.11

Data are presented as mean ± standard deviation (n = 3). Values followed by a single (*) or double asterisk (**) are significantly different from their counterparts obtained by using another extraction solvent at $p \leq 0.05$ or $p \leq 0.01$ (Paired-sample t-test), respectively.

Abbreviation: IS: internal standard; TCA: trichloroacetic acid. Peak area is measured in volts-second (V × s).

3.4.4. Polyamine profiles of barley grains

The polyamine profiles of barley grains were characterized in terms of content and relative abundance of each compound. The molar contents of individual polyamines were calculated based on chromatographic information obtained from the HCl extracts. The levels of putrescine, spermidine and spermine in the examined barley cultivars were 10.2–29.4, 16.9–32.9 and 11.2–25.8 μmol/100 g db, respectively (Figure 3.3A–C). With respect to the proportion of each polyamine, it can be seen from the heatmap that spermidine was the most abundant in barley grains for ten out of thirteen barley cultivars (Figure 3.3D), indicating a transitional role of spermidine in the metabolism of the three polyamines. The conversion of putrescine to spermidine might have taken place to a significant extent during the development of barley seeds. A previous study on the grain filling process of rice reported a noticeably higher activity of spermidine synthase compared to that of enzymes involved in the generation of putrescine (ornithine and arginine decarboxylases) throughout the entire examined period (Yang, Yunying, Zhang, et al., 2008). Nevertheless, the interpretation of data from this work would encounter constraints since the analyses were done on post-harvest grain samples. The high molar proportion of spermidine observed in this study corresponded to the discovery of a substantial amount of N^1, N^8-dicaffeoyl spermidine in the methanolic extract of hulless barley flours (Drawbridge, Apea-Bah, Hornung, et al., 2021). In terms of plant physiology, spermidine was discovered to be more involved than putrescine and spermine in responding to abiotic stresses, such as UV radiation (Xie, Wang, Gu, et al., 2023). This triamine can promote the synthesis of phenolic compounds to mitigate the oxidative damage induced by UV radiation on barley seedlings (Xie, Wang, Gu, et al., 2023), which in turn could be linked to the presence of its caffeic acid conjugate in barley seeds (Drawbridge, Apea-Bah, Hornung, et al., 2021). In the

reproductive organ of another angiosperm, Falasca, Franceschetti, Bagni, et al. (2010) observed that during the development of functional pollens in kiwifruit, the molar content of free spermidine remained considerably higher (>1.5 fold) than that of putrescine and spermine. Hence, it is implied that the combination of pre-anthesis and pre-harvest studies could be useful to better understand the events shaping the polyamine profile in barley grains.

On the other hand, the total polyamine contents of barley cultivars for food were shown to be higher on average (9.73 mg/100 g db) than those of cultivars for malting (7.00 mg/100 g db) and for feeding (7.81 mg/100 g db). It can be noted that most of the barley lines for food used in this study are hulless (five out of six samples). Similarly, among the barley for feed, CDC McGwire was distinguished from other hulled cultivars (AC Ranger and Vivar) by an elevated total polyamine level (31–49%). Although cereal grains are major staples in various countries and cultures, there has been a modesty in the research on polyamines for this food category as reviewed by Muñoz-Esparza, Latorre-Moratalla, Comas-Basté, et al. (2019). The determined total polyamines contents in whole barley grains were higher than that of sorghum (0.58–4.14 mg/100 g db) (Paiva, Evangelista, Queiroz, et al., 2015) and could be comparable to that of fresh sweet corn (11.0 mg/100 g db) (Bandeira, Evangelista, & Gloria, 2012); as calculated by summation of putrescine, spermidine, spermine and cadaverine in both materials. It was revealed that the polyamine intake from dietary sources could help reduce the risk of developing cardiovascular diseases and improve longevity (Muñoz-Esparza, Latorre-Moratalla, Comas-Basté, et al., 2019). These health benefits were attributed to the antioxidant and anti-inflammatory properties of polyamines, as well as the ability to facilitate the renewal of cellular components through the autophagy process (Muñoz-Esparza, Latorre-Moratalla, Comas-Basté, et al., 2019). In this regard, the consumption of barley-based foods with high polyamine content is expected to confer health-

promoting effects. Furthermore, barley grains for food can be identified as a source of polyamines for nutraceutical production purposes.

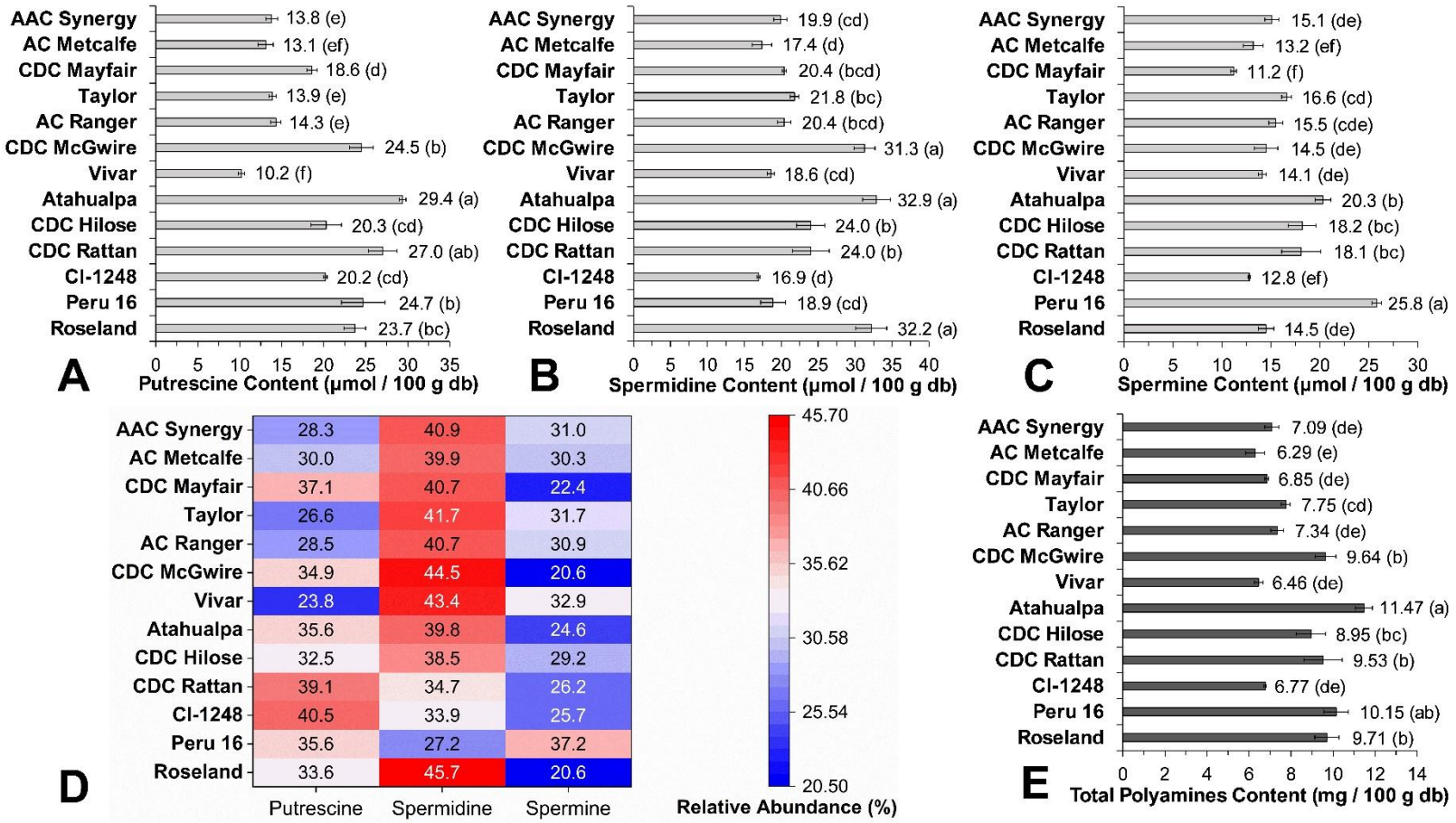

Figure 3.3. Content of individual polyamines (A–C) with relative abundance (D) and content of total polyamines (E) of barley grains

Figure 3.3A–C and 2E: Mean values (n = 3) are displayed at the outside end of each bar and values that do not share a same letter are significantly different (Tukey's comparison test, $p \leq 0.05$).

Abbreviation: db: dry basis.

3.4.5. Relationship between level of free polyamines and protein content

Figure 3.4A-D depicts the relationship between the concentration of free polyamines and the protein content of barley grains. The putrescine, spermine and total polyamines levels were shown to correlate with the protein content ($p < 0.05$). Nonetheless, there was no correlation between the spermidine concentration and the protein amount ($p > 0.05$). According to the criteria proposed by Mukaka (2012), the relationship between spermine content and protein level in barley grains can be regarded as a moderate positive correlation ($0.5 < r < 0.7$), while those of putrescine–protein and total polyamines–protein can be considered as a high positive correlation ($0.7 < r < 0.9$). Different compounds of minute quantities in barley grains were found to be correlated with protein content, including phytochemicals (Dai, Wang, Zhang, et al., 2007) and minerals (Gao, Persson, Vincze, et al., 2022). The total protein content in this work was determined by the Kjeldahl method, and it would be proportional to the total nitrogen content of barley seeds. Polyamine accounted for 0.045–0.071% w/w barley grain protein. The positive correlation observed between level of polyamines and protein content suggested that barley tends to store more free polyamines in their grains at an increased quantity of nitrogen pool, even though the polyamine contents measured in whole barley flour were minor. There was evidence that an enhanced application of nitrogen in fertilization increased the free polyamine level in grape wine berries, whereas this value was reduced under a nitrogen-deficient regime (Gény, Broquedis, Soyer, et al., 1996). On the other hand, Serapiglia, Minocha, and Minocha (2008) reported that the polyamine level of red spruce cell culture remained unchanged despite the supplementation of nitrogenous substrates into the medium. The divergent findings could be partially explained by different conditions between *in vitro* and field cultivation, as well as the threshold of extracellular nitrogen that the cells can tolerate.

Although the partition of nitrogen in barley has not been investigated extensively, it was revealed that this crop had a preference to deposit the absorbed nitrogenous quantity into hordeins during grain development (Egle, Beschow, & Merbach, 2015). Hordeins belong to the prolamins group according to Osborne fractionation and make up 30–50% total nitrogen content of barley grains (Finnie & Svensson, 2014). They serve as the major storage proteins of barley grains (Finnie & Svensson, 2014). Like other cereal grains, storage proteins of barley are synthesized and deposited in the endosperm and aleurone throughout the grain filling stage (Finnie & Svensson, 2014). In rice, an acceleration in cell division and grain filling rate was found to be correlated with an increase in the level of free endogenous polyamines (Yang, Yunying, Zhang, et al., 2008). In this study, to understand how barley grains of different cultivars are distinguished from each other based on their polyamine and protein content, PCA and KCA were performed on the acquired data. The PCA biplot (Figure 3.4E) illustrates the arrangement in two-dimensional space for the loading vectors of the examined variables (putrescine, spermidine, spermine, total polyamines and protein content). It can be noted that the vector of protein content was arranged relatively near to those of putrescine and total polyamine content, visualizing their high correlations. In addition, the three variables mentioned imposed greater loading on the first principal component, since their vectors projected closer to 1.0 on the *x*-axis. On the other hand, the vectors of spermine and spermidine content were positioned in the proximity of the bisectors, indicating a moderate loading on both principal components. The results of KCA demonstrated that the barley samples used in this study can be grouped into two clusters, i.e., cluster 1 composed of six varieties having higher polyamine as well as protein content and cluster 2 encompassing eight varieties with lower polyamine and protein levels. It should be mentioned that the barley cultivars in cluster 1 were mainly hulled and designated for malting and feeding purposes, whereas the members of cluster 2 were principally hulless and destined for food use.

The obtained information would be useful for selecting potential cultivars of high polyamine content in the avenue of dietary incorporation or nutraceutical production.

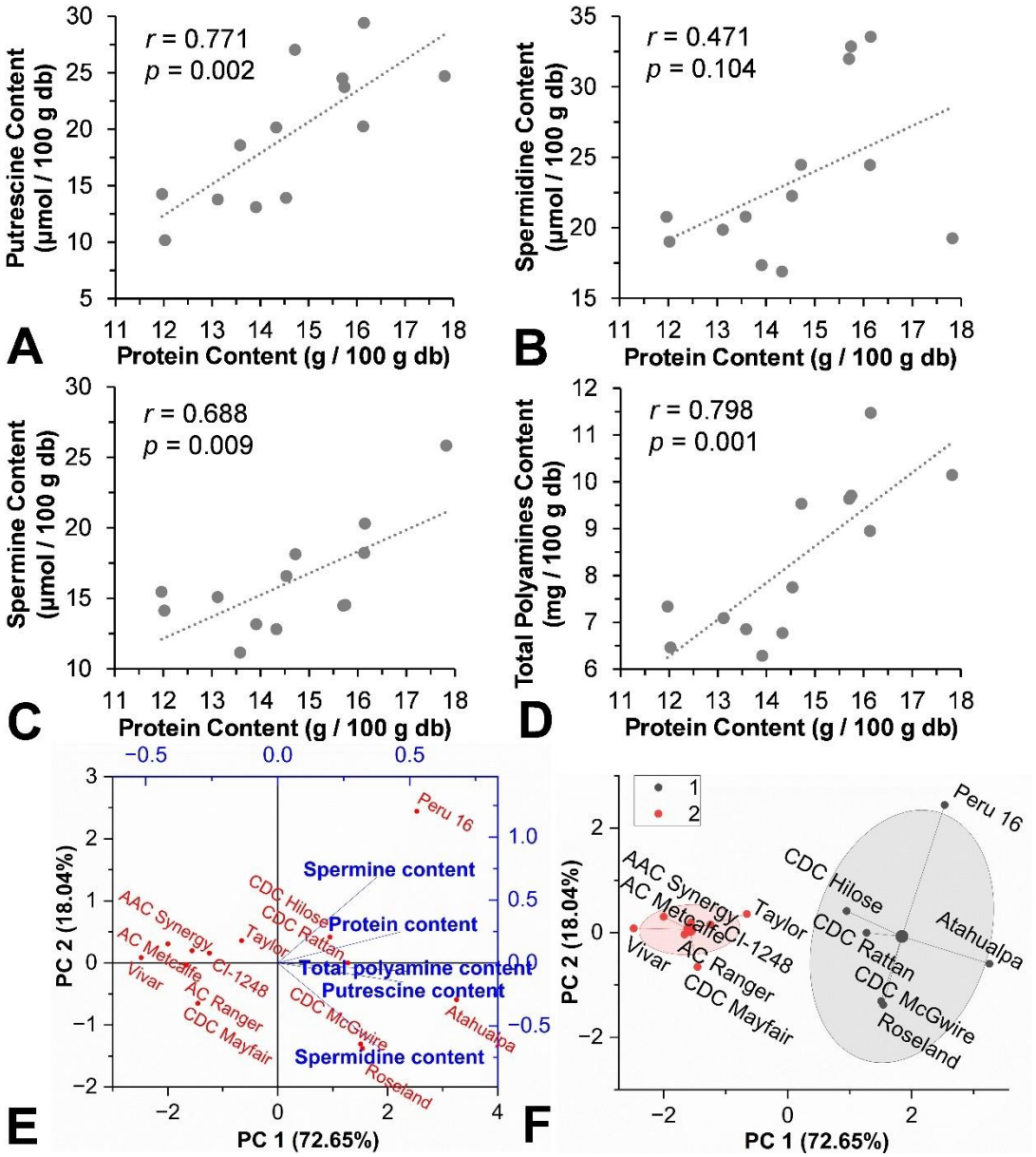

Figure 3.4. Scatterplots for protein and polyamine contents of barley grains (A–D), followed by biplot of Principal Component Analysis (E) and visualization of K-Means Cluster Analysis (F)

Abbreviation: db: dry basis.

3.5. Conclusion

Free polyamines from whole barley flour showed a higher extractability in HCl (1 M) compared to TCA (5% w/w), as demonstrated by greater chromatographic areas of derivatized polyamines. Barley grains were shown to be a significant source of polyamines. Analysis of polyamine profiles showed that spermidine was the predominant compound for the majority of barley lines. The level of putrescine, spermine and total polyamines positively correlated with the protein content. The correlations ranged from moderate to high, suggesting that free polyamines are likely to participate in the nitrogenous reservoir of barley grains. The barley cultivars used for this investigation can be grouped into one cluster of inferior polyamine and protein content (mainly hulled barley for malting and feeding) and another cluster of superior polyamine and protein content (mostly hulless barley for food).

Chapter 4 — Experimental Section 2 (Distribution of polyamines in kernel tissues of Canadian barley cultivars and their association with protein content)

4.1. Chapter Abstract

Polyamines are nitrogen-containing compounds that naturally occur in barley grain and are known to have beneficial effects on human health. This study investigated the distribution of polyamines in barley grains from two covered and two naked Canadian barley cultivars. Five pearling fractions were collected at 5% intervals of kernel weight for all cultivars. Grains from covered cultivars were dehulled before pearling, followed by consecutive sieving to recover the germ, fine hull (<0.5 mm) and coarse hull (>0.5 mm) fractions. The levels of putrescine, spermidine and spermine in barley kernels with 25% kernel weight removed were 69–76, 55–64 and 21–41% lower than those in unpearled kernels. Dehulled and pearled kernels were enriched in spermine and depleted in putrescine. The barley germ showed a notably high total polyamine content (52.0–65.5 mg/100 g). The protein and total polyamine content in the fine hull fraction were 2.8–3.5 and 2.1–3.3 fold higher, respectively, compared to the coarse hull fraction, reflecting differences in their histological composition as shown by microscopic examination. Correlation analysis revealed positive and significant correlations between polyamine and protein content at the sub-kernel level (r = 0.769–0.839), suggesting a potential chemical and physiological synchronization between these components during barley seed development and germination. The insights provided by this study on the obtainment of polyamine-rich fractions from barley grains could be valorized for nutraceutical purposes. The understanding of the polyamine–protein relationship in barley tissues could aid the selection of cultivating or processing conditions for different uses.

Keywords: barley; dehulling; *Hordeum vulgare*; pearling; polyamine; protein

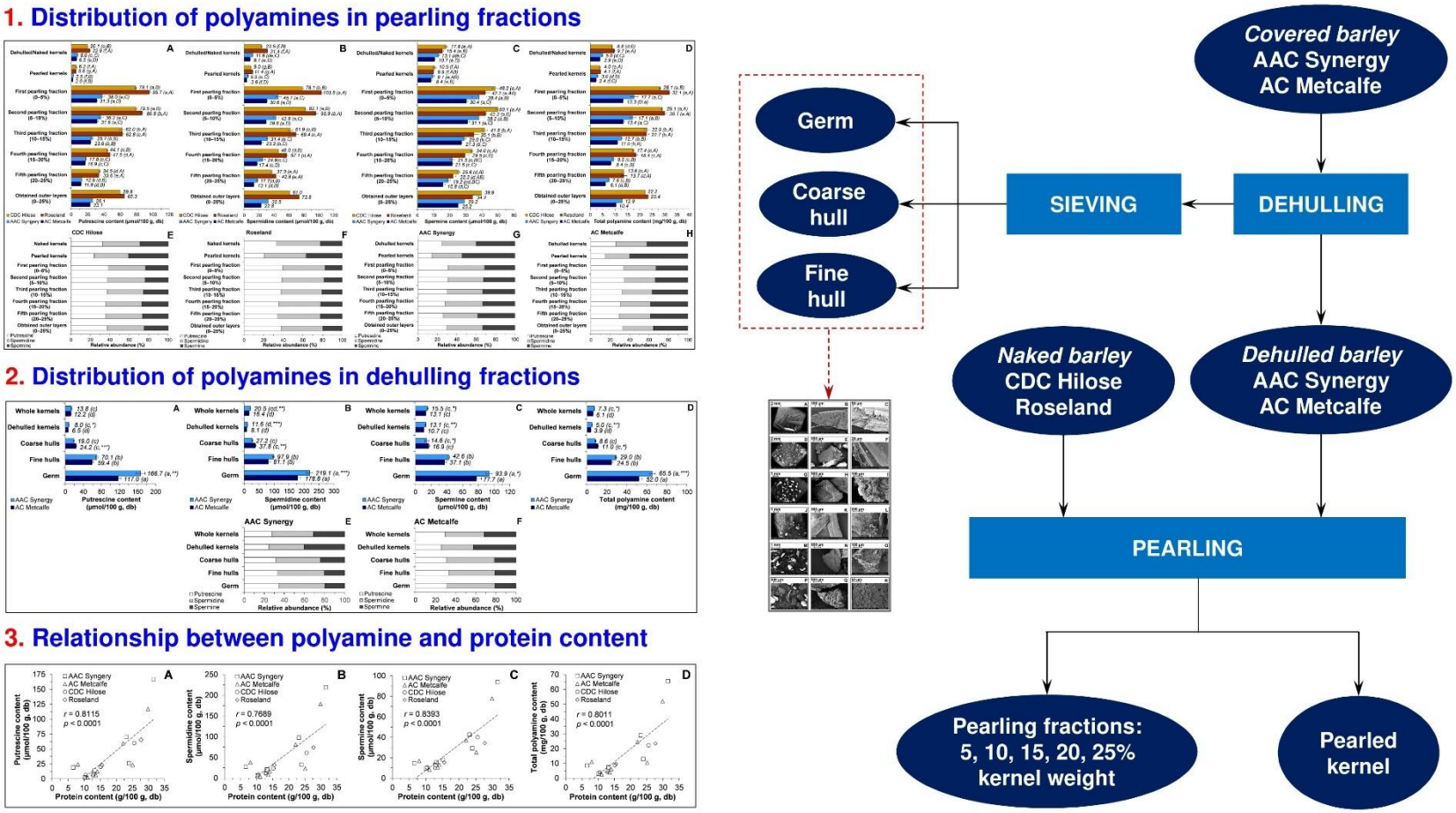

Figure 4.1. Graphical abstract of experimental section 2

4.2. Introduction

Cereal grains, especially whole grains, are known to be a package of macronutrients (starch, proteins and lipids) together with micronutrients (minerals and vitamins) (Garutti, Nevola, Mazzeo, et al., 2022). This package is complemented with dietary fibres and other health-promoting compounds, derived from primary (e.g., polyamines) or secondary metabolisms (e.g., polyphenols and carotenoids) of cereal crops (Garutti, Nevola, Mazzeo, et al., 2022). Regarding polyamines (putrescine, spermidine and spermine), these nitrogenous compounds result from amino acid catabolism and play important regulatory roles in plant growth, along with involvement in defence against biotic and abiotic stresses (Gerlin, Baroukh, & Genin, 2021). It should be mentioned that plants possess an additional pathway to synthesize putrescine from arginine via the formation of agmatine. This would distinguish their polyamine profile from that of animals, where ornithine is the dominant direct precursor of putrescine (Gerlin, Baroukh, & Genin, 2021).

In the human body, the presence of polyamines can help protect nucleic acids from abnormal methylation and can promote the autophagy process, ultimately contributing to enhanced cellular longevity (Soda, 2022). Such physiological effects implicate polyamines as a potential new class of nutraceuticals in the prevention and treatment of cardiovascular, neurodegenerative and aging-related diseases (Arthur, Jamwal, & Kumar, 2024; Kim, Baek, & Lee, 2024). Polyamines have been found in cereal commodities in appreciable quantities, with the total content determined in sorghum seeds (1.7–3.7 mg/100 g dry basis, db), barley seeds (6.5–11.5 mg/100 g db) and wheat germ (33.8–43.0 mg/100 g db) (Mohajeri, Ayatollahi, Kobarfard, et al., 2023; Nguyen & Beta, 2023; Paiva, Netto, Queiroz, et al., 2022).

Previous studies have discovered the uneven partitioning of chemical components in whole cereal grains. A few typical observations for the major components are the decreasing protein level and the increasing starch content from the outer parts to the core of the mature grain (Tosi, He, Lovegrove, et al., 2018). Considering phytochemicals and micronutrients, they were reported to be more concentrated in the bran (phenolic compounds) and the germ (carotenoids and tocopherols) of the cereal grain (Nguyen, Drawbridge, & Beta, 2024). Thus, cereal processing may alter the profile of chemical components that are retained in various processed fractions, due to the peculiar distribution pattern of each compound within the kernel. Mechanical treatments involving friction and abrasive forces, such as dehulling and pearling, are commonly employed in post-harvest processing of cereal grain (Tiwari & Pojić, 2020). On the one hand, these processes can enhance the palatability of cereal grains by removing the inedible or less delectable portions (hull and bran), along with the antinutrients (phytates and tannins) and microbial loads located in these fractions (Tiwari & Pojić, 2020). On the other hand, the separation and exclusion of the outer layers would compromise the health benefits that whole grains can confer due to loss of beneficial substances, notably dietary fibres and phenolic compounds (Garutti, Nevola, Mazzeo, et al., 2022).

Barley is a cereal crop with multiple usages, creating opportunities for different applications of mechanical processing techniques. In general, barley can be divided into covered (hulled) and naked (hulless) varieties, according to the post-harvest presence of the outer hull. Covered varieties of barley are normally destined for malting and feeding, whereas naked varieties are more suitable for human consumption (Lukinac & Jukić, 2022). For the utilization of unmalted covered barley as a brewing adjunct, removal of the outer hull can lower the amount of resultant spent grain, coupled with avoiding haze formation due to a decrease in phenolic compounds that are prone to complex with proteins (van Donkelaar, Noordman, Boom, et al.,

2015). Partial dehulling of covered barley can also be done prior to feeding cattle to improve the nutrient digestibility and metabolizable energy as a result of a reduction in fibre content (Wang, Shi, Xu, et al., 2017). As for pearling, in addition to its refinement purpose, this technique has been applied to obtain fractions rich in arabinoxylans and folates from naked barley (Friero, Martínez-Subirà, Romero, et al., 2024; Ruggeri, De Arcangelis, Aguzzi, et al., 2022). Studying the fractions produced by consecutive abrasions of barley grains can clarify how chemical components are spatially distributed throughout the kernel (Nguyen, Drawbridge, & Beta, 2024).

Following our previous reports of barley as a source of polyamines (Drawbridge, Apea-Bah, Hornung, et al., 2021; Nguyen & Beta, 2023), this research aims to investigate the topological distribution of polyamines across the barley kernel via the analysis of dehulling and pearling fractions. As pearling and dehulling are still relatively common practices in barley processing, the findings of this work will be useful to establish appropriate conditions to balance the loss of polyamines and the treatment objectives, coupled with generating product streams enriched in this type of nutraceutical from barley grains.

4.3. Materials and Methods

4.3.1. Materials

a) Barley cultivars

Two covered (AAC Synergy and AC Metcalfe) and two naked (CDC Hilose and Roseland) barley cultivars registered in Canada were used in this study. AAC Synergy and AC Metcalfe are two-row malting barley varieties, whereas CDC Hilose and Roseland are two-row barley varieties intended for food use. All the cultivars were grown in Brandon (Manitoba, Canada) and harvested in 2021.

b) Chemicals

Polyamine external standards (≥ 99% purity), including putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrachloride, were obtained from Millipore Sigma Corp. (Burlington, MA, USA). Dansyl chloride (≥ 99% purity) was also procured from the same supplier. The internal standard, 1,7-diaminoheptane (98% purity), was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Milli-Q Direct water purification system from Millipore Sigma Corp. was employed to purify water used in the HPLC system and other experimental procedures. Acetonitrile, acetone and ethyl acetate, were all of HPLC grade and from Fisher Scientific Inc. (Pittsburgh, PA, USA); hexamethyldisilazane (HMDS, ≥ 99% purity) and absolute ethanol (≥ 99.8% purity) were from Millipore Sigma Corp. Other chemicals, of at least reagent grade, were sourced from either Millipore Sigma Corp. or Fisher Scientific Inc.

4.3.2. Methods

a) Pearling of naked barley grains

The naked barley samples were pearled using a laboratory pearler (type TM, Satake Corp., Hiroshima, Japan). Initially, each sample weighing 150 g was passed through the pearler, with pearling fractions collected at 5% weight intervals after each pass (approximately 7.5 g). The successive abrasion continued until the kernels reached 75% of their original weight (approximately 112.5 g). Consequently, five pearling fraction ranges (0–5%, 5–10%, 10–15%, 15–20% and 20–25%) were obtained along with the residual pearled grain, which had 25% of its initial weight abraded. Two replicates were conducted per cultivar, and the resultant pearling fractions within each range were combined, followed by sifting through a 0.5 mm sieve. The coarse particles removed after sieving accounted for 1.3–7.2% of the pearling fraction weight. The sieved pearling products were kept at 4 ^{o}C until analysis.

b) Pearling of covered barley grains

Covered barley grains underwent a dehulling process at laboratory scale (dehuller model LH5095, Codema Inc., Minnesota, MN, USA) before proceeding to pearling. Dehulled barley kernels from covered cultivars were pearled using the same procedure as that for the naked cultivars. Trials at Grain Research Laboratory–Canadian Grain Commission (Winnipeg, Manitoba, Canada) demonstrated that grains of the selected cultivars could be dehulled without prior moisturization. For both grain samples, the dehulling rate was 50 g for 2.5 min at compressed air pressure of 100 psi. Dehulling 1,000 g of AAC Synergy grains generated 864 g of dehulled grains, 127 g of hulls and 5.6 g of germ. Dehulling 1,000 g of AC Metcalfe kernels yielded 861 g of dehulled kernels, 135 g of hulls and 3.3 g of germ. The germ fractions were obtained by sifting the finished kernels through a 12-mesh (1.68 mm) sieve. Upon macroscopic observation, the resultant hulls of AAC Synergy and AC Metcalfe were sifted through a 500 µm sieve due to heterogeneity in particle size and had retention proportions of 46% and 61%, respectively. The fraction passing through the sieve was designated as “fine hulls”, while the fraction retained on the sieve was designated as “coarse hulls”. The germ fraction and a portion of the dehulled kernels and the coarse hulls were milled (model ZM200, Retsch Co., Haan, Germany; 0.5 mm sieve ring and operated at 10,000 rpm) prior to storage 4 ºC.

c) Determination of protein and moisture content

The moisture content of samples, excluding the germ and pearling fractions, was determined by drying 2–3 g samples in a hot air oven (model Heratherm, Thermo Fisher Scientific Inc.) at 130 °C until constant weight (AACC International Method 44-15.02). Due to the limited quantity of germ fractions, their moisture contents (denoted as W) were assumed to be

similar to the fractions detached from covered kernels after dehulling. These values were derived from the difference in moisture content between covered and dehulled kernels as follows:

$$W_{\text{detached fractions (AAC Synergy)}} = (W_{\text{whole grains}} - W_{\text{dehulled kernels}} \times 0.864) / (1 - 0.864)$$

$$W_{\text{detached fractions (AC Metcalfe)}} = (W_{\text{whole grains}} - W_{\text{dehulled kernels}} \times 0.861) / (1 - 0.861)$$

Regarding the pearling fractions, our previous results indicated that they did not significantly differ from each other in terms of moisture content as determined by the oven-drying method (Drawbridge, 2023). To ensure sufficient pearling fractions for other analyses, their moisture contents were indirectly estimated using the following formula:

$$W_{\text{pearling fractions (AAC Synergy and AC Metcalfe)}} = (W_{\text{dehulled kernels}} - W_{\text{pearled kernels}} \times 0.75) / 0.25$$

$$W_{\text{pearling fractions (CDC Hilose and Roseland)}} = (W_{\text{naked kernels}} - W_{\text{pearled kernels}} \times 0.75) / 0.25$$

The protein contents of barley samples were quantified following the micro-Kjeldahl method reported by Nguyen and Beta (2023) with a nitrogen-to-protein conversion factor of 6.25.

d) Colour measurement

The tristimulus colour values ($L^*a^*b^*$) of the samples in powder form were assessed using a bench-top spectrophotometer (model CM-3500d, Minolta Co., Osaka, Japan) operated with SpectraMagic software version 3.60 (Minolta Co. and CyberChrome Inc., Branford, CT, USA). L^* (0 to 100), a^* (–128 to +127) and b^* (–128 to +127) values represent the lightness, redness and yellowness, respectively. Before each run, the spectrophotometer was calibrated with dark and white calibration plates. Samples were equilibrated to room temperature, transferred to transparent containers and placed on top of the measurement area. Three measurements were taken per sample. The total colour difference (ΔE) of a pearling/dehulling product from the corresponding pearled/dehulled kernels was calculated as follows:

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

where, L^*, a^* and $b^*$are the values of the examined sample; L_0^*, a_0^* and b_0^* are the colour values of the pearled/dehulled kernel.

e) Extraction and derivatization of polyamines

Extraction of free polyamines from barley samples was done using 1 M HCl, followed by alkalinization and dansyl derivatization using the protocol described by Nguyen and Beta (2023). The content of polyamines was determined using the chromatographic method reported by the same authors. Individual polyamines were identified by comparing the retention time with that of the standards. Calibration curves for extrapolation of polyamine content were constructed by plotting the concentration of the targeted compound (putrescine, spermidine, or spermine) against the peak area ratio of the compound to the internal standard (1,7-diaminoheptane). All calibration curves showed a high linearity with $R^2 > 0.9990$ in the concentration range of 5, 10, 15, 20, 30, 40 and 50 µM (Nguyen & Beta, 2023). The content of each polyamine was calculated on a weight basis and expressed as µmol per 100 g db. The total polyamine content, expressed as mg per 100 g db, was obtained by summing the individual polyamine contents based on their weight. The relative abundance of each polyamine was calculated on a molar basis, which was the percentage of each polyamine relative to the whole.

f) Scanning electron microscopy (SEM) images

SEM images of some dehulling fractions from the two covered barley cultivars were taken to examine their microstructure, including ground coarse hull, unground fine hull and ground germ. About 100 mg of each sample was placed in a 2-mL centrifuge tube and subjected to a dehydration process. Samples were soaked in sequential ethanol solutions of increasing concentration (1 mL each for 30 min; 70%, 80%, 95% and 100%); followed by immersion in hexamethyldisilazane (1 mL for 30 min) and overnight drying in a fume hood with the cap open.

Dehydrated specimens were mounted onto stubs using carbon tape and coated with gold-palladium to a thickness of 20–50 nm. SEM images were acquired using the Quanta FEG 650 Environmental SEM (FEI Co., Hillsboro, OR, USA) located at the Manitoba Institute for Materials (Winnipeg, Manitoba, Canada). The system was operated in high vacuum mode (10^{-2} to 10^{-4} Pa) with the Everhart-Thornley detector (ETD) at an accelerating voltage of 5.00 kV.

g) Statistical analysis

Data were collected in triplicate and expressed as mean ± standard deviation. The software Origin V10.0 (OriginLab Co., Northampton, MA, USA) was used to perform paired sample *t*-test and one-way analysis of variance (ANOVA) with Tukey comparison at $\alpha = 0.05$. Pearson correlation analysis was done using the same software.

4.4. Results and Discussion

4.4.1. Physicochemical properties of pearling products

Table 4.1 shows the physicochemical properties of barley grains and their pearling products, namely colour values, moisture level and protein content. Pearling resulted in various changes in colour values of barley grains, including a reduction in redness (by 64–73%) and yellowness (by 26–37%), accompanied by an increase in lightness (by 3–6%). The differences in colour values of pearling fractions were more noticeable at pearling rates of 5, 10 and 15% for all cultivars, implying that these fractions might contain distinctive grain tissues. Izydorczyk and Dexter (2016) performed pearling experiments on naked barley grains and reported that 5% pearling rate would typically eliminate the pericarp. Additionally, the authors observed that an increment of pearling rate to 10% allowed the removal of one or two aleurone cell layers. Due to the similarity in samples and machinery, these observations could be relevant for the

interpretation of results in our work. Since multiple layers of aleurone are usually found in barley grains, the next pearling fraction (10–15% kernel weight, KW) may still contain aleurone cells as well as those in adjacent regions. The colour values of pearling fractions showed minimal changes as the pearling degree increased to 20 and 25%, implying that these pearling fractions may be histologically similar, with the abrasion process likely reaching the endosperm. The fifth pearling fraction showed the lowest total colour difference against the pearled kernel. Nevertheless, it should be noted that

A paired *t*-test indicated no significant difference between the moisture levels of barley kernels before (9.3–10.6 g/100 g wet basis, wb) and after pearling (8.5–10.2 g/100 g wb) at $\alpha = 0.05$ for all cultivars (statistical annotations not shown in Table 4.1). The moisture levels of removed outer layers were calculated from the mass balance equation and ranged from 9.9 to 11.9 g/100 g wb. Despite the relatively small variation in moisture content among barley samples, these values are necessary for normalizing the content of chemical constituents to a dry basis, allowing for accurate comparisons. Pearling significantly impacted protein content in four cultivars, resulting in notable reductions (by 18–31%, $p < 0.05$) when 25% of KW was abraded. The second and third pearling fractions showed the highest protein content, which could be attributed to their histological composition. Based on the previous discussion, the first pearling fraction was likely made up of pericarp, while the second and third pearling intervals would contain aleurone and sub-aleurone cells. This finding aligns with existing literature, which suggests that protein bodies are more concentrated in the aleurone and sub-aleurone regions of barley grains (Antonini, Zara, Valentini, et al., 2018; Macewicz, Orzechowski, Dobrzyska, et al., 2006), resulting in higher protein content in these areas.

Table 4.1. Physicochemical properties of barley pearling products

Barley cultivar	Pearling product	*L**	*a**	*b**	Δ*E*	Moisture content (g/100 g, wb)	Protein content (g/100 g, db)
AAC Synergy	Dehulled kernels	89.8 ± 0.2 (c,B)	1.06 ± 0.03 (d,B)	8.9 ± 0.05 (e,C)	4.2	10.6 ± 0.2	12.8 ± 0.2 (e,C)
	Pearled kernels	93.2 ± 0.2 (a,B)	0.35 ± 0.02 (d,B)	6.6 ± 0.05 (f,C)	NA	10.2 ± 0.1	10.2 ± 0.2 (f ,C)
	First pearling fraction (0–5%)	80.9 ± 0.2 (f,B)	2.81 ± 0.04 (a,B)	15.6 ± 0.2 (a,B)	15.5	ND	22.5 ± 0.1 (c,B)
	Second pearling fraction (5–10%)	85.5 ± 0.4 (e,A)	1.93 ± 0.03 (b,BC)	13.3 ± 0.1 (b,B)	10.4	ND	26.1 ± 0.2 (a,C)
	Third pearling fraction (10–15%)	88.2 ± 0.3 (d,A)	1.36 ± 0.02 (c,B)	11.4 ± 0.1 (c,C)	7.1	ND	25.8 ± 0.1 (a,C)
	Fourth pearling fraction (15–20%)	89.9 ± 0.2 (c,B)	1.07 ± 0.02 (d,A)	9.9 ± 0.1 (d,C)	4.7	ND	23.8 ± 0.1 (b,C)
	Fifth pearling fraction (20–25%)	90.4 ± 0.2 (b,AB)	0.87 ± 0.06 (e,A)	8.8 ± 0.1 (e,C)	3.7	ND	21.6 ± 0.1 (d,D)
	Obtained outer layers (0–25%)	ND	ND	ND	ND	11.9	23.9
AC Metcalfe	Dehulled kernels	90.8 ± 0.1 (bc,A)	1.05 ± 0.04 (d,B)	8.9 ± 0.2 (e,C)	3.9	10.2 ± 0.2	13.6 ± 0.2 (d,B)
	Pearled kernels	93.7 ± 0.3 (a,A)	0.37 ± 0.03 (f,AB)	6.5 ± 0.2 (f,C)	NA	9.9 ± 0.2	11.1 ± 0.2 (e,B)
	First pearling fraction (0–5%)	82.2 ± 0.3 (f,A)	2.58 ± 0.02 (a,C)	15.4 ± 0.1 (a,B)	14.7	ND	22.9 ± 0.3 (c,B)
	Second pearling fraction (5–10%)	85.9 ± 0.1 (e,A)	1.83 ± 0.07 (b,C)	13.5 ± 0.1 (b,B)	10.6	ND	27.0 ± 0.1 (a,AB)
	Third pearling fraction (10–15%)	88.5 ± 0.2 (d,A)	1.17 ± 0.02 (c,D)	11.3 ± 0.1 (c,C)	7.1	ND	27.2 ± 0.2 (a,B)
	Fourth pearling fraction (15–20%)	90.2 ± 0.3 (c,A)	0.97 ± 0.03 (d,AB)	10.0 ± 0.2 (d,C)	5.0	ND	25.4 ± 0.2 (b,B)
	Fifth pearling fraction (20–25%)	91.0 ± 0.1 (b,A)	0.82 ± 0.04 (e,AB)	8.8 ± 0.1 (e,C)	3.6	ND	23.1 ± 0.1 (c,C)
	Obtained outer layers (0–25%)	ND	ND	ND	ND	11.1	25.1
CDC Hilose	Naked kernels	88.5 ± 0.3 (c,C)	1.20 ± 0.03 (c,A)	12.0 ± 0.4 (d,A)	6.1	9.3 ± 0.3	15.5 ± 0.1 (d,A)
	Pearled kernels	92.9 ± 0.1 (a,B)	0.33 ± 0.02 (f,B)	7.6 ± 0.1 (f,A)	NA	8.5 ± 0.3	12.7 ± 0.4 (e,A)
	First pearling fraction (0–5%)	79.5 ± 0.1 (e,C)	2.60 ± 0.10 (a,C)	16.7 ± 0.2 (a,A)	18.3	ND	26.2 ± 0.1 (c,A)
	Second pearling fraction (5–10%)	80.2 ± 0.3 (e,C)	2.48 ± 0.09 (a,A)	16.7 ± 0.3 (a,A)	12.0	ND	26.8 ± 0.3 (a,B)
	Third pearling fraction (10–15%)	85.8 ± 0.4 (d,C)	1.46 ± 0.02 (b,A)	14.6 ± 0.04 (b,A)	7.5	ND	30.2 ± 0.3 (a,A)
	Fourth pearling fraction (15–20%)	88.5 ± 0.4 (c,B)	0.91 ± 0.06 (d,B)	12.7 ± 0.1 (c,A)	5.1	ND	28.9 ± 0.3 (b,A)
	Fifth pearling fraction (20–25%)	90.0 ± 0.1 (b,B)	0.60 ± 0.09 (e,C)	11.1 ± 0.2 (e,A)	3.4	ND	26.5 ± 0.3 (c,A)
	Obtained outer layers (0–25%)	ND	ND	ND	ND	11.6	27.7
Roseland	Naked kernels	88.0 ± 0.4 (d,C)	1.21 ± 0.04 (c,A)	10.1 ± 0.1 (e,B)	6.3	9.7 ± 0.2	15.2 ± 0.3 (d,A)
	Pearled kernels	93.2 ± 0.1 (a,B)	0.41 ± 0.02 (f,A)	7.1 ± 0.1 (g,B)	NA	9.6 ± 0.2	10.5 ± 0.2 (e,BC)
	First pearling fraction (0–5%)	77.1 ± 0.3 (f,D)	3.18 ± 0.04 (a,A)	15.4 ± 0.1 (a,B)	16.4	ND	22.8 ± 0.2 (c,B)
	Second pearling fraction (5–10%)	83.3 ± 0.3 (e,B)	2.06 ± 0.03 (b,B)	13.7 ± 0.05 (b,B)	15.9	ND	27.4 ± 0.2 (a,A)
	Third pearling fraction (10–15%)	87.4 ± 0.1 (d,B)	1.27 ± 0.05 (c,C)	11.8 ± 0.1 (c,B)	10.1	ND	28.0 ± 0.5 (a,B)
	Fourth pearling fraction (15–20%)	89.6 ± 0.4 (c,A)	0.92 ± 0.03 (d,B)	10.7 ± 0.03 (d,B)	6.8	ND	25.9 ± 0.4 (b,B)
	Fifth pearling fraction (20–25%)	90.8 ± 0.4 (b,A)	0.68 ± 0.01 (e,BC)	9.5 ± 0.1 (f,B)	4.6	ND	23.6 ± 0.2 (c,B)
	Obtained outer layers (0–25%)	ND	ND	ND	ND	9.9	25.6

Data are presented as mean ± standard deviation (n = 3), except for those of the obtained outer layers (n = 1). Values within a single cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values across multiple cultivars that do not share an uppercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: ND: not determined; NA: not applicable; wb: wet basis; db: dry basis.

4.4.2. Distribution of polyamines in pearling products

Figure 4.2 provides information on the individual and total polyamine content in the pearling products of four barley cultivars. Naked barley cultivars (Roseland and Hilose) generally exhibited higher levels of individual and total polyamines, both before and after pearling, compared to dehulled kernels of covered cultivars (AAC Synergy and AC Metcalfe). The average differences in total polyamine content between naked and covered cultivars were 2.1 and 1.5 fold for pearled and unpearled kernels, respectively. The outer tissues of non-pigmented barley grains are known to contain different phytochemicals (e.g., phenolic acids) and micronutrients (e.g., folates, tocopherols and tocotrienols) (Nguyen, Drawbridge, & Beta, 2024). In this study, the first and second abrasion intervals were determined to be the most concentrated in polyamines, potentially corresponding to the pericarp and parts of the aleurone layer as aforementioned. Abrasion of 25% KW resulted in a reduction in concentration of putrescine (69–76%), spermidine (55–64%), spermine (21–41%) and total polyamines (38–58%). The decrease in polyamine content of barley kernels due to abrasion was translated into an augmented content of polyamines in the removed outer layers. The outer layers obtained from naked cultivars were 2.0 and 2.5 times more enriched in total polyamines compared to their unpearled kernels and the outer tissues derived from covered cultivars, respectively. In addition, the extent of reduction in the concentration of putrescine was found to be greater than that of spermidine and spermine. This led to a shift in the polyamine profile after pearling (Figures 1E–H), in which the pearled kernels contained relatively less putrescine and relatively more spermine, whereas the relative abundance of spermidine experienced a minor impact. It is possible that the biosynthetic pathways responsible for polyamine chain elongation are more active in the central regions of the barley caryopsis. To confirm this hypothesis, further research is needed to investigate the

interplay between polyamine biosynthesis and the biological processes occurring in each botanical tissue of the developing barley seed, as well as the detailed histological composition of each pearling fraction.

When accounting for kernel mass, the retention rates of putrescine, spermidine and spermine post-pearling were found to be 18–23%, 29–36% and 44–59%, respectively. Although the reduction in the nutritional value of barley grains due to pearling appeared difficult to avoid, the outer layers obtained through abrasion could serve as a valuable source of polyamines in the context of nutraceutical production. Fermented soybean products such as natto and tempeh are known to be a good source of polyamines; however, they may contain histamine and other food allergens (Toro-Funes, Bosch-Fuste, Latorre-Moratalla, et al., 2015). Therefore, the availability of polyamines in products of cereal grains such as barley bran would offer more selections for consumers in terms of matching dietary preferences and avoiding food allergies. In particular, the average content of putrescine (62.6 μmol/100 g db), spermidine (67.4 μmol/100 g db) and spermine (37.1 μmol/100 g db) in the outer layers obtained from naked barley cultivars was found to be 7.4, 1,5 and 7.4 fold higher than those of the natto product analyzed by Toro-Funes, Bosch-Fuste, Latorre-Moratalla, et al. (2015). It was reported that dietary intake of spermidine and spermine was inversely associated with cardiovascular-related and all-cause mortality (Kim, Baek, & Lee, 2024). The triamine spermidine and tetramine spermine were found to be crucial for the maintenance of human health, as the enzymatic activity of their synthase is diminished with aging (Soda, 2022). Although the role of the diamine putrescine in the human body remains underexplored, an earlier *in vitro* study suggests that dietary or intravenous supplementation with putrescine may have a beneficial impact on embryonic and fetal development in mammals (Kong, Wang, Yin, et al., 2014).

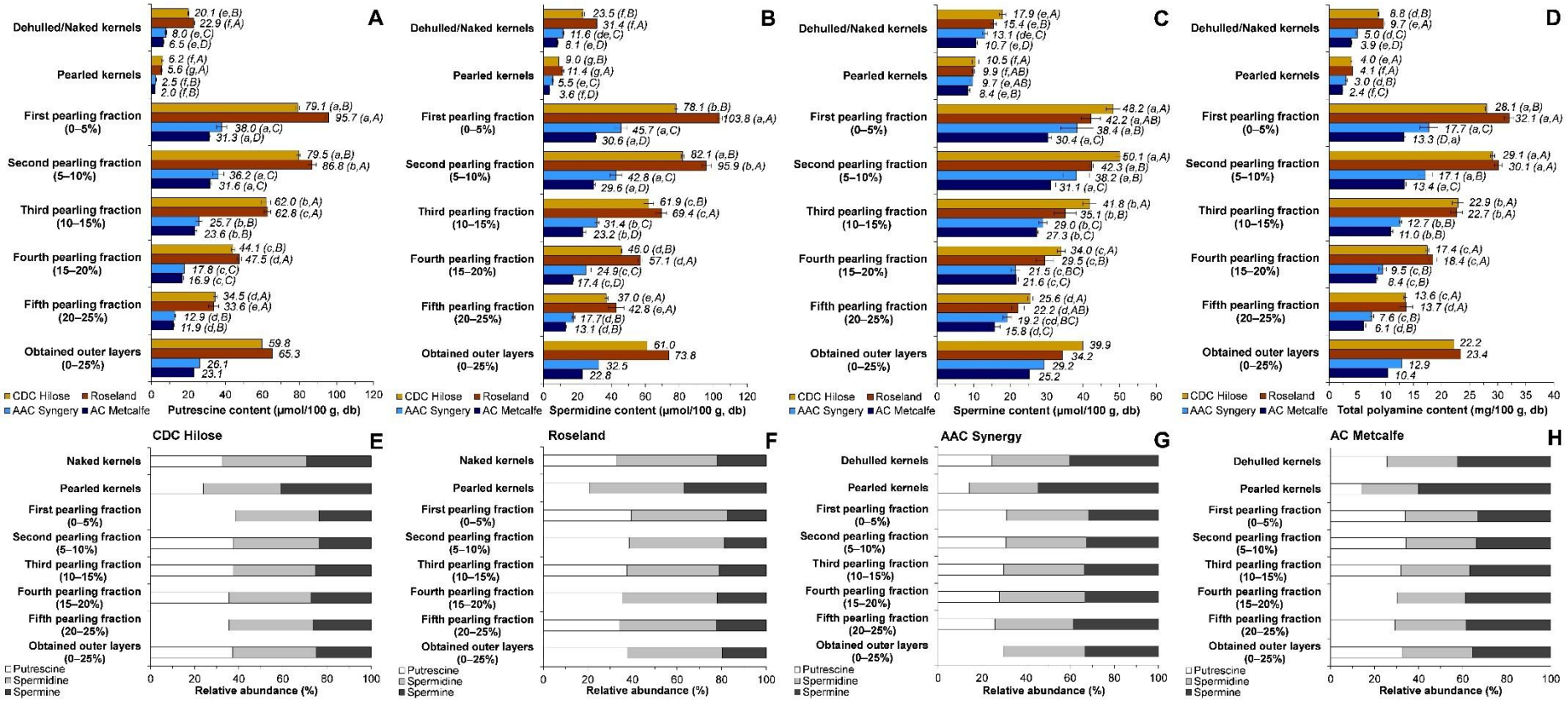

Figure 4.2. Content and profile of polyamines in pearling products of four barley cultivars.

Data are presented as mean ± standard deviation (n = 3). Values within a single cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values across multiple cultivars that do not share an uppercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: db: dry basis.

4.4.3. Physicochemical properties of dehulling products

Table 4.2 displays the physicochemical properties of dehulling products in terms of colour values, moisture level and protein content. The loss of outer husk affected the appearance of barley grains in terms of reduction in redness (by 29–31%) and yellowness (by 23–25%). The changes in colour values after dehulling were noticeable ($\Delta E > 3$). Slight differences in colour values between the dehulling products of the two cultivars were observed. The moisture content of barley kernels ranged from 10.0–10.6%, while that of detached hulls varied between 6.6 and 7.8%. This difference could be attributed to the withering of the husk after its detachment from the caryopsis. Dehulling of covered barley coupled with consecutive sieving of dehulling products yielded fractions with varying protein content, spanning from high-protein fractions (germ and fine hulls) to low-protein fractions (coarse hulls). The germ and fine hull fractions of AAC Synergy had slightly higher protein levels than those of AC Metcalfe, which could be attributed to variations in grain characteristics, dehulling patterns, and histological makeup. Barley hulls would be expected to have a relatively low protein content, as it is primarily composed of dietary fibre components such as cellulose and arabinoxylans (Wang, Shi, Xu, et al., 2017). In the publication of van Donkelaar, Noordman, Boom, et al. (2015), the hull protein content reported by the authors (5.7 g/100 g db) aligns with that of coarse hull fractions in this study (6.6–8.0 g/100 g db). Nevertheless, the notably higher protein level of fine hull fractions in this study (22.1–23.1 g/100 g db) warrants further investigation to understand the discrepancy.

Table 4.2. Physicochemical properties of barley dehulling products

Barley cultivar	Dehulling products	*L**	*a**	*b**	Δ*E*	Moisture content (g/100 g, wb)	Protein content (g/100 g, db)
AAC Synergy	Whole kernels	87.2 ± 0.2 (b,†)	1.49 ± 0.01 (d)	11.5 ± 0.1 (c)	3.7	10.5 ± 0.2	13.2 ± 0.1 (c)
	Dehulled kernels	89.8 ± 0.2 (a,†)	1.06 ± 0.03 (e)	8.9 ± 0.1 (d)	NA	10.6 ± 0.2	12.8 ± 0.2 (c)
	Coarse hulls	75.2 ± 0.3 (d,‡)	3.71 ± 0.08 (a,†)	20.5 ± 0.2 (a,†)	18.8	7.0 ± 0.2	6.6 ± 0.2 (d)
	Fine hulls	75.8 ± 0.5 (d)	3.42 ± 0.08 (b)	18.1 ± 0.1 (b)	16.9	7.8 ± 0.2	23.1 ± 0.1 (b,‡)
	Germ	85.2 ± 0.3 (c,†)	2.80 ± 0.08 (c)	17.9 ± 0.3 (b)	10.3	9.9	31.6 ± 0.1 (a,‡)
AC Metcalfe	Whole kernels	87.6 ± 0.2 (b)	1.53 ± 0.11 (c)	11.8 ± 0.3 (d)	4.3	10.0 ± 0.3	14.1 ± 0.2 (c,†)
	Dehulled kernels	90.8 ± 0.1 (a)	1.05 ± 0.04 (d)	8.9 ± 0.2 (e)	NA	10.2 ± 0.2	13.6 ± 0.2 (d)
	Coarse hulls	77.4 ± 0.1 (d)	3.39 ± 0.04 (a)	19.8 ± 0.1 (a)	17.4	6.6 ± 0.2	8.0 ± 0.1 (e,†)
	Fine hulls	77.1 ± 0.4 (d)	3.26 ± 0.01 (a)	18.1 ± 0.1 (b)	16.6	7.6 ± 0.3	22.1 ± 0.1 (b)
	Germ	83.3 ± 0.6 (c)	2.53 ± 0.18 (b)	16.9 ± 0.3 (c)	11.1	8.8	29.9 ± 0.1 (a)

Data are presented as mean ± standard deviation (n = 3), except for germ moisture content (n = 1). Values within a cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values followed by a single (†) or double dagger (‡) are significantly different from their counterparts of another cultivar at $p \leq 0.05$ or $p \leq 0.01$ (Paired-sample t-test), respectively.

Abbreviations: NA: not applicable; wb: wet basis; db: dry basis.

4.4.4. Distribution of polyamines in dehulling products

Figure 4.3 depicts the distribution of polyamines in dehulling products of barley grains. The germ and fine hull fractions were found to be particularly rich in polyamines, with the germ containing 52.0–65.5 mg total polyamines/100 g db and fine hull fractions containing 24.5–29.0 mg total polyamines/100 g db. On average, the germ and fine hull fractions had polyamine concentrations that were 8.8 and 4.0 times higher, respectively, than those found in whole kernels. In addition, the germ fraction of AAC Synergy showed a higher total polyamine content than that of AC Metcalfe, which could be related to varietal characteristics and environmental factors. Barley germ contains 51% more total polyamines than wheat germ obtained through milling, according to the study of Mohajeri, Ayatollahi, Kobarfard, et al. (2023). Although barley germ is rich in polyamines, its availability as a byproduct of dehulling covered grains is limited, yielding 3.3–5.6 g per 1,000 g of initial grains. According to Lukinac and Jukić (2022), the embryo accounts for approximately 3% kernel weight (KW) of hulled barley, suggesting a theoretical yield of 30 g of germ per 1,000 g of grains. Therefore, future studies can focus on optimizing the degerming techniques to obtain the maximal quantity of cereal germs for polyamine extraction and utilization. In contrast to barley germ, fine hull fractions are more abundant, yielding 53–69 g per 1,000 g of initial grains, making them a promising source of polyamines for potential utilization. Nevertheless, the coarse hulls retained on the 35-mesh sieved had a total polyamine content 2.7 times lower than the finer particle fractions. This disparity may be attributed to differences in tissue composition, warranting further investigation to understand the underlying factors. Dehulled kernels exhibited a shift in their polyamine profile, characterized by an increase in the relative abundance of spermine and a decrease in putrescine and spermidine. This pattern mirrors the changes observed after pearling, suggesting that both processes remove

similar outer tissue layers. The polyamine profiles of the germ and hull fractions were comparable and showed an increased relative abundance of putrescine and spermidine. The polyamine profile of barley seedlings may be associated with that of the embryo. Paiva, Netto, Queiroz, et al. (2022) reported similar findings, noting that putrescine and spermidine are the predominant polyamines in sorghum seedlings, specifically in the leaf, cotyledon and radicle tissues.

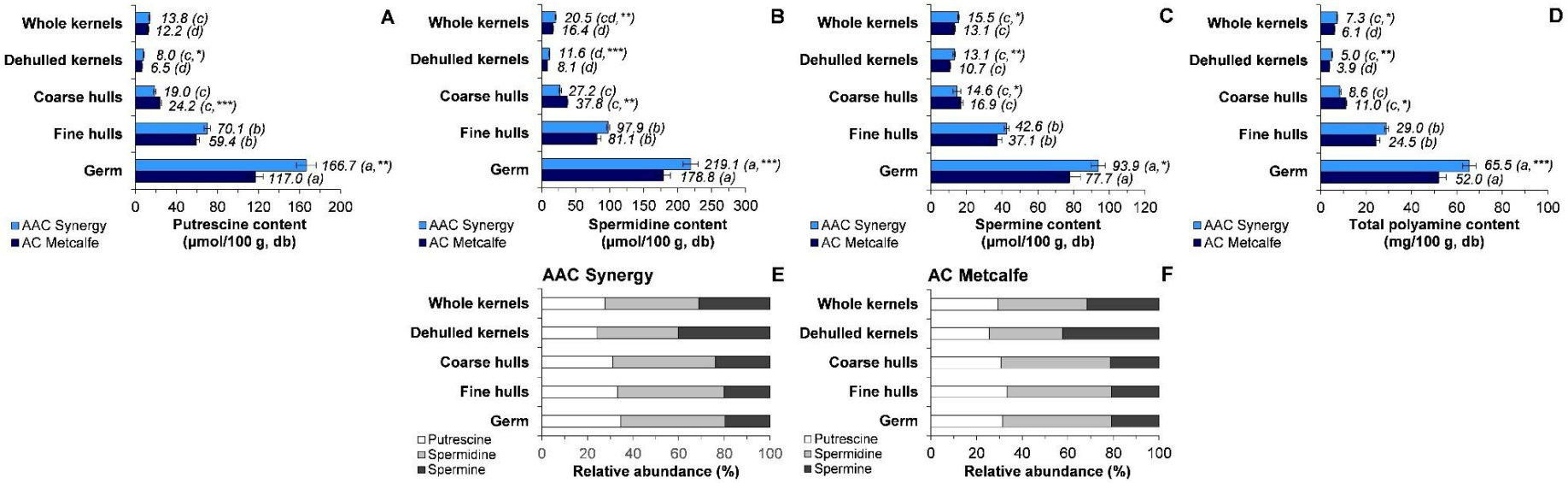

Figure 4.3. Content and profile of polyamines in pearling products of two barley cultivars.

Data are presented as mean ± standard deviation (n = 3). Values within a cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values followed by a single (*), double (**), or triple asterisk (***) are significantly different from their counterparts of the other cultivar at $p \leq 0.05$, $p \leq 0.01$, or $p \leq 0.001$ (Paired-sample t-test), respectively.

Abbreviations: db: dry basis.

4.4.5. Microscopic examination of dehulling products

Figure 4.4 displays the microstructure of some dehulling fractions of AAC Synergy and AC Metcalfe, specifically coarse hull (post-milling), fine hull and germ (post-milling). The removed hulls were of particular interest due to the significant differences in chemical composition observed between the coarse and fine hull fractions. Initial observation at lower magnification levels revealed a more homogenous size and shape of particles in the coarse hull samples (Figure 4.4A&J) compared to those in the fine hull counterparts (Figure 4.4D&M). Upon closer inspection, the coarse hull particles revealed an ordered fibrous structure, characterized by a layered arrangement (Figure 4.4B&C). A comparative analysis of the abaxial and adaxial sides of barley hull revealed distinct structural characteristics (Figure 4.4K&L), with the abaxial layer featuring strips of dead cells of uniform size. In contrast, the fine hull particles examined at higher magnifications consisted of a mix of void and filled cells of varying sizes (Figure 4.4E&F). This suggests that these fractions may contain adjacent tissues that were removed together during the dehulling process. The presence of a distinctly different tissue attached to the hypodermis of the hull supports this observation (Figure 4.4N). The barley pericarp, a thin-walled layer situated directly beneath the husk, was likely co-separated during the dehulling process (Antonini, Zara, Valentini, et al., 2018). The adherence of hull to the barley caryopsis in malting cultivars may be strengthened by a lipid-rich cementing material between the hull and pericarp layer (Hoad, Brennan, Wilson, et al., 2016). This supports the likelihood of their co-separation during the dehulling process. Furthermore, our study observed a honeycomb-like structure occasionally filled with starch granules, suggesting that the detachment may also involve the aleurone and sub-aleurone regions (Figure 4.4O). This is consistent with previous reports of starch granules in the sub-aleurone layer of barley grains (Macewicz, Orzechowski, Dobrzyska,

et al., 2006). The suspected presence of neighbouring tissues, beyond just the husk, in the fine hull fraction likely contributed to the higher polyamine content observed in this fraction. This finding is consistent with the study by van Donkelaar, Noordman, Boom, et al. (2015), which used SEM to examine the removal of outer hulls from covered barley grains through abrasion and found a mixture of fibrous and non-fibrous materials. To isolate fractions enriched in specific compounds, further fractionation of product streams using sieving may be beneficial. The barley germ fraction exhibited particles of relatively uniform size and shape (Figure 4.4G&P). Upon closer examination, their microstructure revealed a dense protein matrix with a mosaic-like pattern (Figure 4.4Q&R) or notable presence of starch granules (Figure 4.4H&I).

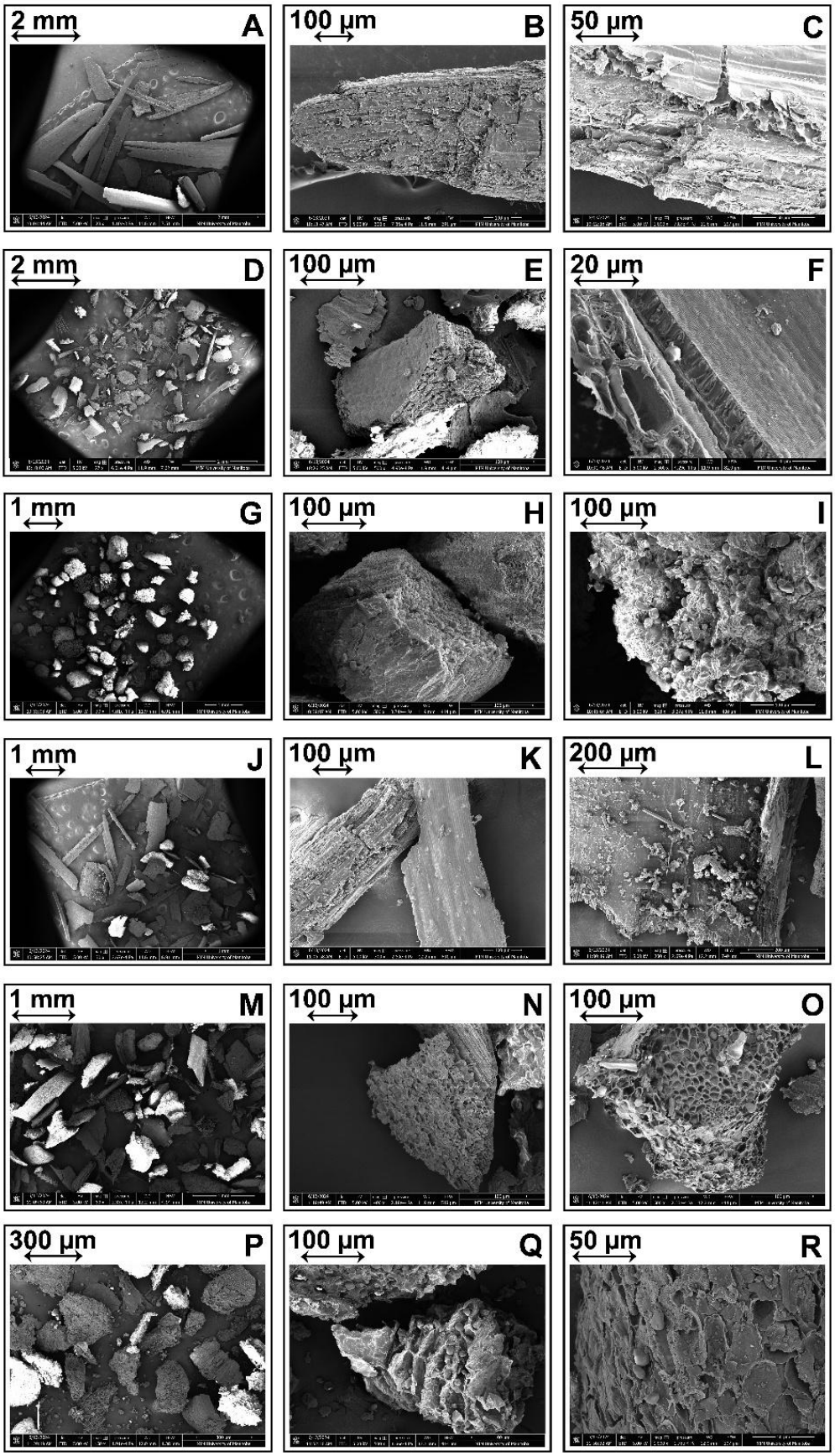
2 mm
A
100 µm
B
50 µm
C
2 mm
D
100 µm
E
20 µm
F
1 mm
G
100 µm
H
100 µm
I
1 mm
J
100 µm
K
200 µm
L
1 mm
M
100 µm
N
100 µm
O
300 µm
P
100 µm
Q
50 µm
R

Figure 4.4. Microstructure of some barley dehulling products observed under scanning electron microscope

Figure 4.4A–C: Ground coarse hulls of AAC Synergy, Figure 4.4D–F: Fine hulls of AAC Synergy, Figure 4.4G–I: Ground germ of AAC Synergy, Figure 4.4J–L: Ground coarse hulls of AC Metcalfe, Figure 4.4M–O: Fine hulls of AC Metcalfe, Figure 4.4P–R: Ground germ of AC Metcalfe.

4.4.6. Relationship between polyamine and protein content

Figure 4.5 depicts relationships between polyamine and protein contents in barley grain fractions. A correlation analysis was conducted on the polyamine and protein content of whole grains, pearling products (outer layers and pearled kernel) and dehulling products (dehulled kernels, coarse hulls, fine hulls and germ). Each pair of variables consists of 20 data points, which were visualized on a scatter diagram. To better represent the entirety of the pearling fractions, which collectively accounted for 25% of the KW, the obtained outer layers were grouped together. The individual and total polyamine content demonstrated a strong and positive correlation ($r > 0.75$) with the protein level. This was in accordance with our previously reported findings, in which positive and significant correlations between the level of polyamines and protein in whole barley seeds were noted (Nguyen & Beta, 2023). The persistent correlations between polyamine and protein content observed in this study, even after fractionation of barley caryopsis, may offer further insights about their co-occurrence. From a physiological perspective, the co-localization of polyamines and proteins at the sub-kernel level suggests a need to re-examine their roles within each specific tissue. Since polyamines are necessary for cellular vitality and proliferation (Gerlin, Baroukh, & Genin, 2021), the high polyamine concentration in the embryo could be related to the vigorous division and development of embryonic cells in the germinating seed. The polyamine reservoir could participate in the generation of cell walls during germination (Torrigiani, Scoccianti, & Bagni, 1988). During this process, polyamines are enzymatically oxidized to yield hydrogen peroxide, which in turn interacts with hemicelluloses to form the cross-linkages (Torrigiani, Scoccianti, & Bagni, 1988). In the barley aleurone layer, polyamines are believed to be involved in the regulation of enzyme-inducing events that rely on growth substances (Lin, 1984). The high levels of proteinaceous compounds in the embryo and aleurone fractions of barley grain can be attributed to the presence of hydrolytic enzymes and

proteins involved in primary metabolism (Finnie & Svensson, 2014). It is likely that proteins and polyamines are more concentrated in the kernel tissues that play an active role in seed germination. This speculation is further supported by the lower levels of polyamines and proteins found in the starchy endosperm, which is a metabolically inactive region primarily containing storage and defence-related proteins (Finnie & Svensson, 2014). However, additional studies are necessary to elucidate the underlying biological mechanisms, particularly the interaction between polyamines and specific proteins that influence the physiological performance of kernel tissues.

From a biochemical perspective, the association between polyamines and proteins could be ascribed to a few amino acids acting as their common building blocks or precursors (i.e., arginine and ornithine). However, nitrogen uptake is likely to be the ultimate factor mediating the interplay between polyamines and proteins. A previous study on barley cultivars grown in Manitoba found glutamate to be the most abundant amino acid in grains (Boila, Stothers, & Campbell, 1996). Glutamate is synthesized in plants from ammonia and α-ketoglutarate via glutamate synthase and serves as a central molecule in amino acid metabolism, including the synthesis of arginine and ornithine (Forde & Lea, 2007). Therefore, it is expected that the concentration of polyamines and protein in cereal grains is dictated by the nitrogen uptake of the plant. This is supported by the study of Kaur and Asthir (2019) on wheat, demonstrating the effects of different nitrogen regimes on grain polyamine content after anthesis. The authors found that supra-optimal nitrogen regimes increased polyamine levels and the activity of enzymes participating in polyamine metabolism. In contrast, suboptimal nitrogen fertilization reduced grain polyamine content by increasing the activity of polyamine-catabolizing enzymes. With the growing interest in polyamine research and the specific protein content requirements for designated purposes of barley grains (Nguyen & Beta, 2023), it is essential to investigate the optimal cultivation conditions for each variety that are needed to achieve the desired quality.

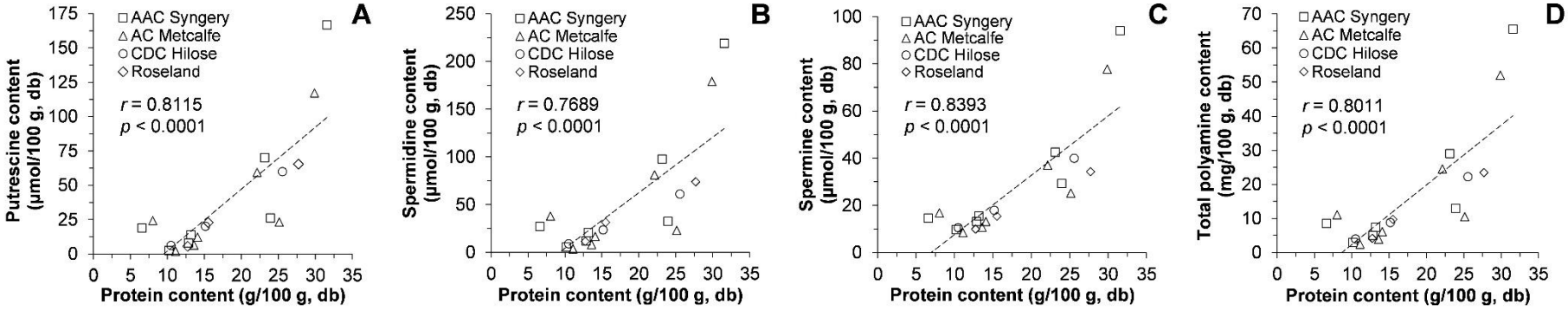

Figure 4.5. Relationships between polyamine and protein content in pearling and dehulling products of barley grains

Each panel includes twenty data points, comprising four of whole-flour samples, two of dehulled samples, four of hull fractions, two of germ fractions, four pearled samples, and four of outer layer fractions (combining all pearling intervals).

4.5. Conclusion

This is the first study to investigate the distribution of polyamines in barley grains through the analysis of pearling and dehulling products. The results showed that pearling decreased the polyamine content in barley kernels of all four cultivars, while the removed outer layers were more concentrated in polyamines compared to naked and dehulled kernels. The first and second pearling fractions, corresponding to the pericarp and aleurone layer, respectively, exhibited the highest levels of polyamines and protein. Dehulling of covered barley grains followed by sieving generated multiple product streams with varying protein and polyamine contents, with barley germ and fine hull fractions having the highest levels. Microscopic examination revealed that other outer tissues of barley caryopsis also contributed to the fine hull fractions, resulting in their higher polyamine concentration compared to the coarse hull fractions. At a sub-kernel level, positive and significant correlations were found between polyamine and protein content, suggesting a biochemical and physiological association between the two components. Given the multiple uses of barley, understanding the distribution of polyamines and their relationship with protein in the barley kernel can facilitate the effective utilization and valorization of products from this crop.

Chapter 5 — Experimental Section 3 (Enrichment of polyamines across barley milling fractions and their correlation with markers of whole-grain content)

5.1. Chapter Abstract

Barley is increasingly recognized for its health-promoting constituents, e.g., polyamines, phenolic compounds and micronutrients concentrated in the germ and outer kernel layers. This study investigated the potential of dry fractionation, combining milling and size-based sieving, to enrich polyamines and whole-grain markers in barley milling products. Six hulled and hulless barley cultivars were used, encompassing malting, feed, and food types. Whole barley grains were milled and separated into three particle-size fractions, namely fraction A (>500 μm), fraction B (200–500 μm), and fraction C (<200 μm). Across all cultivars, the intermediate fraction B consistently exhibited polyamine enrichment, with total polyamine concentrations averaging 25% higher than those in whole flour. Specifically, spermidine increased by 20–70%, putrescine by 10–68% and spermine by 20–36%, depending on genotype. In contrast, the fine fraction C and coarse fraction A showed reductions in total polyamines and a compositional shift toward the polyamine-poor starchy endosperm and hull materials, respectively. Hand dissection confirmed that polyamines are concentrated in the outer layers and germ tissues of the barley kernel, which were present in greater proportion in the intermediate fraction B, as indicated by physicochemical properties. Correlation analysis revealed positive associations between polyamines and markers of whole-grain status, including protein (r = 0.70–0.84), ash (r = 0.44–0.63), barley germ agglutinin (r = 0.67) and total phenolics (r = 0.44–0.66). Overall, dry fractionation effectively concentrated polyamines and whole-grain constituents into a nutritionally superior milling fraction, highlighting its potential to produce barley ingredients with enhanced bioactive content.

Keywords: barley; dry fractionation; *Hordeum vulgare*; milling; polyamine; correlation analysis

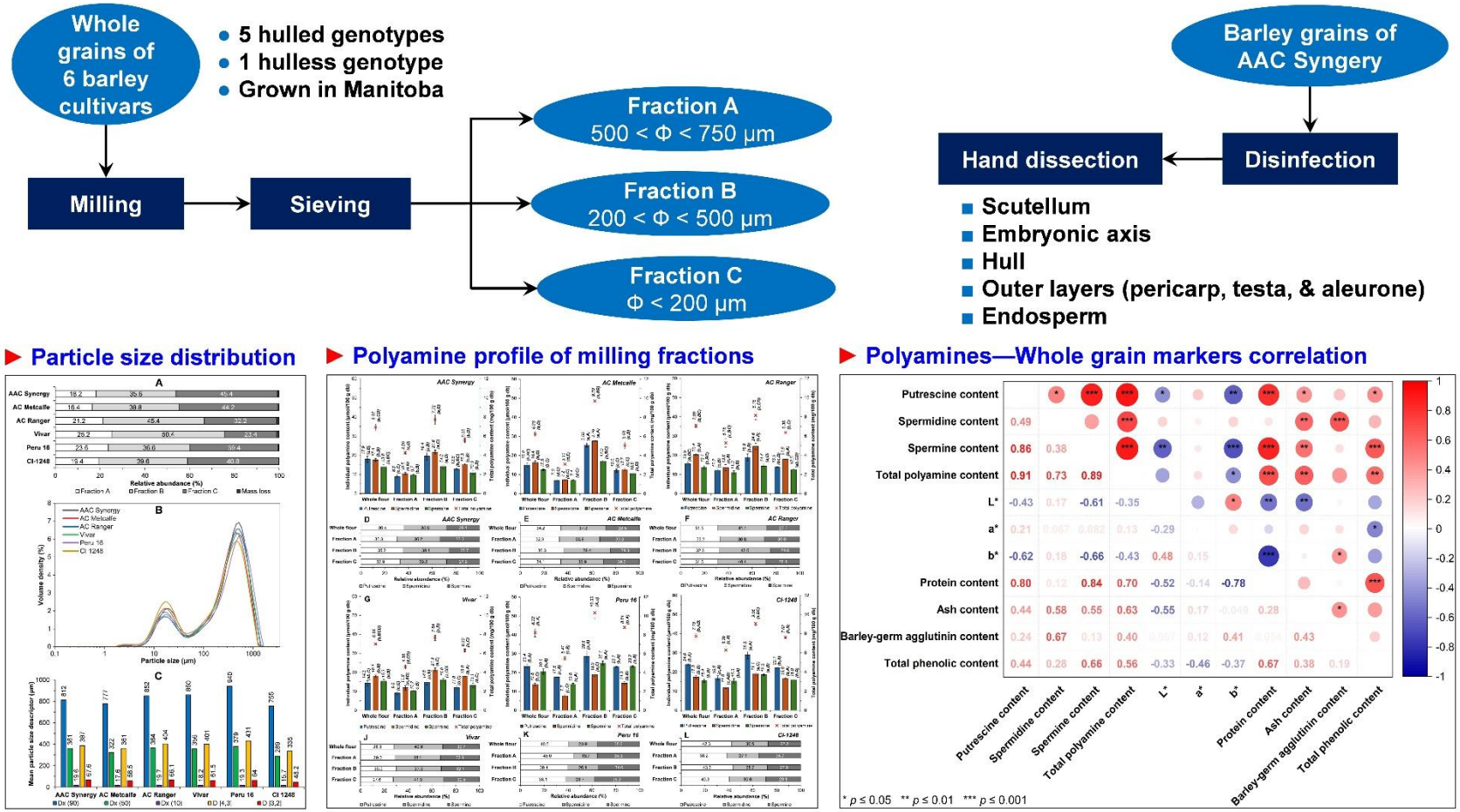

Figure 5.1. Graphical abstract of experimental section 3

5.2. Introduction

The provision and delivery of recommendations for healthy diets have experienced certain shortcomings, resulting in a diminished quality of life among vulnerable populations and an increased burden on health care systems (Ensaff, 2021). In this context, the concept of "nudging" in food choices has emerged as a collective effort to guide consumers toward healthier and more sustainable eating habits. Whole cereal grains have been promoted as part of healthy eating patterns due to the widespread popularity of cereal-based diets in many cultures and their provision of health-promoting components concentrated in the germ and outer layers of the grain (Allai, Azad, Gul, et al., 2022). Cereal crops primarily include grains from the *Gramineae* family, such as corn, rice, wheat, oat and barley. Among these, barley has attracted renewed research attention because of its health benefits, largely attributed to its high content of fibres (e.g., β-glucans and arabinoxylans), micronutrients (e.g., B-complex vitamins, tocols and tocopherols) and phytochemicals (e.g., phenolic compounds) (Lukinac & Jukić, 2022). The interest in barley and its varieties also highlights the ongoing need to develop climate-resilient crops for diverse agri-food applications (Nguyen & Beta, 2023).

Considerable scientific focus has been devoted to β-glucans in barley grains and their milling fractions (Izydorczyk, Nam, Sharma, et al., 2021; Liu, Barrows, & Obert, 2009; Šimić, Horvat, Lalić, et al., 2019). As part of a broader effort to integrate barley and barley-based products into the food system, research attention has also focused on the phytochemical components of barley kernels. Barley has been reported to contain various phenolic acids and polyamines, which are predominantly concentrated in the germ and bran tissues (Drawbridge, Herrera-Balandrano, & Beta, 2025; Nguyen, Bakker, & Beta, 2025). Polyamines are small aliphatic molecules containing two or more amino groups, such as putrescine ($C_4H_{12}N_2$),

spermidine ($C_7H_{19}N_3$) and spermine ($C_{10}H_{26}N_4$). These compounds play essential roles in numerous physiological processes across biological systems. In plants, polyamines have been implicated in regulating development and mediating responses to different biotic and abiotic stresses (Chen, Shao, Yin, et al., 2019). From a human health perspective, polyamines have been recognized as a novel class of nutraceuticals due to their association with the improvement in age-related conditions and the prolongation of life expectancy (Mohajeri, Mokhtari, Khandan, et al., 2025; Spremo, Radišić, Kebert, et al., 2025). Regarding barley grains, Nguyen and Beta (2023) characterized the polyamine profiles of several Canadian varieties and established a relationship between polyamine levels and grain protein content. The authors also fractionated hulled and hulless barley varieties using a dehulling and pearling approach, revealing a polyamine reservoir in the germ and outer kernel layers (Nguyen, Bakker, & Beta, 2025).

Although its applications have been limited, milling barley grains has increasingly been investigated for new novel processing purposes, particularly for the enrichment of valuable compounds (Izydorczyk, Nam, Sharma, et al., 2021; Liu, Barrows, & Obert, 2009; Šimić, Horvat, Lalić, et al., 2019; Wang, Xue, Newman, et al., 1993). Barley grains can be milled in several ways, including milling hull-free kernels of naked cultivars, milling dehulled kernels of covered cultivars, and milling intact kernels of covered cultivars without prior dehulling. The availability of existing wheat-milling equipment and the feasibility of adapting it for barley justifies this approach. However, there has been no report evaluating the potential of milling barley grains to obtain polyamine-enriched products. In this study, a fractionation strategy combining milling and sieving was employed to assess the potential to obtain polyamine-enriched fractions from hulled and hulless barley cultivars. The resulting fractions were analyzed for polyamine content and physicochemical properties associated with markers of whole-grain status (i.e., chemical components of the cereal grain indicating the presence of tissues other than the starchy

endosperm). Interpretation of polyamine profiles was supported by a complementary hand-dissection study.

5.3. Materials and Methods

5.3.1. Materials

a) Barley cultivars

Six barley cultivars were used in this study and supplied by Agriculture and Agri-Food Canada (AAFC). These included two malting cultivars (AAC Synergy and AC Metcalfe, both harvested in 2021), two feed cultivars (AC Ranger and Vivar, both harvested in 2016) and two food cultivars (CI-1248 and Peru 16, harvested in 2008 and 2012, respectively). All cultivars were grown in Brandon, Manitoba, Canada. CI-1248 is hulless, whereas the other five cultivars are hulled. CI-1248 and Peru 16 are pigmented varieties with purple and black grains, respectively. In the study, the term "whole barley grains" refers to hulled cultivars with hulls adhering to the caryopsis and hulless cultivars without hulls.

a) Chemicals

Polyamine external standards (≥ 99% purity) were obtained from Millipore Sigma Corp. (Burlington, MA, USA), including putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrachloride. Dansyl chloride (≥ 99% purity), *trans*-ferulic acid (99% purity) and Folin-Ciocalteu reagent were also purchased from the same supplier. The internal standard, 1,7-diaminoheptane (98% purity), was acquired from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Reagents used for the enzyme-linked immunosorbent assay (ELISA) were obtained from Millipore Sigma Corp. unless otherwise specified, including lectin from *Triticum vulgaris* (wheat germ agglutinin, WGA), Anti-WGA antibody produced in rabbit, *p*-nitrophenyl phosphate,

ovalbumin, bovine serum albumin (BSA) and phosphate-buffered saline (PBS). Goat alkaline phosphatase-conjugated and affinity-purified anti-rabbit immunoglobulin targeting heavy and light chains (IgG H+L) was sourced from Rockland Immunochemicals Inc. (Limerick, PA, USA). Water used in the HPLC system and other experimental procedures was purified using a Milli-Q Direct water purification system (Millipore Sigma Corp.). HPLC-grade acetonitrile, acetone and ethyl acetate were obtained from Fisher Scientific Inc. (Pittsburgh, PA, USA). All other chemicals, of at least reagent grade, were procured from either Millipore Sigma Corp. or Fisher Scientific Inc.

5.3.2. Methods

a) Preparation of barley milling fractions

Approximately 150 g of barley grains were milled into whole flour using a laboratory centrifugal mill (Model ZM200, Retsch Co., Haan, Germany) fitted with a 0.75-mm ring sieve and operated at 10,000 rpm, which produced no noticeable sieve retention aside from minor dust. The resulting whole barley flour was then sifted using a Rotex^{TM} sieve shaker (Tripette & Renaud Co., Villeneuve la Garenne, France) with a 50 g subsample and equipped with 200 µm and 500 µm sieves, operated at a rotational speed of 180 rpm for 10 min. The resulting fractions were designated as fraction A (retained on the 500 µm sieve), fraction B (retained on the 200 µm sieve) and fraction C (sieve bottom). Preliminary macroscopic examination discerned that fraction A consisted mainly of large fragments of barley hulls (in hulled varieties) and endosperm (in all varieties), while fraction C contained fine, floury particles. The relative abundance of each sieved fraction was defined as the proportion of its mass (wet basis, wb) relative to the total mass (wb) of the original whole flour. All milling fractions were stored at 4 °C until further analysis.

b) Hand dissection of barley grains

Grains of the hulled barley cultivar AAC Synergy were manually fractionated using the method described by Ndolo and Beta (2013), with modifications. The hand-dissected fractions obtained were the embryonic axis, scutellum, starchy endosperm and outer layers comprising aleurone, testa and pericarp. Approximately 25 whole kernels were dissected per session, for a total of 250 grains. Initially, barley grains were placed on a 0.5 mm sieve and washed under running reverse osmosis water to remove dirt and reduce microbial load. Disinfection was achieved by soaking intact grains in 15 mL of 80% (v/v) ethanol for 10 min, followed by soaking in 15 mL of sodium hypochlorite solution (0.1% available chlorine) for 20 min. The grains were rinsed twice with 20 mL of Milli-Q water after each soaking step. Disinfected grains were placed on a sterile surface and cut longitudinally into halves using a scalpel. The hull, embryonic axis and scutellum were sequentially removed from the half-grains using tweezers and a scalpel with the aid of a jewelry magnifying glass. Dissecting tools were disinfected by wiping with 80% (v/v) ethanol before and during each dissection. The hull, embryonic axis and scutellum fractions were collected separately into aluminum containers, covered with perforated aluminum foil and dried overnight in a laboratory desiccator. The de-embryonated half-grains were placed in 10-cm sterile Petri dishes containing two layers of Whatman filter paper, pre-disinfected by soaking in 80% (v/v) ethanol and dried in a clean chamber. Each Petri dish contained approximately 25 half-grains, moistened by adding 7 mL of Milli-Q water. The dishes were covered with aluminum foil and kept under ambient conditions. After 48 h of moisturization, the starchy endosperm was removed from the half-grains by scraping with a scalpel and tweezers. The remaining outer layers consisted of aleurone, testa and pericarp. The pericarp was not collected separately due to its scarcity. Regarding the moisturization step, preliminary trials revealed that a 24 h imbibition

period was insufficient to achieve a soft texture in the starchy endosperm of the de-embryonated half-barley grains, which is conducive to manipulation. However, a 24 h duration could minimize the polyamine metabolisms potentially induced by moisture supply, as observed in isolated and moisturized barley aleurone layers in the presence of gibberellic acid (Lin, 1984). Meanwhile, when the imbibition time was prolonged to 72 h, the starchy endosperm became paste-like and facile to remove. However, the findings of Lin (1984) reported significant changes in polyamine levels when barley aleurone layers were soaked for 72 h. The 48-h soaking time was therefore chosen to balance the softness of aleurone texture and the extent of polyamine metabolism. The endosperm and outer layer fractions were dried overnight in a desiccator. All collected fractions were milled (Model ZM200, Retsch Co. run at 10,000 rpm, incorporated with a 0.5-mm sieve as required for operation). No retention of large particles (>0.5 mm) was observed after milling, and the products were stored at -18 °C.

c) Analysis of particle size distribution

The particle size distribution of whole barley flours was analyzed using a laser diffraction instrument (Mastersizer MS3000, Malvern Panalytical Co., Worcestershire, England). Dry dispersion was applied during each run, and approximately 3 g of sample was fed into the instrument. Results for each sample represent the average of two measurements. The volume-based particle size distribution as well as particle size descriptors, were recorded, including Dx (90), the particle diameter below which 90% of the sample's volume lies; Dx (50), the median particle diameter; D [4,3], the volume-weighted mean, which is sensitive to coarse particles; and D [3,2], the surface area-weighted mean, which is sensitive to fine particles.

d) Measurement of colour values

The tristimulus colour values, including lightness (L*), redness (a*) and yellowness (b*) of whole barley flour and its milling fractions were measured using a handheld colourimeter (Model CR400, Konica Minolta Co., Osaka, Japan). The colourimeter was calibrated with a black plate prior to measurement, and three readings were taken for each sample. The total colour difference (ΔE) of each milling fraction relative to the corresponding whole flour was calculated using the following equation:

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2}$$

where, L^*, a^* and $b^*$are the colour values of the sieved fraction; L_0^*, a_0^* and b_0^* are the colour values of the corresponding whole flour sample.

e) Determination of proximate composition

The proximate composition of whole barley flours and milling fractions, including moisture, protein and ash content, was characterized. Moisture content was measured using an adaptation of AACC International Method 44-15.02, in which approximately 0.5 g of sample in a lidded stainless-steel tin was oven-dried (Chopin EM10 NG, KPM Analytics Co., Villeneuve la Garenne, France) at 130 °C for 2 h until a constant weight was achieved. Due to the limited availability of hand-dissected fractions, the calculation of polyamine content for these samples was based on the moisture content of the whole flour of AAC Synergy. Protein content was determined using the Kjeldahl method (AOAC Official Method 992.23). Briefly, mineralization was carried out by digesting approximately 50 mg of sample in concentrated H_2SO_4 with a K_2SO_4–$CuSO_4$ catalyst (FisherTab™, Fisher Scientific Inc.) at 380 °C for 2 h using a DK digestion unit (VELP Scientifica Co., Usmate Velate, Italy). Ammonia released from the digested sample was distilled by adding 40% NaOH and infusing steam, captured in boric acid and

subsequently titrated with standardized 0.1 M H_2SO_4. Distillation and titration were performed automatically using the Kjeltec 8400 distillation–titration system (FOSS Co., Hilleroed, Denmark). Nitrogen values were converted to protein using a factor of 6.25. Total ash content was determined by incineration at 550 °C for 18 h in a muffle furnace (Lindberg/Blue M Moldatherm, Thermo Fisher Scientific Inc.).

f) Quantification of total phenolic content

The total phenolic content of whole flours and milling fractions were quantified using the protocol outlined by Apea-Bah, Drawbridge, and Beta (2022). Phenolics were extracted by adding 1 mL of acidified methanol (methanol:water:hydrochloric acid, 80:20:0.5) to 100 mg of sample, followed by vortexing and sonication for 60 min (B5510-MTH, Branson Ultrasonics Corp., Danburry, CT, USA). The mixture was centrifuged at 20,000 × g and 4 ºC for 5 min (Sorvall Legend Micro 21 R, Thermo Scientific Inc) and the supernatant was collected. For the assay, 18.2 µL of each extract was pipetted into individual wells of a 96-well microplate, followed by the addition of 36.4 µL of 10% (v/v) aqueous Folin–Ciocalteu reagent and 145.4 µL of 700 mM sodium carbonate. The mixtures were incubated in the dark at room temperature for 2 hours. Absorbance was subsequently measured at 750 nm using a microplate reader (Varioskan™ LUX Multimode, Thermo Scientific Inc). Calibration curves were constructed by plotting absorbance against the concentration of ferulic acid standard (25–200 ppm, R^2 = 0.9944) and the total phenolic content of each sample was determined by extrapolation. Results were expressed as mg of ferulic acid equivalents per 100 g dry basis (db).

g) Quantification of barley germ agglutinin content

The total barley-germ agglutinin (BGA) content was quantified using the ELISA method described by Barron, Samson, Lullien-Pellerin, et al. (2011). Germ agglutinins of plants in the

Gramineae family have been reported to exhibit cross-antigenicity, including those of wheat and barley (Killilea, McQueen, & Abegania, 2020). To extract BGA, approximately 500 mg of whole barley flour or milling fraction was mixed with 5 mL of 0.05 M HCl, vortexed and agitated for 30 min at room temperature. The suspension was centrifuged at 1690 × g for 10 min at room temperature (Avanti J-26S XP, Beckman Coulter Inc., Brea, CA, USA) and the supernatant was collected for ELISA analysis. Microplate wells (NUNC MaxiSorp^{TM}, Millipore Sigma Corp.) were coated overnight at 4 °C with 100 µL of ovalbumin solution (20 µg/mL in PBS, pH 7.2). After washing with PBS, free binding sites were blocked with 0.5% BSA–PBS for at least 30 min at 37 °C. Aliquots (100 µL) of diluted extracts and WGA standards (prepared by serial dilution in 0.1% BSA–PBS, ranging from 0.125 to 10 µg/mL) were added to the wells and incubated for 2 h at 37 °C. Following three washes (Wellwash Versa, Thermo Scientific Inc.), 100 µL of rabbit anti-WGA serum (1:6000 in 0.1% BSA–PBS) was added and incubated for 1 h at 37 °C. Subsequently, after washing, 100 µL of alkaline phosphatase-conjugated goat anti-rabbit IgG H+L serum (1:1000 in 0.1% BSA–PBS) was added and incubated for another hour at 37 °C. After washing, 100 µL of *p*-nitrophenyl phosphate substrate was added and incubated for 45 min at 37 °C. The reaction was stopped by adding 50 µL of 1 N NaOH and absorbance was measured at 450 nm using a microplate reader (Tecan Ltd., Männedorf, Switzerland). Absorbance data from WGA standards of known concentrations were fitted using a four-parameter logistic regression to generate the calibration curve. BGA levels in the extracts were determined by applying the inverse of the fitted response function (Sigma Plot V13.0, Systat Software Inc., Point Richmond, CA, USA) and expressed as mg per 100 g db.

h) Determination of polyamine profile

Free polyamines from barley samples (approximately 100 mg) were extracted using 1 mL of 1 M HCl. For the embryonic axis and scutellum fractions obtained by hand dissection, the sample size was reduced to about 30 mg due to their limited availability and the anticipated high concentration of polyamines (Nguyen, Bakker, & Beta, 2025). The extracts were then subjected to alkalinization and dansyl derivatization following the procedure reported by Nguyen and Beta (2023). Polyamine quantification was performed using the chromatographic method described by the same authors. Individual polyamines were identified by matching their retention times with those of the corresponding commercial standards. Calibration curves were established by plotting the molar concentration of each target polyamine (putrescine, spermidine, or spermine) against the peak area ratio of the analyte to the internal standard (1,7-diaminoheptane). All calibration curves within the working concentration range (≤ 50 μM) demonstrated high linearity ($R^2 \geq$ 0.9996) (Nguyen & Beta, 2023). The content of individual polyamine was expressed as μmol per 100 g db. Total polyamine content, expressed as mg per 100 g db, was obtained by summing the gravimetric content of individual polyamines. The relative abundance of individual polyamines was calculated as their molar proportion within the total polyamine pool.

i) Statistical treatment

All data were collected in triplicate (n = 3) and expressed as mean ± standard deviation, except for BGA content determination, which was performed in duplicate (n = 2). Statistical analyses were conducted using Origin V10.0 software (OriginLab Co., Northampton, MA, USA), including paired sample t-tests and one-way analysis of variance (ANOVA) with Tukey comparison at $\alpha = 0.05$. Pearson correlation analysis was also performed using the same software.

5.4. Results and Discussion

5.4.1. Relative yield and particle size distribution

Figure 5.2 provides information on the relative yield and particle size distribution of barley milling fractions. Variations were observed in the relative yield of milling products across different barley genotypes (Figure 5.2A), indicating the potential genetic effects on kernel hardness. The intermediate fraction B (200–500 μm) was predominant in the two feed cultivars (AC Ranger and Vivar), whereas the fine fraction C (<200 μm) was the major milling product of the two malting genotypes (AAC Synergy and AC Metcalfe), suggesting a higher friability of the kernel structure. Fractions B and C could be considered as co-dominant milling products in Peru 16 and CI 1248, given their approximately equal contribution to the whole flour. The coarse fraction A (>500 μm) was the least abundant across all barley cultivars, except for Vivar. In general, the kernel hardness and milling technique are the primary factors controlling the relative yield of milling fractions. In this study, centrifugal milling was used, in which particle size reduction occurs through combined impact and shear forces generated by high rotational speeds (Eze, Ekaette, & Ngadi, 2024). Centrifugal milling generally yields a broader particle size distribution due to variations in energy transfer and fracture mechanisms during processing, whereas other techniques, such as ball milling, can produce finer particles (Ali, Richards, & Alzard, 2025). On the other hand, the hardness of barley kernels is mainly influenced by endosperm texture, which is believed to be partially determined by intrinsic properties, including the continuity of the starch–protein network and cell wall thickness (Nair, Knoblauch, Ullrich, et al., 2011). In wheat and barley grains, friabilin proteins are thought to contribute to kernel softness by reducing the adhesion of starch granules to the protein matrix (Heinze, Kiszonas, Murray, et al., 2016; Nair, Knoblauch, Ullrich, et al., 2011). Environmental factors may also

affect kernel hardness (Nair, Knoblauch, Ullrich, et al., 2011), although their effects were not demonstrated in the present work.

Considering the particle size distribution curves of whole barley flours, a bimodal characteristic was observed for all cultivars, with the first peaks located at around 15–20 μm and the second ones recorded near 400–550 μm (Figure 5.2 B). Although volume-based particle size distribution may not correspond directly to gravimetric sieve fractions, particles contributing to the first peak generally fell within the <200 μm range (fraction C), while a substantial portion of those forming the second peak lay within the 200–500 μm range (fraction B). This alignment helps explain the greater relative abundance of fractions B and C among the milling products. A bimodal particle size distribution is commonly observed in flours derived from grains with a soft texture, whereas milling grains of increased hardness, such as durum wheat, typically results in a unimodal distribution pattern (Heinze, Kiszonas, Murray, et al., 2016). Nevertheless, the findings of Zhang, Hu, Xu, et al. (2025) demonstrated that the technological factors influence the particle size distribution of flours. The authors reported a bimodal distribution when roller and hammer mills were used for hard wheat, with the initial peak attributed to free starch granules and the latter to starch–protein aggregates. In our study, co-fragmentation of barley hull and adjacent tissues may have increased the histological diversity of milling products. Along with variation in endosperm texture, this phenomenon could have contributed to the minor shifts in peak intensity across the distribution curves of different varieties, despite the overall similarity in shape. Notably, the hulless genotype CI-1248 displayed a particle distribution curve with the highest intensity of the first peak and the lowest of the second compared to other varieties. The absence of the outer hull layer likely reduced kernel resistance to mechanical forces, resulting in finer particles (Olkku, Kotaviita, Salmenkallio-Marttila, et al., 2005). Additionally, the moisture

content of CI-1248 whole flour was the lowest among all cultivars (Table 5.1), which may have contributed to its increased friability.

The observed variations were consistent with the mean particle size descriptors (Figure 5.2C), where the CI-1248 variety exhibited reduced values for all particle size parameters. In contrast, the hulled genotype Peru 16 showed elevated Dx (90) and Dx (50) values compared to other samples, indicating a greater abundance of coarse particles in its whole flour. The Dx (90) values of whole flour samples exceeded 750 μm despite the use of a 750-μm ring sieve in the centrifugal mill. This may be explained by rod-shaped particles with lengths exceeding, but widths smaller than, the cutoff size passing through the sieve under strong centrifugal force. Additionally, volume-based measurement of particle size distribution may be prone to deviation when non-spherical and larger particles are present in the sample (Islam, Hawkins, Meyer, et al., 2022). All the whole barley flours had their D [4,3] (335–431 μm) much larger than D [3,2] (48.2–67.6 μm) but closer to Dx (50) value (289–379 μm), implying the predominance of particles with increased size within the distribution. From a product formulation perspective, the particle size range of milled grain products plays a critical role in determining their functional properties and, consequently, the quality of the final food products (Zhang, Hu, Xu, et al., 2025). From a compound valorization standpoint, understanding particle size distribution can facilitate other assessments to identify fractions enriched in target constituents (Liu, Barrows, & Obert, 2009).

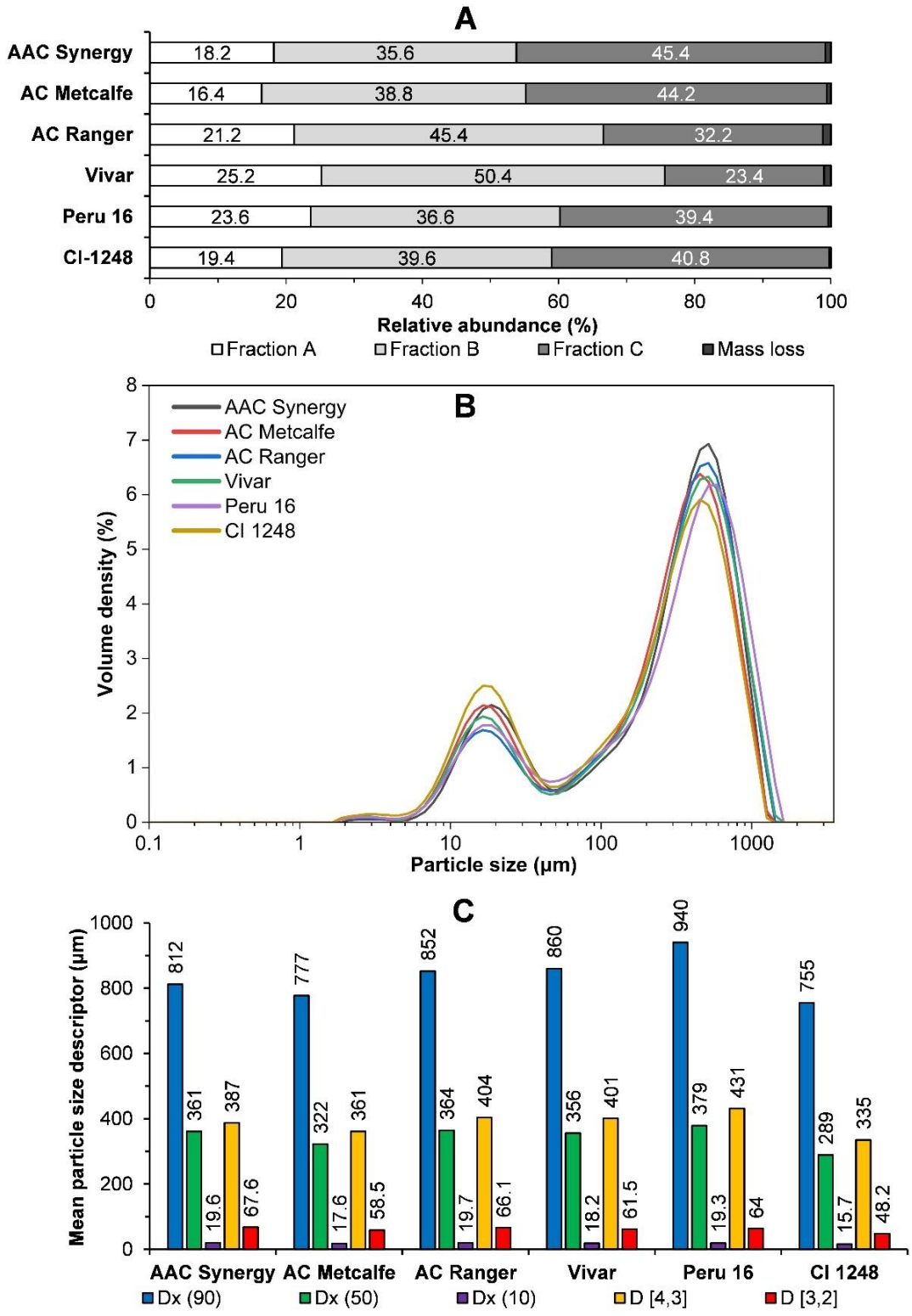

Figure 5.2. Relative yield (A), particle size distribution (B) and particle size descriptors (C) of milling fractions and whole barley flours

5.4.2. Physicochemical properties: Colour values and proximate composition

Table 5.1 presents the physicochemical properties of whole barley flours and milling fractions in terms of colour values and proximate composition. Comparing these properties provides insights into the histological composition of the samples. Within each barley cultivar, the finer fraction C exhibited higher lightness (L^*) and lower redness (a^*) and yellowness (b^*) than the whole flour and coarser fractions, suggesting a greater proportion of starchy endosperm. In contrast, the coarser fractions A and B showed lower lightness but higher redness and yellowness, likely due to the presence of outer grain layers such as bran and husk, which contain pigmented components. Four of the six cultivars displayed total colour differences (ΔE) greater than 3 for all milling fractions, indicating that colour variation relative to the corresponding whole flour would be perceptible to the naked eye. Regarding moisture content, fractions A and C tended to have lower water levels than the whole flour and fraction B. The fibrous structure of the hull in fraction A, with its limited specific surface area, may not favour water retention, while the finer particle size and greater surface area of fraction C could facilitate mass transfer and result in greater moisture loss during handling.

Protein and ash contents can be linked to the whole-grain status of milling products due to their greater abundance in non-endosperm tissues of the cereal grain, where the germ typically contains elevated protein levels and the bran is rich in both protein and minerals (Allai, Azad, Gul, et al., 2022). Protein content ranged from 12.1–17.8% for whole flour, 10.2–15.8% for fraction A, 13.2–18.3% for fraction B and 12.9–18.5% for fraction C. For the hulled cultivars, an increase in protein content was observed in the finer fractions B and C, while the coarse fraction A was relatively depleted of this component. Conversely, the coarser fractions A and B of the hulless genotype CI-1248 exhibited higher protein content, leaving the fine fraction C depleted. Within each cultivar, the intermediate fraction B showed the highest protein level compared to

the corresponding whole flour and other milling products, indicating an enhanced presence of non-endosperm and non-hull tissues. On average, fraction B, with a relative yield of 41.1%, was 8.5% more enriched in protein than whole flour. In three hulless cultivars, an increment in protein content of fraction C relative to whole barley flour was also recorded, with an average difference of 3.4%. This may result from removing the protein-poor hull fraction from the endosperm-rich milling product, coupled with the possible presence of finely shattered barley bran in this fraction. From a practical perspective, protein-enriched barley fractions can be used for enhancing the nutritional value of cereal-based products (Boukid, 2026). Regarding the fracture mechanism, Izydorczyk, Nam, Sharma, et al. (2021) reported that the outer layers of barley grains (i.e., aleurone and surrounding tissues) can have a brittle texture and be susceptible to pulverization during dry fractionation, leading to the formation of fine particles that are difficult to separate from starch granules in the sieve-bottom fraction. Turning to ash content, values ranged from 2.15 to 3.19% for whole flour, 1.88 to 2.77% for fraction A, 2.68 to 3.74% for fraction B and 2.00 to 2.58% for fraction C. The enrichment of ash in fraction B suggests that a considerable portion of mineral-rich aleurone was likely recovered in this milling product, whereas the fine fraction C was mainly composed of starchy endosperm and displayed the lowest ash level. These observations align with the findings of Šimić, Horvat, Lalić, et al. (2019), who reported a decline in ash content as the refinement level of barley milling products progressed from bran to short flour and then fine flour.

Table 5.1. Colour values and proximate composition of barley flours and their milling fractions

Cultivar		AAC Synergy	AC Metcalfe	AC Ranger	Vivar	Peru 16	CI 1248
*L**	Whole flour	85.4 ± 0.3 (b,A)	86.1 ± 0.3 (b,A)	81.1 ± 0.6 (b,B)	80.0 ± 0.6 (b,B)	68.3 ± 0.3 (b,C)	81.1 ± 0.4 (b,B)
	Fraction A	81.8 ± 0.6 (c,B)	84.2 ± 0.4 (c,A)	75.4 ± 0.7 (d,D)	77.8 ± 0.3 (c,C)	63.6 ± 0.6 (c,E)	81.9 ± 0.6 (ab,B)
	Fraction B	80.9 ± 0.1 (d,B)	84.0 ± 0.1 (c,A)	78.8 ± 0.2 (c,D)	79.9 ± 0.4 (b,C)	63.5 ± 0.2 (c,E)	78.9 ± 0.3 (c,D)
	Fraction C	88.9 ± 0.1 (a,A)	88.5 ± 0.2 (a,A)	84.4 ± 0.3 (a,B)	82.3 ± 0.4 (a,D)	72.1 ± 0.3 (a,E)	83.0 ± 0.3 (a,C)
*a**	Whole flour	1.53 ± 0.07 (b,C)	1.51 ± 0.06 (b,C)	1.76 ± 0.10 (b,B)	1.87 ± 0.09 (b,B)	1.85 ± 0.03 (a,B)	2.33 ± 0.07 (a,A)
	Fraction A	2.08 ± 0.19 (a,B)	1.86 ± 0.15 (a,B)	2.60 ± 0.21 (a,A)	2.09 ± 0.07 (a,B)	1.78 ± 0.02 (b,B)	1.75 ± 0.06 (c,B)
	Fraction B	2.22 ± 0.04 (a,A)	1.87 ± 0.01 (a,C)	2.04 ± 0.02 (b,B)	1.82 ± 0.02 (b,C)	1.48 ± 0.02 (c,D)	2.27 ± 0.03 (ab,A)
	Fraction C	0.82 ± 0.02 (c,F)	1.02 ± 0.01 (c,E)	1.31 ± 0.07 (c,D)	1.59 ± 0.02 (c,C)	1.86 ± 0.03 (a,B)	2.17 ± 0.06 (b,A)
*b**	Whole flour	12.2 ± 0.2 (b,A)	12.0 ± 0.3 (b,A)	11.8 ± 0.2 (c,A)	11.9 ± 0.3 (b,A)	5.2 ± 0.2 (a,C)	6.4 ± 0.04 (a,B)
	Fraction A	15.8 ± 0.3 (a,A)	14.0 ± 0.3 (a,C)	15.0 ± 0.4 (a,B)	13.6 ± 0.2 (a,C)	5.1 ± 0.1 (a,D)	5.6 ± 0.04 (c,D)
	Fraction B	15.4 ± 0.1 (a,A)	13.5 ± 0.1 (a,B)	13.3 ± 0.3 (b,B)	11.9 ± 0.2 (b,C)	5.1 ± 0.2 (a,E)	6.4 ± 0.01 (ab,D)
	Fraction C	9.7 ± 0.3 (c,C)	10.2 ± 0.2 (c,BC)	10.3 ± 0.2 (d,B)	11.0 ± 0.2 (c,A)	5.2 ± 0.1 (a,E)	6.0 ± 0.3 (b,D)
Δ*E*	Fraction A	5.11	2.85	6.57	2.84	4.74	1.32
	Fraction B	5.58	2.63	2.75	0.19	4.82	2.17
	Fraction C	4.35	3.00	3.66	2.38	3.77	2.02
Moisture content (g/100 g, wb)	Whole flour	13.0 ± 0.4 (a,A)	11.7 ± 0.4 (a,BC)	11.8 ± 0.2 (a,BC)	12.1 ± 0.2 (a,B)	11.3 ± 0.1 (a,CD)	10.9 ± 0.1 (a,D)
	Fraction A	9.1 ± 0.3 (c,D)	11.9 ± 0.4 (a,A)	11.2 ± 0.2 (ab,B)	11.9 ± 0.4 (a,A)	8.1 ± 0.1 (c,E)	10.4 ± 0.1 (bc,C)
	Fraction B	10.1 ± 0.3 (b,C)	12.2 ± 0.3 (a,A)	11.4 ± 0.3 (ab,B)	12.4 ± 0.2 (a,A)	8.8 ± 0.3 (b,D)	10.6 ± 0.2 (ab,C)
	Fraction C	9.2 ± 0.1 (c,C)	9.4 ± 0.5 (b,C)	10.9 ± 0.3 (b,B)	12.2 ± 0.3 (a,A)	7.8 ± 0.1 (c,D)	10.2 ± 0.1 (c,B)
Protein content (g/100 g, db)	Whole flour	13.4 ± 0.1 (b,C)	13.7 ± 0.4 (c,C)	12.5 ± 0.1 (ab,D)	12.1 ± 0.2 (b,D)	17.8 ± 0.2 (b,A)	14.6 ± 0.2 (bc,B)
	Fraction A	11.1 ± 0.4 (c,C)	12.2 ± 0.2 (d,B)	10.2 ± 0.4 (b,D)	11.0 ± 0.3 (c,C)	15.8 ± 0.2 (c,A)	15.1 ± 0.3 (b,A)
	Fraction B	14.7 ± 0.5 (a,C)	15.2 ± 0.1 (a,C)	13.3 ± 0.1 (ab,D)	13.2 ± 0.1 (a,D)	18.3 ± 0.2 (a,A)	16.4 ± 0.2 (a,B)
	Fraction C	13.1 ± 0.6 (b,D)	14.5 ± 0.1 (b,B)	13.4 ± 0.1 (a,CD)	12.9 ± 0.2 (a,D)	18.5 ± 0.2 (a,A)	14.1 ± 0.1 (c,BC)
Ash content (g/100 g, db)	Whole flour	2.15 ± 0.06 (b,E)	2.27 ± 0.03 (b,DE)	3.19 ± 0.07 (b,A)	2.69 ± 0.03 (b,C)	3.01 ± 0.04 (b,B)	2.29 ± 0.03 (b,D)
	Fraction A	1.88 ± 0.04 (c,D)	1.96 ± 0.04 (d,CD)	2.77 ± 0.04 (c,A)	2.53 ± 0.05 (c,B)	2.64 ± 0.03 (c,B)	2.04 ± 0.04 (c,C)
	Fraction B	2.68 ± 0.06 (a,D)	2.90 ± 0.04 (a,C)	3.74 ± 0.08 (a,A)	3.46 ± 0.03 (a,B)	3.71 ± 0.07 (a,A)	2.77 ± 0.05 (a,CD)
	Fraction C	2.02 ± 0.04 (b,D)	2.05 ± 0.03 (c,C)	2.58 ± 0.05 (d,A)	2.35 ± 0.03 (d,B)	2.58 ± 0.02 (c,A)	2.00 ± 0.04 (c,C)

Data are presented as mean ± standard deviation (n = 3). Values within a cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values that do not share an uppercase letter are significantly different from their counterpart of another cultivar (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: wb: wet basis; db: dry basis.

5.4.3. Physicochemical properties: Total phenolic and germ agglutinin content

Table 5.2 shows the distribution of phenolic compounds and BGA in the whole flour and milling fractions of the barley cultivars. Cereal germ agglutinins have been used as markers for the whole-kernel status in grain products due to their exclusive localization within the embryonic axis of the kernel (Barron, Samson, Lullien-Pellerin, et al., 2011). Killilea, McQueen, and Abegania (2020) reported that nearly 95% of wheat germ agglutinin (WGA) is localized in the germ tissue of the wheat kernel. The authors also successfully estimated the level of whole wheat in pasta formulations based on WGA content. With the exception of Vivar and Peru 16, BGA content in this study was most concentrated in the intermediate fraction B of both hulled and hulless cultivars, indicating that most of the barley germ tissue resided in this fraction. Notably, the BGA content in the intermediate fraction of AAC Synergy and AC Metcalfe was the highest among all genotypes, being 2.2 and 2.6 times greater, respectively, than in the corresponding whole flour. Occasional and elevated standard deviations in BGA content could be ascribed to variations in antibody affinity, antigen extractability, and histological composition in the sub-samples. Combining the data on BGA content with the relative abundance of milling fractions (Figure 5.2) and hand-dissected fractions (Table 5.3), fraction B of AAC Synergy was estimated to contain 6.4% germ, representing 79% of the germ present in the entire kernel. In barley processing, portions of germ tissue are typically recovered by sifting the dehulling by-products (Liu, Barrows, & Obert, 2009; Nguyen, Bakker, & Beta, 2025). Comminution milling followed by sieving has also been applied to hulless barley, enabling the removal of up to 99% of viable germ for the generation of an oil-rich barley germ fraction (Moreau & Hicks, 2013).

Phenolic compounds are known to localize in the outer layers of the cereal kernel, including aleurone, testa and pericarp (Nguyen, Drawbridge, & Beta, 2024). Drawbridge,

Herrera-Balandrano, and Beta (2025) examined the pearling fractions of hulless barley cultivars and reported a high concentration of phenolic acids in the outer layer fractions (up to 25% kernel weight). In addition, certain phenolic compounds have been proposed as markers for specific anatomical fractions of the barley kernel, such as alkylresorcinols for the testa and *p*-coumaric acid for the hull layer (Barron, Holopainen-Mantila, Sahlstrom, et al., 2017). In the present work, the coarse fraction A exhibited the lowest total phenolic content among barley milling products. Conversely, phenolic compounds were expected to be more pronounced in fraction B, which was observed in five cultivars, given the higher abundance of the outer layers. However, fraction C demonstrated an increased total phenolic content, which could be attributed to the presence of finely ground outer layers. Further reduction in particle size could expose the matrix and improve the extractability of phenolic compounds. There could have been possible interference from other substances during the quantification of phenolic compounds. Although the Folin–Ciocalteu assay is useful for a preliminary evaluation of total phenolics, it may overestimate values due to its lack of specificity toward reducing substances such as thiols or reducing sugars (Apea-Bah, Drawbridge, & Beta, 2022). Under acidic and ultrasonic conditions, starch hydrolysis may occur during phenolic extraction, given the higher level of starch damage associated with the finer particle size in fraction C (Zhang, Hu, Xu, et al., 2025). To address the limitation of this work, future studies employing chromatographic techniques would provide more detailed information on phenolic composition. Additionally, a notable proportion of phenolic acids in barley grain occurs in the bound fraction, owing to their conjugation with cell wall components such as lignins and polysaccharides (Drawbridge, Herrera-Balandrano, & Beta, 2025). Treatment with a strong alkaline is usually needed to cleave the covalent linkages (primarily esters), making the bound phenolic compounds more extractable for subsequent quantification (Drawbridge, Apea-Bah, Silveira Hornung, et al., 2021).

Table 5.2. Germ agglutinin and total phenolic content of barley flours and their milling fractions

Cultivar		AAC Synergy	AC Metcalfe	AC Ranger	Vivar	Peru 16	CI 1248
Barley-germ agglutinin content (mg/100 g, db)	Whole flour	28.7 ± 6.9 (b,AB)	27.3 ± 1.1 (bc,AB)	27.3 ± 2.5 (ab,AB)	39.7 ± 11.5 (a,A)	28.8 ± 0.5 (a,AB)	12.9 ± 0.4 (b,B)
	Fraction A	14.8 ± 1.2 (b,BC)	15.6 ± 0.6 (c,BC)	18.4 ± 0.7 (bc,B)	29.6 ± 4.6 (a,A)	22.4 ± 1.4 (a,AB)	9.4 ± 0.1 (b,C)
	Fraction B	63.4 ± 13.5 (a,AB)	71.7 ± 8.2 (a,A)	37.3 ± 0.6 (a,BC)	40.4 ± 0.7 (a,BC)	26.9 ± 0.3 (a,C)	30.6 ± 8.1 (a,C)
	Fraction C	10.4 ± 2.0 (b,B)	34.0 ± 0.5 (b,A)	14.6 ± 4.6 (c,B)	28.6 ± 2.9 (a,A)	10.5 ± 1.3 (b,B)	15.7 ± 1.2 (ab,B)
Total phenolic content (mg FAE/100 g, db)	Whole flour	173 ± 4 (a,AB)	160 ± 9 (a,BC)	147 ± 3 (a,CD)	144 ± 7 (b,D)	187 ± 7 (bc,A)	127 ± 2 (a,E)
	Fraction A	104 ± 4 (c,C)	114 ± 4 (b,C)	114 ± 2 (b,C)	128 ± 2 (c,B)	155 ± 4 (c,A)	112 ± 6 (b,C)
	Fraction B	155 ± 6 (b,CD)	172 ± 3 (a,B)	151 ± 6 (a,D)	168 ± 3 (a,BC)	207 ± 11 (ab,A)	127 ± 2 (a,E)
	Fraction C	174 ± 4 (a,B)	169 ± 7 (a,BC)	146 ± 5 (a,CD)	158 ± 7 (ab,BC)	235 ± 22 (a,A)	129 ± 2 (a,D)

Data are presented as mean ± standard deviation ($n = 2$ and $n = 3$ for barley-germ agglutinin and total phenolic content, respectively). Values within a cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values that do not share an uppercase letter are significantly different from their counterparts of another cultivar (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: wb: wet basis; db: dry basis.

5.4.4. Distribution of polyamines in whole barley flours and milling fractions

Figure 5.3 illustrates the distribution of polyamines in milling fractions of six barley cultivars. Overall, consecutive milling and sieving of barley grains produced fractions with unequal polyamine levels. Except for AAC Synergy, fraction B, composed of intermediate-size particles, exhibited the highest level of total polyamines compared to whole flour and other milling fractions ($p < 0.05$), being 9–56% more concentrated than the corresponding whole flour. The fraction B of the AAC Synergy exhibited a higher spermidine content than its whole flour, despite the insignificant difference in contents of putrescine, spermine, and total polyamines ($p > 0.05$). This particularity could be partially attributed to some intrinsic factors, such as kernel shape and weight. These parameters would dictate the grinding force experienced by each kernel, which is proportional to kernel weight in the case of centrifugal milling (Zhang, Hu, Xu, et al., 2025). AAC Synergy had the highest 1000-kernel weight in this cultivar panel (Nguyen & Beta, 2023), which could have contributed to the variations in fractionation pattern. Moreover, preharvest and postharvest conditions that modify kernel hardness would play a certain role. It can be noted that the moisture content of AAC Synergy was the highest among the cultivars, which could have altered the breakage pattern of kernels (Muhamad & Campbell, 2004), making enrichment less effective due to the shifted recovery of polyamine-rich tissues into finer or coarser fractions. The greatest enrichment in total polyamine content for fraction B was observed in AC Metcalfe (56%), followed by Peru 16 (26%), CI-1248 (17%) and AC Ranger (15%). In contrast, fraction A, consisting of coarse particles, was the most depleted in polyamines across all six cultivars, with 19–50% lower total polyamine content than whole flour. The total polyamine level in fraction C, containing fine particles, was lower than that of whole flour in AC Synergy, AC Metcalfe and Vivar, while no significant difference ($p > 0.05$) was observed for the remaining three cultivars. As noted earlier, the three milling fractions differ in particle size range,

physicochemical properties and histological composition, resulting in partitioned polyamine concentrations. A similar distribution pattern was observed for individual polyamines, with fraction B exhibiting 20–70%, 10–68% and 20–36% higher concentrations of putrescine, spermidine and spermine, respectively, compared to whole flour. Some trends were noted in the relative abundance of polyamines across milling fractions of six genotypes, despite minor inconsistencies. Generally, fraction A showed an increased proportion of spermine, whereas spermidine was reduced. Meanwhile, the relative abundance of spermidine in fraction B was slightly higher in AAC Synergy, AC Metcalfe, AC Ranger and Peru 16.

In the context of valorizing nutraceutical compounds, dry fractionation combining barley grain milling and flour sieving can be employed to obtain fractions enriched in polyamines. The advantages of this process include higher yields of recovered fractions and the ability to operate using existing milling industry equipment. These fractions can be further refined to increase polyamine levels and used as ingredients for nutraceutical extraction and production. Additionally, they can be incorporated into food products to enhance nutritional value, given their higher contents of proteins, minerals and beneficial phytochemicals. Similar workflows combining milling and sieving have been employed by other authors to isolate barley fractions enriched in health-promoting compounds, such as vitamin E (Wang, Xue, Newman, et al., 1993), β-glucan (Liu, Barrows, & Obert, 2009) and phenolic acids (Šimić, Horvat, Lalić, et al., 2019). It is anticipated that the degree of enrichment in the compound of interest depends on the fractionation technique applied to cereal grains (Izydorczyk, Nam, Sharma, et al., 2021). Therefore, further research on the design of milling and separation strategies is desirable to improve phytochemical enrichment in barley fractions. Since strong positive relationships between protein and polyamines have been demonstrated in previous studies (Nguyen & Beta,

2023), protein enrichment strategies for barley flour may also be applied to obtain fractions with higher polyamine contents, as reported elsewhere (Izydorczyk, Nam, Sharma, et al., 2021).

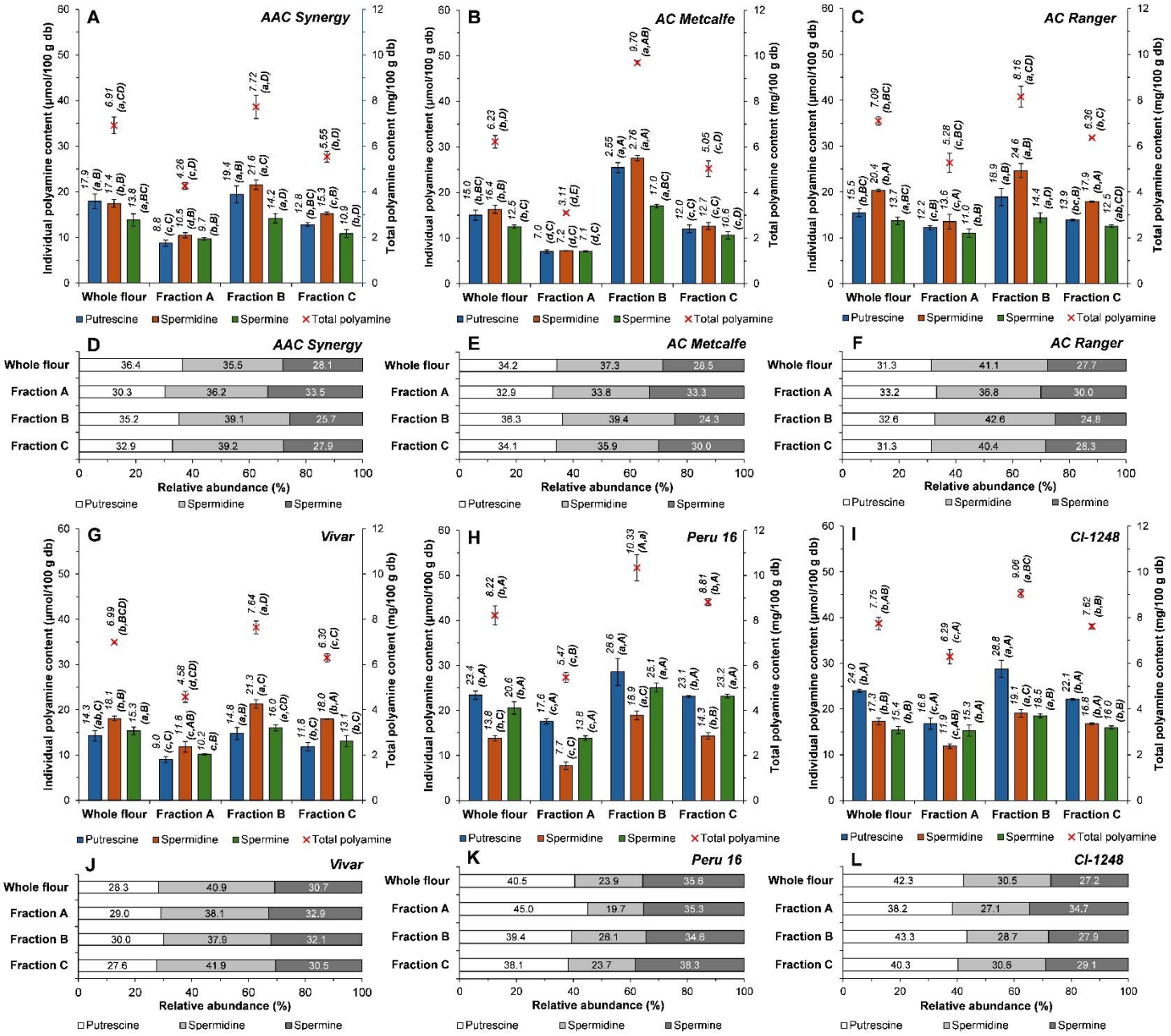
A
AAC Synergy
B
AC Metcalfe
C
AC Ranger
D
AAC Synergy
E
AC Metcalfe
F
AC Ranger
G
Vivar
H
Peru 16
I
CI-1248
J
Vivar
K
Peru 16
L
CI-1248
Individual polyamine content (µmol/100 g db)
Total polyamine content (mg/100 g db)
Whole flour
Fraction A
Fraction B
Fraction C
Putrescine
Spermidine
Spermine
Total polyamine
Relative abundance (%)

Figure 5.3. Content and profile of polyamines in whole barley flours and milling fractions

Data are presented as mean ± standard deviation (n = 3). Values within a cultivar that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$). Values that do not share an uppercase letter are significantly different from their counterpart of another cultivar (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: db: dry basis.

5.4.5. Distribution of polyamines in hand-dissected fractions of barley grain

Table 5.3 summarizes the distribution of polyamines in anatomical fractions of AAC Synergy barley grain obtained by hand dissection. Manual separation is a well-established method for studying the partition of phytochemicals and micronutrients within the cereal kernel. Despite its labour-intensive nature, hand dissection yields fractions with a higher degree of histological uniformity compared to mechanical processes such as milling and pearling (Nguyen, Drawbridge, & Beta, 2024). The proportions of anatomical fractions obtained in this study were comparable to those reported by Barron, Holopainen-Mantila, Sahlstrom, et al. (2017), who manually dissected barley grains from two covered Norwegian cultivars. The malting cultivar AAC Synergy was selected for this experiment due to its hulled characteristic and popularity in Canadian barley production. The embryonic axis exhibited the highest polyamine concentration, followed by the scutellum and outer layers (aleurone, testa and pericarp). The weight-adjusted total polyamine content of the barley germ (embryonic axis and scutellum combined) was 82.7 mg/100 g db. A discrepancy was noted when comparing the polyamine content of the AAC Synergy germ fraction obtained by hand dissection with that obtained by mechanical dehulling (65.5 mg/100 g db) in the previous study by Nguyen, Bakker, and Beta (2025). However, the relative abundance of the germ fraction produced by mechanical dehulling was considerably lower than that obtained by hand dissection (0.33% vs. 2.9%), suggesting that the former fraction may have contained germ tissue with incomplete anatomical integrity. Moreover, the mechanically isolated fraction may have included adjacent tissues that were co-dislodged during dehulling, such as aleurone and endosperm, contributing to the observed discrepancy in polyamine content between the two methods. In this context, the germ fraction recovered through dehulling could be considered a proxy for determining tissue-specific polyamine content, warranting confirmatory studies using hand dissection. Cereal germs, such as wheat germ, are

recognized for their high polyamine concentrations and have attracted research interest aimed at optimizing the extraction protocols for nutraceutical production (Mohajeri, Mokhtari, Khandan, et al., 2025). Recently, the portfolio of plant-based ingredients rich in polyamines has expanded. Floral pollens have been reported to contain substantial amounts of polyamines, including those of *Ailanthus altissima* (56.4 mg/100 g db), *Salix alba* (54.0 mg/100 g db) and *Zea mays* (39.2 mg/100 g db) (Spremo, Radišić, Kebert, et al., 2025). Polyamines in angiosperms are typically accumulated in reproductive organs or metabolically active tissues during germination, likely linked to their essential roles in embryonic hypocotyl elongation (Li, Li, Han, et al., 2024; Nguyen, Bakker, & Beta, 2025).

The outer layers obtained by hand dissection in this study contained a total polyamine level comparable to that obtained by abrading 15% of the kernel weight from dehulled AAC Synergy barley (15.8 mg/100 g db), as reported by Nguyen, Bakker, and Beta (2025). Similarly, the total polyamine level was consistently low in the hand-isolated endosperm fraction and in the 25% weight-removed barley kernels (3.0 mg/100 g db) of the same cultivar. After adjusting for the proportional contribution of each anatomical fraction, the sum of total polyamine contents across all fractions did not differ from that of the whole AAC Synergy barley grain. This suggests that polyamine metabolism during manual dissection was minimal, likely due to the removal of the germ prior to imbibition and the maintenance of low water activity during desiccation. However, under certain conditions, such as in the presence of the plant hormone gibberellic acid, aleurone cells of de-embryonated barley half-seeds have been shown to continue metabolizing polyamines during imbibition (Lin, 1984). Regarding the relative abundance of polyamines, spermidine was the predominant polyamine in the scutellum and embryonic axis of the barley kernel. This finding aligns with the distribution observed in milling fractions, where the proportion of spermidine was slightly higher in the germ-rich intermediate fraction B.

Spermidine has also been reported as the most abundant polyamine in wheat germ (Mohajeri, Mokhtari, Khandan, et al., 2025) and floral pollens (Spremo, Radišić, Kebert, et al., 2025). In contrast, spermidine was virtually absent in the endosperm, where putrescine was predominant. In a previous study, the molar concentration and relative abundance of putrescine, spermidine, and spermine in the 25% weight-abraded, outer layer-free kernels of AAC Synergy were 2.5 µmol/100 g db (7.5%), 5.5 µmol/100 g db (26.9%) and 9.7 µmol/100 g db (65.6%), respectively. This suggests a possible conversion of spermidine and spermine into putrescine during imbibition of half-grains in water. This hypothesis is supported by Paiva, Netto, Queiroz, et al. (2022), who reported an increase in the relative abundance of putrescine in the seed, leaf, cotyledon and radicle of germinating sorghum. Such interconversion of polyamines may partially explain the similar total polyamine content in manually isolated endosperm and pearled kernels. Future research should focus on elucidating polyamine metabolism under germination-like conditions.

Table 5.3. Content and profile of hand-dissected fractions of AAC Synergy grains

Kernel tissue	Quantity obtained by hand dissection (g, wb)	Proportion within the kernel (%)	Content of polyamines				Relative abundance (%)		
			Putrescine (µmol/100 g, db)	Spermidine (µmol/100 g, db)	Spermine (µmol/100 g, db)	Total polyamines (mg/100 g, db)	Putrescine	Spermidine	Spermine
Scutellum	0.0983	1.2	135.0 ± 4.1 (b)	226.5 ± 2.9 (b)	117.1 ± 2.9 (b)	68.5 ± 0.7 (b)	28.2	47.3	24.5
Embryonic axis	0.1385	1.7	239.8 ± 15.8 (a)	327.0 ± 22.2 (a)	135.3 ± 10.4 (a)	92.8 ± 4.6 (a)	34.2	46.6	19.3
Hull	0.7796	9.5	37.2 ± 5.5 (d)	12.2 ± 1.4 (c)	10.7 ± 1.1 (d)	7.2 ± 0.3 (cd)	62.0	20.3	17.7
Outer layers	1.1124	13.6	66.2 ± 9.3 (c)	22.4 ± 3.3 (c)	26.3 ± 3.2 (c)	14.2 ± 2.7 (c)	57.6	19.5	22.9
Endosperm	6.0421	73.9	14.5 ± 1 (d)	1.0 ± 0.2 (c)	5.6 ± 0.2 (d)	2.6 ± 0.1 (d)	68.8	4.6	26.6

Data are presented as mean ± standard deviation (n = 3). Values within a column that do not share a lowercase letter are significantly different (Tukey's comparison test, $p \leq 0.05$).

Abbreviations: wb: wet basis; db: dry basis.

5.4.6. Relationship between polyamine contents and other physicochemical properties

Figure 5.4 illustrates the associations between levels of total and individual polyamines with other physicochemical properties of whole barley flours (6 samples) and milling fractions (18 samples). Excluding the *a** value, significant correlations were observed between polyamine content and various physicochemical parameters, ranging from weak ($0.3 \leq |r| < 0.5$) to moderate ($0.5 \leq |r| < 0.7$) and high ($0.7 \leq |r| < 0.9$), based on the criteria defined by Mukaka (2012). As the abundance of polyamines in the germ and outer layers of barley grains was confirmed by the analysis of hand-dissected fractions, these correlations can be partially attributed to their co-localization within these grain tissues. Such relationships may be useful for selecting barley milling fractions with increased polyamine content based on known physicochemical properties. Furthermore, certain underlying mechanisms can be inferred from the correlations, providing insights into barley grain physiology. Regarding the relationship between polyamine levels and colour values, putrescine content showed significant and negative correlations with *L** ($r = -0.43$, $p < 0.05$) and *b** value ($r = -0.62$, $p < 0.01$). Similar but stronger associations were noted for the panels of spermine content with *L** ($r = 0.61$, $p < 0.01$) and b* value ($r = -0.66$, $p < 0.001$). A negative and weak correlation between total polyamine content and *b** value was also recorded ($r = -0.43$, $p < 0.05$). Previous studies reported that lightness inversely correlates with the occurrence of aleurone and pericarp fractions in milling streams of cereal grains (Symons & Dexter, 1993). Moreover, the yellowness of milling products is proportional to carotenoid content, which is typically more concentrated in the polyamine-poor endosperm fraction of cereal grains (Nguyen, Drawbridge, & Beta, 2024).

Protein content demonstrated high and positive correlations with putrescine ($r = 0.80$, $p < 0.05$), spermidine ($r = 0.84$, $p < 0.001$), and total polyamine concentration ($r = 0.70$, $p < 0.001$). This correlation was the most prominent among the physicochemical attributes of whole barley

flour and milling fractions examined in this study. The association between protein and polyamine contents in barley grains has been observed at both kernel and sub-kernel levels (Nguyen, Bakker, & Beta, 2025; Nguyen & Beta, 2023), highlighting their synchronized roles in the physiological processes of this crop. Nguyen, Bakker, and Beta (2025) proposed that the co-occurrence of polyamines and proteins in metabolically active tissues of barley grains (i.e., germ and aleurone) facilitates biochemical reactions essential for germination, such as the formation of polyphenol crosslinks to strengthen cell walls via polyamine oxidation and the synthesis of hydrolytic enzymes to utilize the nutrient reserves. With respect to ash content, this attribute exhibited weak to moderate and positive correlations with individual and total polyamines (r = 0.44–0.63). In milling products of cereal grains, ash levels can reflect the presence of aleurone and pericarp (Symons & Dexter, 1993), which may account for their positive association with polyamine content. However, these correlations were weaker than those between protein and polyamine concentrations. This could be explained by contributions of ash (primarily silicon) from husk fractions (Lukinac & Jukić, 2022), which typically contain limited protein. This speculation is supported by the absence of a significant correlation between protein and ash contents in whole barley flours and milling fractions ($p > 0.05$). Despite the significant but weak correlation for ash and putrescine contents ($r = 0.44$, $p < 0.05$), the appreciable correlation coefficients detected in other panels, namely spermidine–ash contents ($r = 0.58$, $p < 0.01$), spermine–ash contents ($r = 0.55$, $p < 0.01$), and total polyamine–ash contents ($r = 0.63$, $p < 0.01$), suggested that these associations could be valuable to explore for industrial application.

Apart from the association with protein and ash content in the proximate composition, polyamine concentrations showed significant and positive correlations with BGA and phenolic compounds. The BGA level did not correlate with putrescine and spermine ($p > 0.05$); however, it exhibited a positive, moderate correlation with spermidine ($r = 0.67$, $p < 0.001$), likely due to the

predominance of spermidine in the germ fraction. Although a direct relationship between BGA and polyamines has not been reported, both components are believed to play important roles in the barley germ. Agglutinins are known for their protective function in cereal germs (Killilea, McQueen, & Abegania, 2020), whereas spermidine is essential for cell proliferation (Mohajeri, Mokhtari, Khandan, et al., 2025). Regarding phenolic compounds, which represent a major class of plant secondary metabolites, the correlation coefficients between total phenolic content and the levels of putrescine, spermine and total polyamines were 0.44, 0.66, and 0.56, respectively. Beyond their co-localization in the outer layers of barley grains (Drawbridge, Herrera-Balandrano, & Beta, 2025; Nguyen, Bakker, & Beta, 2025), the relationship between polyamines and phenolics may be attributed to their coordinated roles in crop physiology, particularly in response to pathogenic infections through the formation of covalent conjugates with phenolic acids or phenolamides (Chen, Shao, Yin, et al., 2019). In hulless barley cultivars, Drawbridge, Herrera-Balandrano, and Beta (2025) documented the occurrence of N^1,N^8-dicaffeoyl spermidine and characterized its distribution within the kernel. This compound was more concentrated in the outer layers and showed a positive correlation with ferulic and *p*-coumaric acids, suggesting a possible interplay among phenolic acid, polyamine and phenolamide (Drawbridge, Herrera-Balandrano, & Beta, 2025). Due to the hybrid nature of phenolamides—comprising a polyamine moiety that enhances water solubility, a phenolic acid moiety that serves as the chromophore for chromatographic detection and an amide linkage between the two moieties that may be labile under acidic conditions—different strategies are required for their extraction, identification and quantification to better elucidate their contribution to the polyamine and phenolic acid profile.

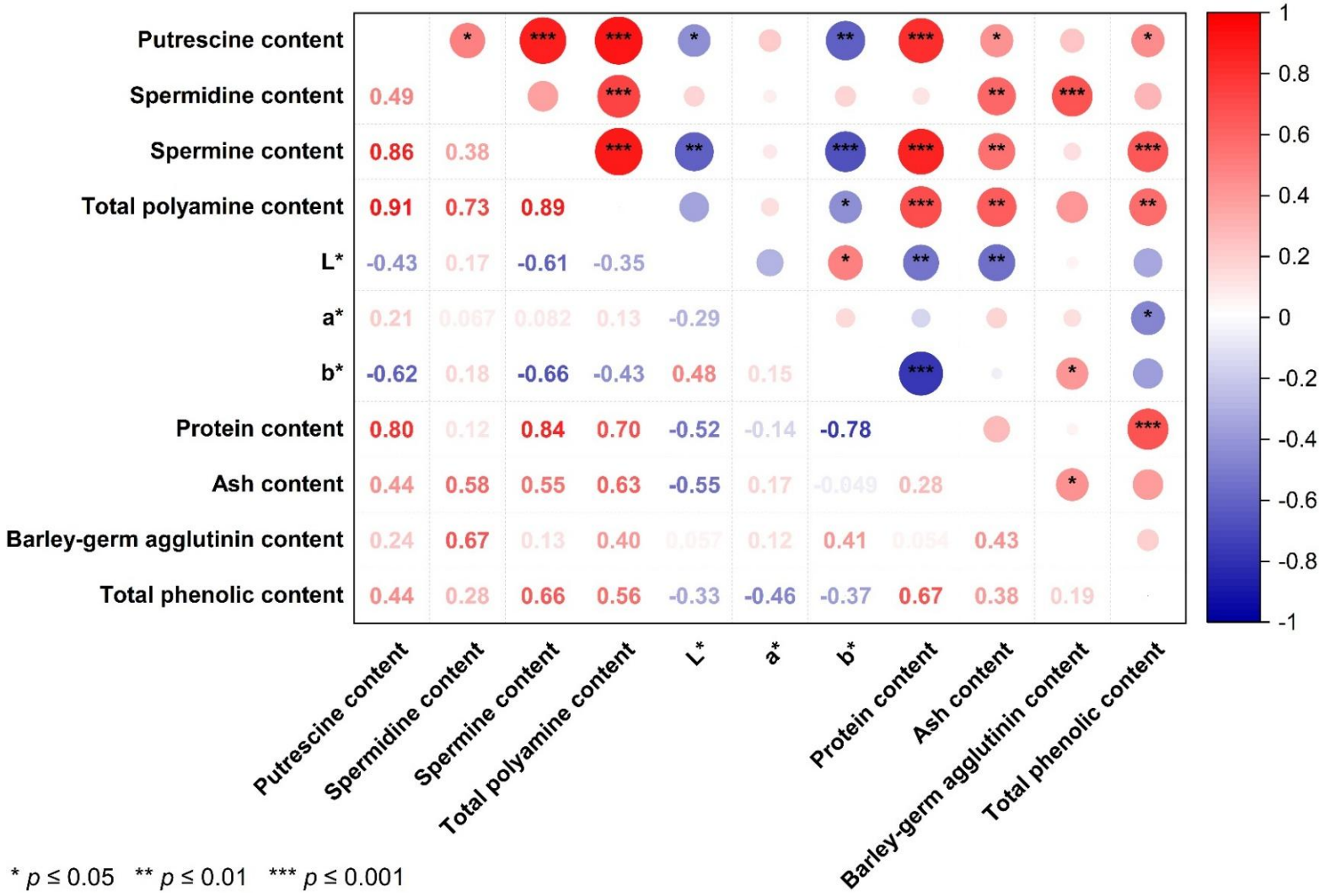

Figure 5.4. Pearson correlation between polyamines and markers of whole-grain status

Each panel include twenty-four samples, including one whole flour and three milling fractions of each cultivar (six cultivars in total) .

5.5. Conclusion

This study demonstrated that dry fractionation, combining milling and size-based sieving, is a practical approach for enriching polyamines and other constituents associated with whole-grain status in barley. Hand dissection conducted on the AAC Synergy cultivar confirmed the localization of polyamines in the germ and outer kernel tissues of the barley kernel, and this anatomical distribution was reflected in the milling fractions. The intermediate fraction B (200–500 μm) consistently exhibited the greatest enrichment of polyamines, protein, ash, phenolics and BGA, highlighting its potential as a value-added ingredient for functional food applications. In particular, the level of polyamine enrichment in this fraction, relative to whole flour, was 24.6% on average. In contrast, the fine fraction C (<200 μm) primarily represented starchy endosperm, while the coarse fraction A (>500 μm) was influenced by hull content in hulled varieties. The observed genotype-specific patterns also highlighted the role of kernel structure in fractionation behaviour. Collectively, the findings of this work supported dry fractionation as an efficient and scalable strategy for producing polyamine-enriched ingredients from barley grains.

Chapter 6 — General Conclusion and Future Studies

6.1. General Conclusion

This thesis provided a comprehensive examination of the occurrence, distribution and enrichment of polyamines in barley (*Hordeum vulgare* L.), situating these nitrogen-rich bioactive compounds within the broader context of cereal grain physiology, nutrition and food processing. By employing analytical chemistry, kernel histology, and mechanical fractionation, this work gained new understandings of polyamines as phytochemical components of barley grain and highlighted their importance for both applied research and agri-food system development. At the analytical level, this thesis first addressed the methodological aspects of extracting polyamines from the barley flour matrix. Examination of hydrochloric acid (1 M) as an extraction solvent demonstrated higher recovery rates than trichloroacetic acid (5% w/v), providing a reliable method for quantifying free polyamines in barley flour. This analytical method would be relevant to future studies comparing polyamine levels across cultivars, environments, or processing conditions. A recent publication utilized the polyamine derivatization protocol developed in this thesis to quantify polyamines in an herbaceous sample (Mai, Van Quan, Doan, et al., 2026).

Among thirteen Canadian barley cultivars representing various end uses, the study revealed that barley grains contain appreciable and variable amounts of putrescine, spermidine and spermine. Total polyamine levels were comparable to or higher than those in other commonly consumed cereals, suggesting barley as a dietary source of these bioactive compounds. Notably, hulless food-grade varieties exhibited higher polyamine levels than malting or feed types, reflecting differences in nitrogen allocation and grain composition. These results highlighted how genotypes could influence polyamine accumulation and the need for cultivar selection or breeding to enhance nutritional value without compromising agronomic performance.

A key and consistent finding is the positive, significant correlation between polyamine content and protein levels, observed at both the whole-kernel and sub-kernel levels. Correlation coefficients ranged from moderate to high across cultivars, pearling fractions and milling streams, indicating an interrelated relationship between polyamine biosynthesis and protein synthesis, both of which rely on nitrogen uptake and amino acids such as arginine and ornithine. In this sense, polyamines appear to be part of a coordinated nitrogen metabolism within the barley grain. This finding provided new insights into nitrogen partitioning and underscores the physiological importance of polyamines in cereal biology.

The spatial distribution of polyamines within the kernel further affirms their functional role in grain development and germination. Using hand dissection, pearling, dehulling and milling, the study revealed a gradient, with the highest concentrations in metabolically active tissues, such as the germ and aleurone layers. These tissues also contain enzymes and nutrients vital for early seedling growth. Elevated polyamine levels in these areas support roles in cell division and nitrogen mobilization during germination. In contrast, the starchy endosperm, which is primarily a storage tissue in dormant grains, exhibited much lower polyamine levels. This histological pattern aligned with observations in other nitrogen-rich compounds and emphasized the biological significance of localizing polyamines in barley.

Another practical contribution is the report of protein and ash contents as indicators of polyamine enrichment. Since these measures are routinely used in cereal processing and quality control, their strong association with polyamine levels provides a simple and cost-effective means of monitoring polyamine content during fractionation and manufacturing. This approach would reduce the need for specialized analysis and make polyamine-focused ingredient development more accessible to the food industry. Gowen, Galani, Hauser, et al. (2026) mentioned the barley products in this research as a supplement to improve the polyamine profile of infant formula.

This thesis highlighted the importance of polyamines as key nutritional, physiological and technological components of barley grain. Through the exploration of plant metabolism, kernel structure and food processing, the research expanded knowledge about the nutritional and nutraceutical benefits of barley grains beyond the traditional focus on dietary fibre components. In addition, the discussed solvent-free methods for polyamine enrichment would also align with clean-label standards, sustainability goals and the growing market for functional foods made from climate-resilient crops. Overall, this research contributed to the advancement in cereal science and supports the development of sustainable food systems that improve public health.

6.2. Future Studies

While this thesis established a strong foundation for understanding polyamines in barley grains, several avenues for future research emerge that could further enhance both scientific knowledge and practical application.

From a plant physiology and agronomy perspective, future studies can investigate how environmental factors influence polyamine accumulation in barley grains. Nitrogen fertilization regimes, soil quality, water availability and abiotic stresses such as drought, salinity and temperature extremes are known to affect nitrogen metabolism and can significantly modulate polyamine biosynthesis. In addition to these factors, the effects of biotic stresses, including pathogen infection and pest pressure, warrant further investigation, as polyamines participate in plant defence responses and stress signalling pathways. Evaluating genotype-by-environment interactions would help determine the stability of polyamine traits across growing conditions and identify cultivars that consistently accumulate higher levels. Such information would be valuable for breeding programs seeking to optimize both resilience and nutritional quality.

At the molecular level, further elucidation of the genetic and regulatory mechanisms governing polyamine metabolism in barley grains and their relationship to barley proteins is warranted. Concerning the connection between polyamines and protein, studies in *E. coli* demonstrated that polyamines promote the translation of several distinct proteins (Igarashi & Kashiwagi, 2018). In barley, transcriptomic and metabolomic approaches could be employed to examine the expression of key enzymes involved in polyamine biosynthesis, catabolism and transport during grain development and germination. Attention would be given to the coordination between polyamine metabolism and nitrogen assimilation pathways, as well as the potential role of polyamine transporters in determining tissue-specific accumulation. Integrating these data with existing barley genomic resources, such as genome-wide association studies (GWAS), would allow more targeted breeding strategies.

Delving into the identified relationship between protein levels and polyamine content, proteomic approaches would be useful for categorizing grain proteins to pinpoint groups of proteins that show a stronger association with polyamines. In general, proteins in barley grains can be classified into storage proteins (primarily hordeins) and metabolic proteins (including albumins, globulins, enzymes, and stress-response proteins) (ElShamey, Yang, Yang, et al., 2025). While hordeins are mainly localized in the endosperm, metabolic proteins are more concentrated in the outer layer regions of the barley kernel (ElShamey, Yang, Yang, et al., 2025). Certain proteomic methods, such as MS-based ones, can be performed at the tissue level (Bahmani, O'Lone, Juhász, et al., 2021), helping elucidate this correlation at the sub-kernel scale.

Another area for future research is the characterization of conjugated polyamines and phenolamides in barley fractions. While this thesis focused on free polyamines, barley grains are known to contain phenolamides that may confer additional bioactive properties. Investigating how these conjugates are distributed within the kernel, how they respond to mechanical

fractionation and how they behave during food processing would provide a more complete picture of polyamine-related phytochemical profile in barley grains.

From a nutrition and health perspective, studies examining the bioaccessibility and bioavailability of polyamines from barley-based foods are needed. In vitro digestion models and ultimately clinical studies could assess how processing techniques and matrix composition influence polyamine release and absorption. Given emerging evidence linking dietary polyamines, particularly spermidine, to reduced inflammation, improved cardiovascular health and healthy aging, understanding how polyamines originating from barley grains contribute to systemic polyamine pools is essential to substantiate functional food claims.

From an industrial perspective, future work can focus on scaling up dry fractionation strategies (pearling and milling) and assessing their economic feasibility. Attention should be paid to the geometry of the barley kernel when adapting existing equipment or designing new equipment, as well as choosing the technique to yield products with the desired particle size. Pilot-scale trials using industrial milling equipment would help validate the enrichment patterns observed at the laboratory scale and identify process parameters that maximize polyamine concentration while preserving functional and technological properties. Additionally, exploring the incorporation of polyamine-enriched barley fractions into consumable food systems, such as breads, extruded products, fermented foods, or plant-based protein formulations, would provide insight into their technological performance and consumer acceptance.

In conclusion, this thesis opened avenues for interdisciplinary research across plant science, nutrition, food processing and sustainability. By highlighting polyamines as both biologically important and practically useful in barley, this work laid the groundwork for ongoing innovations that enhance human health through the strategic use of resilient cereal crops.

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