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EFFECT OF PROCESSING ON IN-VITRO PROTEIN DIGESTIBILITY AND OTHER NUTRITIONAL ASPECTS OF NEBRASKA CROPS

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EFFECT OF PROCESSING ON *IN-VITRO* PROTEIN DIGESTIBILITY AND OTHER NUTRITIONAL ASPECTS OF NEBRASKA CROPS

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EFFECT OF PROCESSING ON *IN-VITRO* PROTEIN DIGESTIBILITY AND OTHER NUTRITIONAL ASPECTS OF NEBRASKA CROPS

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Among plant based agricultural products, Nebraska ranks first nationwide in the production of Great Northern beans (GNB), second in proso millet production, and eighth in production of winter wheat. The present research was focused on the effect of processing on nutritive components and *in vitro* protein digestibility of these crops with the aim of promoting their human consumption. Proso millet based extrudates had physical properties similar to commonly extruded rice but had lower expansion than corn. GNB extrudates had limited expansion and high bulk density mostly due to high fiber and protein content. Extrusion significantly reduced the anti-nutritional components in GNB flour while moderately reducing the essential folate. Extrusion significantly increased the dialyzability of the essential mineral elements Mg, P, K, and Fe in GNB, while significantly reducing dialyzability of the heavy metal Cd. Extrusion also improved the *in vitro* protein digestibility of GNB flour. In contrast, extrusion had a significant negative impact (almost 50% reduction) on *in-vitro* digestibility of proso millet proteins. Formation of hydrophobic aggregates was the main reason identified for the low digestibility in proso millet proteins. The effect was not specific to extrusion but was observed in all the processing techniques that involved heating above 55 °C. Among various mitigation strategies explored, enzymatic modifications of millet proteins with transglutaminase, heating in low a_w solutions, or heating in chaotropic salts (e.g., $CaCl_2$)

at high concentration proved to be beneficial in at least partially preventing the low digestibility effect. Further, the results obtained from comparing in vitro digestibility of breads made from legacy and modern wheat cultivars indicated a significant improvement in digestibility of cultivars released after 1931. The old cultivar, Red Chief, and the land race cultivars, Kharkof and Turkey, had significantly lower digestibility than newer released lines. The changes incorporated by controlled breeding in the proteins of early wheat cultivars were preserved and successfully transferred to modern wheat cultivars resulting in wheats with better yield, end use characteristics and high protein digestibility.

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"No one can whistle a symphony alone. It takes a whole orchestra to play it"

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PREFACE

Introduction

Nebraska ranks fourth in the United States in terms of agricultural profits--outranked only by California, Texas, and Iowa (USDA-NASS, 2017). The state ranks first nationwide in the production of Great Northern beans; second in proso millet production; and eighth in winter wheat production (USDA ERS, 2017, Nebraska department of agriculture).

Wheat is a nutritionally important crop, rich in micronutrients, including minerals and B-vitamins, and accounts for 20% of calories in our diets (Cummins & Thomson, 2009). The crop has been a human dietary staple for centuries mainly due to its adaptability to various geographical locations, nutritional abundance, and presence of viscoelastic gluten proteins.

In the past few years, wheat has earned a bad reputation, especially in developed countries, due to unsubstantiated claims linking wheat consumption to adverse health effects like obesity, metabolic diseases, and gluten sensitivities (Davis, 2011). Some advocates of such claims have blamed modern wheat breeding programs as the main culprit for destroying the ancient crop by modifying the gluten proteins, making them less digestible and contributing to gluten sensitivities. Nebraska has a successful wheat breeding program and majority of wheat produced are byproducts of such initiatives. Thus, one objective of this dissertation was to establish if there is any truth to claims accusing wheat breeding programs for producing inferior wheat, especially in terms of protein digestibility.

Further, the negative press surrounding wheat has encouraged a trend in consumption of gluten-free diets, which in turn has caused a boom in the gluten-free

foods industry (Terazono, 2017). While a gluten-free diet is a necessity for some, for others there is no proven benefit of consuming a gluten-free diet and if not carefully monitored it can result in nutritional inadequacies (Miranda et al., 2014). Most of the gluten free products are rice or corn based, which have low amount of protein, fiber, and other nutrients when compared to wheat (whole). Thus, it is important to identify new wholesome sources of gluten free foods.

Great North beans (*Phaseolus vulgaris*) and proso millet (*Panicum miliaceum*) are the top two ranked agricultural commodities produced in Nebraska when compared with other states in the US. These crops are suitable candidates for the gluten-free foods market. While proso millet is a cereal with a protein content similar to wheat (~12%), Great Northern beans (GNB) belong to legume family and have a protein content of ~21%. Apart from the protein content, both these crops are good sources of functional compounds, such as polyphenols and resistant starch, that are known for their low glycemic index, anti-oxidant, anti-cancer, prebiotic, cholesterol lowering and other health promoting properties (Wang et al.,2010). Also, GNB is a good source (>10% DV) of micronutrients such as folate, Mg, K, P, and Fe. Despite several nutritional benefits, human consumption of these two crops is limited mainly due to lack of versatile products and mass appeal. Thus, another objective of the present research was to explore if proso millet and GNB can be promising candidates for the gluten free foods market, specifically targeting the nutritional quality of these processed crops.

Objectives and hypotheses of current research

1) ***Determine the extrusion performance of proso millet and Great Northern beans to develop snack products.***

Extrusion is a resourceful food processing technique that has been used to develop various products ranging from cereals to puffed snacks utilizing a variety of raw materials. At present, there are no extruded puffed snack products in the market made with either proso millet or GNB. Thus, the first objective was to determine if puffed snack products could be developed using extrusion from proso millet and GNB flour that might promote the consumption of these crops.

Among cereal grain components, starch polymers play a significant role in expansion during extrusion processing while high fiber and protein can disrupt the cell wall formation by the starch film and negatively affect the expansion and texture of the product (Guy, 2000). Proso millet flour has a starch content similar to commonly extruded rice and maize while GNB has comparatively lower starch content but high protein and fibers. In this study, it was hypothesized that proso millet would extrude similar to other cereals resulting in good expansion and texture. In contrast, while expansion of GNB flour would be comparatively lower due to the low starch and high fiber and protein contents, it was hypothesized that manipulating processing conditions like moisture content, barrel temperature, and screw speed, can produce a protein rich product with decent expansion from GNB.

2) Determine the effect of processing on in-vitro protein digestibility and other nutritional components of proso millet and Great Northern beans.

In order to claim that proso millet and GNB are better than commonly used gluten free raw materials it is important to make sure that their protein quality is good and is not adversely affected by processing. *In-vitro* protein digestibility is one step in evaluating the protein quality of cereal proteins. Extrusion processing has shown to improve

digestibility of various cereal grains (Dahlin and Lorenz, 1993). It was hypothesized that extrusion would significantly improve digestibility of both proso millet and GNB proteins by thermal denaturation and/or inactivation of interfering substances like trypsin inhibitors.

Additionally, since these crops have an abundance of minerals and vitamins, attention was also given to these components and effect of extrusion on them. Further, the high temperature and shear conditions in extrusion which are responsible for inactivation of anti-nutritional compounds can also lead to destruction of vital nutrients. Thus, it was hypothesized that extrusion would improve the dialyzability of abundant minerals in GNB flour but simultaneously decrease important labile vitamins like folate. In order to tackle that issue it is crucial to identify the optimum extrusion conditions which would result in maximum reduction in anti-nutritional components without sacrificing on the heat/shear sensitive vital nutrients. Thus, the second objective was directed to achieve that.

3) Compare in-vitro protein digestibility of legacy and modern wheat cultivars grown in Nebraska.

The final objective of this research was to establish if the protein digestibility of modern wheat produced using targeted breeding programs in Nebraska is any different from that of legacy wheat cultivars that were introduced in US prior to the establishment of wheat breeding programs. Thus, protein content and digestibility of 21 different cultivars of wheat released between 1870 and 2014 was compared in the final chapter. It was hypothesized that the *in vitro* digestibility of breads made with legacy and modern

cultivars would be the same and thus breeding initiatives did not induce any change in nutritional quality of wheat proteins.

Organization of the thesis

The current thesis is focused on three Nebraska crops, i.e. proso millet, Great Northern beans (GNB) and wheat. The first 5 chapters are based on proso millet, followed by two on GNB, and finally one chapter on wheat. The first and sixth chapters are based on the first objective, i.e., to explore the extrusion performance of proso millet and GNB flour. While performing experiments for chapter 1, I discovered that the protein digestibility of proso millet declines drastically upon extrusion, a finding not previously reported in the literature. Therefore, chapters 2-5 address objective 2, with focus on protein digestibility of proso millet, specifically looking into the 'what' and 'why' of the observed decline in digestibility of proso millet during processing and 'how' to prevent it. Chapter 7 targets the abundant minerals (Mg, P, K, Fe) and vitamins (folate) in GNB flour, as well as some anti-nutritional compounds (phytate and Cd) and reports on how they change during extrusion. The final chapter is based on the third and last objective of the research i.e. to compare legacy and modern wheat cultivars to determine if modern wheat breeding efforts have changed protein digestibility of wheat. The dissertation concludes (chapter 9) with overall findings from each study.

The first three chapters have been published in *International Journal of Food Science and Technology*, *Journal of Agricultural and Food Chemistry*, and *Journal of Cereal Science*, respectively, while the fourth chapter is under review in *Food Research International*. Thus, the first four chapters have been formatted according to their

respective journals. The final four chapters have been formatted using guidelines from International Journal of Food Science and Technology.

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1.1. ABSTRACT

The objective of this work was to determine the impact of extrusion variables [moisture (17–25%), screw speed (170–250 rpm) and temperature (90–150 °C)] on the physical properties and antioxidant activity of proso millet extrudates. Extrusion variables were adjusted using an inscribed central composite rotatable design. Response variables were bulk density (BD), radial expansion ratio, water absorption index, water solubility index, hardness, color (L*, a*, b*) and antioxidant activity. Moisture and screw speed were the most influential variables affecting millet extrusion: their linear, quadratic and interaction terms accounted for more than 50% of the variability in all responses except for b*. Expansion was greatest at severe conditions of low moisture and high screw speed. These conditions were also consistent with the highest antioxidant activity. This study demonstrated that high expansion and antioxidant activity can be obtained by extruding proso millet under low moisture and high screw speed conditions.

1.2. INTRODUCTION

Millets (finger, proso, pearl, and foxtail millet) are a diverse group of small seeded crops belonging to the Poaceaea family and are commonly termed ‘ancient grains’. Various types of millets are consumed as porridges, soups, flat breads, and in other forms in Asian and African countries (Zhu *et al*., 2014), but are comparatively new to the western world. Proso millet (true millet, common millet, hog millet, yellow hog, etc.) is the only millet grown as a grain crop in US, with main production in states of Nebraska, Colorado, and South Dakota, where it is often employed as rotational crop with winter wheat (Graybosch and Baltensperger, 2009). A short growing period, lower water requirement, and improvement in wheat, corn, and sorghum yield when rotated with these crops makes growth of proso millet desirable from an agricultural standpoint (Lyon and Baltensperger, 1995).

In the US, Proso millet is mostly used as animal and bird feed. Exploring novel food applications of proso millet for US market can promote the growth and consumption of this crop. Some applications of proso millet reported in the literature include weaning mix (Srivastava *et al*., 2001), pasta (Sudhadevi *et al*., 2013), and breads and cookies in combination with wheat flour (Schoenlechner *et al.,* 2013).

Extrusion is a versatile, cost effective process that that can be used to create ready-to-eat cereals and snacks from grains. Currently, the only reported work on proso millet extrusion is to evaluate the effect of extrusion on proso lipids (Anderson, 2009). Due to a similar proximate composition with other commonly extruded grains (rice, corn, wheat) (Gopalan *et al.,* 2010), extrusion of proso millet may be a viable way of increasing proso millet utilization in human foods.

The extrusion process is a multi-variable technique that involves the interaction among flour characteristics, such as moisture content and chemical composition, as well as processing parameters, including temperature, screw speed, feed rate, and screw configuration. Flour moisture influences the degree of cook and shear experienced by flour components, especially the starch, which has a major impact on expansion volume (Thymi *et al*., 2005). As reported by various authors, temperature and screw speed are the most commonly studied and easily varied extrusion parameters with significant impact on physical changes in cereal flours inside an extruder barrel (Chinnaswamy and Hanna, 1988).

Millets are also a good source of phenols and flavonoids, contributing to their antioxidant activity. Chandrasekara *et al*. (2012) reported significant superoxide and peroxide radical scavenging activity of a de-hulled proso millet extract. Understanding how extrusion variables affect the antioxidant activity of proso millet extrudates could be important knowledge for creating snacks for health-conscious consumers. Thus, the aim of this work was to determine the effects of flour moisture and extruder screw speed and temperature on physical characteristics and antioxidant activity of extruded proso millet flour.

1.3. MATERIALS AND METHODS

1.3.1. Preparation of millet flour and analysis

De-hulled proso millet was obtained from Clean Dirt Farms (Sterling, CO, USA) and milled using a pilot scale hammer mill (20SSHMBD, C.S. Bell, Tiffin, OH, USA) with screen size of 0.7 mm. The flour was analyzed for moisture, fat, and ash following approved methods (AACC International, 2015). Protein content was analyzed using a

nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25. Total starch content was analyzed using total starch assay kit (K-TSTA, Megazyme, Bray, Ireland) following the KOH format. Millet flour was stored at 4°C until extrusion.

1.3.2. Experimental design

The effects of three extrusion factors: barrel temperature (90-150°C), feed moisture content (17-25%), and screw speed (170-250 rpm), on proso millet extrusion were studied while keeping other factors such as feed rate and screw configuration constant. The levels of these factors were determined based on preliminary trials. Response surface methodology with an inscribed central composite rotatable design (CCRD; α=1.682) with 8 factorial points, 6 star points, and 3 central points was used (Table 1.1). Apart from the 17 CCRD experimental runs generated by the statistical software (JMP version 10.0.0, SAS Institute, Cary, NC, USA), 6 additional treatment combinations for validation of the statistical model were run. The additional points were generated through the Latin hypercube sampling (LHS) technique. Thus, a total of 23 combinations of extrusion conditions were run.

1.3.3. Extrusion process

A laboratory scale co-rotating conical twin screw extruder with mixing zones was used for extrusion (CTSE-V, C.W. Brabender, Hackensack, NJ, USA). The diameter of the conical screws decreased from 43 mm to 28 mm along the length of 370 mm from the feed to die end. The extruder barrel had 4 temperature zones with temperatures of the first two zones maintained at 50 and 80 °C, respectively, while the temperatures of the last two zones were set according to the experimental design. Due to frictional heat generated during extrusion, the indicated temperatures of last two sections of barrel were higher

than the set value. Hence, the actual temperature was recorded and used in all calculations. The exit die internal diameter was 3 mm. The extruder was operated by a direct current drive unit (Intelli-Torque, Pastic Corder Lab-station, C.W. Brabender) with a 7.5 hp motor. The extruder software (Measurement and extrusion program for control systems, version 3.0.2, C.W. Brabender) displayed the measured torque readings. To adjust the moisture content of proso millet flour batches (3 kg) representing each experimental run were blended in an upright blender (H-600-D, Hobart, Troy, Ohio, USA) at medium speed with the required water to obtain the target moisture content as per experimental design. The moist samples were sealed in polyethylene bags and tempered for 16 h at 4°C. The flour was then fed into the extruder barrel using a single screw volumetric feeder (FW 40 Plus, C. W. Brabender) set at a constant delivery rate of 76 g/min.

The extrudate sample for each experimental condition was collected after a stable temperature and torque reading was observed. The collected samples were dried in a belt drier (4800 series Wenger, Sabetha, KS, USA) at 100°C for 10 min. Half of the samples were sealed in plastic bags for measuring texture, expansion index, and bulk density, whereas the other half was ground using cyclone sample mill (UDY, Fort Collins, CO, USA) with a screen size of 1 mm and used for measuring water absorption index (WAI) and water solubility index (WSI).

1.3.4. Process responses

Five torque readings were recorded during the course of sample collection for a particular condition and the average reading was used to calculate specific mechanical energy (SME). The SME (kJ/kg) was calculated as: $SME = (2\pi * N * T)/\dot{m}$, where N=

screw speed (rpm), T = motor torque (N m) calculated by subtracting the no-load torque from the average torque recorded for a particular run, and $\dot{m}$ = mass flow rate (g/min) (Godavarti and Karwe, 1997).

1.3.5. Product responses

The volume of the extrudates was measured using rapeseed displacement method, which was further used to calculate the apparent bulk density (g/cm^3) of the extrudates. An average of 5 readings were reported for each experimental condition.

Radial expansion ratio (RER) was calculated by dividing the cross-sectional diameter of each extrudate, measured using a Vernier caliper (Mitutoyo Co., Kawasaki, Japan), by the extruder die diameter. An average of 10 readings were reported for each extrusion condition.

WAI and WSI were measured following Anderson *et al*. (1969) method. Ground extrudate (2.5 g) was suspended in 30 mL water at 30 °C for 30 min with intermittent stirring, centrifuged at 3000 × g for 10 min. The supernatant was decanted into a tared evaporating dish. The WSI was recorded as percent weight of dry solids in the supernatant to the original weight of sample. The WAI was the weight of gel obtained after removal of the supernatant per unit weight of original dry solids. The average of three readings were reported.

The texture of extrudates was measured in terms of hardness which was recorded as the amount of force (N) required to crush the samples using texture analyzer (TA-XT Plus, Texture Technologies, Scarsdale, NY, USA) equipped with a 5-blade Kramer shear cell attachment. The probe cell was filled to half volume with extrudates approximately 7 cm long. The 5 blades crushed the samples at a speed of 2 mm/s to a distance of 48 mm

with a load cell of 25 kg. The peak force obtained from force-time curve was analyzed using the texture analyzer software (Texture Exponent 32, Texture Technologies). Ten measurements were performed for each experimental condition and their average value was recorded (Meng *et al*., 2010).

Color of ground extrudates placed in glass petridishes was measured in terms of lightness (/darkness) (L*), redness (/green) (a*) and yellowness (/blue) (b*) using a hand-held Minolta chromameter (CR-200b, Minolta Camera Co. Ltd, NJ, USA). An average of 5 readings was reported for each sample.

Antioxidant activity of ground extrudates were measured using ABTS (2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) assay according to Serpen *et al*., (2007) with slight modification. 1.7 ml of ABTS radical pre-oxidized using potassium persulfate and diluted with 50% ethanol was added directly to 5-7 mg of ground extrudate. The mixture was vortexed for 2 minutes and kept in dark for 6 minutes. Absorbance of clear supernatant was measured at 734 nm following centrifugation at 9200 g for 2 minutes. The antioxidant activity was expressed as mmol of Trolox equivalent antioxidant capacity (TEAC) per gram sample by means of a standard curve (0-0.21 mmol) for Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Trolox, Sigma-Adrich, St. Louis, MO).

1.3.6. Data analysis

Statistical software (JMP version 10.0.0, SAS institute) was employed to model the behavior of the responses (torque, SME, bulk density, RER, WAI, WSI and hardness) as a function of the processing variables (moisture content, temperature and screw speed) using RSM. The fitted models for process and product responses were validated to ensure

that the models used were capable of predicting the effect of the extrusion variables on measured responses. The fit of each model was evaluated by observing the adjusted R^2 while considering actual versus predicted plots and residual versus predicted plots. Model errors were evaluated using the model fit error (MFE) and the model representation error (MRE) functions. The MFE was the relative error of the model with respect to actual values. The MRE was the error associated with values obtained for the LHS (space filling) runs compared with the RSE predicted values. An error range within ±5% for both MFE and MRE indicated that the fit was acceptable. The estimates of regression coefficients were also checked for their statistical significance (α=0.05) in impacting the model terms. Since the magnitude of regression coefficients are highly dependent on the scale of corresponding factor, scaled estimates were calculated by the software to determine the relative contribution of each factor to model variance. Correlations among response variables were calculated using Pearson's method. Principal component analysis (PCA) was conducted based on the correlation matrix to visualize relationships among responses.

1.4. RESULTS AND DISCUSSION

1.4.1. Proso millet composition

The de-hulled proso millet flour used in the study had the following proximate composition (mean of 3 replicates ± standard deviation, dry basis): 9.02±0.07% moisture; 13.6±0.0% protein; 3.13±0.10% crude fat; 1.26±0.01% ash, and 71.9±0.0% starch. Results obtained for all response variables were similar to values reported in literature (Altan *et al*., 2008; Mezreb *et al*., 2003; Ali *et al*., 1996; Ilo *et al*., 1996; Hagenimana *et al*., 2006; Ding *et al*., 2005; Ryu and Walker; 1995).

1.4.2. Model validation

Significant F-ratios and high adjusted R^2, coupled with MFE and MRE ranges within ±5% indicate that the RSE models fit the data well for all variables except WSI. For WSI, both the MFE and MRE were slightly outside the range of ±5%. From visual inspection of fitted models, actual versus predicted plots showed some clustering of points and deviation from fit for WSI (data not shown). Results obtained for WSI were retained for completeness; however, it should be recognized that the model for WSI did not fit the data as well as for other variables.

1.4.3. Influence of extrusion parameters on process responses

During extrusion of proso millet flour, torque ranged from 28.6 to 60.3 N m, while SME ranged from 533 to 857 kJ/kg (Table 1.1). The maximum torque was recorded for the lowest screw speed (170 rpm), lowest temperature (90° C) and intermediate moisture content (21%), whereas the highest SME was recorded at the lowest moisture content and intermediate screw speed and temperature. The regression coefficients for linear effects of screw speed and temperature; the quadratic effect of moisture; and the screw speed*temperature interaction were significant for both torque and SME. The linear effect of temperature for torque and quadratic effect of screw speed for SME were also significant (Table 1.2). After scaling, moisture content contributed the greatest (26%) to variation in torque followed by temperature and screw speed and their interaction (Fig. 1.1). The main effects of all variables (linear or quadratic) had greater effect on SME compared to interactions among variables. Increasing screw speed, temperature, and moisture were associated with reductions in torque (Fig. 1.2), while

SME showed a maximum at moderate screw speed and decreased as moisture and temperature increased.

Changes in torque and SME during extrusion were consistent with literature. At a constant feed rate, an increase in screw speed results in a reduction in fill inside the barrel which reduces the motor load and reduces the torque (Meng *et al*. 2010). Also with increasing screw speed and temperature, the viscosity of the melt decreases as a result of starch depolymerization, resulting in lower torque (Pansawat *et al*., 2008). Torque can also be decreased by increasing moisture due to the plasticizing effect of water and the corresponding reduction in viscosity. The initial increase in SME with increasing screw speed is related to the increased power input required to rotate the screw shaft, while the decrease in SME at higher screw speeds, moisture contents, or temperatures can be related to lower melt viscosity (Ding *et al*., 2006).

1.4.4. Influence of extrusion parameters on product responses

Bulk density of the extrudates ranged from 0.20 g/cm^3 to 0.48 g/cm^3 (Table 1), with the highest and lowest density obtained for products extruded under the highest (25%) and lowest moisture (17%) contents. From the regression estimates, screw speed had significant linear and quadratic effects on bulk density (Table 1.2), and the interactions of temperature and moisture with screw speed were also significant. After scaling, moisture content contributed to 34% of the model variability in bulk density followed by quadratic effect of screw speed and the interaction of screw speed with temperature (Fig. 1.1). Increasing screw speed showed increased density until 210 rpm, beyond which there was a decrease in bulk density (Fig. 1.2). Moisture had a strong positive relationship with bulk density, while temperature had little effect. The bulk

densities were slightly higher than the values reported for extruded corn grits but were similar or lower than extruded rice flour (Table 1.3). The trends obtained for effect of moisture, screw speed, and temperature on extrusion of proso millet were similar to the trends obtained while extruding corn grits (Ilo *et al*., 1996) and wheat based snacks (Ding *et al*., 2006). The similarity in bulk density of proso millet flour on extrusion with rice and rice blends may be due to similarity in starch granule size (Kumari *et al*., 1998).

The RER of proso millet extrudates ranged from 1.4 to 3.1 (Table 1.1), with the highest expansion observed at the lowest moisture condition (17%). The linear and quadratic regression coefficients for moisture content and interaction term of screw speed and moisture content were significant (Table 1.2). Scaled estimates showed that moisture contributed to about 40% of the model variability in RER which was followed by screw speed and temperature (Fig. 1.1). Screw speed and temperature had positive linear effects on RER, while moisture had a negative quadratic effect (Fig. 1.2). The RER for proso millet extrudates was less than extruded corn grits and high amylose rice flour but higher than extruded barley and similar to extruded wheat flour (Table 3). The dramatic effect of moisture on RER is consistent with literature showing that moisture reduces the melt elasticity of starch and prevents it from expanding (Ilo *et al*., 1999).

The WAI values obtained for proso millet extrusion ranged from 3.8 to 4.5 g/g while WSI was between 3.7% and 8.1% (Table 1.1). The linear and quadratic effects of screw speed had significant effects on both WAI and WSI (Table 1.2), while the linear and quadratic effects of moisture also affected WSI. The combined effect of screw speed with temperature and moisture affected WAI and WSI, respectively. From the scaled estimates, it was observed that both moisture and temperature contributed to a substantial

portion of the variation in WAI and WSI (Fig. 1.1). Screw speed explained a substantial portion of the variation in WAI, but was not an important contributor to changes in WSI, whereas quadratic effect of moisture was the main contributing factor for WSI. As moisture content increased, WAI showed an increasing trend while with increasing screw speed there was an initial increase in WAI and then a decrease (Fig. 1.2). The values obtained for WSI were lower than values obtained by other researchers for extruded samples, but the trends of various extrusion parameters on WSI were similar to extruded rice (Ding *et al*., 2005). The dramatic influence of moisture and temperature on WAI were in agreement with other researchers (Ding *et al*., 2005; Yagci and Gogus, 2008). Increasing moisture and temperature promotes internal mixing and uniform heating, which enhances starch gelatinization and increases WAI (Lawton *et al.*, 1972). On the other hand, excessive temperature or low moisture significantly decreases the WAI which can be explained by prevalence of dextrinization over gelatinization (Ding *et al*., 2006). WSI was maximum at lower moisture content and increasing moisture to 21% resulted in a decrease in WSI beyond which an increase was observed. An increase in screw speed resulted in increase in WSI. This can be explained by greater shear degradation of starch at lower moistures making starch fragments more soluble in water (Yagci and Gogus, 2008) while high moisture can have plasticizing effect on starch granules thus preventing them from degradation by shear (Hegenimina *et al*., 2006).

The hardness of extrudates ranged from 160 to 251 N with minimum hardness recorded at the lowest moisture condition. The regression coefficients of the linear, quadratic, and interaction terms for moisture and screw speed were significant (Table 1.2) and together they contributed to 88% of the variation in hardness (Fig. 1.1). This

highlights the important role of screw speed and moisture content in causing changes in hardness of extruded products. The prominent effect of moisture and quadratic effect of screw speed on hardness is consistent with extrusion of corn-lentil (Lazou and Krokida, 2010) and rice based snacks (Ding *et al*., 2005). As reported previously, higher moisture results in a less expanded extrudate with thicker cell walls and lower porosity, which is directly related to the hardness of a sample (Barrette *et al*., 1994).

The L* values of ground extrudates ranged from 74.5 to 80.2, a* values from -0.28 (green) to 1.98 (red), and b* values from 17.0 to 18.3 (yellow) (Table 1). For all three-color coordinates, the lightest, least red, and most yellow products, all suggesting a lesser degree of cook, were observed for high moisture conditions, thus indicating the protective action of moisture towards extrusion cooking of proso flour. The regression coefficients of temperature and its interaction with moisture and screw speed significantly affected the color coordinates (Table 1.2). The scaled estimates plot (Fig. 1.1) indicated moisture to be main factor in affecting L* and a* values while moisture in interaction with temperature was the major factor in affecting b* value. The b* value was also highly influenced by the main effect of screw speed. The effect of these variables was also evident from the prediction profilers (Fig. 1.2) with increasing moisture resulting in higher L* values or lighter product and decreasing a* value. At higher moisture conditions the effect of temperature is reduced which results in lesser browning of sample. Also, the increasing b* value with increasing screw speed can also be linked to lower residence time in barrel, thus less cooking of sample. The results obtained are consistent with extruded rice flour (Hegenimina *et al*., 2006).

The antioxidant activity of extruded millet flour ranged from 16.5 to 31.4 mmol/g (Table 1.1). The regression coefficients of screw speed, moisture, their interaction with each other and with temperature was significant in affecting the antioxidant capacity (Table 1.2). After scaling, the linear, quadratic, and interaction terms of moisture and screw speed contributed most to model variability (62%; Fig. 1.1). Increasing moisture content resulted in a decrease in antioxidant capacity while increasing screw speed showed an increasing effect on antioxidant activity (Fig. 1.2). Previous studies have reported loss of antioxidant activity of cereals on extrusion (Ozer *et al*., 2006; Korus *et al*., 2007; Altan *et al.,* 2009) while others have indicated an increase (White *et al*., 2010; Sharma *et al.,* 2012). The difference in results can be attributed to difference in techniques used for measuring the antioxidant activity. In our study, a direct method was used which resulted in interaction of ABTS radical with both soluble and insoluble antioxidant compounds. Other studies have reported antioxidant activity of extracts from extrudates. The increase in antioxidant activity with increasing screw speed can be linked to shear resulting in greater breakdown of cellular components and better interaction between ABTS radical and the insoluble antioxidant compounds. Another explanation for an increase in antioxidant activity is the production of maillard reaction products under more severe conditions contributed to an increase in antioxidant activity (Yilmaz and Toledo, 2005; see 'Relationships among response variables' section).

1.4.5. Relationships among response variables

Except for b*, product response variables were highly correlated among each other (Fig. 1.3A). Because of the many significant correlations, data interpretation was difficult. Therefore, we used PCA to more clearly explain the relationships among the

variables. The first two principle components (PC) explained 75% of the variation in the data. PC1 was a contrast between samples processed under mild conditions (high moisture, low screw speed and temperature) with negative loadings and those processed under severe conditions (low moisture, high screw speed and temperature) with positive loadings. Samples processed under more severe conditions were associated with higher RER, WSI, TEAC, and a*, while those processed under mild conditions were associated with a hard product with high bulk density, WAI, and L*. Similar results for physical properties have been reported previously (Meng *et al*., 2010; Altan *et al*., 2008). One notable finding revealed by the correlation table and PCA biplot is the inverse relationship between TEAC and L* (Fig. 1.3B). These variables were highly negatively correlated ($r=-0.7$; $p=0.002$). Because lower L* would indicate a darker product, this suggests that the increase in antioxidant activity was likely due to maillard reaction products produced under more severe extrusion conditions.

1.5. CONCLUSION

Moisture content had the most significant impact on both antioxidant activity and physical properties (bulk density, RER, and hardness) of proso millet extrudates, with lower moisture conditions giving a more desirable extruded product, i.e., one with greater expansion and antioxidant activity and lower hardness. This was followed by screw speed, which either had a linear or quadratic impact on all product variables. Among the various interactions, the most significant interaction that affected process responses was between screw speed and moisture.

1.6. REFERENCES

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Table 1.1: Experimental design and process and product responses obtained for extrusion of proso millet.[a]

Variables					Process responses		Product responses								
Run	SS (rpm)	MC (%)	Set T (°C)	Actual T (°C)	Torque (N m)	SME (kJ/kg)	BD (g/cm^3)	RER	Hardness (N)	WAI (g/g)	WSI (%)	L*	a*	b*	TEAC mM/g sample
CCRD runs															
1	210	21	90	107	44.6	774	0.36	1.78	235	4.35	4.20	77.56	0.54	17.59	22.61
2	186	18.6	102	110	57.4	803	0.27	2.20	186	4.10	5.20	77.43	0.70	17.49	21.98
3	186	23.4	102	115	39.4	647	0.39	1.40	208	4.34	4.41	77.88	-0.12	17.32	17.75
4	210	21	150	155	29.2	544	0.31	2.50	230	3.83	4.71	77.01	0.48	17.00	21.18
5	170	21	120	121	47.8	613	0.28	1.41	180	3.96	3.70	77.54	-0.02	17.33	17.67
6	250	21	120	121	36.0	720	0.29	2.33	202	4.12	4.40	76.12	1.26	18.16	23.83
7	210	21	120	122	40.8	712	0.36	2.02	220	4.20	4.39	76.98	0.62	17.54	20.78
8	233	18.6	138	140	39.5	769	0.25	3.10	181	3.85	6.84	75.59	1.54	17.94	25.26
9	186	18.6	138	140	40.7	620	0.21	2.56	180	3.89	5.55	74.50	1.01	16.62	21.10
10	186	23.4	138	138	31.1	533	0.32	1.70	208	4.13	4.99	78.45	0.08	17.34	23.03
11	233	18.6	102	117	42.4	826	0.24	2.83	178	4.13	6.81	75.38	1.75	18.09	31.35
12	233	23.4	102	118	31.0	695	0.42	1.56	239	4.43	4.39	77.92	0.58	17.77	19.88
13	210	17	120	132	44.8	857	0.20	3.18	160	3.93	8.10	74.72	1.98	17.53	30.73
14	210	25	120	121	30.1	705	0.48	1.25	231	4.52	5.51	80.20	-0.28	17.48	16.53
15	210	21	120	124	41.6	760	0.34	1.91	220	4.20	4.40	77.25	0.62	17.62	18.65
16	210	21	120	123	42.0	733	0.35	1.89	230	4.14	4.45	76.92	0.62	17.63	20.12
17	233	23.4	138	139	28.6	627	0.40	1.89	251	4.16	4.56	78.68	0.60	18.30	19.56
Space filling (validation) runs															
18	170	21	90	93.6	60.3	717	0.31	1.68	184	4.09	3.98	78.14	0.18	17.17	18.75
19	223	18.8	122	126	44.5	831	0.27	2.67	190	4.03	6.29	76.10	1.21	17.90	20.89
20	183	23	121	120	39.2	599	0.37	1.41	210	4.30	4.30	78.18	-0.08	17.10	17.51
21	220	24.5	98.6	107	31.6	705	0.44	1.41	219	4.60	4.53	78.60	0.22	17.69	17.98
22	222	21.2	119	124	37.8	700	0.36	1.96	226	4.13	4.49	78.07	0.74	17.70	20.80
23	250	21	120	130	34.7	711	0.29	2.10	216	4.06	4.67	76.60	1.15	18.30	23.92

[a]SS, screw speed; MC, moisture content (%, wet basis); T, temperature; SME, specific mechanical energy; BD, Bulk density; RER, radial expansion ratio; WAI, water absorption index; WSI, water solubility index; L*, a*, b*, color coordinates; TEAC, Trolox equivalent antioxidant capacity; CCRD, central composite rotatable design.

Table 1.2: Regression coefficients for each response surface equation and model fit parameters.

	Process responses						Product responses				
Parameter[a]	Torque	SME	BD	RER	WAI	WSI	Hardness	L*	a*	b*	TEAC
Intercept	320	799	-0.94	7.28	-0.27	38.1	-489	165	-0.50	36.18	83.00
SS	-1.18*	17.2*	0.007*	0.06	0.049*	0.258*	4.67*	-0.11	0.05	-0.062	1.31*
MC	-0.41	-150.5	0.054	-0.64*	-0.187	-5.66*	40.5*	-3.22	-0.99	-0.614	-13.37*
T	-1.46*	0.952	-0.005	-0.06	0.018	0.003	-5.31	-0.75*	0.11	-0.112	-0.81
SS^2	-0.0001	-0.052*	-0.00004*	-0.00004	-0.00009*	-0.0002	-0.021*	-0.0003	0.00002	-0.00007	0.0004
SS*MC	0.009*	-0.093	0.0002*	-0.002*	0.0002	-0.008*	0.175*	0.0015	-0.001	-0.0014	-0.037*
MC^2	-0.156*	3.41*	-0.001	0.020*	0.005*	0.155*	-1.76*	0.038	0.018	0.0023	0.306*
SS*T	0.007*	0.066*	0.00005*	-0.00005	-0.0001*	-0.0002	0.007	0.0014*	-0.0003	0.0006*	-0.005*
MC*T	0.021*	0.003	-0.0003	0.0009	-0.0003	0.003	0.049	0.015*	0.0014	0.0065*	0.057*
T^2	-0.003*	-0.075	-5E-06	0.0002	-3E-06	-0.00008	0.011	0.0005	-0.0003	-0.0006*	0.0025
Adjusted R^2	0.97	0.95	0.95	0.97	0.98	0.95	0.96	0.89	0.95	0.93	0.87
MFE (% range)	(-3.45,2.41)	(-2.14,3.17)	(-4.01,3.17)	(-5.01,4.48)	(-1.18,0.73)	(-4.87,6.77)	(-3.28,4.67)	(-1.01,0.65)	(-1.34,2.93)	(-0.54,0.80)	(-4.75,5.11)
MRE (% range)	(-3.09,3.63)	(-4.13,2.91)	(-1.56,1.29)	(-3.63,2.67)	(-1.50,1.32)	(-7.74,6.27)	(-1.83,3.56)	(-1.88,1.40)	(-1.22,4.55)	(-1.64,3.57)	(-1.93, 5.5)

[a] SS, screw speed; MC, moisture content; T, temperature; SME, Specific Mechanical Energy; BD, Bulk Density; RER, Radial expansion ratio; WAI, water absorption index; WSI, water solubility index; L*,a*,b*, color coordinates; TEAC, Trolox equivalent antioxidant capacity *significant at $p<0.05$.

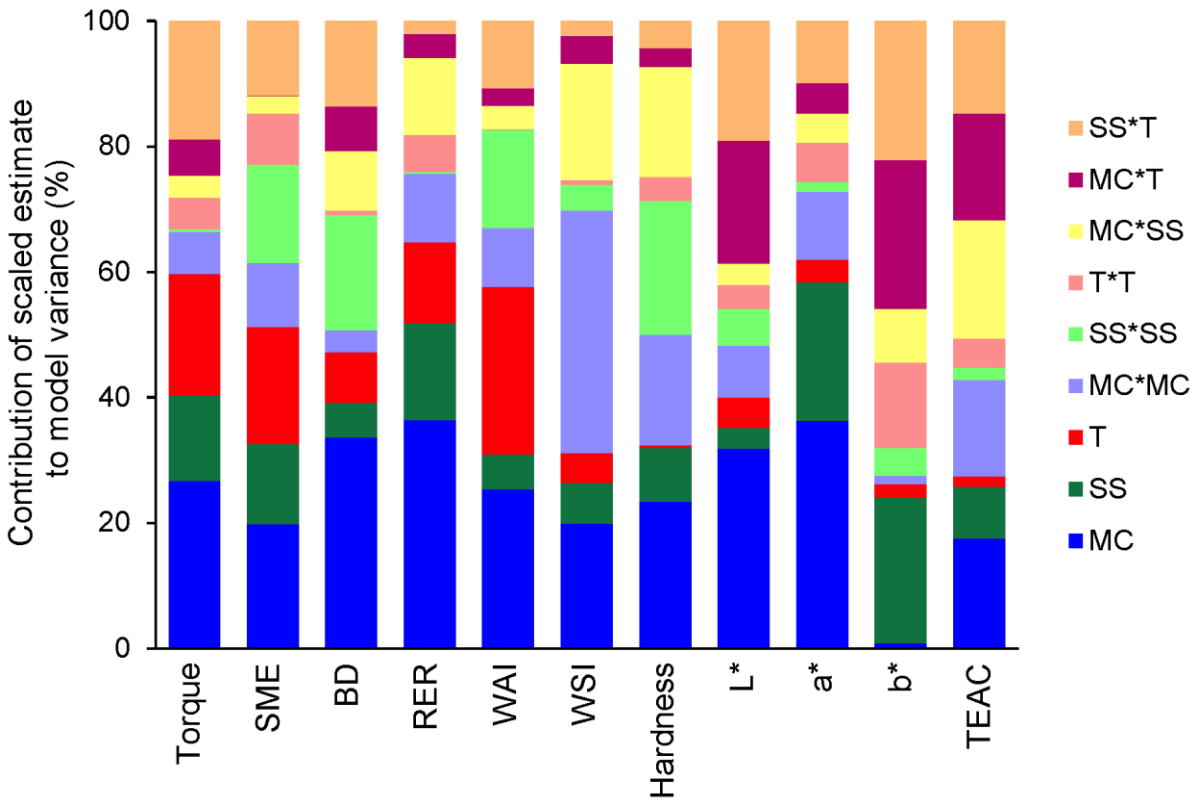

Figure 1.1: Scaled estimates representing the relative contribution of each processing variable to product and process responses[1]

[1] MC, moisture content; SS, screw speed; T, temperature; SME, specific mechanical energy; BD, Bulk density; RER, radial expansion ratio; WAI, water absorption index; WSI, water solubility index; TEAC, Trolox equivalent antioxidant capacity.

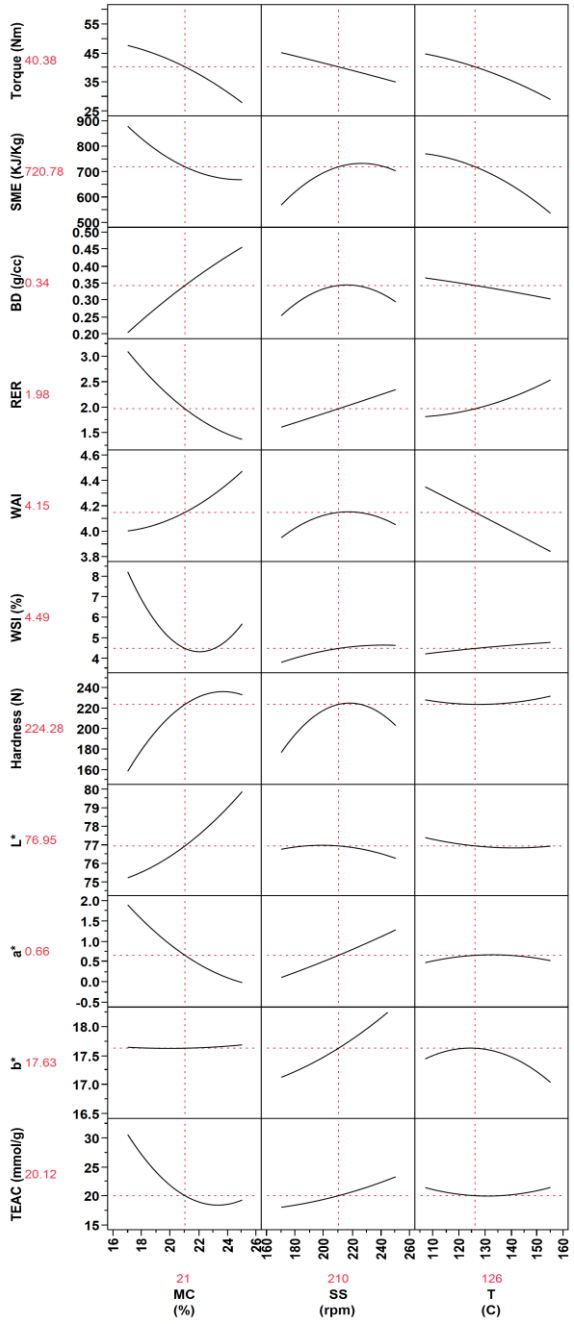

Figure 1.2: Influence of each processing variable on response variables when other processing variables are held constant[2]

[2] Numbers next to axes labels correspond to the value of each variable at the dotted lines; MC, moisture content; SS, screw speed; T, temperature; SME, specific mechanical energy; BD, bulk density; RER, radial expansion ratio; WAI, water absorption index; WSI, water solubility index; TEAC, Trolox equivalent antioxidant capacity

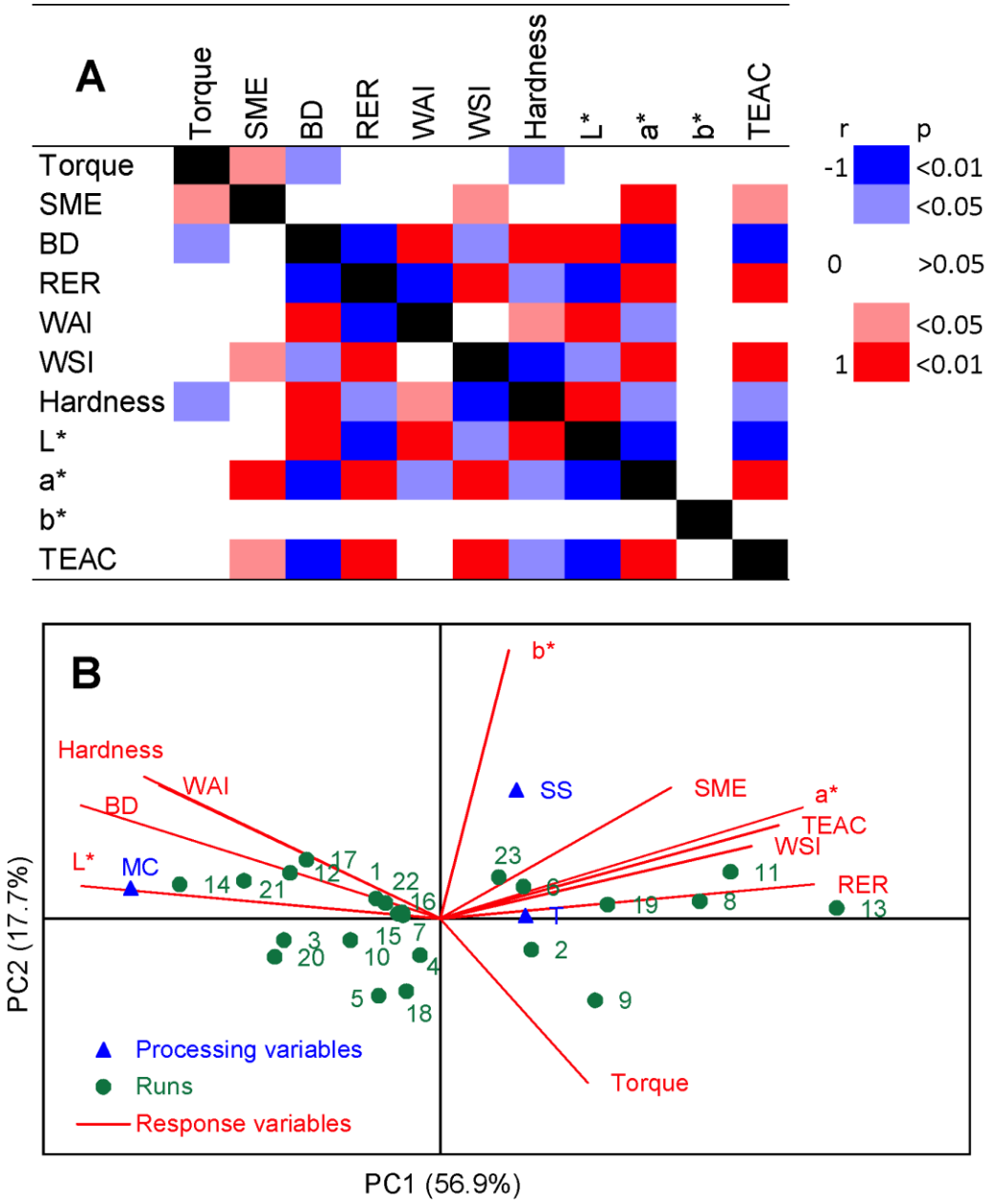

Figure 1.3: Correlation matrix of response variables (a) and principal components (PC) bi-plot showing Eigenvectors for response variables and loadings for processing variables and individual runs (b)[3]

[3] MC, moisture content; SS, screw speed; T, temperature; SME, specific mechanical energy; BD, bulk density; RER, radial expansion ratio; WAI, water absorption index; WSI, water solubility index; TEAC, Trolox equivalent antioxidant capacity.

CHAPTER 2: HEATING REDUCES PROSO MILLET PROTEIN DIGESTIBILITY VIA FORMATION OF HYDROPHOBIC AGGREGATES

2.1. ABSTRACT

Proso millet protein has reported structural similarities with sorghum. In order to explore the potential of this crop as an alternative protein source for people with gluten sensitivity, in vitro protein digestibility was analyzed. De-hulled proso millet flour was subjected to various processing techniques (dry heating and wet heating). The results indicated that regardless of the processing technique there was a significant decline (p value ≤ 0.05) in digestibility of protein in proso millet flour when compared with unprocessed flour (from 79.7±0.8% to 42.0±1.2%). Reduced digestibility persisted even when cooking with reducing agents. Heating in the presence of urea (8 M) and guanidine HCl (4.5 M) prevented the reduction in observed digestibility (urea cooked: 77.4±0.8%; guanidine HCl cooked: 84.3±0.9), suggesting formation of hydrophobic aggregates during heating in water. This was supported by an increase in surface hydrophobicity upon cooking. Thus, the proso millet protein, termed Panicin, forms hydrophobic aggregates that are resistant to digestion when subjected to heat.

2.2. INTRODUCTION

Gluten-free foods are an important and expanding market. In a recent survey, it was found that the sales of gluten-free foods grew by 34% annually in the last five years[1] and is expected to continue to rise. The driving force for this rise is the increase in diagnosed gluten intolerant cases and changing mindsets towards gluten in the diet. In the US, this has created a demand for alternative gluten-free crops that can replace wheat in familiar foods with the added advantage of nutrition, cost effectiveness and availability.[2] Among gluten-free grains (e.g., rice, millets, teff, sorghum), rice is the most widely produced and consumed crop, but has a low protein content compared with wheat. Lately millets (e.g., finger, pearl, proso, foxtail) have been utilized as gluten-free alternatives.

Among different millet varieties, proso millet is the only type of millet grown on a commercial scale in the US. Proso millet (e.g., true millet, common millet, hog millet) is an important crop from an agricultural standpoint in the Great Plains of the US due to its short growing duration, low water requirement, and resistance to pests and diseases. Furthermore, millet can lead to improvement in yields of wheat, corn and sorghum when grown in rotation with these crops.[3-4] The crop belongs to the genus *Panicum* and is more closely related to inedible grasses like switchgrass and panicgrass than to other millet species. Different millet types do not bear species or genus similarity (although pearl, proso, and foxtail millets belong to same subfamily, Panicoideae, which also includes maize and sorghum) and are grouped together based only on the basis of their small grain size and drought-resistance. Thus, different millets would be expected to have different physical and chemical properties.

Nutritionally, proso millet is a good source of protein, vitamins and minerals and its nutritive parameters are comparable or better than common cereals.[5] Reports have suggested that the quantities of nutrients in millet are very similar to the recommended ratio of protein, carbohydrate, and lipid.[6] Also, research on proso millet protein has provided evidence for its beneficial role in cholesterol metabolism and liver injuries.[7]

The protein content of proso millet (13%) is similar to wheat with the added advantage of being gluten-free. This makes proso millet a potential candidate as an alternative protein crop. However, there is dearth of information on the composition and quality of proso millet protein. While Lorenz[8] found lower lysine content in proso millet protein compared to wheat, Kalinova[6] reported the opposite results. Since sorghum and proso millet belong to the same subfamily a generalized statement is usually made for their protein structure, implying that these cereals contain similar types of proteins.[9] Furthermore, at times the protein behavior of different millet types (finger, foxtail, pearl etc) are assumed to be same despite belonging to different genera.[10] There is also contradiction pertaining to proso millet protein digestibility. Ravindaran[11] reported an improvement in digestibility of proso millet proteins on cooking, while Kovalev[12] found a reduction in protein digestibility upon cooking. These discrepancies demand a thorough understanding of proso millet protein quality.

In vitro protein digestibility techniques are often a first step in measuring cereal protein quality due to their rapidity and sensitivity. A number of in vitro techniques with varying protease types and concentration, incubation conditions and end product analysis techniques have been used to measure protein digestibility of various foods.[13-14] Among these techniques, the residue method, which employs pepsin as the main proteolytic

enzyme,[15] has been successfully used for measuring protein quality of mainly sorghum, but has also been applied to other cereals.[10] Thus, the established technique for sorghum digestibility was also employed as a first step studying digestibility of proso millet flour.

Since all cereals are subjected to some kind of processing technique before being consumed, this study was mainly focused on understanding the effect of different processing techniques on the in vitro protein digestibility of proso millet flour. The secondary objective was to determine the cause of any changes in digestibility of proso millet protein upon cooking.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Commercially available de-hulled proso millet was obtained from Clean Dirt Farms (Sterling, CO, USA). Eight pure cultivars of proso millet (Early Bird, Sunrise, Cope, Snobird, Plateau, Horizon, Sunup and Huntsman) were also used in this study. These samples were grown at Scottsbluff, Nebraska, USA. For comparison, whole finger millet, [University of Nebraska-Lincoln (UNL) NeFm #1], pearl millet (UNL NM-4B), and foxtail millet (UNL N-Si-7), grown in 2013 or 2014 at Mead, NE, USA, were used in this study. Whole white sorghum (UNL3016) and wheat (variety McGill) were grown in Lincoln, NE USA. Sorghum grains were decorticated using a laboratory scale decorticator (Venables Tangential Abrasive Dehulling Device, Venables Machine Works, Ltd, Saskatoon, Canada) for 60 seconds before being milled. All grains were milled using a pilot scale hammer mill (20SSHMBD, C.S. Bell, Tiffin, OH, USA) with screen size of 0.7 mm.

Chemicals. The following chemicals and enzymes were used in the study: α-amylase (3,000 U/mL) and amyloglucosidase (3,260 U/mL), each from Megazyme (Bray, Ireland); pullulanase (E2412), pepsin (P7000), pancreatin (8X USP) sodium azide, sodium hydroxide, phosphoric acid (85%), sodium bisulfite, 2-mercaptoethanol, sodium chloride, potassium phosphate dihydrate, sodium dodecyl sulfate, acrylamide, 2,4,6-trinitrobenzenesulfonic acid (TNBS, P-2297), Folin-Ciocalteu phenol reagent (2 N), sodium dihydrogen phosphate, 8-anilinonaphthalene-1-sulfonic acid (ANS), urea, phenol, sodium bicarbonate, and sodium carbonate, each from Sigma-Aldrich (St. Louis, MO USA); and guanidine HCl, tetramethylethylenediamine(TEMED), ammonium persulphate, and coomassie brilliant blue (R-250), each from Thermo Fisher (Waltham, MA USA).

2.3.2. Extraction of millet proteins

Millet proteins were separated from starch and fiber components by a wet milling method.[16] Modifications and additions to the published method to improve purity of millet protein were made based on preliminary experimentation. Briefly, 250 g de-hulled millet grains were steeped in distilled water for 3 h at 40 °C followed by overnight steeping at 4 °C. The steeped grains were washed and then blended with 500 mL fresh water in a Waring blender (Dynamic Corp. of America, New Hartford City, CT, USA) with the blades reversed for 4 min. The blender was attached to an autotransformer (Staco Energy Products Co., Dayton, OH, USA) and power (120 V) was adjusted to 80%. The slurry obtained after blending was filtered through a #16 mesh sieve (1.18 mm openings; Air Jet Sieve, Hosokawa micron powder systems, NJ, USA). The slurry passing through the filter was passed through a plate mill (Quaker City model no. 4-E,

The Straub Co., Warminster, PA). The finely ground slurry was then filtered through #100 mesh sieve (150 µm openings). The filtrate obtained was centrifuged at 5,000 x g for 15 min at 4 °C. The supernatant obtained was discarded and the top gray layer (protein) was scarped from white (starch) bottom layer of pellet. The scraped off protein fractions were mixed from different centrifugations and washed with water followed by repeated centrifugation and protein layer separation. When there was no more visible distinction left between protein and starch layers, the mixture was subjected to hydrolysis by α-amylase (0.05 mL / g solids), amyloglucosidase (0.7 mL/g solids) and pullulanase (0.12 mL/g solids) for 48 h at 37 °C and pH 5 (sodium acetate buffer) with 0.01% sodium azide. The tube was then washed twice and freeze dried and the protein content was measured by combustion.

2.3.3. Amino acid profile

For amino acid analysis, samples were hydrolyzed for 24 h using 6N HCl containing 0.5% phenol and derivitized.[17] Briefly, the hydrolyzed samples were dried and amino acids were reconstituted with 1 mL of 20 mM HCl. The constituted amino acids and hydrolysate standard amino acid mixture were reacted with the AccQ-Tag derivatization chemistry (Waters, Milford, MA, USA), following this they were separated detected and quantified using reversed phase HPLC (Agilent Infinity 1290 HPLC-DAD). The concentrations were calculated using a series of standard dilutions run alongside the samples and reported as g/kg protein. Under the acid hydrolyzed conditions cysteine is converted into cysteic acid and methionine into methionine sulfone. During the process tryptophan is destroyed, hence tryptophan was not determined.

2.3.4. Phenolics

Phenolics in acidified methanol extracts from different samples were quantified using Folin-Ciocalteu reagent.[18] Results were expressed as mg of gallic acid equivalents per gram of sample.

2.3.5. Heating in excess water

Samples (400 mg) of de-hulled proso millet, whole proso millet, wheat, de-hulled sorghum, finger millet, pearl millet, and foxtail millet were suspended in 5 mL of water and then heated at 25-100 °C (in 15 °C increments) for 20 min. After heating, the samples were cooled to room temperature and then subjected to digestibility measurements (see 'In vitro protein digestibility').

2.3.6. Heating in limited water

The moisture contents of de-hulled millet flour, sorghum and wheat flour were adjusted at 10, 20 and 30% by the addition of a calculated volume of water. The flours were then hermetically sealed in a glass vial (2 mL) and subjected to oven heating (VWR Scientific, 1350F Forced Air Oven, IL) or pressure-cooking (Deni Electric Pressure Cooker, Model 9780, Keystone Mfg company, Buffalo, NY) at 120 °C for 20 min. After cooling, 400 mg of sample was subjected to digestibility measurements (see 'In vitro protein digestibility').

De-hulled millet flour, sorghum flour, and wheat flour were extruded using a laboratory scale co-rotating conical twin screw extruder at a screw speed of 210 rpm (CTSE-V, C.W. Brabender, Hackensack, NJ, USA).[19] The extruder barrel had 4 temperature zones with temperatures of the first two zones maintained at 50 and 80 °C, respectively and last two zones at 120 °C. The moisture of the flours was adjusted to 17% (wet basis) and equilibrated overnight before extruding. The extrudates were ground

using cyclone sample mill (UDY, Fort Collins, CO, USA) with a screen size of 1 mm and subjected to pepsin digestibility measurement (see 'In vitro protein digestibility').

2.3.7. Heating in the presence of reducing agents

Millet, sorghum and wheat flour (400 mg) were heated (100 °C/ 20 min) in 5 mL of either 0.1 M sodium bisulfite or 0.1 M 2-mercaptoethanol,[20] and then subjected to in vitro digestion (see 'In vitro protein digestibility').

2.3.8. Heating in the presence of chaotrops

In order to explore any hydrophobic interactions, millet flour (400 mg) was heated (100 °C/ 20 min) in 5 mL of 1 M or 8 M urea or 4.5 M guanidine hydrochloride. Following cooking, urea and guanidine hydrochloride were removed by dialysis (MW 12-14 kDa) overnight against water. After dialysis, the samples were subjected to in vitro digestion (see 'In vitro protein digestibility').

2.3.9. In vitro protein digestibility

Pepsin digestibility was measured using residue method developed by Mertz et al., 1984.[15] with slight modifications.[21] After digestion, the nitrogen remaining in the pellet was measured and pepsin digestibility was calculated according to the following equation: PD (%) = $[(N_i - N_f)/N_i] \times 100\%$, where N_i was the concentration of N in the sample before digestion and N_f was the concentration of N in the recovered pellet after digestion.

Gastrointestinal proteolysis was also simulated using the method described by Mandalari et al.[22] with some modifications. Individual tubes were maintained in duplicate for various time periods between 0-120 min for gastric digestion and 0-240 min for intestinal digestion (following 2 h of gastric digestion; 120-360 min total digestion time)

along with a blank (no enzyme tube). In each tube, 100 mg of flour was suspended in simulated gastric fluid (SGF; 4 mL, 0.5 M NaCl, pH:2.5) to achieve a pH of 2.5 (approximately 4 mL) and incubated at 37 °C for 10 min. To analyze the effect of heating, flour samples (100 mg) were first suspended in water 1 (mL) and heated at 100 °C for 20 min prior to adding SGF and proceeding with the gastric digestion phase. The contents were then mixed with pepsin dissolved in SGF to give an activity of 200 U of pepsin/mg of protein in sample and incubated for the specified time. Gastric digestion was stopped by raising the pH to 7 using 0.5 M sodium bicarbonate. For intestinal digestion, pepsin hydrolyzed samples were mixed with 1 mL simulated intestinal fluid (SIF) (0.05 M KH_2PO_4, pH: 7.0) and warmed at 37 °C. Meanwhile, pancreatin (1.5 mg/mL) was suspended in SIF and centrifuged. The supernatant (3 mL) was added to flour mixture and incubated for the specified time. Intestinal digestion was stopped by plunging the tubes into a boiling water bath for 5 min and then placing on ice. Gastric and intestinal digested tubes were centrifuged and the supernatants were used to quantify degree of hydrolysis (DH) while the pellets were analyzed for insoluble nitrogen (protein).

The DH, or extent of proteolytic hydrolysis, was determined by the reaction of free amine groups with TNBS using leucine as the standard.[23] DH was then calculated using the following equation: DH (%) = $(h_s/h_{total}) \times 100\%$, where h_s was defined as the mmol of free amine groups (leucine equivalents) per gram of protein (initial) in the sample and h_{total} was the mmol of free amino groups per gram of protein assuming complete hydrolysis of the protein (7.96 mmol/g protein). This was calculated by summing the molar concentrations of individual amino acids per gram of protein (see

'Amino acid profile' section and Supplementary Table 1). All tubes (representing duplicate samples from above) were measured thrice.

2.3.10. Electrophoresis

Proteins before and after digestion or with and without heating were extracted from 50 mg of sample. If samples were wet, they were dried overnight at 50 °C. First, samples were heated at 100 °C for 20 min in 1.5 mL of 0.0125 M sodium tetraborate buffer (pH 10) containing 1% SDS and 2% 2-mercaptoethanol and then extracted for 2 h at room temperature followed by centrifugation.[24] Alternatively, 8 M urea was added to the extraction buffer when testing the effects of urea on protein extraction. The supernatant was subjected to SDS-PAGE analysis using a vertical mini gel system (Mini protein II cell tetra system, Biorad, CA). Protein extract was mixed with sample buffer in the ratio of 4:1 and loaded along with molecular weight markers (BioRad Precison Plus Dual color protein standard, 10-250 KDa) on to gel system with following specifications. The resolving gel consisted of 12% polyacrylamide in 1.9 M Tris HCl buffer (pH 8.8), and 1% SDS (w/v). The stacking gel contained 5% polyacrylamide in 0.63 M Tris HCl buffer (pH 6.8), and 1% SDS (w/v). TEMED (0.05% v/v) and ammonium persulfate (0.1 v/v) were used to polymerize the gels. Electrophoresis was done at 100 V for 60 min in tank buffer consisting of 1.9 M Tris, and 1% SDS (w/v). After electrophoresis, the gels were stained with 20 mL of coomassie brilliant blue reagent (0.25%) containing isopropanol and acetic acid for 60 min. De-staining was achieved by washing gels several times in a solution of 10% acetic acid and 30% methanol in water. Gel images were captured and analyzed using Image Analyser (BioRad Molecular Imager, Gel Doc-XR system, CA).

2.3.11. Surface hydrophobicity

Fluorescence intensity of millet proteins were measured both intrinsically and using an external fluorescence probe (8-anilinonaphthalene-1-sulfonic acid, ANS) as described.[25] Intrinsic fluorescence of proteins is mainly attributed to tryptophan, a hydrophobic amino acid when the excitation wavelength is higher than 290nm. Two sets of samples of millet proteins were dispersed in 0.01 M phosphate buffered saline (PBS; pH 7, 10% NaCl, 2.5% sodium dihydrogen phosphate) with concentrations ranging from 0.01% to 0.1%. One set of millet proteins were heated at 100 °C for 20 min while other was maintained at room temperature. For measuring intrinsic fluorescence, both heated and unheated samples were excited at 295 nm and the fluorescence intensity recorded at 415 nm using a spectrofluorometer (LS55, PerkinElmer Inc., Walthman, MA). This was the maximum intrinsic fluorescence intensity based on a scan from 300-700 nm. For extrinsic fluorescence, the heated and unheated samples were mixed with 20 μL of ANS (8 mM in 0.01 M PBS) and incubated for 2 h at room temperature in dark. The fluorescence intensity was recorded at an excitation wavelength of 365 nm and emission wavelength of 520 nm.

2.3.12. Statistical analysis

The overall treatment effects were first analyzed using ANOVA. Following ANOVA, the specific difference among treatments were assessed using either t-test, Tukey's test or Dunnett test at a significance level of 0.05. The multiple comparison test used was specified in footnotes. All descriptive statistics were computed using JMP statistical software (JMP version 12.0.1, SAS Institute Inc.)

2.4. RESULTS AND DISCUSSION

2.4.1. In vitro protein digestibility of grains heated in excess water

Proso millet, sorghum, and wheat flours were heated in excess water at temperatures ranging from 25 °C to 100 °C for 20 min. Following the holding time all flours were first cooled to room temperature (approximately for 10 min) and then prepared for pepsin digestibility by warming at 37 °C. Holding the flour-water slurries at temperatures above 40 °C before digestion resulted in a significant decrease in digestibility of sorghum and proso millet proteins while no change in wheat protein digestibility was observed (Figure 2.1). At the higher temperatures (85 °C and 100 °C), the digestibility of proso millet protein was significantly less than for sorghum. At 100 °C, a 50% decline in digestibility of millet flour was observed when compared with unheated flour.

Poor protein digestibility may be positive or negative depending on perspective. Poor protein digestibility of foods consumed as staples in a limited diet can have negative consequences on health. In contrast, in the developed world, where protein is often consumed far in excess of dietary requirements, low protein digestibility could constitute a lower calorie food that could help reduce energy intake. Under these circumstances, however, the same undigested protein may serve as substrate for large bowel fermentation resulting in toxic metabolites that can trigger disease.[26]

In general, cooking cereal flours or proteins in excess water has little impact on pepsin digestibility, [27] as seen for wheat flour. However, reduction in pepsin digestibility upon cooking has been reported for sorghum.[20] This unusual property of sorghum grain has been attributed mainly to extensive disulfide cross-linking of proteins upon heating. [28] A decrease in pepsin digestibility upon heating proso millet flour has not been reported previously. On the contrary, one report suggested an increase in digestibility upon heating

proso millet[9] These contradictory results could be due to varietal differences or differences in assay technique. Therefore, both possibilities were explored.

Eight cultivars of whole proso millet flour were assayed for pepsin digestibility along with three other types of millet (Table 2.1). The pepsin digestibility of raw proso millet cultivars was significantly different (p value ≤ 0.05) and ranged from 77 to 85%. But upon cooking all proso millet cultivars showed similar dramatic decreases in digestibility. Heating also reduced the digestibility of pearl millet and foxtail millet flour but the reduction was not as drastic as observed for proso millet. In contrast, cooking actually improved the digestibility of finger millet protein. These results were in accordance with previous studies.[29-31]

Protein digestibility was also assayed using a sequential digestion procedure mimicking both gastric and small intestinal digestion (Figure 2.2). This showed that heating reduced digestibility of proso millet flour during both the gastric (pepsin) and small intestinal phases (pancreatin). Thus, both the pepsin digestibility assay and the sequential digestion assay confirmed that heating of proso millet flour had a significantly detrimental effect on its protein digestibility.

2.4.2. In vitro protein digestibility of grain heated in limited water

Previous studies have suggested that heating sorghum flour in limited water has less of a negative impact on protein digestibility than heating in excess water.[21,32] The above experiments demonstrated that, like sorghum, proso millet protein digestibility also decreases when heating in excess water. Therefore, proso millet, sorghum, and wheat flours were adjusted to 10%, 20%, or 30% moisture (wet basis) and then heated in sealed containers in an oven or autoclave and protein digestibility was subsequently assayed.

Oven heating and autoclaving of proso millet flour, even at low moisture contents, gave similar results to heating in excess water with a 28% to 46% reduction in digestibility (Table 2.2). It is remarkable that the digestibility of millet flour reduced so dramatically even when heated with as little as 10% moisture. Heating sorghum flour with limited water also decreased digestibility, but the effect was not nearly as dramatic as for proso millet.

Extrusion, being a common low-moisture food processing technique, was also tested. Extrusion resulted in a dramatic decrease in digestibility of proso millet proteins (Table 2.2). In accordance with previous studies, only minimal reduction in digestibility was observed when sorghum was extruded. It has been suggested that the lower impact of dry heating on sorghum protein digestibility is due to limited formation or hydrolysis of disulfide linkages during heating or high shear.[13] As expected, there was no change in digestibility of wheat flour on processing.

2.4.3. Effects of exogenous factors on protein digestibility of proso millet protein

Poor digestibility of sorghum protein in general has been attributed to protein interactions with dietary fiber, starch, polyphenols, phytic acid, and other cell wall constituents.[33] The de-hulled proso millet flour used in this study had the lowest concentration of extractable phenolics among the grains studied herein (data not shown). This was expected because the grain was de-hulled. To determine if interactions among other flour components were affecting proso millet protein digestibility, proso millet protein was partially purified using a wet-milling procedure. The focus of wet-milling is usually to isolate the starch from a grain; however, it is also an efficient way to isolate protein without denaturing it (as happens when extracting with aqueous alcohols). Wet-milling of proso millet resulted in a fraction that contained 80% protein (wet basis) (%N X

6.25). Upon heating this material, a decline in digestibility similar to heated proso millet flour was observed (Figure 2.3). These results suggested that the reduction in digestibility of proso millet protein upon heating was not due to exogenous interactions. Therefore, further experiments focused on intermolecular interactions among proso millet proteins as the cause for the decreased digestibility.

2.4.4. In vitro protein digestibility of grain heated in the presence of reducing agents

Proso millet, sorghum, and wheat flours were heated in the presence of 2-mercaptoethanol or sodium bisulfite and their pepsin digestibility was measured. There was an improvement in digestibility of sorghum flour when heated in presence of either 2-mercaptoethanol or sodium bisulfite (Figure 2.4). In contrast, heating in the presence of these reducing agents did not improve the digestibility of proso millet protein. There was no significant change observed in digestibility of wheat flour when heated in reducing agents compared to water.

The improvement in digestibility upon heating sorghum with reducing agents has been reported previously.[20] Sorghum kafirins are more resistant to proteases than other prolamins due to extensive disulfide linkages that form during heating.[28] Thus, treating sorghum flour with reducing agent like 2-mercaptoethanol improved the digestibility during heating by reducing internal di-sulfide bond formation. However, because these reducing agents had no effect on the digestibility of proso millet, the reduction in digestibility of proso millet proteins upon heating was probably not due to disulfide bond formation.

2.4.5. In vitro protein digestibility of millet flour heated in the presence of chaotropes

We hypothesized that heating proso millet flour denatures protein thereby exposing hydrophobic amino acids that form compact hydrophobic aggregates at partial state of denaturation that limit proteolytic hydrolysis. At high concentrations chaotropes are known to prevent aggregation of proteins by binding to exposed hydrophobic amino acids.[34] Therefore, millet flour was heated in the presence of 1 M and 8 M urea followed by extensive dialysis to remove the urea and then digestibility was assayed.

While heating in the presence of 1 M urea had no effect on digestibility, heating in the presence of 8 M urea resulted in a significant improvement in digestibility when compared to heating in water. This was likely due to the interaction of urea with less polar amino acid residues by H-bonding, which stabilizes the partially unfolded protein conformation during denaturation by heating and impedes hydrophobic interactions between protein chains.[35] With continuous heating the denaturation of millet proteins is completed and on removal of urea they go back to original conformation which is digestible by *in-vitro* assays.

Similar to urea, guanidine HCl is also a known denaturant involving hydrophobic interactions but with a different mechanism. It is thought that guanidium ions coat protein hydrophobic surfaces preventing the aggregation of hydrophobic amino acid residues.[34] When millet flour was heated in presence of 4.5 M guanidine HCl it was found that the digestibility was same as uncooked flour (Table 2.3). Thus, two reagents that prevent hydrophobic aggregation of proteins supported our hypothesis of formation of hydrophobic aggregates in millet proteins during heating in presence of water.

2.4.6. Surface hydrophobicity

To evaluate the change in surface hydrophobicity upon heating, proso millet protein was mixed with ANS, a fluorescent probe that binds to the exposed hydrophobic regions on a protein which leads to an increase in its fluorescence quantum. For unheated proso millet protein there was no change in fluorescence intensity of ANS as protein concentration increased (Figure 2.5). In contrast, when millet protein was heated there was an increase in fluorescence intensity of ANS, suggesting more hydrophobic groups were exposed on heating and could readily bind with ANS.

Intrinsic fluorescence (IF) of millet protein was also measured, which is mostly due to exposed tryptophan residues on the protein surface. Though the IF value for the system is very low but the IF intensity was greater for heated millet protein compared with unheated millet protein. This suggests that there is a change in millet protein on heating which is potentially due to the exposure of tryptophans to aqueous phase due to opening of protein structure. Cumulatively both these results supported the hypothesis that the reduced digestibility could be due to formation of hydrophobic aggregates in millet proteins during heating in presence of water.

2.4.7. Changes in proso millet protein profiles upon heating

Change in proso millet protein profiles upon cooking and digestion were visualized on SDS-PAGE. After digestion of uncooked proso millet flour, a decrease in the prolamin band (20 kDa) intensity was evident (Figure 2.6; lanes 1 and 3). This indicated good digestibility of unheated proso millet flour. In contrast, only faint prolamin bands were seen in the cooked proso millet protein extracts both before and after digestion, and did not show noticeable changes upon digestion (Figure 2.6; lanes 2 and 4). The low intensity of the

prolamin band in the cooked samples was not due to loss of the protein; it was due to the inability of the reducing buffer to extract the protein after cooking. This was evident because 91% of the protein remained in the pellet after extraction of the cooked flour (data not shown). The inability of the extraction buffer, which contained reducing agents, to extract proso millet prolamins after cooking supported our previous results that suggested the changes in millet protein upon cooking are not driven by disulfide linkage formation. Furthermore, when urea was added to the extraction buffer, extraction of the prolamin fraction was greatly improved in heated proso millet flour (Figure 2.6; lanes 2 and 6); thus, conforming the ability of urea to prevent hydrophobic aggregation. Finally, when the sample was cooked in urea and then digested (following removal of urea), digestibility was good for both unheated and heated samples as evidenced by lack of prolamin bands (Figure 2.6; lanes 7 and 8).

2.4.8. Amino acid profile

On comparing the amino acid profile of proso millet flour, sorghum flour and wheat flour it was found that hydrophobic amino acids contributed to 51% of total amino acids in proso proteins while for sorghum and wheat, hydrophobic amino acids contributed 48 and 37%, respectively, of total amino acids (Supplementary Table 2.4). The results obtained for sorghum and wheat are consistent with previous findings.[36] Although the proportion of hydrophobic amino acids in sorghum and millet proteins were similar, the different causes for lower digestibility post cooking could be due to the sequence of amino acids in the primary structure of the protein

Proso millet storage proteins (prolamins) have been reported to be structurally similar to sorghum prolamins, or kafirins, and thus have been commonly grouped together.

However, while both sorghum and proso millet proteins possess the unique property of low digestibility upon cooking, the present study has shown that although the observed effect is similar the mechanism involved is different. Thus, proso millet prolamins are unique among the cereal proteins and deserve their own nomenclature. In following the pattern used for some other cereal proteins, including corn (zein), rye (secalin), and barley (hordein), we propose that proso millet prolamins should be named ‘panicin’. This name is particularly appropriate because some old manuscripts from India indicate that proso millet as a staple crop in the Neolithic era was called ‘Pani’.[37-38]

The objective of the present study was to assess the potential of proso millet as an alternate crop for gluten intolerant people with prime focus on protein digestibility as affected by processing. The results indicated that regardless of processing technique, there was a 50% decline in digestibility of proso millet protein when compared with unprocessed flour. Heating the flour in the presence of reducing agents did not improve the digestibility but denaturants like urea and guanidine HCl did. The improvement in protein digestibility of proso millet in the presence of these agents suggested formation of hydrophobic aggregates in proso protein upon cooking. This indicates a unique property of proso millet protein among other cereal proteins.

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Table 2.1. Effect of heating in excess water on protein digestibility (%) of millets[a]

Sample	Unheated	Heated[b]	% change
Proso millet			
Early Bird	84.6 ± 0.3	37.5 ± 1.1	-56
Sunrise	75.8 ± 1.5	32.8 ± 0.9	-57
Cope	83.4 ± 0.8	39.1 ± 2.7	-53
Snobird	81.6 ± 0.9	37.9 ± 0.4	-54
Plateau	77.9 ± 1.3	42.8 ± 0.6	-46
Horizon	81.5 ± 1.1	37.7 ± 0.9	-54
Sunup	80.9 ± 0.2	37.7 ± 1.2	-54
Huntsman	77.4 ± 1.8	42.1 ± 2.3	-46
Other millets			
Finger millet (UNL NeFm #1)	62.3 ± 2.0	77.1 ±1.6	+24
Pearl millet (UNL NM-4B)	89.4 ± 0.8	79.7 ±0.8	-11
Foxtail millet (UNL N-Si-7)	79.6 ± 1.3	60.7 ± 1.2	-23

[a]Heating at 100 °C for 20 min; mean ± SD (n=3); [b]all heated samples were significantly different from their unheated counterpart (t-test, α=0.05).

Table 2.2. Effect of dry heating and extrusion on protein digestibility (%) of selected grain samples[a]

		Autoclave (121 °C, 20 min)			Oven (121 °C, 20 min)			
Sample	Unheated	10% moisture	20% moisture	30% moisture	10% moisture	20% moisture	30% moisture	Extrusion
Proso millet	79.7 ± 0.8	52.2 ± 1.8*	34.4 ± 1.3*	44.3 ± 0.8*	49.1 ± 1.4*	34.4 ± 1.6*	45.5 ± 1.2*	38.3 ± 1.1*
Sorghum	76.1 ± 2.8	66.6 ± 0.3*	40.6 ± 2.7*	39.2 ± 1.9*	49.7 ± 0.4*	37.3 ± 2.4*	38.6 ± 0.8*	69.9 ± 0.9*
Wheat	94.2 ± 0.6	92.4 ± 0.0	89.3 ± 0.8*	89.3 ± 1.3*	91.6 ± 1.2	89.3 ± 0.8*	88.6 ± 2.2*	93.5 ± 0.9

[a] Mean ± SD (n=3); *Means marked with asterisks are significantly different from their unheated counterpart (Dunnett Test at α=0.05).

Table 2.3. Effect of heating in urea and guanidine-HCl on protein digestibility (%) of proso millet[a]

Additive in heating water	Unheated proso millet	Heated proso millet
Urea (1M)	82.3 ± 0.8	37.9 ± 2.0[b]
Urea (8M)	82.0 ± 1.9	78.6 ± 0.2
Guanidine-HCl (4.5 M)	83.0 ± 1.4	84.3 ± 1.1

[a]Heating at 100 °C for 20 min; mean ± SD (n=3); [b]means marked with asterisks are significantly different from their unheated counterpart (t-test at α=0.05).

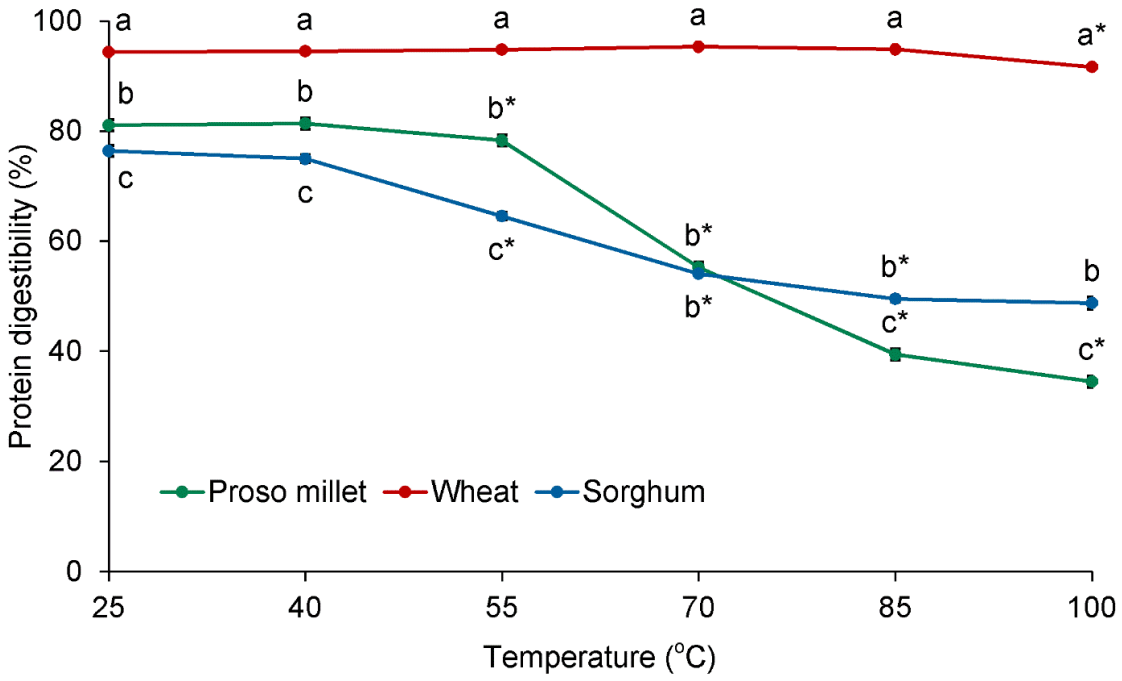

Figure 2.1. Effect of heating in excess water on protein digestibility of proso millet, sorghum, and wheat flours[4]

[4] Error bars show standard deviation (n=3); some error bars were too small to plot; a,b,c Means marked with different letters indicate significant differences among grains within temperature (Tukey's adjustment; α=0.05); *Means marked with asterisks indicate significant difference within grain type with its previous temperature.

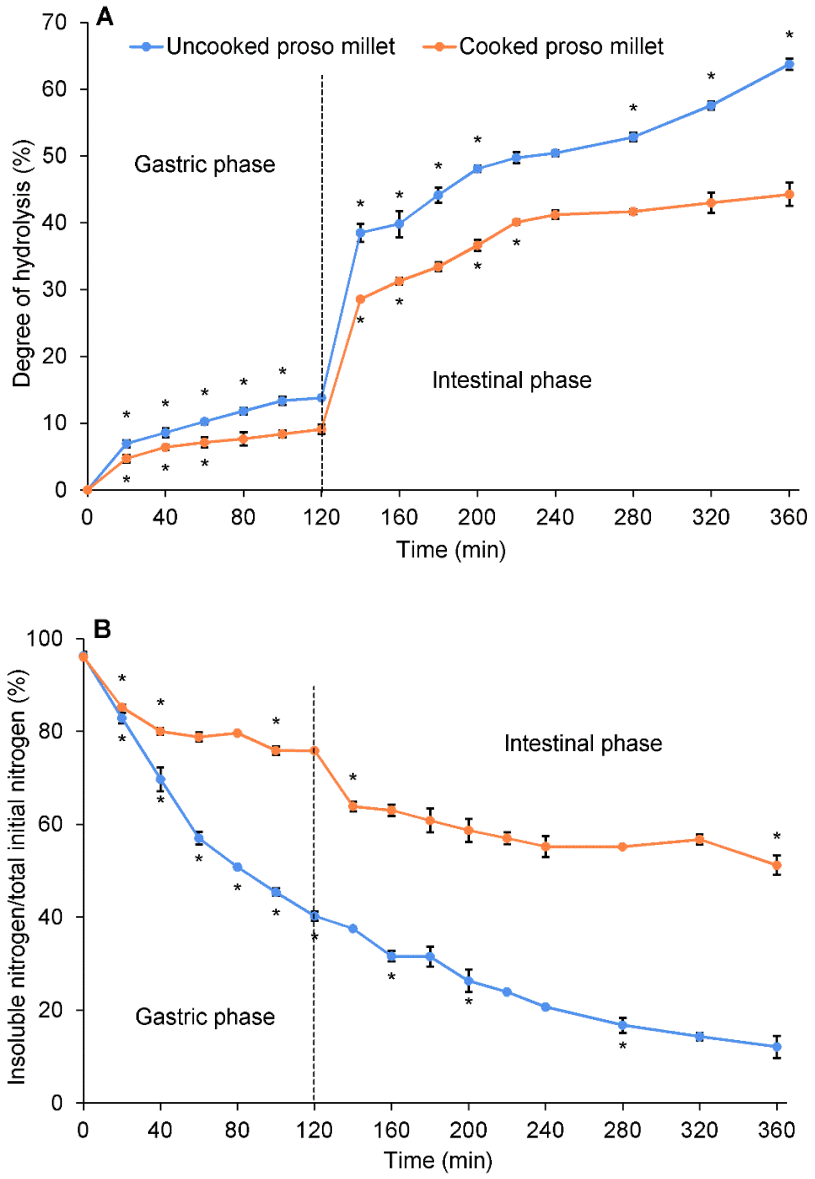

Figure 2.2. Sequential in vitro digestion of uncooked and cooked proso millet flour: (a) degree of hydrolysis and (b) insoluble nitrogen; dotted line indicates the cut-off between gastric and small intestinal phase of digestion[5]

[5] Error bars show standard deviation (n=2); (*) Means marked with asterisks indicate significant difference within heating condition with its previous temperature (t-test; α=0.05).

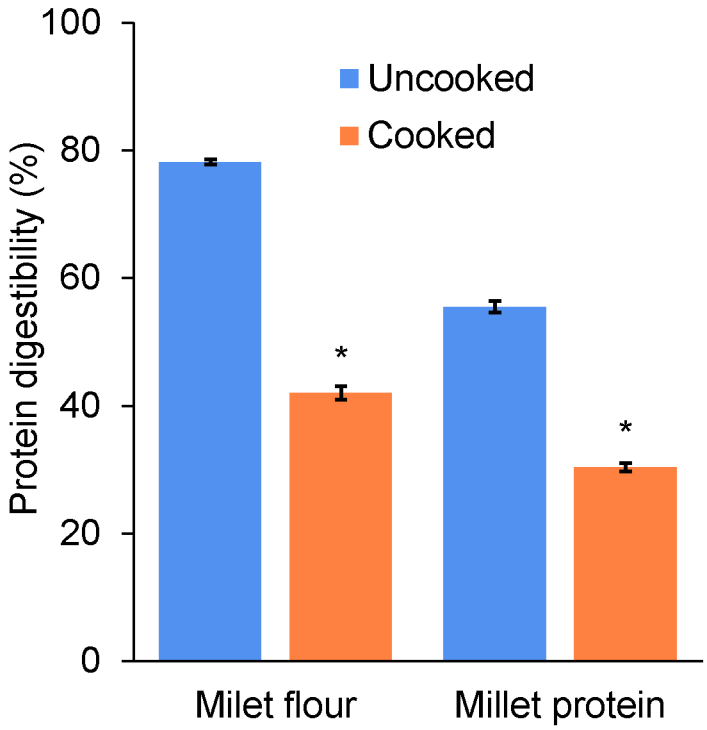

Figure 2.3. Effect of heating in excess water on protein digestibility of proso millet flour and semi-purified proso millet protein[6]

[6] Error bars show standard deviation (n=3); *means marked with asterisks indicate significant difference with its unheated counterpart

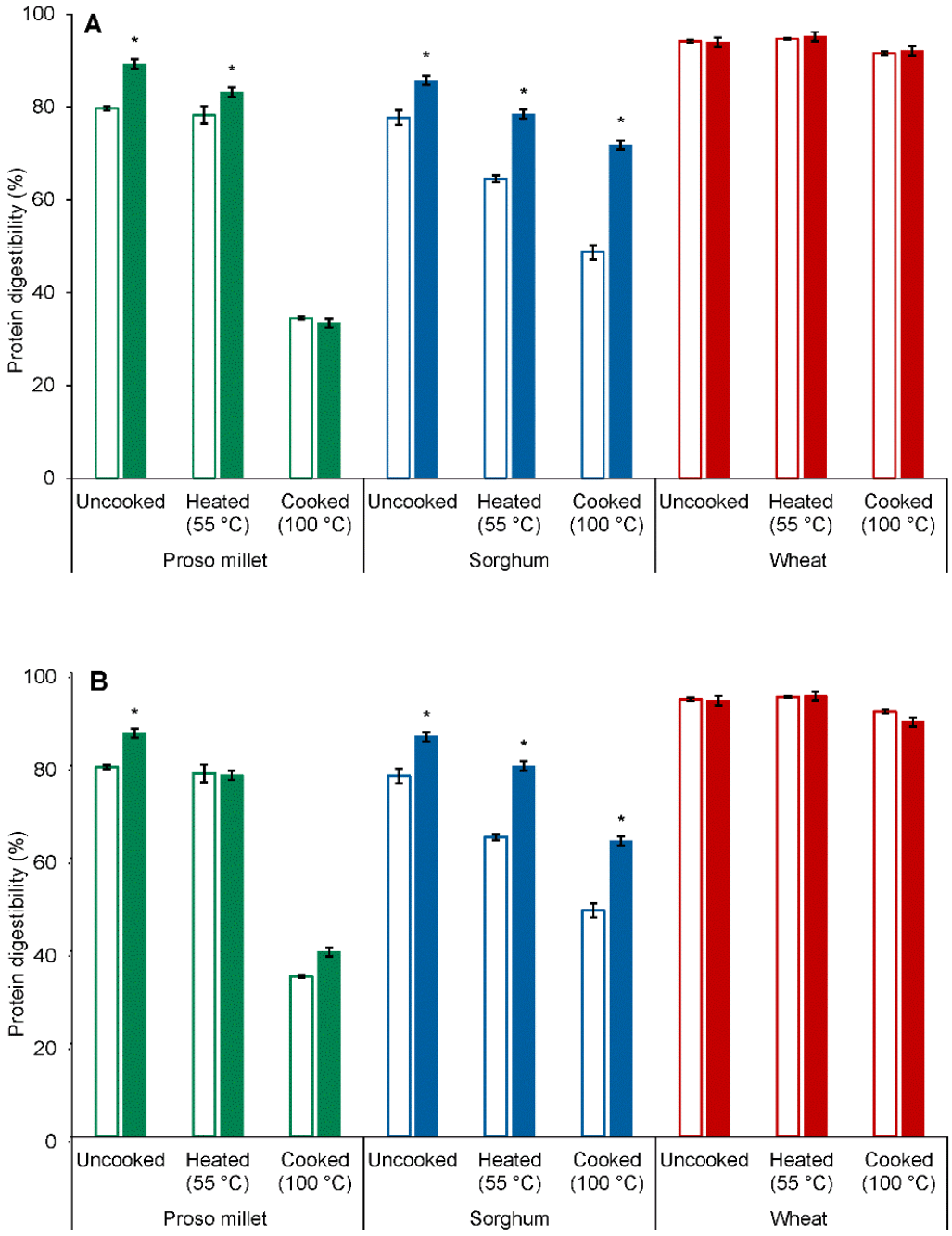

Figure 2.4. Effect of reducing agents on protein digestibility of proso millet, sorghum and wheat flour: (A) heating with 2-mercaptoethanol; (B) heating with sodium bisulfite, open bars: flour in water; solid bars: flour in reducing agents.[7]

[7] Bars marked with asterisks indicate a significant difference from the corresponding sample heated in water only (t-test, α=0.05).

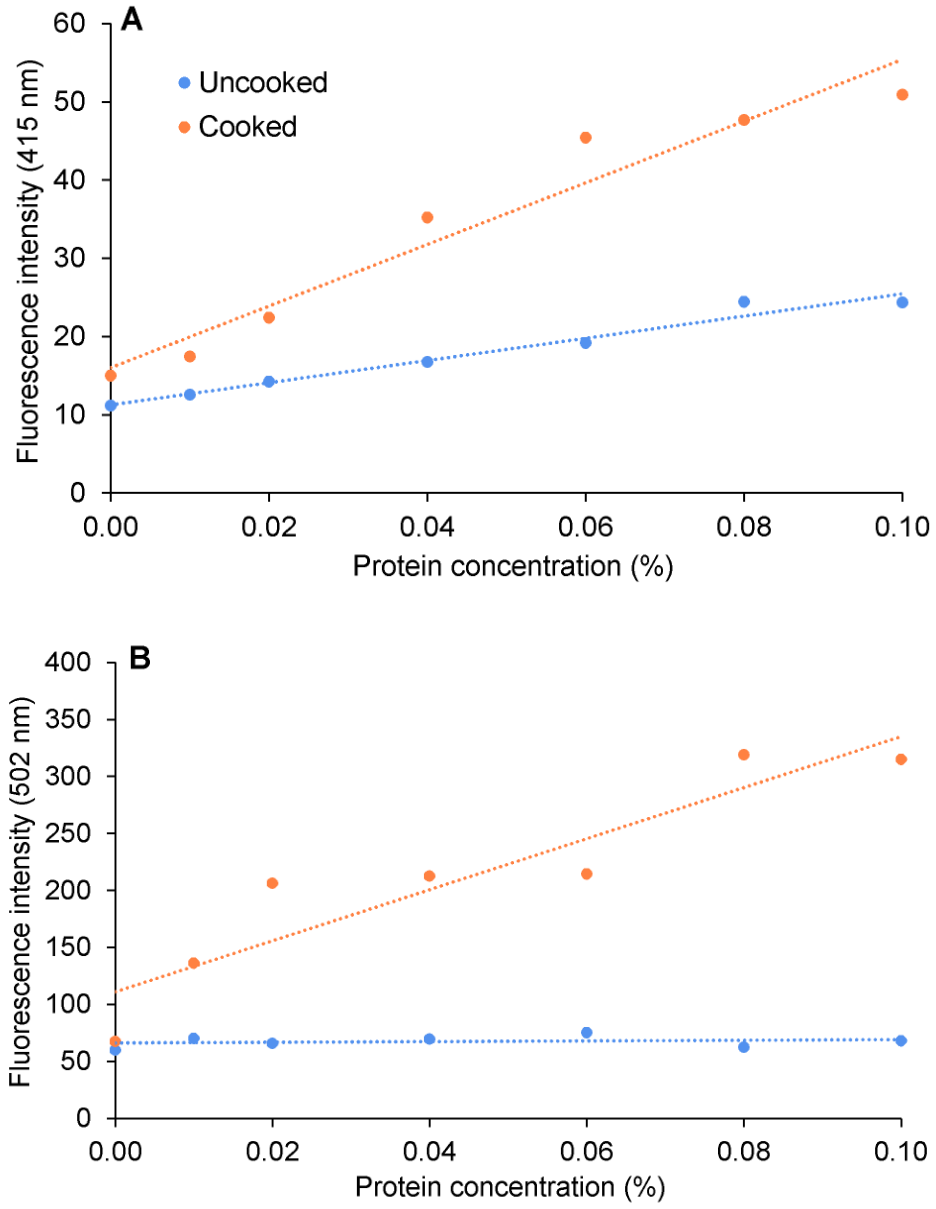

Figure 2.5. Effect of cooking on (A) intrinsic and (B) ANS based fluorescence of proso millet protein.

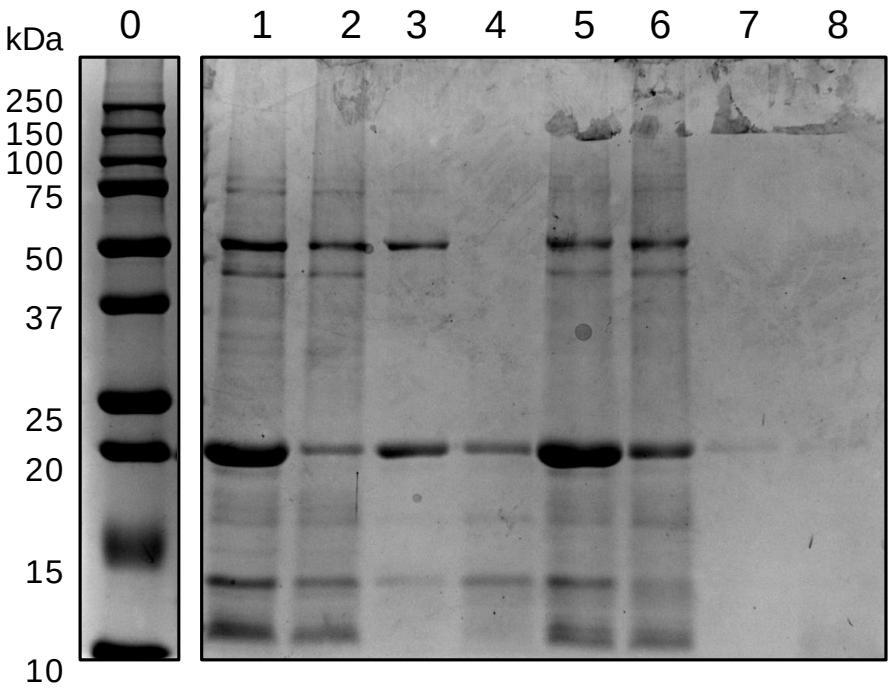

Figure 2.6. SDS-PAGE gels of proso millet flour[8]

[8] Lane 0, MW marker; 1, uncooked before digestion; 2, cooked before digestion; 3, uncooked after digestion; 4, cooked after digestion; 5, uncooked before digestion, extracted with buffer containing 8 M urea; 6, cooked before digestion, extracted with buffer containing 8 M urea; 7, uncooked placed in 8M urea and digested; 8, cooked in 8M urea and then digested (urea removed before digestion)

Table 2.4. (Supplementary) Amino acid composition of different flours (g/kg)

Amino Acid	Proso millet	Proso millet protein	Sorghum	Wheat
His	1.85	11.03	2.87	1.70
Ser	2.54	26.60	6.51	4.10
Arg	6.19	12.11	4.72	3.54
Gly	3.74	8.16	5.28	2.89
Asp	2.57	28.48	7.08	8.89
Glu	27.10	102.04	64.99	22.82
Thr	3.49	13.66	4.05	3.27
Ala	13.33	55.79	4.76	9.61
Pro	8.70	38.17	19.90	8.17
Lys	1.71	3.97	4.05	3.23
Tyr	1.42	13.05	0.04	1.31
Val	6.38	27.32	6.86	5.39
Ile	5.02	23.19	6.07	4.31
Leu	15.11	65.74	11.28	12.63
Phe	8.03	32.87	7.23	4.76
CysA	1.85	11.03	3.48	1.58
MetSO2	3.07	14.71	2.56	1.63
% hydrophobic amino acids	50.5	51.9	37.8	39.2

CHAPTER 3: MICROSTRUCTURAL CHANGES TO PROSO MILLET PROTEIN BODIES UPON COOKING AND DIGESTION

3.1. ABSTRACT

Cooking results in a drastic decline in digestibility of proso millet proteins, panicins. Scanning electron and confocal microscopy were used to observe morphological changes in proso millet protein bodies upon cooking and digestion that could be associated with the loss in digestibility. Spherical protein bodies (1-2.5 μm) were observed in proso millet flour and extracted protein. Cooking did not result in any noticeable change in the size or shape of the protein bodies. However, upon digestion with pepsin the poor digestibility of cooked proso millet protein was clearly evident from the differences in microstructure of the protein bodies: large cavities were observed in the uncooked protein bodies while cooked protein bodies had only tiny holes. When proso millet was cooked in 8 M urea and then digested, the protein bodies appeared similar to uncooked digested protein bodies. The morphological changes observed in proso millet protein upon cooking and digestion did not show any visible aggregates, but the inability of pepsin to digest cooked protein bodies was clearly evident under microscopy and is in agreement with the chemical analyses reported previously.

3.2. INTRODUCTION

Millets are a group of small seeded grains known for sustaining agriculture and ensuring food security in semi-arid regions (Amadou et al., 2013). The production and cultivation of millets is comparatively new to the western world and they are mostly cultivated to provide agricultural benefits rather than nutritional advantages (Lyon and Baltensperger, 1995). In the past few years there has been a rising interest in the nutritional quality of millets mainly due to the abundance of phytochemicals (phenolics and flavonoids) and their gluten free protein profile (Amadou et al., 2013).

Among different millet varieties (finger, foxtail, little, pearl etc.), proso millet (*Panicum miliaceum)* is the only millet variety grown on a commercial scale in the US. The majority of this crop is used as bird feed but recently there has been an increased interest in proso millet for human food due to the rapidly growing gluten free foods market (McDonald et al., 2003). Being gluten free with a protein content similar to wheat and higher than commonly consumed gluten free crops, proso millet is an ideal food choice for people with Celiac disease and individuals with gluten sensitivity. Thus, many researches are focused on ensuring nutritional adequacies of proso millet as human food or developing novel foods from proso millet (Taylor et al., 2014; Gulati et al., 2016; McSweeney et al., 2017).

Previously, we reported a unique property of proso millet protein that could be a matter of concern when promoting the crop as a gluten free food (Gulati et al., 2017). Specifically, we found that there was a significant decline in digestibility (more than 50%) of proso millet protein when it was heated above 55 °C. The effect observed was similar to the decrease in digestibility reported for sorghum proteins (Hamaker et al.,

1986), but more dramatic and with a different mechanism of action. Rather than being driven by disulfide bond formation as in sorghum, the digestibility of proso millet proteins declines upon heating due to intramolecular hydrophobic protein aggregation (Gulati et al., 2017).

The storage proteins of cereals are present along with minerals and enzymes required during seed germination in subcellular spherical organelles called protein bodies. Protein bodies typically have diameters ranging from 0.5-2.5 μm. Cereal protein hydrolysis by enzymes appears as protein body degradation initiated either at the periphery (from external enzymes) or internally which leaves behind large cavities (Ashton, 1976). Several researchers have reported the presence of spherical protein bodies (up to 2.5 μm in diameter) in proso millet and their association with starch granules (Jones et al., 1970; Zarnkow et al., 2007) but there has been no report on the morphological changes or appearance of these protein bodies when subjected to heating or enzymatic hydrolysis.

In the present study, microscopy was used to examine morphological changes that occur in proso millet protein bodies upon cooking both in water and urea. Based on our chemical findings we expected to observe 1) aggregates of protein bodies upon cooking as a result of hydrophobic association and 2) visual evidence of the inability of enzymes to hydrolyze cooked proso millet protein bodies. The objective of this research was to strengthen our understanding of temperature-induced changes in panicins that can help in preventing the loss in digestibility.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Commercially available de-hulled proso millet grains were obtained from Clean Dirt Farms (Sterling, CO, USA) and milled using cyclone sample mill (UDY, Fort Collins, CO, USA) with a screen size of 1 mm. The flour was stored at 4 °C until analysis. Proso millet protein and starch were extracted from proso millet grains using a wet milling method (Xie and Seib, 2000) as modified by Gulati et al. (2017).

The flour and protein and starch fractions were analyzed for ash, fat, moisture, protein, and starch using approved methods (AACC International, 1999). Protein content was analyzed using a nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25. Total starch content was analyzed using total starch assay kit (K-TSTA, Megazyme, Bray, Ireland) following the KOH format.

3.3.2. Cooking

Four hundred milligrams of flour, 200 mg protein, or 2 g starch, were suspended in 5 mL of water or 8 M urea in a centrifuge tube and heated at 100 °C for 20 min (time recorded after reaching boiling temperature) with intermittent mixing. After heating, the samples were cooled to room temperature and then either used directly for digestibility measurements or frozen at -80 °C for further analysis.

3.3.3. In vitro protein digestibility

Pepsin digestibility of cooked (water and urea) and uncooked proso millet flour and protein was measured using the residue method developed by Mertz et al. (1984) as described by Gulati et al. (2017). After digestion, the pellet was freeze dried (FreeZone 6, Labconco, Kansas City, MO) and used for microscopic analysis.

3.3.4. Scanning electron microscopy

A thin uniform layer of freeze-dried sample (cooked and uncooked millet flour, protein and starch) was fixed on an aluminum stub (26 mm diameter, 6 mm height) by tapping the sample tubes on adhesive conductive carbon tape (EMS, Hatfield, PA) and gently blowing off the extra sample using pressurized air. Samples fixed on the stub were kept overnight in a vacuum oven (Model 5831; NAPCO scientific, Tualatin, OR) at 20 KPa and 40 °C to remove any residual moisture. The dried samples were then sputter coated with chromium under an argon atmosphere using a Denton desk V TSC sputter apparatus (Denton Vacuum LLC, Moorestown, NJ) for 15 min (mean thickness of coating was 4-5 nm).

A field-emission scanning electron microscope (SEM) was used to study the morphological changes in millet proteins and starch upon cooking and digestion (Hitachi, S4700, Hitachi America Ltd., Tarrytown, NY) at an accelerating voltage of 5 kV and an emission current of 5μA. Samples were studied under different magnifications ranging from 500x to 10,000x and images were captured using built-in software (HI-S027-0003, Version 3.8). The size of protein bodies was determined using image processing and analysis software (ImageJ, 1.51s, National Institute of Health, USA).

3.3.5. Confocal laser scanning microscopy

A thin uniform smear of millet flour, protein or starch sample in water was placed on a clean glass slide and covered with a cover glass and observed under Nikon A1 confocal laser scanning microscope (CLSM) mounted on a Nikon 90i upright fluorescence microscope (Nikon Instruments Inc., Melville, NY) at approximately 1200x magnification. The samples were subjected to an excitation wavelength of 405 nm and the protein auto-fluorescence was detected using a pseudo green colored filter at emission

wavelength ranging between 425-475 nm. The transmitted light detector was used with a 561.4 nm laser. In order to confirm the observed auto-fluorescence was emitted by proteins in millets and not other substances, the protein and starch samples were stained with Fast Green FCF (Sigma-Aldrich, St. Lois, MO USA) at a concentration of 0.025 µg/mL in water for at least 15 min. The stained samples were excited at 561.6 nm and red fluorescence was detected at 570-620 nm. Images were processed using confocal acquisition software (NIS-Elements 4.4.0, Nikon Instruments Inc., Melville, NY).

3.4. RESULTS AND DISCUSSION

3.4.1. Sample composition

The proximate composition of de-hulled proso millet flour and protein and starch fractions is shown in Table 3.1. Similar to other cereal grains, starch was the major component of millet flour while proteins constituted the second largest component. The protein fraction obtained by wet milling of millet grains was composed of 80% protein and 11% fat while no starch was detected. On the other hand, the starch fraction contained about 90% starch, 6% protein and trace amounts of inorganic matter and lipids. The high protein content in the starch fraction was likely because of the similarity in size and density of some of the starch granules and protein bodies, which made their physical separation difficult.

3.4.2. Morphology of proso millet flour, protein, and starch

Starch granules and protein bodies were the main components visible when proso millet flour was observed under SEM (Fig. 3.1a and 3.1b). The starch granules were polygonal in shape and were cohesively joined to one another resulting in compound starch granules similar to those found in oats and rice (Thomas and Atwell, 1999). In

proso millet flour, mainly two size of starch granules were observed: A-type (>9.9 µm) and B-type (<9.9 µm) (Yu et al., 2014). Spherical protein bodies were observed in crevices of compound starch granules observed mainly at higher magnifications.

The protein bodies isolated using wet milling appeared as non-uniform clusters (Fig. 3.1c and 3.1d). The clustering of the protein bodies may have been important, but was more likely a result created during extraction or sample preparation. Protein bodies with <2.5 µm diameter and 2.5-5 µm diameter were observed. Starch was not detected in protein samples by chemical analysis (Table 3.1), but granules that were probably starch, with a distinct hexagonal shape and large size when compared with protein bodies, were observed in SEM. These were likely present below the limit of detection of the chemical analysis.

In the starch sample both compound starch granules and individual starch granules, probably broken from their compound structure during milling, were observed (Fig. 3.1e and 3.1f). A third size of starch granule similar to that of the protein bodies (<2.5 µm) was also observed in these samples, but was not noticed in the flour samples. Some of the larger starch granules had depressions on their surface that have been reported as a characteristic of starches from the Panicoideae subfamily to which proso millet belongs (Fannon et al., 1992). These indentations have been claimed as sites where small starch granules and protein bodies associate with larger starch granules (Zarnkow et al., 2007). The results obtained for morphology of starch granules and protein bodies of proso millet were similar to previous reports (Zarnkow et al., 2007; Serna-Saldivar and Rooney, 1995).

3.4.3. Morphological changes upon cooking

Upon cooking, proso millet flour and starch samples appeared as web-like networks of gelatinized starch when observed under SEM (Fig. 3.2a and 3.2e). At higher magnifications, the protein bodies could be seen intact and embedded in the starch network (Fig. 3.2b and 3.2f). In the protein samples the protein bodies did not show any visible change in structure upon cooking (Fig. 3.2c and 3.2d).

The loss in starch granular structure upon cooking due to gelatinization has been reported for other grains like maize and rice (Hu et al., 2011; Utrilla-Coello et al., 2013). Also, researchers have reported through microscopic and chemical analyses that protein bodies in cereals do not lose their structure upon cooking (Tanaka et al., 1978), which supports our observations. This is likely because the storage proteins of cereals are arranged along with other components in crystalloid subunits inside spherical protein bodies (Ashton, 1976). Thus, changes taking place inside the protein bodies could not be viewed by SEM as it is a tool for surface visualization rather than internal imaging.

The structures observed by SEM before and after cooking were supported by images from CLSM (Fig. 3.3). Cooked and uncooked samples of protein fractions were observed using both auto-fluorescence and fluorescence after protein staining. For auto-fluorescence, a green filter was used to detect proteins (Fig 3.3a and 3.3e), while fluorescence of stained samples was observed in the far-red region (Fig. 3.3b and 3.3f). The auto-fluoresced and stained images merged well when fitted on top of one another (Fig. 3.3c and 3.3g), suggesting that the spherical bodies observed under both SEM and CLSM were indeed protein bodies. There were no differences observed in light images of uncooked and cooked protein bodies.

One striking difference observed in stained and unstained images of protein bodies (both cooked and uncooked) was that the dye stained protein bodies only on the periphery while auto-fluorescence (green) was mainly concentrated in the core (inserts in Fig. 3.3c and 3.3g). This could be due to the arrangement of amino acids in protein bodies. Fast green dye has a greater affinity towards basic amino acids (Tas et al., 1980) which may be exposed on the surface of the protein bodies while intrinsic fluorescence of proteins is mostly linked to aromatic amino acids (Eftink, 2000) which are hydrophobic and would likely be embedded in the core. Auto-fluorescence can also be linked to other aromatic compounds like phenolic acids and tannins, although it was unlikely that these compounds were responsible for the auto-fluorescence in the present experiment due to the low concentrations of phenolics and tannins in these samples (Gulati et al., 2017).

3.4.4. Microstructural change to proso millet flour and protein after digestion

A significant change in protein body morphology was observed when uncooked and cooked samples were digested with pepsin (Fig. 3.4). In uncooked proso millet protein samples (Fig. 3.4a and b), the protein bodies appeared shrunken with huge cavities or craters on their surface that appeared as a result of enzymatic hydrolysis. Similar peripheral enzymatic degradation of protein bodies has been reported previously for sorghum and yellow foxtail grass (Rost, 1972; Rom et al., 1992), but they were not as intense as those observed in our study. Similar structures have been observed when starches are digested by amylolytic enzymes, which suggests a common mode of hydrolysis by these enzymes on their substrates (Uthumporn et al., 2010;).

On the other hand, when the protein bodies were digested after cooking (Fig. 3.4c and d) they displayed only tiny holes on their surface indicating the inability of pepsin to

digest cooked proso millet protein bodies. Based on the nitrogen content in these samples before and after digestion, protein digestibility of 79.7 ± 0.8% and 36.6 ± 1.5% was recorded for uncooked samples and cooked samples, respectively.

When these samples were observed using CLSM (images not shown), the results were not as prominent as SEM but there was diminished auto-fluorescence of proteins in uncooked proso millet flour and protein when compared to cooked samples following digestion. The reduced fluorescence is an indication that protein bodies do not have the same fluorescence properties as intact protein bodies. Also, since cooked samples were not digested they still maintained their auto-fluorescence.

When samples were cooked in 8 M urea (Fig. 3.5), the protein bodies appeared to have digested the same way as raw protein bodies. We have reported that heating initiates denaturation of proso millet protein which exposes hydrophobic amino acids during a partial state of denaturation (Gulati et al., 2017). However, when the water is replaced by 8 M urea it prevents the formation of hydrophobic interactions and results in high digestibility of protein even after cooking. The present microscopic images are a visualization of those previous findings.

3.5. CONCLUSION

Small, spherical protein bodies ranging from 1-2.5 μm in diameter were observed in proso millet flour and protein samples by both SEM and CLSM. Based on our chemical analyses we expected to observe aggregates of protein bodies upon cooking as a result of hydrophobic association and some visual evidence of the inability of enzymes to hydrolyze cooked proso millet protein bodies. When observed under SEM, protein bodies appeared as random clusters that were visually unchanged upon cooking. However, there

was clear evidence that cooking reduced pepsin hydrolysis, which was observed as tiny holes on the surface of protein bodies after digestion compared with large craters appearing in the uncooked digested protein bodies. As expected, when samples were cooked in 8 M urea and then digested the protein bodies had the same large cavities as observed for uncooked protein bodies after digestion. Thus, the visualization of proso millet protein bodies using microscopy provided conformational support for our chemical findings regarding the unique structure of millet storage proteins, panicins, and demands that future work should be focused on mitigating this effect.

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Table 3.1. Compositional analysis of proso miller flour and protein and starch fractions.[a]

Sample	Moisture	Protein	Starch	Fat	Ash
Flour	8.12 ± 0.03	13.6 ± 0.0	71.9 ± 0.1	3.32 ± 0.06	1.17 ± 0.00
Protein	2.43 ± 0.01	82.5 ± 0.6	ND	11.9 ± 0.3	0.57 ± 0.00
Starch	1.22 ± 0.05	5.72 ± 0.21	89.9 ± 0.2	0.35 ± 0.07	0.37 ±0.01

[a]Mean ± SD (% wb); n=3; ND, not detected.

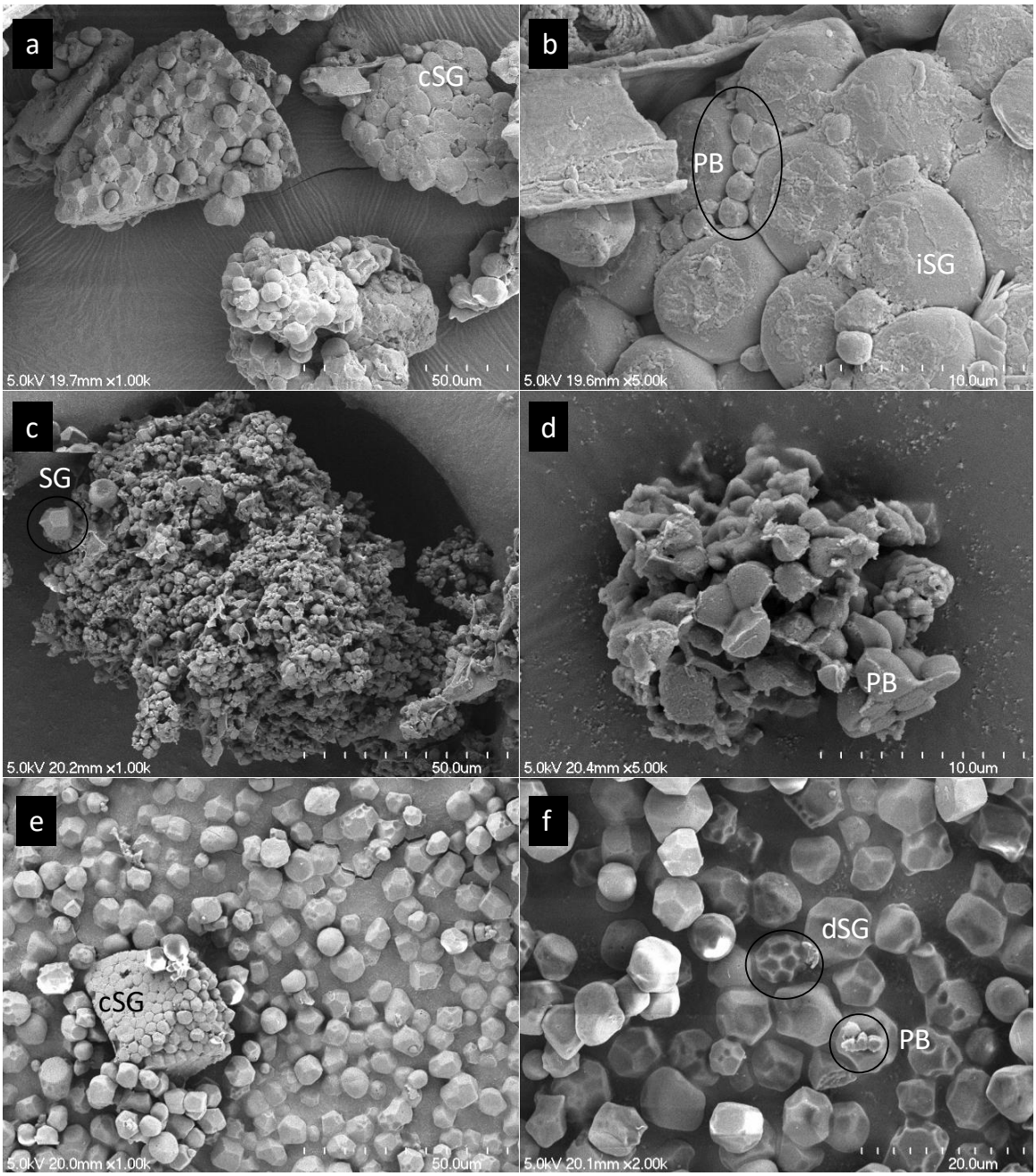

Figure 3.1. Morphology of uncooked proso millet flour[9]

[9] 1000x (a) and 5000x (b) magnification, proso millet protein at 1000x (c) and 5000x (d) magnification, and proso millet starch at 1000x (e) and 2000x (f) magnification. PB, protein body; SG, starch granule; iSG, individual starch granule; cSG, compound starch granule; dSG, dented starch granule.

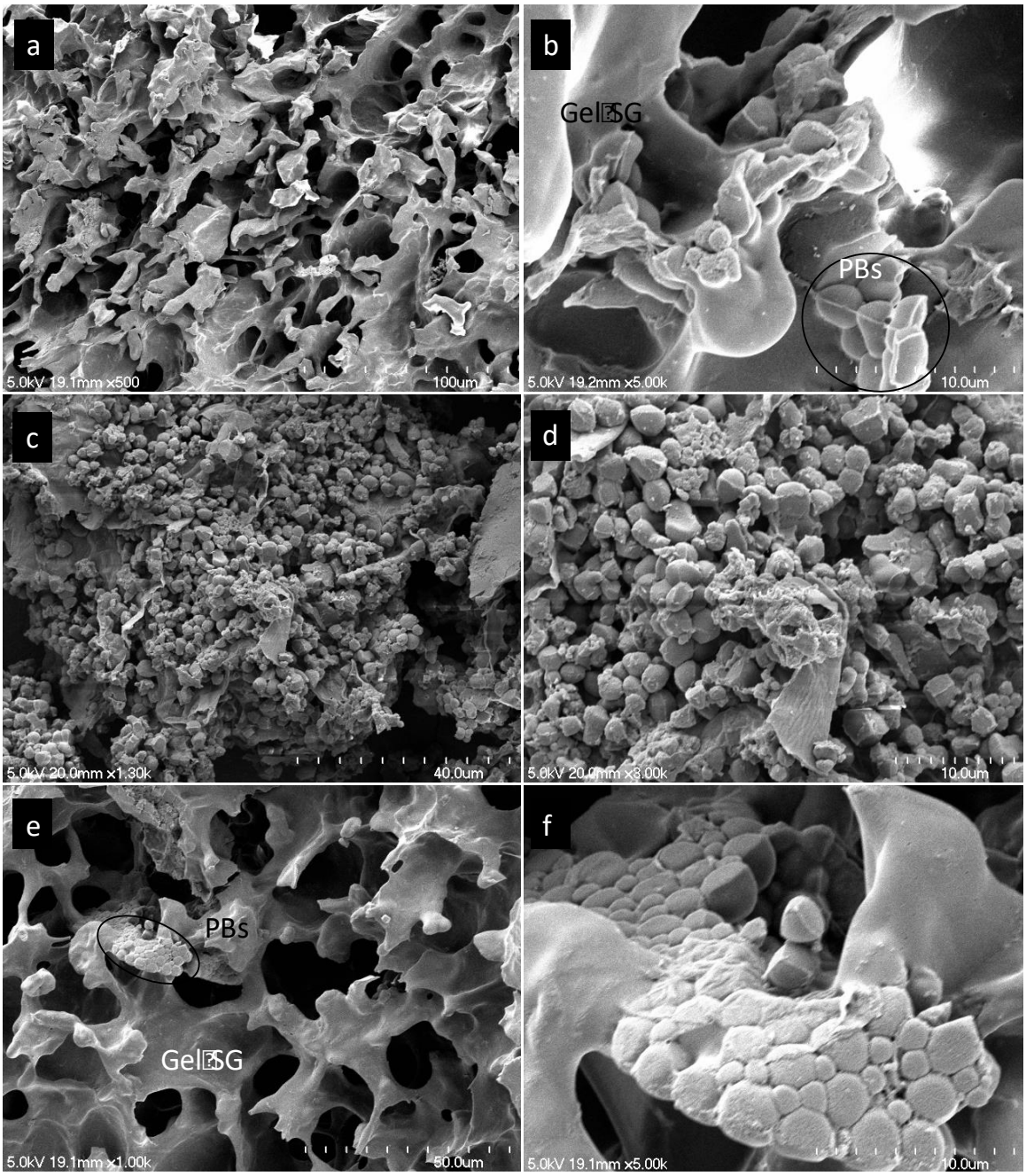

Figure 3.2. Morphology of cooked proso millet flour[10]

[10] 500x (a) and 5000x (b) magnification, proso millet protein at 1300x (c) and 8000x (d) magnification, and proso millet starch at 1000x (e) and 5000x (f) magnification. PB, protein body; Gel SG, gelatinized starch granule.

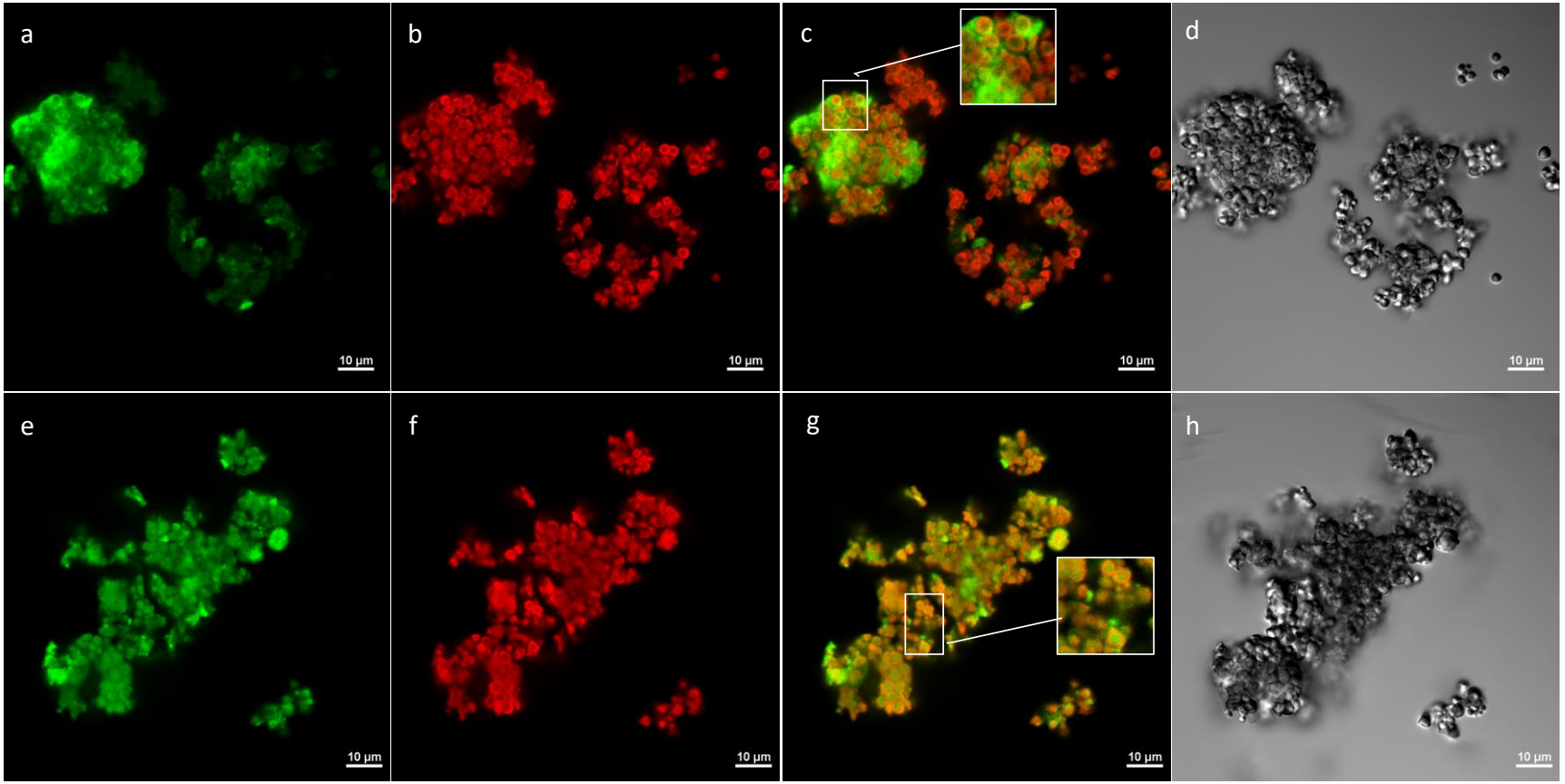

Figure 3.3. Light images of proso miller proteins using confocal microscopy[11]

[11] Images of uncooked (a-d) and cooked (e-h) proso millet protein bodies under confocal microscopy at 1200x magnification; auto-fluorescence (a, e), red fluorescence after staining (b, f), merged auto- and stained fluorescence (c, g), and transmitted light image (d, h); scale bar: 10 µm.

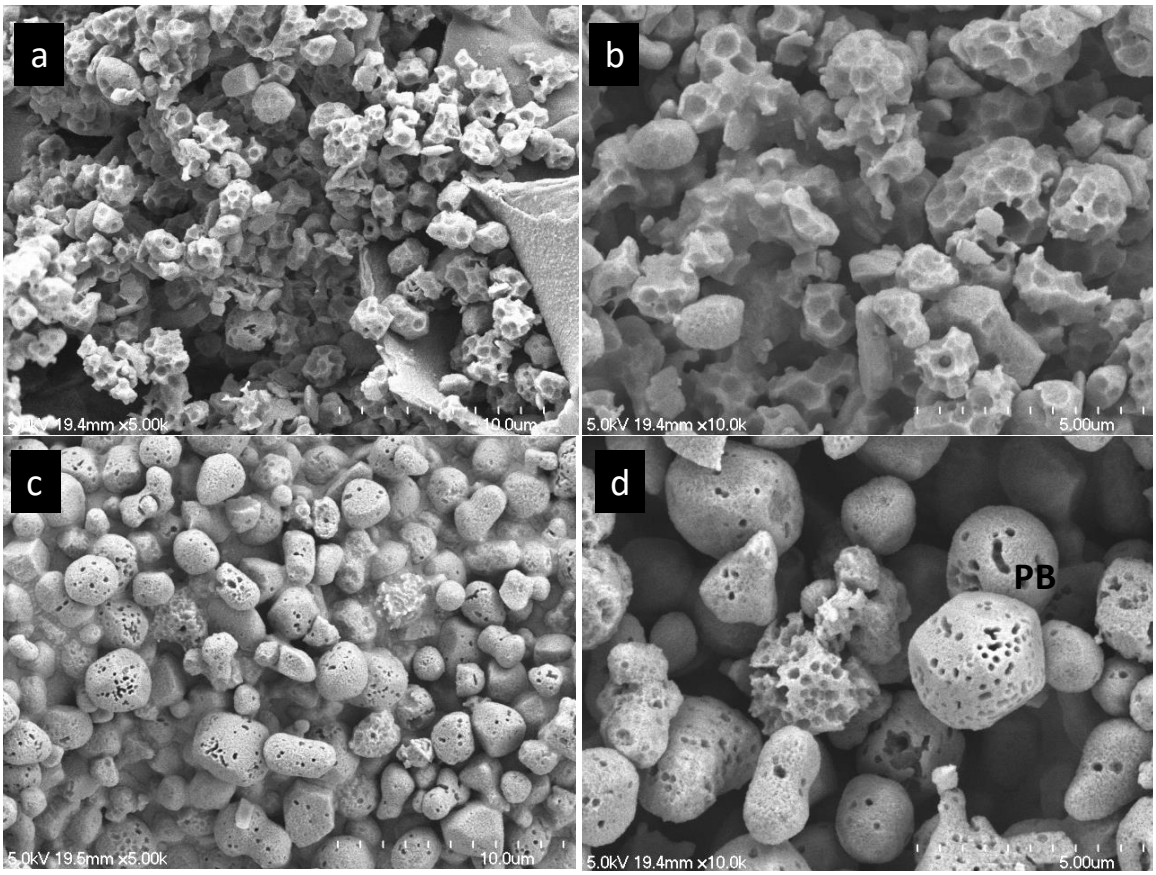

Figure 3.4. Morphology of uncooked proso millet protein after digestion[12]

12 5000x (a) and 10000x (b) magnification, and cooked proso millet protein after digestion at 5000x (c) and 10000x (d) magnification. PB, protein body.

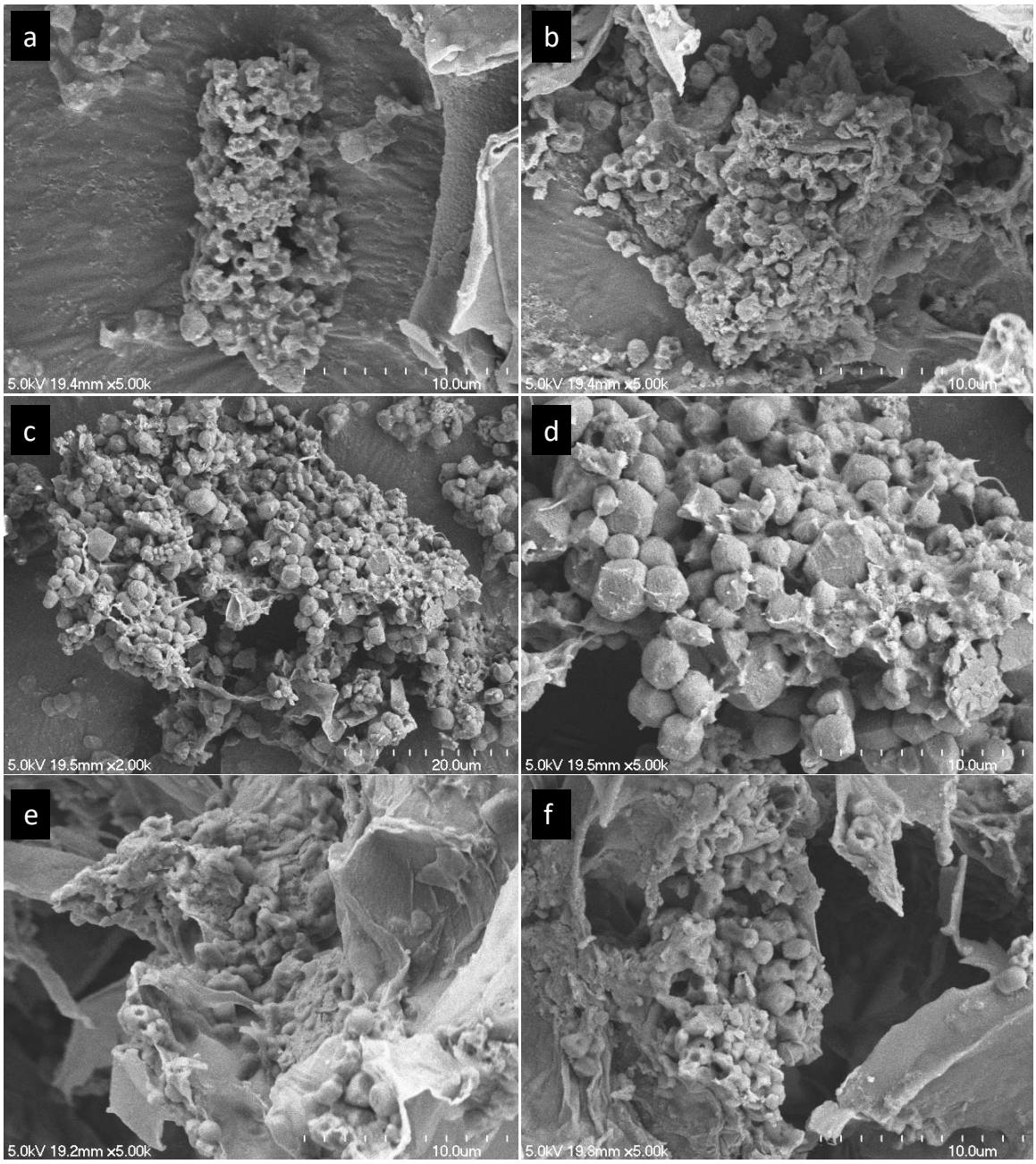

Figure 3.5. Morphology of uncooked proso millet protein bodies in 8 M urea after digestion[13]

[13] 2000x (a) and 5000x (b) magnification, cooked proso millet protein bodies in 8 M urea before digestion at 2000x (c) and 5000x (d) magnification, and cooked proso millet protein bodies in 8 M urea after digestion at 2000x (e) and 5000x (f) magnification.

CHAPTER 4: EFFECTS OF PROCESSING METHOD AND SOLUTE INTERACTIONS ON PROTEIN DIGESTIBILITY OF COOKED PROSO MILLET FLOUR

4.1. ABSTRACT

Previous studies have reported a substantial decline in *in vitro* digestibility of proso millet protein upon cooking. In this study, several processing techniques and cooking solutions were tested with the objective of preventing the loss in protein digestibility. Proso millet flour was subjected to the following processing techniques: high pressure processing (200 and 600MPa for 5 and 20 min); germination (96 h); fermentation (48 h); roasting (dry heating); autoclaving (121 °C, 3 h), and treatment with transglutaminase (160 mg/g protein, 37 °C, 2 h). To study the interaction of millet proteins with solutes, millet flour was heated with sucrose (3-7 M); NaCl (2-6 M); and $CaCl_2$ (0.5-3 M). All processing treatments failed to prevent the loss in protein digestibility except germination and treatment with transglutaminase, which resulted in 23 and 39% increases in digestibility upon cooking, respectively, when compared with unprocessed cooked flours. Heating in concentrated solutions of sucrose and NaCl were effective in preventing the loss in protein digestibility, an effect that was attributed to a reduction in water activity (a_w). $CaCl_2$ was also successful in preventing the loss in digestibility but its action was similar to chaotrops like urea. Thus, a combination of enzymatic modification and cooking of millet flour with either naturally low a_w substances or edible sources of chaotropic ions may be useful in processing of proso millet for development of novel foods without loss in digestibility. However, more research should be done to determine optimum processing conditions.

4.2. INTRODUCTION

In order to promote proso millet (*Panicum miliaceum*) for human food, our previous work focused on quality of proso millet storage proteins, panicins. Unexpectedly, we found a drastic decline in digestibility of panicins upon cooking due to formation of hydrophobic aggregates (Gulati et al., 2017). This discovery could prove to be a stymie in the promotion of proso millet as human food. Hence, it is important to explore strategies that could prevent the observed decline in digestibility of proso millet flour.

Potential processing methods that would not result in poor protein digestibility could be focused on either inhibiting the formation of the hydrophobic aggregates, or breaking of the hydrophobic aggregates after they are already formed. The formation of the hydrophobic aggregates may be inhibited either by modifying the structure of proteins prior to cooking or by creating an unfavorable environment for their formation during cooking. Hydrophobic aggregates may be broken after being formed by external stress like high pressure.

Several processing techniques [e.g., roasting, autoclaving, high pressure processing (HPP), fermentation, germination] are known to improve protein digestibility, functionality, and flavor of cereals by modifying protein structure (Poutanen et al., 2009; Tiwari & Awasthi, 2014; Hugo et al., 2003). HPP and autoclaving have been useful in either breaking protein aggregates or making them more soluble by disrupting the ionic and hydrophobic forces that are essential for the tertiary and quaternary structure of proteins (Galazka et al., 2000). Germination and fermentation, on the other hand, result in proteolysis and denaturation of storage proteins by intrinsic or microbial enzymes,

respectively, which may change protein digestibility (Ghumman et al., 2016; Hammes et al., 2005; Szewińska et. al, 2016; Li et al., 2017; Ganzle et al., 2008). Among fermented cereal products, sourdoughs formed by lactic acid bacteria and yeast fermentation of cereal flour slurries are widely recognized for imparting changes in cereal starches and proteins that result in a product with improved structure and stability (Poutanen et al., 2009).

Enzymes can also modify protein structure by crosslinking. Transglutaminase (TGase) is an enzyme approved for food processing that catalyzes the formation of an iso-peptide bond between the ε-amine group of lysine and the γ-amide group of glutamine residues (Motoki & Seguro, 1998). TGase has been used either to crosslink proteins (Nonaka et al., 1992) or create internal covalent crosslinks (Renzetti et al., 2008). In our study, we hypothesized that TGase could crosslink panicins and "lock" the panicins in their native state so the hydrophobic amino acids would not aggregate upon cooking and avert the observed loss in digestibility.

Protein aggregation can also be inhibited in the presence of co-solutes (e.g., salts and sugars). These compounds can create an unfavorable environment for aggregate formations by either reducing the water activity or interacting with hydrophobic amino acids and preventing them from associating (Ohtake et al., 2011)

Thus, the objective of this research was to identify techniques that would result in high digestibility of proso millet protein after processing. The above processing strategies to either break hydrophobic aggregates after they are formed or to prevent the formation of the aggregates altogether were tested.

4.3. MATERIALS AND METHODS

4.3.1. Samples

Commercially available dehulled proso millet grains were obtained from Clean Dirt Farms (Sterling, CO, USA) and milled using a cyclone sample mill (UDY, Fort Collins, CO, USA) fitted with a screen size of 1 mm. The milled flour had a mean particle size of 374 μm. The flour was kept under refrigerated conditions until further analysis. The flour was analyzed for moisture, fat, and ash content using approved methods (AACC International, 1999a, b, c, d, e). Protein content was analyzed using a nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25. Total starch content was analyzed using a total starch assay kit (K-TSTA, Megazyme, Bray, Ireland) following the KOH format. Whole proso millet grains (variety: sunrise) used in the study were grown at Scottsbluff, Nebraska, USA.

4.3.2. Preliminary heating in water

Four hundred milligrams of proso millet flour were dispersed in 10 mL of water and heated from 25 °C to 100 °C in 15 °C increments on a magnetic stir plate with constant stirring. The temperature was recorded constantly during heating and once the temperature was reached the flasks were cooled immediately over ice. *In vitro* protein digestibility was measured on cooled samples. For cooking, before and after various treatments discussed in subsequent sections, 400 mg flour/treated samples were dispersed in 10 mL water and heated at 100 °C for 20 min after accounting for time required to reach the temperature.

4.3.3. In vitro protein digestibility

Protein digestibility was measured using the residue method developed by Mertz et al. (1984) with modifications as reported by Gulati et al. (2017). Briefly the cooked

flour was dispersed in 60 mL of phosphate buffer (pH:2) containing pepsin (1.5mg/ml) and digested for 2 hours at 37°C with intermittent mixing. After 2 hours, the reaction was stopped by adding 4 mL of 2N NaOH and contents of the flask were centrifuged and pellet washed twice with deionized water. After digestion, the pellet was oven dried at 40 °C overnight and used to measure protein digestibility according to the following equation: PD (%) = $[(N_i - N_f)/N_i] \times 100\%$, where N_i was the total concentration of N in the sample before digestion and N_f was the concentration of N in the recovered pellet after digestion.

4.3.4. Processing of millet flour and grains

Millet flour or un-milled grains were subjected to either high temperature processing (i.e., dry roasting and autoclaving) or non-thermal processing (i.e., HPP, fermentation, germination). Depending on processing technique, heating of the sample to test for changes in protein digestibility upon cooking was performed before, during, or after the processing step was completed as described in this section.

Dry Roasting

Ten grams of proso millet flour was dry roasting by heating at 250-300 °C in a skillet for 10 min while stirring continuously, cooled, and stored for further analysis.

Autoclaving

Four hundred milligrams of proso millet flour was dispersed in 35 mL of water in a flask, covered, and autoclaved at 120 °C for 3 h. The samples were cooled to room temperature and analyzed immediately for protein digestibility.

Sourdough Fermentation

De-hulled millet flour (100 g) was mixed with water (100 g), covered, and allowed to stand at room temperature for 24 h, whereupon fresh flour (100 g) and water (100 g) were added and the mixture was allowed to stand for another 24 h. One hundred grams of this slurry was retained and fresh flour (100 g) and water (100 g) were added and fermented for another 24 h. This step was repeated twice more before the sourdough starter was ready to be used. To prepare the bread dough 75 g of sourdough starter was mixed at speed 2 in a Kitchen Aid stand mixer (Benton Harbor, MI) for 5 min with 150 g millet flour, 75 g water, 3 g salt, 22 g sugar, and 10 g oil. Batters were deposited directly into 20 X 10 cm greased loaf pans, covered, and proofed for 4 h at room temperature before baking at 190 °C for 25 min. After cooling, the bread was sliced, frozen, and freeze-dried before measuring protein digestibility without further cooking.

High Pressure Processing

Millet flour slurry in water (1:10 w/v) was vacuum sealed (Impulse sealer, Wu-Hsing Electronics, Ltd, Taiwain) in a bag and then placed in a new bag and vacuum sealed again at 100 mbar (Model C200, Multivac Inc., Kansas City, MO). The double bagged samples were further placed in another vacuum pouch and sealed to avoid any contamination or leakage (VacMaster VP215, Ovaerland Park, KS). Some samples were heated (100 °C, 20 min) prior to vacuum sealing and others were not. The triple bagged samples were then subjected to either 200 MPa or 600 MPa pressure for 5 or 20 min at 20 °C in a complete 2 X 2 factorial design. Pressure treatments were carried out using a 2.0 L Stansted ISO Lab high pressure processing unit (FPG 9400:922, Stansted Fluid Power Ltd., Essex, UK). The pressure-transmitting fluid used was a mixture of ethylene glycol and water. The temperature increase due to the adiabatic heating effect during processing

was approximately 3 °C per 100 MPa. After pressure treatment, the millet flour slurry was centrifuged at 4,000 x g / 10 °C for 15 min and the pellet was freeze dried. The dried material was then subjected to protein digestibility assays directly or after cooking in water. Thus, HPP treatment was compared for unprocessed flour, flour cooked before HPP, flour cooked after HPP, and flour cooked both before and after HPP.

Germination

Two hundred and fifty grams of whole proso millet grains were cleaned by hand to remove any chaff or dirt and then rinsed in water. The cleaned grains were then soaked in 600 mL distilled water for 4 h. Soaked grains were then drained and washed several times and then spread onto a double layer of wet paper towels and covered with single layer of cheese cloth and paper towels. The covered grains were kept moist by sprinkling with water every 2-3 h during the day. The grains were germinated for 24, 48, and 96 h. Germinated grains were oven dried at 40 °C overnight and milled using cyclone mill. The germinated flours (400 mg) were then cooked (100 °C, 20 min) in water (35 mL) prior to analysis of protein digestibility.

Treatment of Transglutaminase

Five grams of proso millet flour or 1 g proso millet protein (80% purity) obtained from wet-milling of proso millet according to Gulati et al. (2017) was incubated with 10 mL of transglutaminase (TGase) (RM transglutaminase, Modernist Pantry, Eliot, ME; 10 mg/mL in 0.1 M phosphate buffer, pH 7) for 2 h at 37 °C in a shaking water bath. After 2 h, the contents of the tube were centrifuged and washed with phosphate buffer twice and centrifuged. The pellet was freeze dried and used for protein and protein digestibility measurements under uncooked and cooked conditions.

4.3.5. Electrophoresis

Electrophoretic patterns of proteins from TGase-treated samples were compared with untreated samples both before and after cooking and before and after digestion experiments. Proteins were extracted from 50 mg of sample using 1.5 mL of 0.0125 M sodium tetraborate buffer (pH 10) containing 1% SDS and 2% 2-mercaptoethanol for 2 h at room temperature followed by centrifugation. The supernatant was subjected to SDS-PAGE analysis using a vertical mini gel system (Mini protein II cell tetra system, Biorad, CA) as described by Gulati et al. (2017).

4.3.6. Heating with solutes

Four hundred milligrams of proso millet flour was heated in 10 mL of sucrose solution (3-7 M); sodium chloride solution (2-6 M); calcium chloride solution (0.5-3 M); honey; maple syrup; and 2% fat milk in a boiling water bath for 20 min. Following cooking, the co-solutes were removed by dialysis (MW 12-14 kDa) overnight against water. Dialyzed samples were subjected to digestibility studies (cooked and uncooked). The water activity (a_w) of the cooking solutions was measured using a water activity meter (Aqua lab, Model 3TE, Decagon devices Inc., Pullman, WA) at 25 °C.

4.3.7. Statistical analysis

The overall treatment effects were first analyzed using ANOVA. Following ANOVA, the specific difference among treatments were assessed either by using t-test, when comparing two means, or using Tukey's test for multiple mean comparisons at a significance level of 0.05. All measurements were done in triplicate. All descriptive statistics were computed using JMP statistical software (JMP version 12.0.1, SAS Institute Inc.).

4.4. RESULTS AND DISCUSSIONS

4.4.1. Samples and preliminary heating in water

Like other cereals, starch (71.9±0.05%) was the main component of the proso millet flour, which was followed by protein (13.6±0.0%), fat (3.3±0.1%), and ash (1.17±0.00%). The millet protein fraction extracted using wet milling had protein content of 82.5±0.6%, fat content of 11.9±0.3%, and ash content of 0.57±0.00% while no starch was detected.

A slurry of millet flour in water (1:10 w/v) took on an average 42 s to rise by 15 °C (Supplementary Fig. 4.6). The decline in protein digestibility was significant when millet flour was heated beyond 70 °C, which took about 2.7 min to reach. At 100 °C, which was reached in 4 min, there was a 20% decline in digestibility of panicins. In our previous work, we found that protein digestibility declined when proso millet flour was heated at 55 °C or above for 20 min without considering come-up time. Because we did not observe a decline in digestibility in this experiment until the sample was heated to 70 °C and then immediately cooled, the results suggest that the decline in digestibility starts the moment proso millet proteins achieve high temperature, but continues to decline with increased heating time. Thus, for all the control cooking experiments in this study, millet flour was cooked at 100 °C for 20 min after accounting for a 4-min come-up time.

4.4.2. Dry heating

Dry heating proso millet flour resulted in lower digestibility of proso millet proteins compared with wet heating the flour (Table 4.1). Our previous work reported decline in digestibility of proso millet proteins, panicins, when cooked in presence of even 10% water (Gulati et al., 2017). We expected that heating millet flour by dry heating

in the absence of any external water would not show a reduction in protein digestibility. Prior to roasting, the proso millet flour had 8% moisture, which must have been enough to instigate the same hydrophobic aggregation responsible for causing lower digestibility as we have reported before. The low moisture and high heat caused Maillard browning in millet flour that may have further reduced the digestibility (Seiquer et al., 2006). This confirms that even trace amount of water in millet flour can result in lower digestibility when heated.

4.4.3. Autoclaving

Millet flour was subjected to autoclaving for 3 h with the aim of breaking hydrophobic aggregates that may form during shorter heating intervals. Prolonged heating under pressure did not improve protein digestibility of millet proteins (Table 4.1) unlike reports for other cereal proteins (Xia et al., 2012), suggesting very strong aggregation in proso millet protein aggregates.

4.4.4. Fermentation (sourdough)

When proso millet flour was made into sourdough bread there was no change in protein digestibility compared with proso millet flour simply boiled in excess water (Table 4.1). Anecdotally, the sourdough breads made with proso millet flour were extremely bitter, potentially due to peptides released as a result of the fermentation process; however, this modification did not change protein digestibility. This is contrary to other reports where sourdough fermentation results in an appealing flavor and also an improvement the protein digestibility of breads made from other grains (Bartkiene et al., 2012; Hugo et al.,2003).

4.4.5. High-pressure processing

Millet flour was subjected to HPP under two pressure settings (200 and 600 MPa) for two time periods (5 and 20 min). HPP significantly improved the digestibility of uncooked millet flour treated at 600 MPa when compared with unprocessed flour (Fig. 4.1). However, there were no significant differences among HPP processing conditions and no conditions resulted in improved digestibility compared with flour that had been cooked without HPP. The improvement in digestibility in uncooked flour subjected to high pressure could be due to protein denaturation by internal bond breaking (Mozhaev et al., 1996) which made the proso protein more digestible by pepsin. However, the change caused by HPP was unable to modify the protein structure extensively enough to improve digestibility upon cooking.

4.4.6. Germination

Proso millet grains displayed a visible plumule within 12 h of initiating germination, which continued to elongate during the 96h experiment. Protein digestibility was measured on both uncooked and cooked flour that had been germinated for 24, 48, and 96 h. There were no significant changes in protein digestibility of uncooked, germinated flours compared with the ungerminated flour (Fig. 4.2). However, there was a significant improvement in digestibility of cooked germinated proso flours when compared with cooked ungerminated proso millet flour. This suggests that germination did induce some change in panicins, either by hydrolysis, denaturation, increased solubility, or some other modification, that partially improved digestibility (Singh et al., 2017; Szewinska et al., 2016).

4.4.7. Treatment with transglutaminase

Millet flour and extracted protein fraction were both treated with commercial TGase and their nitrogen content before and after digestion was measured (Fig. 4.3). There was a significant increase in the protein concentration of enzymatically treated millet flour. This increase could be due to the cross-linking of albumins in the flour (Renzetti et al., 2008), rendering the proteins insoluble and thus not washed away following enzyme treatment and before protein digestibility experiments. In contrast, there was no change in protein content of treated or un-treated protein fraction, which did not contain albumins.

There was significant improvement in digestibility of both uncooked and cooked flour and protein samples after TGase treatment (Fig. 4.3). In the uncooked samples, protein digestibility increased by 19% and 29% in the enzymatically treated flour and protein samples, respectively, compared with untreated flour, suggesting a structural modification of proso proteins making them easily accessible for hydrolysis by pepsin. There was still decline in digestibility of both flour and protein TGase-treated samples upon cooking, but the decline was not as severe as observed for untreated samples when cooked. Indeed, the protein digestibility increased by 39% and 52% in the enzymatically treated flour and protein samples, respectively, compared with cooked flour that had not been treated with TGase. There have been contradictory reports on effects of transglutaminase treatment on digestibility of proteins. While Romano et al., (2016) reported a decline in digestibility of transglutaminase treated proteins, Havenaar et al, (2013) observed no difference in digestibility, further, Hassan et al., (2007) observed that TGase treatment can resist heat induced aggregation in pearl millet proteins but they didn’t measure the digestibility of crosslinked polymers. In our study, we observed an

improvement in protein digestibility after TGase treatment potentially due to modification of protein structure with reduced aggregation ability and partial loss in digestibility upon cooking.

To visualize changes that occurred in proso millet proteins upon treatment with TGase, SDS-PAGE was run on both treated and untreated samples (Fig. 4.4). Unfortunately, treatment of proso millet flour with TGase did not show any noticeable change in proso millet protein profile (lane 2) other than among some faint albumin bands when compared with untreated millet proteins (lane 1). However, the improvement in digestibility was clearly evident by the near disappearance of all protein bands of digested millet flour after treatment with TGase (lane 6) while for untreated millet flour there was still some protein observed in the 20 kDa range (lane 2).

In our previous study, we showed that the main prolamin band (20 kDa) of proso millet protein is not extracted after cooking using borate buffer with SDS and 2-ME. Here, similar results were seen with cooked TGase treated millet flour proteins. This suggests that there was formation of hydrophobic aggregates even after TGase treatment; however, based on the observations digestibility is still slightly restored. Thus, TGase modification could be a potential technique to improve protein digestibility of cooked proso millet and develop novel millet based products, but requires some further investigation.

4.4.8. Co-solutes

Cooking in increasing concentrations of sugar resulted in improved protein digestibility when compared with cooking in water (Fig. 4.5). This reduction was a function of the a_w of the sugar solution, which ranged from 0.93 (3 M) to 0.68 (7 M).

Further, when proso millet flour was heated in honey or maple syrup, which have naturally low a_w, the protein digestibility fell right along the regression line for the sugar solutions.

Various studies have reported that sugars and polyols can reduce aggregation of proteins by enhancing the stability of proteins (Ohtake et al., 2011). These co-solvents are categorized as 'protecting osmolytes' (Liang et al., 2010). The presence of these osmolytes increases internal interactions in proteins and thereby increases the denaturation temperature such that even with continuous heating the protein remains in its native conformation (Arakawa et al., 1985; Sharma et al., 2012).

In order to analyze the effect of other co-solutes, millet flour was heated in NaCl, and $CaCl_2$ solutions at different concentrations and their a_w was used as a predictor of digestibility of cooked millet flour (Table 2). The measured digestibility of millet flour following cooking in these solutions was predicted well by the regression equation created with the sucrose solutions, except for 3 M $CaCl_2$ and urea. Thus, NaCl probably improved protein digestibility simply by a reduction in a_w as explained for sucrose. In contrast, cooking in 3 M $CaCl_2$ resulted in 80% digestibility which was higher than the predicted value (67%) based upon a_w of the solution. This difference could be due to interactions of Ca^{2+} ions with proteins (Ohtake et al., 2011). Specifically, at high concentrations Ca^{2+} has an affinity towards hydrophobic groups in proteins, which prevents aggregation in a manner similar to urea and guanidine HCl (Timasheff, 1993; Tuhumury et al., 2016).

Proso millet flour was cooked in 2% fat milk, which is a good edible source of calcium (0.03 M Ca^{2+}; USDA nutrient database, NDB # 01079). We found that cooking

in milk resulted in a slight, but significant, improvement in digestibility (40.3 ± 0.6 %) when compared with cooking in water (34.1 ± 1.0%). These results were contradictory to Kovalev et al. (1974) who reported only slight loss in protein digestibility of proso millet when cooked in milk, although the researchers used a combination of pepsin and trypsin for digestion and the method used for digestibility measurements was very different from that employed herein.

4.5. CONCLUSION

To prevent the decline in digestibility of proso millet proteins upon cooking, various processing strategies were tested. Subjecting millet proteins to severe conditions such as high pressure or prolonged heating did not prevent or break the cooking-induced hydrophobic aggregates responsible for lower digestibility. This confirms a very strong interaction between hydrophobic amino acids in proso millet proteins. Germination slightly improved the digestibility upon cooking when compared to cooked non-germinated flour, while fermentation had no effect. Treatment of millet proteins with TGase modestly improved the digestibility of proso millet proteins upon cooking. Cooking in low water activity solutions of sucrose and NaCl prevented the loss in digestibility. Solutions containing Ca^{2+} ions also prevented the loss in digestibility, and the improvement in digestibility was better than would be predicted based on water activity alone. These strategies suggested that a combination of different processing techniques and cooking conditions may help in the development of novel proso millet-based food products without the low protein digestibility characteristic.

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Table 4.1. Effect of different processing techniques on protein digestibility of proso millet flour.[A]

Processing technique	**Protein digestibility (%)**
Unprocessed	75.2 ± 0.8 a
Cooked in water	34.1 ± 0.9 b
Dry heating	28.2 ± 1.0 c
Autoclaving	35.7 ± 1.5 b
Sourdough bread	32.3 ± 2.1 bc

[A]Mean±SD, n=3; means marked with different lower-case letters are significantly different; Tukey's test, α=0.05.

Table 4.2. Protein digestibility of proso millet flour in solutions of different water activity.[A]

Sample	Water activity	Protein digestibility (%)			Diff. actual vs. predicted (%)
		Uncooked	Cooked actual	Cooked predicted[B]	
2 M NaCl	0.93	78.4 ± 1.4 a	41.9 ± 1.6 f	43.8	4.53
4 M NaCl	0.83	80.3 ± 1.0 a	53.3 ± 1.6 e	54.9	3.02
6 M NaCl	0.75	80.6 ± 1.0 a	61.6 ± 0.7 d	63.4	2.92
0.5 M $CaCl_2$	0.98	76.9 ± 1.9 ab	40.6 ± 0.5 f	38.4	5.42
1 M $CaCl_2$	0.95	73.5 ± 1.0 bc	39.9 ± 1.5 f	42.0	5.26
2 M $CaCl_2$	0.86	72.7 ± 2.2 c	50.5 ± 1.0 e	52.2	3.37
3 M $CaCl_2$	0.72	79.4 ± 1.2 a	80.2 ± 1.3 a	66.9	16.6
8 M Urea	0.89	82.0 ± 1.9 a	78.6 ± 0.2 a	48.9	37.8

[A]Mean±SD, n=3; means marked with different lower-case letters are significantly different; Tukey's test, α=0.05.
[B]Predicted based on regression equation % Protein digestibility = $-108*A_w + 144.7$ (Fig. 4.5).

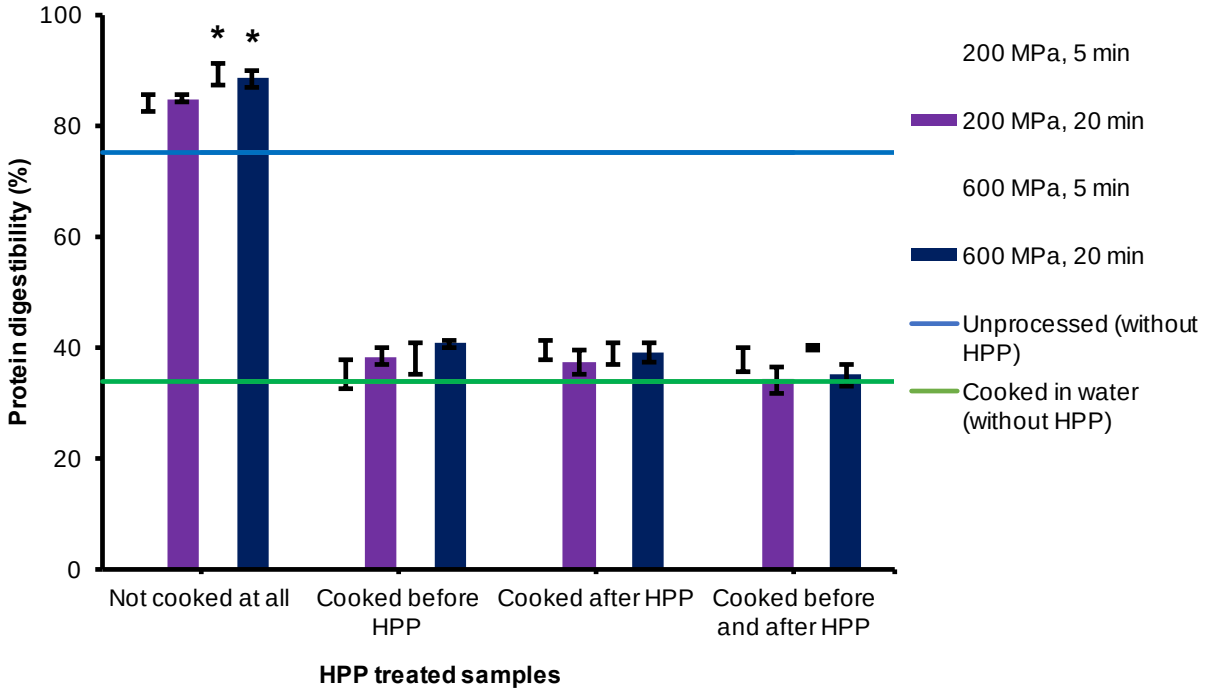

Figure 4.1. Effect of high pressure processing (HPP) on protein digestibility of millet flour[14]

[14] Error bars denote standard deviation (n=3); *significantly different from unprocessed flour; there were no differences among cooked samples (both HPP-treated and untreated).

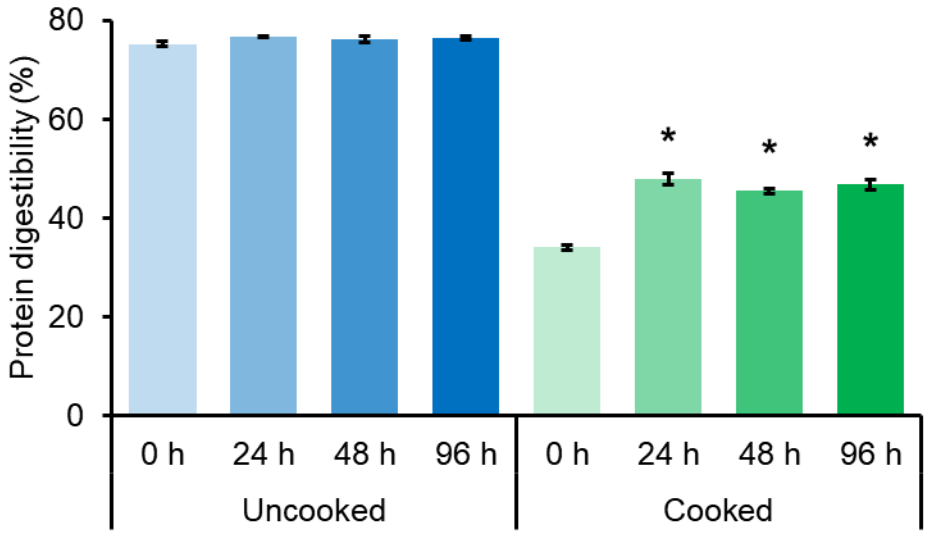

Figure 4.2. Effect of germination time on protein digestibility of uncooked and cooked proso millet flour[15]

[15] Error bars denote standard deviation (n=3); *significantly different from un-germinated (0 h) cooked millet flour; Tukey's multiple comparison test; α=0.05.

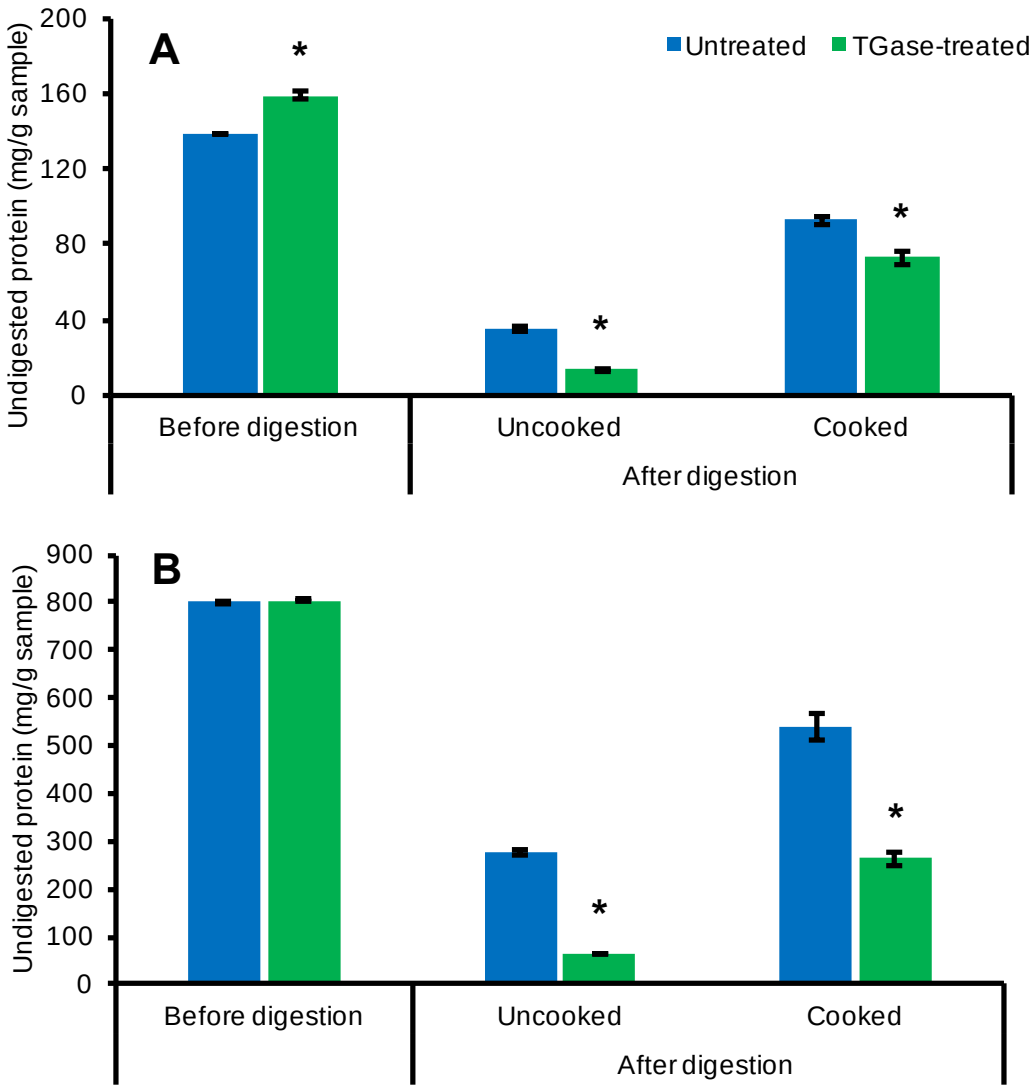

Figure 4.3. Effect of transglutaminase treatment on protein digestibility of millet flour (A) and protein (B)[16]

[16] Error bars denote standard deviation (n=3); *significantly different from the untreated counterpart.

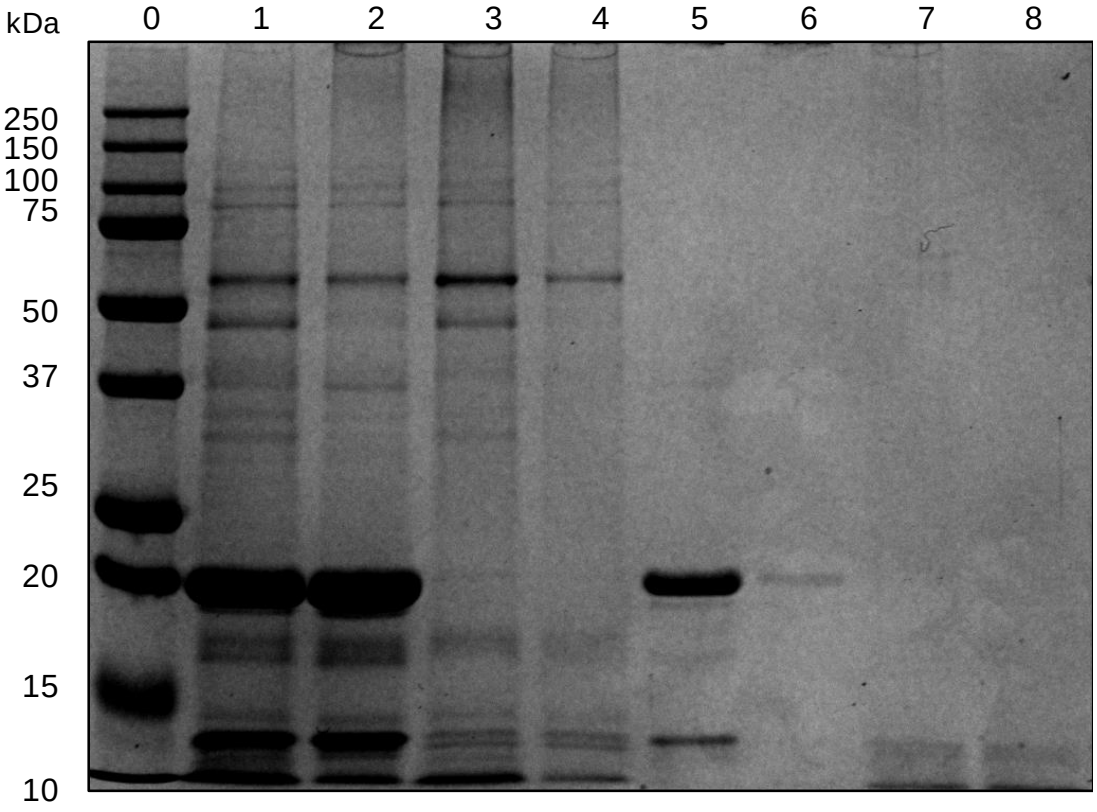

Figure 4.4. SDS-PAGE gel of proso millet flour[17]

[17] Lane 0, MW marker; 1, proso millet flour; 2, proso millet flour treated with transglutaminase; 3, cooked untreated flour; 4, cooked treated flour; 5, uncooked untreated flour after digestion; 6, uncooked treated flour after digestion; 7, cooked untreated flour after digestion; 8, cooked treated flour after digestion.

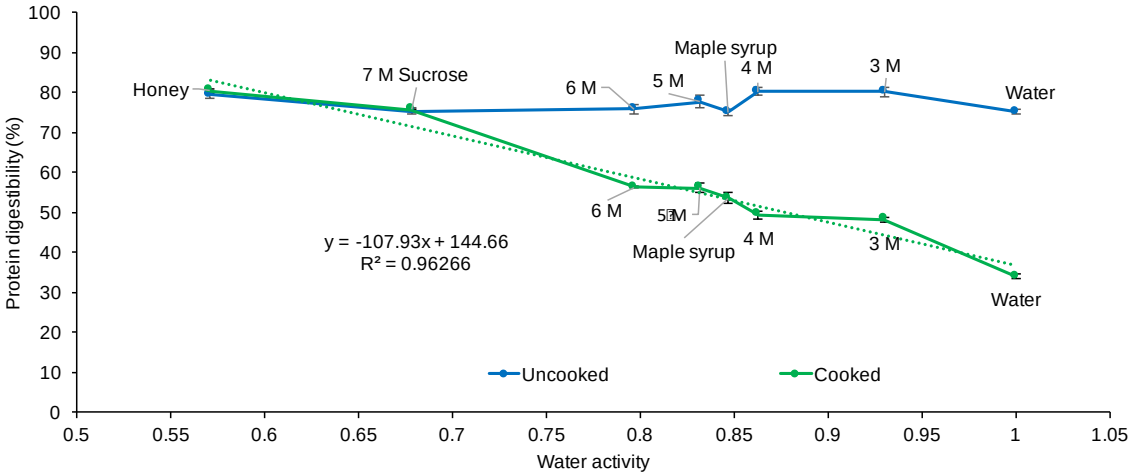

Figure 4.5. Effect of sugar solutions and their water activity on protein digestibility of proso millet flour[18]

[18] Error bars denote standard deviation (n=3); some error bars were too small to plot.

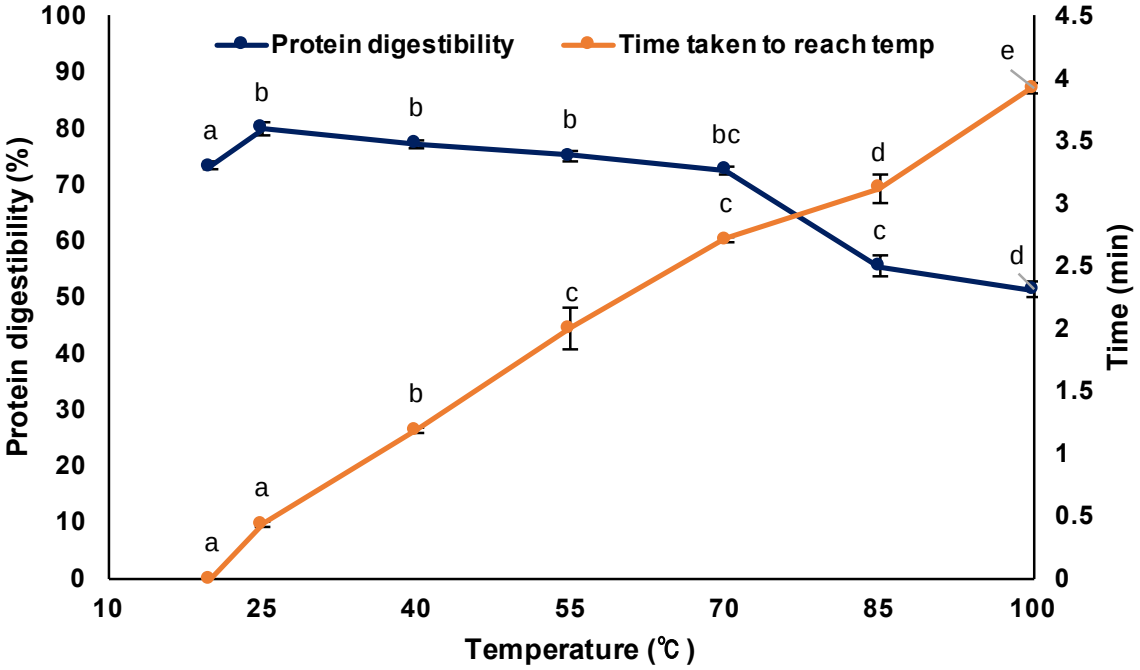

Figure 4.6. (Supplementary) Time taken by millet flour slurry to reach respective temperature and protein digestibility of millet flour at that temperature[19]

[19] Error bars show standard deviation (n=3); some error bars were too small to plot; a,b,cMeans marked with different alphabets indicate significant differences among digestibility series and time series with increasing temperature (Tukey's adjustment; α=0.05)

CHAPTER 5: COMPARING *IN-VITRO* PROTEIN DIGESTIBILITY OF COOKED PROSO MILLET (*PANCIUM MILIACEUM* L.) CULTIVARS AND RELATED SPECIES FROM 21 DIFFERENT COUNTRIES.

5.1. ABSTRACT

Thirty-three accessions of proso millet with different countries of origin were screened for their protein digestibility after cooking in order to identify samples with high digestibility. Seeds were also sown in the greenhouse and were evaluated for protein content and digestibility. The protein digestibility of all samples ranged from 26 to 57% with an average digestibility of 32%. Only 26 of the 33 accessions could successfully grow in the greenhouse. The protein content and digestibility of all greenhouse grown seeds were higher than originally procured seeds. There were no apparent differences in protein profiles of samples with lowest, intermediate and highest digestibility among all cooked samples when analyzed using SDS-PAGE. The main prolamin band of these samples were further digested by chymotrypsin-trypsin and subjected to LC-MS/MS analysis. Maximum peptides in all samples matched with proteins from *Panicum hallii*. There was a positive correlation between the 18-20 kDa peptides identified with the digestibility of samples and a negative correlation with 24 kDa peptides and digestibility. Some other species of genus *Panicum*, such as little millet, switchgrass and panicgrass were also analyzed in the study. These species had protein content slightly higher than proso millet but a low digestibility upon cooking. This suggests that the low digestibility upon cooking could be an inherent property of genus *Panicum*. The results from this study showed variability in digestibility of proso millet samples grown in different

countries, this variation in the data can form basis for in-depth analysis of proso proteins that may help in developing new cultivars with higher digestibility upon cooking.

5.2. INTRODUCTION

Panicum is a large plant genus that includes more than 400 species of grasses (Roshevits, 1980) with common ones being *P. miliaceum (*proso millet*); P. sumatrense (*little millet*); P. virgatum (*switchgrass*), P. capillare (*witchgrass*); P. halli;* and *P. hirticaule (*panicgrass*).*

Among these, proso millet, and to some extent little millet, have economic importance given their application mostly as bird feed and some share in human food industry (Sabir and Ashraf, 2007).

Proso millet is one of the oldest domesticated crops with recorded origin in China (Bettinger et al., 2007) and wide culinary presence in Asian countries. From Asia, the cultivation of proso millet spread to Eurasia and eastern Europe and was introduced in the US by German-Russian immigrants at the end of 19th century (Habiyaremye et al, 2017). At present USDA- National Plant Germplasm System (USDA-ARS GRIN Global) has record of almost 700 accessions of proso millet grown in almost every country in the world. This suggests the excellent adaptability of proso millet to grow in diverse conditions. Apart from that, proso millet is an excellent agricultural aid especially for wheat, corn and sorghum. It is often employed as a rotational crop as it can replenish the soil nutrients, and preserve deeper soil water. It is also used to manage grass weeds, diseases etc. and have been a common choice as an emergency catch crop (Rajput and Santra, 2016). Moreover, proso millet is a gluten free crop with good amount of protein which is rich in essential amino acids, and also contain good amount of fiber, digestible

starches and non-nutritive beneficial compounds like phenolics etc. Over the years, the food uses of proso millet has declined and at present the majority of proso grown in US is used as bird or animal feed. The limited human consumption of proso millet restricts its growth resulting in agricultural losses.

In order to promote proso millet for human consumption, our previous work focused on quality evaluation of proso millet proteins. We found a drastic decline in protein digestibility under thermal processing due to hydrophobic aggregation (Gulati et al., 2017). This could prove to be a great hurdle in promoting the crop as human food. One way to combat this issue is to develop proso based products with novel processing techniques that could modify the proteins and prevent loss in digestibility. Alternatively, we can look for proso millet cultivars with naturally mutated proteins not susceptible to form hydrophobic aggregates and use them to modify the commonly grown cultivars. The current research was focused on the latter.

Given the adaptability of proso millet to grow in different geographical regions and climates we expected to find variation in protein digestibility of proso millet samples upon cooking with some potentially highly digestible mutants too. Thus, 33 accessions of proso millet with different seed colors and geographical origin were screened for their protein digestibility after cooking. Further, *Panicum* is a big genus with non-edible grasses having good protein content, in order to understand if the observed property of proso millet proteins is restricted to the millet variety or is a characteristic of the genus we also tested some non-edible grasses from genus *Panicum* like witchgrass, switchgrass, panic grass etc. for their protein digestibility. Also, since proso millet is usually grouped with other millet varieties (finger, pearl, foxtail etc.) owing to their efficient growth

parameters, we wanted to investigate the protein digestibility of other millets to understand their protein behavior after cooking. Thus, the principal objectives of this study were to find 1) Is there a significant difference in digestibility of proso millet cultivars with different countries of origin? If so, can we expect samples with natural mutation leading to high digestibility? and 2) Is the observed low digestibility upon cooking a property of the genus *Panicum* or just a peculiar characteristic of proso millet proteins? The ultimate objective was to better understand the proteins in proso millet in order to correct the undesirable loss in digestibility and promote its consumption as human food.

5.3. MATERIALS AND METHODS

5.3.1. Millet seeds procurement, preparation and growth in greenhouse

In total, 35 samples of proso millet and 10 samples of related species were used in the study (Table 5.1). In short, 33 of the proso millet samples with different countries of origin were obtained from the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA, USA). Two commercial de-hulled samples of proso millet were also used in the study, one of them was obtained from Clean Dirt Farms (Sterling, CO, USA) and other one was obtained from Ukraine. Three samples of related species (little millet, panicgrass, and witchgrass) were obtained from the USDA-ARS North Central Regional Plant Introduction Station. Additionally, three samples of switchgrass were obtained from the USA (two from Nebraska and one from Illinois), and whole finger millet, pearl millet, and foxtail millet samples were obtained from the USA (Nebraska). Finally, a commercial sample of de-hulled foxtail millet was obtained from China. Different

countries from which proso millet or related species samples were obtained has been highlighted in Fig 5.1.

The 33 proso millet accessions that were obtained from USDA-ARS were grown in a greenhouse at the University of Nebraska-Lincoln (First 33 samples in Table 5.1). Ten seeds of each cultivar were planted in a soil mixture (Sunshine MVP, Sun Gro Horticulture, Agawam, MA). The seeds started germinating one week after planting and were moved to four different pots, one germinated seed per pot and grown under 16 h day length cycle with day time temperature of 28-30 °C and night time temperature of 20-23 °C and were watered every alternate day. The flowering stalks of each plant were covered with paper bags to facilitate self-pollination. The developed seeds were allowed to dry on the plant and the seeds were harvested 110-120 d after planting. All seeds were kept at 4 °C until further analysis. Different characteristics like plant height, yield, flowering days etc. were recorded for plants growing in greenhouse and is shown in Table 5.1. The original seeds and greenhouse-grown seeds were milled using a ball mill (Genogrinder; Thermoscientif, Waltham, MA) for 120 s at 1600 rpm. The milled samples were saved in polyethylene bags at 4°C until further analysis.

5.3.2. Protein concentration

Protein concentration of all samples (original and greenhouse grown) were analyzed by combustion using a nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25.

5.3.3. In-vitro protein digestibility

Two hundred milligrams of milled sample were cooked in 10 mL water at 100 °C for 20 min (time recorded after temperature was reached). The cooked samples were

cooled to room temperature and immediately used for digestibility measurements. Protein digestibility of milled seeds from originally procured samples and greenhouse-grown samples were analyzed after cooking using the residue method described in Gulati et al. (2017). In brief, 25 mL of 0.1 M phosphate buffer (pH: 2) containing pepsin (2.2 mg/mL) was added to each cooked sample tube and digested for 2 h at 37 °C. The reaction was stopped using 2 M NaOH and the contents centrifuged with repeated washing of the pellet with water. The pellet was dried overnight and used for measuring residual nitrogen content by combustion. Protein digestibility was calculated according to the following equation: PD (%) = $[(N_i - N_f)/N_i] \times 100\%$, where N_i was the total N in the sample before digestion and N_f was the amount of N in the recovered pellet after digestion.

5.3.4. Electrophoresis

Total proteins from milled un-cooked samples (50 mg) were extracted with 1.5 mL of 0.0125 M sodium tetraborate buffer (pH 10) containing 1% SDS and 2% 2-mercaptoethanol for 2 h at room temperature followed by centrifugation. The supernatant was subjected to SDS-PAGE analysis using a vertical mini gel system (Mini protein II cell tetra system, Biorad, CA). Protein extract was mixed with sample buffer in the ratio of 4:1 and loaded along with molecular weight markers (BioRad Precison Plus Dual color protein standard, 10-250 KDa) on to gel system with following specifications. The resolving gel consisted of 15 % polyacrylamide in 1 M Tris HCl buffer (pH 8.8), and 1% SDS (w/v). The stacking gel contained 5% polyacrylamide in 0.63 M Tris HCl buffer (pH 6.8), and 1% SDS (w/v). TEMED (0.05% v/v) and ammonium persulfate (0.1 v/v) were used to polymerize the gels. Electrophoresis was done at 70 V for 120 min in tank buffer

consisting of 1.9 M Tris, and 1% SDS (w/v). After electrophoresis, the gels were stained with 20 mL of coomassie brilliant blue reagent (0.25%) containing isopropanol and acetic acid for 60 min. De-staining was achieved by washing gels several times in a solution of 10% acetic acid and 30% methanol in water. Gel images were captured and analyzed using Image Analyser (BioRad Molecular Imager, Gel Doc-XR system, CA).

5.3.5. In gel protein digestion and LC-MS/MS analysis

23 kDa bands from SDS-PAGE gels were used for in-gel chymotrypsin-trypsin digestion and then subjected to LC-MS/MS analysis in duplicate. Gel bands were excised from the gels, reduced with 10 mM DTT, alkylated with 20 mM iodoacetamide and then fully de-stained before digestion. The solutions were removed by centrifugation. For digestion, 200 ng of chymotrypsin was added to each sample and incubated overnight at 37 °C. The peptides were then liberated from the gel pieces using 2% acetonitrile/1% formic acid solution, then 60% acetonitrile. To increase coverage, the extracted peptides and the gel pieces left from the chymotrypsin digestion were re-combined and 200 ng of trypsin was added and the tubes incubated at 37°C overnight. The chymotrypsin/trypsin digested peptides were pooled and separated on a rapid separation liquid chromatography system (Dionex U3000 nano) equipped with a C18 column (0.075 mm x 250mm Waters CSH) using a 1 h gradient of 0.1% formic acid and 100% acetonitrile at a flow rate of 300 nl min^{-1} and detected using a mass spectrometer (Q-Exactive HF; Thermo Fisher Scientific).

Data were analyzed with Mascot software (Matrix Science, London, UK; version 2.6.1), which was set up to search the cRAP_20150130 and NCBI databases (selected for *Viridiplantae*, January 2018, 5845301 entries). Mascot was searched with a fragment ion

mass tolerance of 0.060 Da and a parent ion tolerance of 10.0 PPM. De-amidation of asparagine and glutamine, oxidation of methionine and carbamidomethyl of cysteine were specified in Mascot as variable modifications. Scaffold (version 4.7.5, Proteome Software Inc., Portland, OR) was used to validate the MS/MS based peptide and protein identifications from both replicates. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides using a false discovery rate of <1%.

5.3.6. Statistical analysis

The overall difference between protein concentration and protein digestibility of all samples were first analyzed using ANOVA. Following ANOVA, the specific difference among all samples were assessed using Tukey's test and differences among the original seeds and greenhouse grown seeds for same samples were analyzed using t-test, at a significance level of 0.05. Pearson's correlations were computed between protein content, digestibility, percent change in protein content and digestibility of original and greenhouse-grown seeds. Separate correlations were computed between each identified protein from MS analysis and digestibility after cooking for selected samples. The results obtained was used to construct a principal component analysis (PCA) score plot. All descriptive and inferential statistics were computed using JMP statistical software (JMP version 12.0.1, SAS Institute Inc.)

5.4. RESULTS AND DISCUSSION

Protein content and digestibility after cooking was measured in 45 different samples including proso millet and related species (inedible grasses from the *Panicum* genus and other millet varieties) with different geographical origins. From the original

pool of seeds, 33 proso millet samples were planted in the greenhouse to have understanding of growth pattern of proso millet and observe morphological characteristics of varieties with different origin. Also, by growing seeds under controlled environmental conditions of green-house we can be sure that the observed results are genetic traits of plants and not environment driven effects. The ultimate objective of this work was to identify even a single proso millet sample whose digestibility was not adversely affected by cooking as observed earlier (Gulati et al., 2017, Gulati et al., 2018).

5.4.1. Morphological characteristics of original seeds and greenhouse-grown seeds

The 35 proso millet samples included 5 samples with a red seed color, 2 with an orange seed color, 2 with a grey seed color, and the remaining with a white seed color. The other samples studied that belonged to the *Panicum* genus were little millet and the inedible grasses switchgrass, witchgrass and panicgrass, which are mostly used for bio-fuel production (McLaughlin and Kszos, 2005). The other millet varieties studied were foxtail, finger, and pearl millets, which do not belong to the *Panicum* genus but are characterized as drought-resistant, sustainable crops similar to proso millet (Table 5.1).

Only the proso millet samples were planted in the greenhouse (First 33 samples in Table 5.1). All proso millet varieties grew in the greenhouse with variable yield (low to good) except sample #11 from Afghanistan, which did not germinate. Additionally, sample #4 (China, red), #8 (Canada, grey), #9 (Czech, grey), #22 (Kazakhstan, white), #31 (US, white), and G32 (US, white) did not produce enough seeds for analysis. Thus, 26 greenhouse-grown proso millet samples were available for analysis of protein concentration and protein digestibility.

The height of the greenhouse plants ranged between 38 cm and 109 cm with a mean height of 61 cm, similar to other reports for proso millet (Trivedi et al., 2015). All plants from Afghanistan and Turkey were tall (above average height) while plants from China and US had below average height. Some plants started flowering within 26 d of planting while others took up to 48 d, with an average of 37 days to flowering. This is similar to observations by Upadhyaya et al. (2011). Plants with different panciles (lateral, vertical loose, and dense type) were observed (Wang et al., 2016) with most plants having a lateral panicle. There was no relation between the plant yield with region of origin, seed color, or plant height. The maximum yield was observed for sample #15, which also had a relatively large seed size, which was followed by sample #30. On the other hand, a sample from Ukraine (#17) flowered first (26 d) but produced the smallest seeds and had shortest stature. Apart from the common characteristics, a peculiar feature was observed for sample #3 from China, in which two seeds developed in one spikelet. The plant had very low yield with 5-6 spikes and only 8-10 spikelets per spike, but each spikelet had two mature seeds arranged back to back. While there is no report of such observation in proso millet or other millet varieties, similar multiple seed spikelets have been observed in maize and sorghum varieties from Asia (Kempton, 1913; Karper, 1931), which is considered a growth abnormality due to insufficient utilization of resources by the plant. The broad range of different characteristics of plants in our study suggests wide genetic diversity in used samples.

5.4.2. Protein concentration of proso millet samples

The protein concentration of the originally procured proso millet seeds ranged from 9.3 to 15% with an average value of 12.1%. The protein concentration of the

greenhouse-grown seeds was generally higher and ranged between 12.2 and 18.1% with an average value of 14.4% (Fig. 5.2). There was no correlation between original seed protein content and greenhouse-grown protein content, but there was a significant negative correlation (r^2 = -0.74) between original seed protein and the percent change in protein content [(original-greenhouse)/original*100%] while a positive correlation (r^2 = 0.51) was observed between greenhouse seed protein and percent change in protein (Supplementary Fig 1). The highest increase in protein content was observed for samples from Middle East with up to 40% increase in protein content but the increase was not consistent for all the samples of the same region suggesting a genotype effect.

The increase in protein content in greenhouse-grown seeds compared with the originally procured samples can be due to known negative correlation between grain yield and grain protein content as observed for various cereals (Simmonds, 1995; Bogard, 2010) which can be associated with N dilution effect by carbon based compounds (Acreche and Slafer, 2009). The growth of these plants in the greenhouse was done under commonly used soil and fertilizer conditions with no modifications to optimize millet plant growth. The objective of this experiment was to observe growth pattern of different varieties of proso millets under same environmental conditions without any focus on yield, this resulted in reduced yield when compared to field growth and thus increased protein content. The deviations from this common phenomenon can be linked to genotype by environment interactions which at times can result in no change or have more adverse effect (Oury et al, 2003).

5.4.3. In vitro digestibility of proso millet samples after cooking

As with protein concentration, the protein digestibility of greenhouse-grown samples was higher than the originally procured seeds except for samples 2, 6, 12, 15, 16, 21, 27, and 30, for which there was no difference (Fig. 5.3). There was a positive correlation between digestibility of the originally procured seeds and the greenhouse-grown seeds ($r^2 = 0.78$); original seed protein and percent change in protein digestibility; greenhouse seeds protein digestibility and % change in protein digestibility ($r^2 = 0.58$) but no correlation between original seeds protein digestibility and percent change in protein digestibility (Supplementary Fig 5.7).

The protein digestibility after cooking for various proso millet samples ranged between 26% and 57% with an average digestibility of 32%. There was a significant loss in digestibility of all proso millet samples on cooking when compared with the uncooked control (data from Gulati et al., 2017 for commercial proso millet sample from US) (Fig. 5.3.) due to hydrophobic aggregation as reported before (Gulati et al., 2017). Sample #6 (US, orange) had the lowest digestibility for both original and greenhouse grown sample while sample #20 (China, white) had highest digestibility, 35% more than average digestibility. A peculiar observation was made for one sample from India (#19) which had the average digestibility when measured from originally procured seeds but after growth in the greenhouse the digestibility was much higher than the average, almost similar to sample #20. This sample took the longest time to flower (48 d) but was allowed to mature for the same length of time as all other plants, the observed high digestibility in this sample could be due to highly digestible water-soluble albumins and globulins rather than storage proteins, these water-soluble fractions of cereal proteins are mostly present during early stages of grain development and are down-regulated towards grain maturity

when prolamins (storage proteins) start increasing (Dong et al., 2012). Nonetheless, the relatively high digestibility of samples 19 and 20 suggested that these samples might have different protein profiles compared with the other samples and demands further investigation to understand the changes that could be used to develop a proso variety with high digestibility even on cooking.

5.4.4. Comparing protein content and in vitro digestibility of proso millet samples to related species

The protein concentration of edible little millet and inedible grasses from the *Panicum* genus (switchgrass, panicgrass) was either higher or similar to average protein content in proso millet samples (Fig. 5.4A). On the other hand, all the other millet varieties not belonging to the *Panicum* genus (foxtail, finger and pearl) had significantly lower protein than the average protein in proso millet.

On comparing the protein digestibility of these samples after cooking the observed results were completely reversed (Fig. 5.4B). All *Panicum* samples (edible and inedible) had low protein digestibility upon cooking when compared with average digestibility from proso samples. The lowest digestibility was for the switchgrass samples, with the sample from the United States having the highest protein (16%) but lowest digestibility (16%). The protein digestibility of the other millet varieties was higher than cooked proso millet average and for pearl and finger millet it was similar to uncooked proso millet. Both commercial and non-commercial samples of foxtail millet had about 60% digestibility, which was higher than cooked proso millet, but not as high as observed for other cereals (Kulp et al., 2000). Similar results in digestibility for pearl and finger millet has been reported before (Ramachandra et al, 1977; Ejeta et al., 1987).

Further, the low digestibility of foxtail millet when compared with other millets could be a characteristic of the Panicoideaae subfamily to which both foxtail and proso millet belong but a digestibility of only up to 35% as observed for majority of proso samples and related species of *Panicum* genus indicates a more specific characteristic of this genus.

5.4.5. SDS-PAGE

Based on the digestibility results, commercial de-hulled proso millet sample from US (#38) with almost average digestibility; #6 from US with low digestibility; #30 from US with intermediate digestibility; and #19 and #20 from India and China with high digestibility were selected for SDS-PAGE. Proteins were extracted from both original and green house grown samples selected based on digestibility, except for commercial sample for which only original seed flour was used (Fig 5.5A), also protein profiles of non-proso samples were compared (Fig 5.5B). There were no prominent differences in protein profiles of the control sample (lane 1) with other proso samples (lane 2-9) except slightly lighter high MW bands in the control. These represent albumins and globulins, which may have been removed during the de-hulling process of the control, which did not take place for the other samples. For all the proso millet samples, the major protein band at ~23 KDa, representing proso millet prolamins, panicin, was previously identified as the protein fraction that was associated with the poor digestibility upon cooking (Gulati et al. 2017). There were no visual differences in banding pattern for lanes 2-5 which were proteins from selected samples with the low and moderate digestibility. Lanes 6 and 7 were proteins from the sample from India (#19) that showed a huge change in digestibility when measured from originally procured seeds

compared with greenhouse-grown seeds. The proteins from these samples appeared to have a slightly thinner prolamin band. Lanes 8 and 9 were the proteins from sample 20 from China, which had consistently the highest digestibility among all samples for both original and greenhouse-grown seeds. The protein profile from these samples was similar to the other proso samples, but may have a slightly thinner prolamin band compared with other samples.

Overall, the protein profiles of all the proso samples did not indicate a major difference in proteins even though the samples had different digestibility upon cooking (Fig. 5.5A). This indicates that the differences in proteins might be at a molecular level that SDS-PAGE could not capture. Further, based upon the protein profile of related species almost all of the samples from the panicodeae subfamily samples [lane 10-12 and 15-16 in Fig 5.5(B)] had lower MW prolamins similar to proso proteins which also appeared as one thick band. Lane 11(little millet) and 15 (witchgrass) had a protein profile very similar to proso proteins due to genus similarity but not so for lane 16 (switchgrass). This was not observed for the other samples, such as pearl millet (lane 13) and finger millet (lane 14), which contained many protein bands between 20 and 25 kDa. All this suggests that that the MW of prolamin storage proteins is similar and appears as one thick band. This band could be a single low digestible protein with no subunits but could also be a cluster of many subunits with very similar MW, migrating together upon electrophoresis.

5.4.6. Protein identification

The main prolamin band of SDS-PAGE gel (23 KDa) from one commercial (#38) and 3 proso millet samples grown in the green house (#6: lowest digestibility; #30:

intermediate digestibility; and #20: highest digestibility) were subjected in duplicates to LC-MS/MS analysis after trypsin/chymotrypsin digestion.

Thirty-nine proteins from different plant species were identified that had matching peptides to at least one of the proso millet sample tested (Table 5.2). About half of the identified proteins matched to different accessions of *Panicum hallii*. *P. hallii*, commonly known as Hall's panicgrass is an inedible grass commonly used as a genetic model for studying biofuel crop switchgrass (Meyer et al., 2012). At present, it is the only crop with publically available transcriptomic data from the genus *Panicum*. Among the four proso samples we tested, #20 had the maximum peptide matches with *P. hallii* but other three samples were not far behind. There was also a significant correlation between protein digestibility of samples and proteins identified from three accessions of *P. hallii*. There was a positive correlation of digestibility with 18 and 19 kDa peptides of *P. hallii* and negative correlation with higher 24 kDa band (Table 5.2). This could be an indication that the prolamin band from proso proteins might be a cluster of sub units and high MW subunit is actually responsible for lower digestibility but this hypothesis needs to be validated. Further, three common unique peptide sequence identified from *P. halli* protein matching with all proso samples were: S**PIAAV**GYEH**PIV**QSY; NQ**LAVAVA**NS**AAF**; and NQ**LAVA**N**PAAIL**. One striking observation from the amino acid sequence was that half of the amino acids were hydrophobic and arranged in clusters (*hydrophobic amino acids in bold*). This was not observed for proteins identified from other plants but only *P. hallii*. In our previous study, we showed that hydrophobic aggregation was the major cause of low digestibility of cooked proso millet proteins (Gulati et al., 2017). These

peptide sequences with many conjunctive hydrophobic amino acids could be a potential reason for the formation of hydrophobic aggregates.

After *P. hallii*, the next highest match was to proteins from foxtail millet (*Setaria italic*) and few-flowered panicgrass (*Dicanthelium oligosanthes).* Both these species belong to the same sub family as proso millet. A significantly high positive correlation (0.92) was observed between a 20 kDa protein band of a foxtail millet accession with digestibility of the samples. Further, a high positive correlation (0.95) was also observed between a 9 kDa peptide of panicgrass with digestibility but this protein had peptide sequence matching only with sample #20. Also, interestingly only one replicate of sample #20 had matching peptides to the starch synthase enzyme of proso millet. Both these observations might point to the uniqueness of sample 20 which had highest digestibility among all samples.

Proteins from maize and sorghum were also identified in our proso millet samples; however, the matches were not to the storage proteins but instead to the histones and embryo specific proteins, suggesting that storage proteins of proso millet are indeed quite different from zeins and kafirins.

Further, the correlations between identified proteins and digestibility was used to plot a PCA score plot (Fig. 5.6) and based upon the sample loadings, both replicates of sample #20 had positive loadings of component 1 while all other samples had negative loadings but there was also a huge variation in replicates of #20 and #30. Cluster analysis was used to group the samples on PCA plot and based upon the proximity of their distance, both replicates of commercial sample grouped together along with one of the replicate of sample #6 while the other replicate of #6 grouped with one replicate of #30,

the other replicate of #30 grouped with one replicate of #20 while the other replicate of #20 stood by its own. There is definitely an indication of similarity between commercial proso millet sample and sample with low digestibility while given the variation in the data for intermediate and high digestibility sample it is difficult to draw a conclusion about them but given the positive loadings of #20 and earlier described observations it is evident that sample #20 from China with highest digestibility has different protein profile from other proso samples. This sample could be used as a potential cultivar to develop a proso millet with high digestibility after cooking but requires some more investigation.

5.5. CONCLUSION

The observed protein content and protein digestibility (%) after cooking for greenhouse grown seeds were higher than original seeds. The protein digestibility of different proso millet samples ranged between 26 and 57% with most samples having a digestibility around 34%. For other species belonging to the *Panicum* genus the protein content was slightly higher than proso millet but the digestibility of all *Panicum* samples was lower than 35% after cooking. The highest digestibility among cooked proso millet samples was observed for a white seeded sample from China. There were no major differences in protein profile of different proso samples under SDS-PAGE. Peptides from trypsin/chymotrypsin digestion of proso millet panicins matched most closely with *P. hallii,* with low MW peptides having a positive correlation with digestibility and the peptides contained strings of connected hydrophobic amino acids. The results from this study suggest that low digestibility upon cooking may be a unique property of genus *Panicum*. However, the presence of one sample with significantly higher digestibility

than the other samples suggests that highly digestible cooked proso millet may be possible.

5.6. REFERENCES

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Table 5.1. Proso millet samples and related species used in the study and the observed characteristics after growth in greenhouse

Sample#	Original seeds characteristics					Characteristics during growth in greenhouse			
	Accession# /cultivar name	Scientific Name	Common name	Country of Origin	Seed Color	Height of main stalk	Special morphological traits	Observed yield	Days to flower
1	PI 531426	P. miliaceum	Proso Millet	Hungary	Red	45.7	Bushy plants	Low	30
2	PI 436623	P. miliaceum	Proso Millet	China	Red	48.2	Bushy plants	Low	46
3	PI 436625	P. miliaceum	Proso Millet	China	Red	50.8	Twin seeded spikelet	Low	38
4	PI 662288	P. miliaceum	Proso Millet	China	Red	40.6	Spikelet at the base of stalk	Not enough seeds	48
5	PI 531423	P. miliaceum	Proso Millet	Poland	Red	49.5	Lateral Panicle	Good	26
6	Cerise	P. miliaceum	Proso Millet	United States (Nebraska)	Orange	41.9	Lateral panicle	Intermediate	27
7	PI 531425	P. miliaceum	Proso Millet	Hungary	Orange	46.5	Lateral panicle	Intermediate	27
8	PI 296376	P. miliaceum	Proso Millet	Canada	Grey	60.9	Vertical loose panicle	Not enough seeds	31
9	Pi 531406	P. miliaceum	Proso Millet	Czech Republic	Grey	58.4	Vertical loose panicle	Not enough seeds	30
10	PI 220535	P. miliaceum	Proso Millet	Afghanistan	White	81.3	Lateral Panicle	Intermediate	39
11	PI 212862	P. miliaceum	Proso Millet	Afghanistan	White	-	Couldn't grow		-
12	PI 223794	P. miliaceum	Proso Millet	Afghanistan	White	80.0	Lateral panicle	Good	35
13	PI 179380	P. miliaceum	Proso Millet	Turkey	White	109	Vertical loose panicle	Intermediate	48
14	PI 170595	P. miliaceum	Proso Millet	Turkey	White	73.7	Vertical loose panicle	Intermediate	36
15	PI 173750	P. miliaceum	Proso Millet	Turkey	White	63.3	Big seeds	Good	32
16	PI 250979	P. miliaceum	Proso Millet	Former Serbia (Yugoslavia)	White	55.9	Lateral panicle	Intermediate	41
17	PI 346942	P. miliaceum	Proso Millet	Ukraine	White	38.1	Small seeds	Intermediate	25
18	PI 517018	P. miliaceum	Proso Millet	Morocco	White	88.9	Lateral dense panicle	Good	42
19	Ames 11680	P. miliaceum	Proso Millet	India	White	64.8	Lateral panicle	Intermediate	48
20	PI 436626	P. miliaceum	Proso Millet	China	White	55.9	Lateral panicle	Intermediate	38
21	PI 251389	P. miliaceum	Proso Millet	Iran	White	63.5	Lateral panicle	Intermediate	42
22	PI 346938	P. miliaceum	Proso Millet	Kazakhstan	White	45.7	Vertical loose panicle	Not enough seeds	29
23	PI 516181	P. miliaceum	Proso Millet	Romania	White	86.4	Dense panicle	Very Good	36
24	PI 346936	P. miliaceum	Proso Millet	Kyrgyzstan	White	53.3	Dense panicle	Intermediate	33
25	PI 531400	P. miliaceum	Proso Millet	Hungary	White	60.9	Lateral panicle	Intermediate	36
26	PI 202295	P. miliaceum	Proso Millet	Argentina	White	83.8	Lateral panicle	Intermediate	39
27	PI 531400	P. miliaceum	Proso Millet	Hungary	White	63.5	Lateral panicle	Intermediate	33
28	PI 430742	P. miliaceum	Proso Millet	Australia	White	78.7	Lateral panicle	Intermediate	42

29	Farmers entry	P. miliaceum	Proso Millet	Austria	White	50.8	Lateral dense panicle	Good	31
30	PI 649383	P. miliaceum	Proso Millet	United States (Nebraska)	White	50.8	Big seeds	Good	29
31	Abarr	P. miliaceum	Proso Millet	United States (Nebraska)	White	45.7	Lateral panicle	Not enough seeds	27
32	PI 649384	P. miliaceum	Proso Millet	United states (Minnesota)	White	43.2	Lateral panicle	Not enough seeds	27
33	PI 253421	P. miliaceum	Proso Millet	Spain	White	81.3	Lateral panicle	Intermediate	37
34	PI 436720	P. sumatrense	Little Millet	India	White	-	Not grown in green house		-
35	PI 654448	P. hirticaule	Panicgrass	Mexico	White	-	Not grown in green house		-
36	-	P. capillare	Witchgrass	United States	White	-	Not grown in green house		-
37	Commercial	Setaria Italica	Foxtail millet de-hulled	China	White	-	Not grown in green house		-
38	Commercial	P. miliaceum	Proso Millet de-hulled	United States (Colorado)	White	-	Not grown in green house		-
39	Commercial	P. miliaceum	Proso Millet de-hulled	Ukraine	White	-	Not grown in green house		-
40	-	P. virgatum	Switchgrass Octoploid	United States (Nebraska)	White				
41	-	P. virgatum	Switchgrass Tetraploid	United States (Nebraska)	White	-	Not grown in green house		-
42	-	P. virgatum	Switchgrass Octoploid	United States (Illinois)	White	-	Not grown in green house		-
43	UNL N-Si-7	Setaria Italica	Foxtail millet	United States (Nebraska)	White	-	Not grown in green house		-
44	UNL NM-4B	Pennisteum glaucum	Pearl millet	United States (Nebraska)	Grey	-	Not grown in green house		-
45	UNL NeFM#1	Eleusine coracana	Finger millet	United States (Nebraska)	Red	-	Not grown in green house		-

Table 5.2. Proteins identified in different samples of proso millet after LC-MS/MS.

#	Matched Protein Id	Organism	Accession Number	MW (kDa)	Unique Peptide Counts								Correlations with protein digestibility
					#38a	#38b	#6a	#6b	#30a	#30b	#20a	#20b	
1	PAHAL_H00455	*Panicum hallii*	PAN43368.1	24	8	9	9	9	6	11	8	8	-0.23
2	PAHAL_J00793	*Panicum hallii*	PAN53324.1	18	0	0	0	0	4	4	5	4	0.68*
3	PAHAL_D02270	*Panicum hallii*	PAN49697.1	24	3	2	2	4	2	3	5	2	0.29
4	PAHAL_H00450	*Panicum hallii*	PAN43373.1	24	3	2	2	3	2	3	0	2	-0.68*
5	PAHAL_E03585	*Panicum hallii*	PAN31676.1	22	5	6	4	6	0	4	5	4	0.06
6	PAHAL_G01245	*Panicum hallii*	PAN37607.1	49	4	2	2	3	5	4	3	4	0.16
7	PAHAL_E02207	*Panicum hallii*	PAN29292.1	21	3	0	2	0	3	0	0	2	-0.12
8	PAHAL_D02338	*Panicum hallii*	PAN49796.1	58	2	0	0	0	0	2	0	0	-0.2
9	PAHAL_C04165	*Panicum hallii*	PAN21013.1	22	0	3	2	0	3	3	3	4	0.59
10	PAHAL_J00522	*Panicum hallii*	PAN52119.1	17	0	0	0	0	0	0	0	2	0.6
11	PAHAL_B00733	*Panicum hallii*	PAN49407.1	19	3	2	0	2	3	0	2	3	0.4
12	PAHAL_C02647	*Panicum hallii*	PAN19039.1	19	0	0	0	0	2	2	2	4	0.74**
13	PAHAL_B02528	*Panicum hallii*	PAN12130.1	24	0	0	2	0	0	0	0	0	-0.41
14	PAHAL_C00211	*Panicum hallii*	PAN41165.1	20	0	0	0	3	0	0	0	0	-0.37
15	PAHAL_A00847	*Panicum hallii*	PAN04747.1	20	2	0	2	0	3	0	3	3	0.55
16	PAHAL_I02334	*Panicum hallii*	PAN45805.1	62	2	0	0	0	2	0	2	2	0.6
17	PAHAL_F01989	*Panicum hallii*	PAN35492.1	25	0	0	0	0	0	2	0	3	0.46
18	hypothetical protein PAHAL_C02115	*Panicum hallii*	PAN18358.1	19	2	0	0	0	0	0	0	2	0.37
19	PAHAL_C01299	*Panicum hallii*	PAN17201.1	37	0	0	0	0	2	0	0	0	-0.16
20	PAHAL_C01409	*Panicum hallii*	PAN17346.1	21	3	0	0	3	0	0	0	0	-0.37
21	S-type granule-bound starch synthase 1,	*Panicum miliaceum*	ACJ49212.1	37	0	0	0	0	0	0	0	2	0.6
22	Kafirin PSKR2	*Setaria italica*	XP_012703493.2	44	4	4	5	3	4	6	5	6	0.51
23	LOC101757961	*Setaria italica*	XP_004984201.2	20	0	0	0	0	0	0	2	3	0.92**
24	60S ribosomal protein L9	*Setaria italica*	XP_004964297.1	21	0	0	0	0	0	0	0	4	0.6

25	ATP synthase subunit	*Setaria italica*	XP_004973702.1	20	0	0	0	0	0	0	0	3	0.6
26	LOC101758756	*Setaria italica*	XP_004961786.1	18	0	0	0	0	0	0	0	3	0.6
27	Vicilin-like seed storage protein	*Dicanthelium oligosanthes*	OEL15976.1	57	2	3	0	0	0	0	0	3	0.36
28	Late embryogenesis protein B19.4	*Dicanthelium oligosanthes*	OEL21938.1	17	3	2	3	4	3	5	2	3	-0.44
29	BAE44_0014842	*Dicanthelium oligosanthes*	OEL24138.1	9	0	0	0	0	0	0	2	2	0.95**
30	Vicilin-like seed storage protein	*Dicanthelium oligosanthes*	OEL15976.1	57	2	3	0	0	0	2	0	3	0.3
31	60S ribosomal protein L21	*Zea Mays*	ACG30052.1	19	0	0	0	2	0	0	2	2	0.6
32	embryo specific protein	*Zea Mays*	NP_001105349.2	12	3	2	0	3	3	3	0	5	0.12
33	Peroxidase 12	*Zea Mays*	ONM34315.1	38	0	0	0	0	0	2	0	0	-0.14
34	zein-alpha A20	*Sorghum bicolor*	XP_002451115.1	29	0	0	0	0	0	2	0	0	-0.14
35	histone H2B.1 isoform X2	*Sorghum bicolor*	XP_021312576.1	29	0	0	2	3	0	3	2	4	0.3
36	hypothetical_protein	*Oryza brachyantha*	CBX25323.1	17	2	2	0	2	0	0	0	2	0.05
37	YK426	*Oryza sativa*	BAA19798.1	21	0	0	0	0	0	0	0	4	0.6
38	At1g07930/T6D22	*Arabidopsis thaliana*	AAK32834.1	50	0	0	0	0	0	0	0	3	0.6
39	histone H4 replacement, isoform C	*Drosophila melanogaster*	NP_524352.1	11	0	0	0	0	0	0	0	4	0.6

#38: commercial sample; #6: low digestibility; #30: intermediate digestibility; #20: high digestibility. a and b are duplicates of same samples, *significant at alpha=0.05; **significant at alpha = 0.01. n=8.

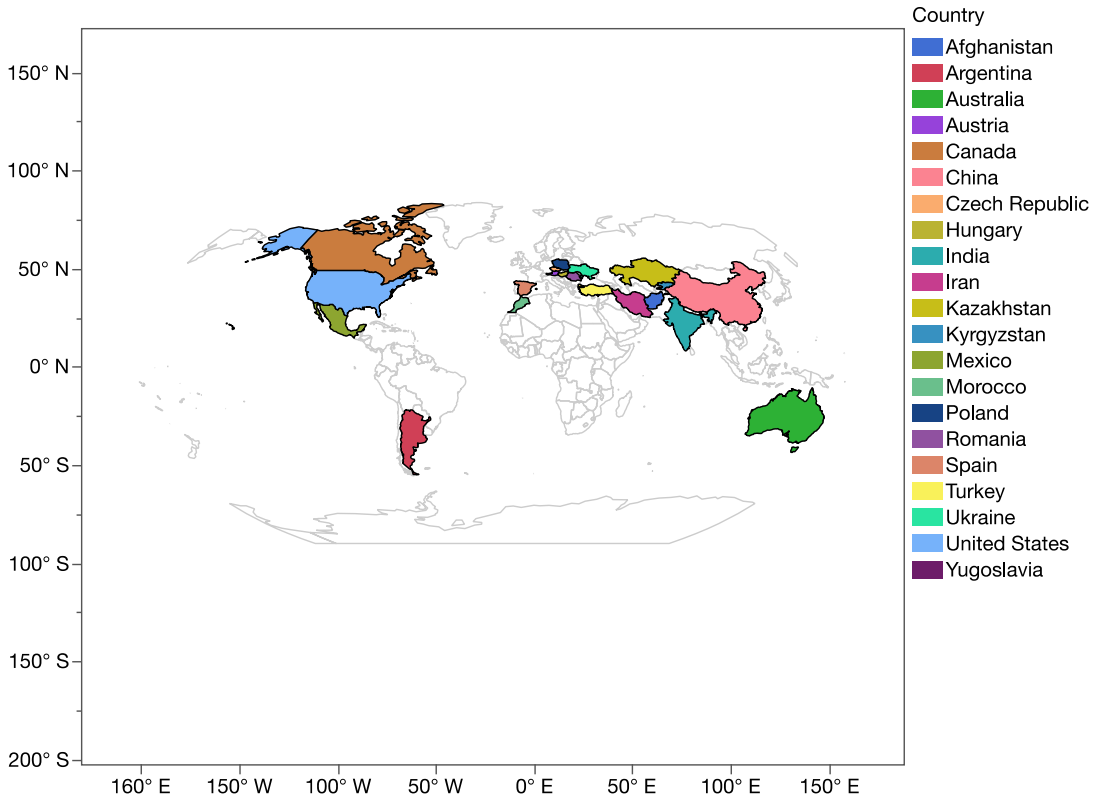

Figure 5.1. World Map highlighting the different countries of origin from which samples were used in the study

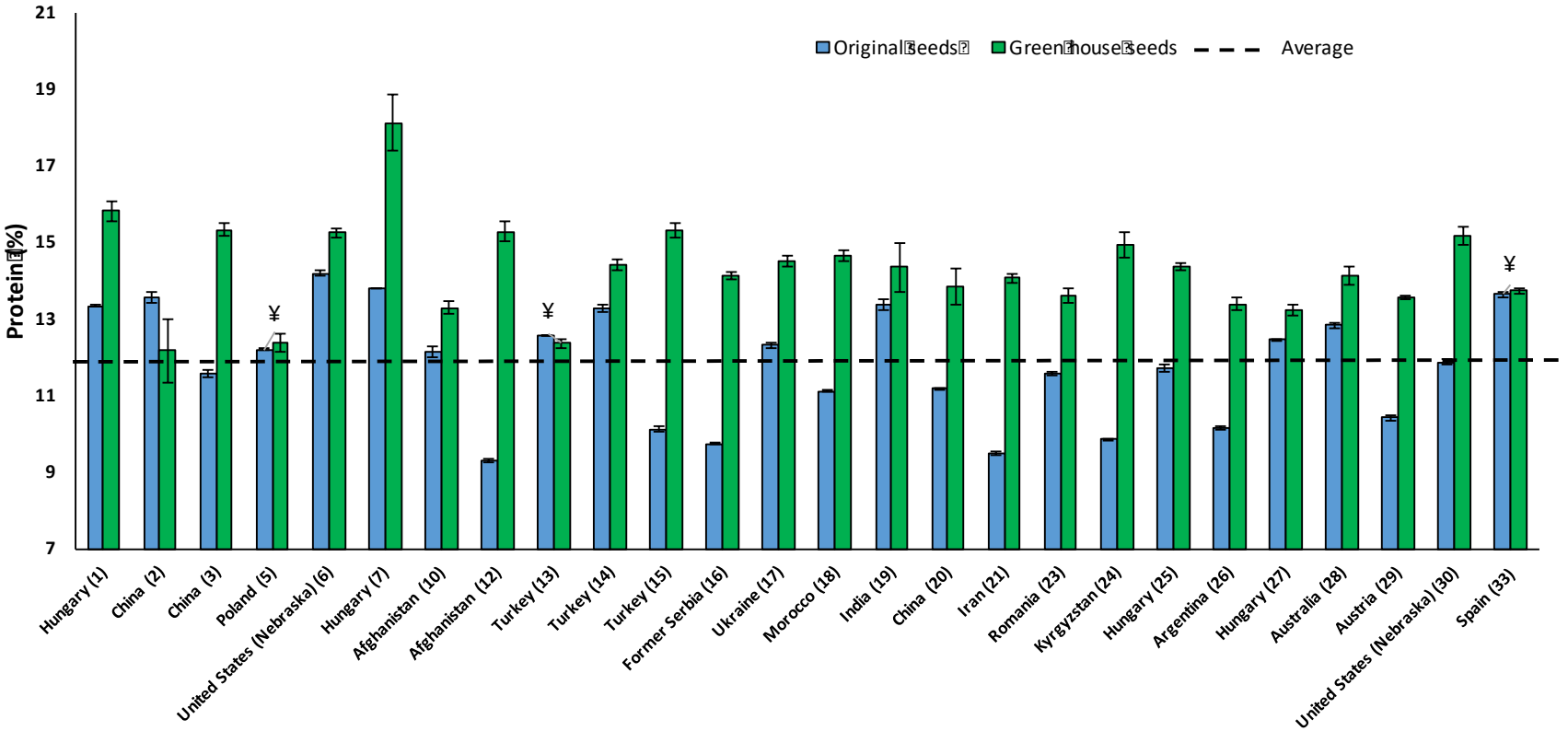

Figure 5.2. Average protein concentration of originally procured seeds and greenhouse-grown seeds.[20]

[20] Error bars show standard deviation (n=3); some error bars were too small to plot; * Bars marked with, '¥', represents samples which has the same protein content for original seeds and greenhouse seeds, compared by t-test (P value ≥ 0.05); dotted line represents average protein content of original seeds. Numbers in parenthesis of horizontal axis are the sample # from Table 5.1.

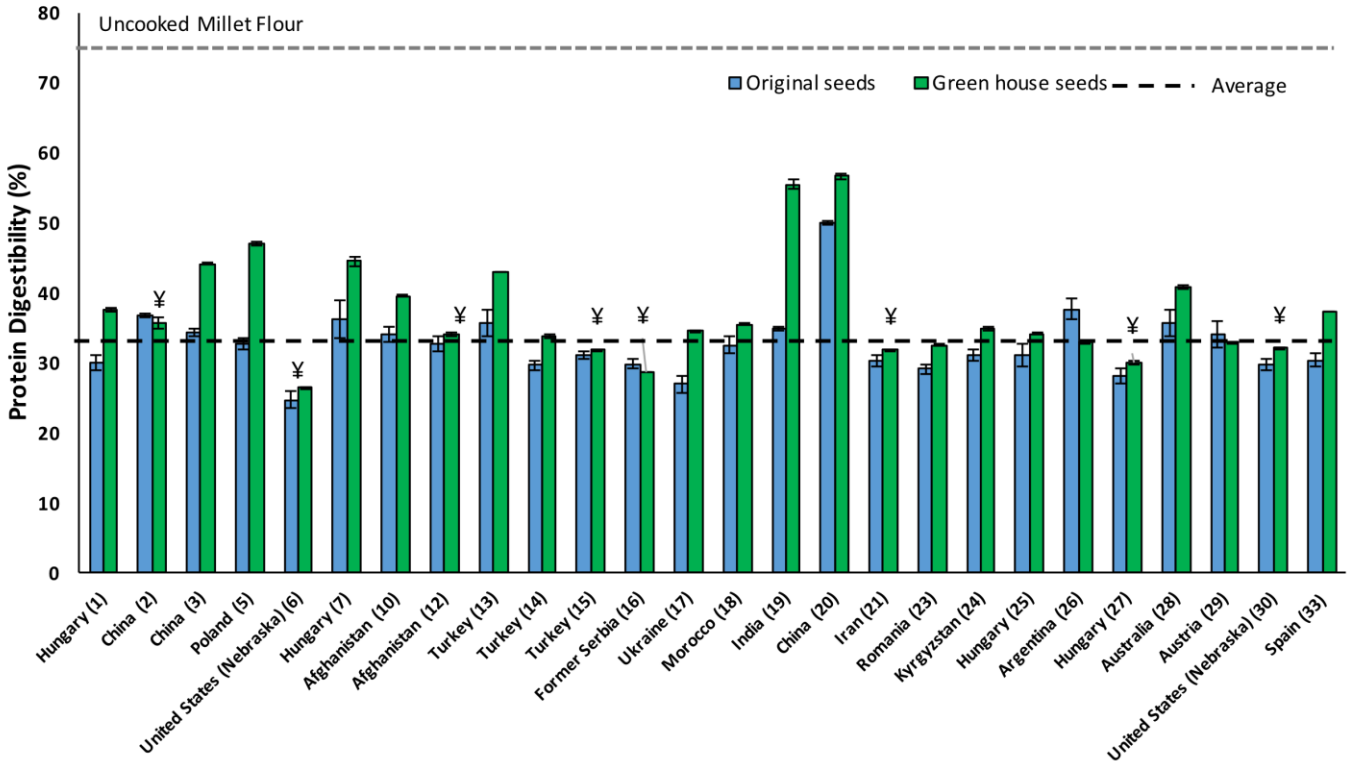

Figure 5.3. Average protein digestibility after cooking from original seeds greenhouse-grown seeds.[21]

[21] Error bars show standard deviation (n=3); some error bars were too small to plot; Bars marked with, '¥', represents samples which has the same digestibility for original seeds and greenhouse seeds, compared by t-test (P value ≥ 0.05); dotted line represents average protein digestibility of original seeds. Numbers in parenthesis of horizontal axis labels are the sample # from Table 5.1.

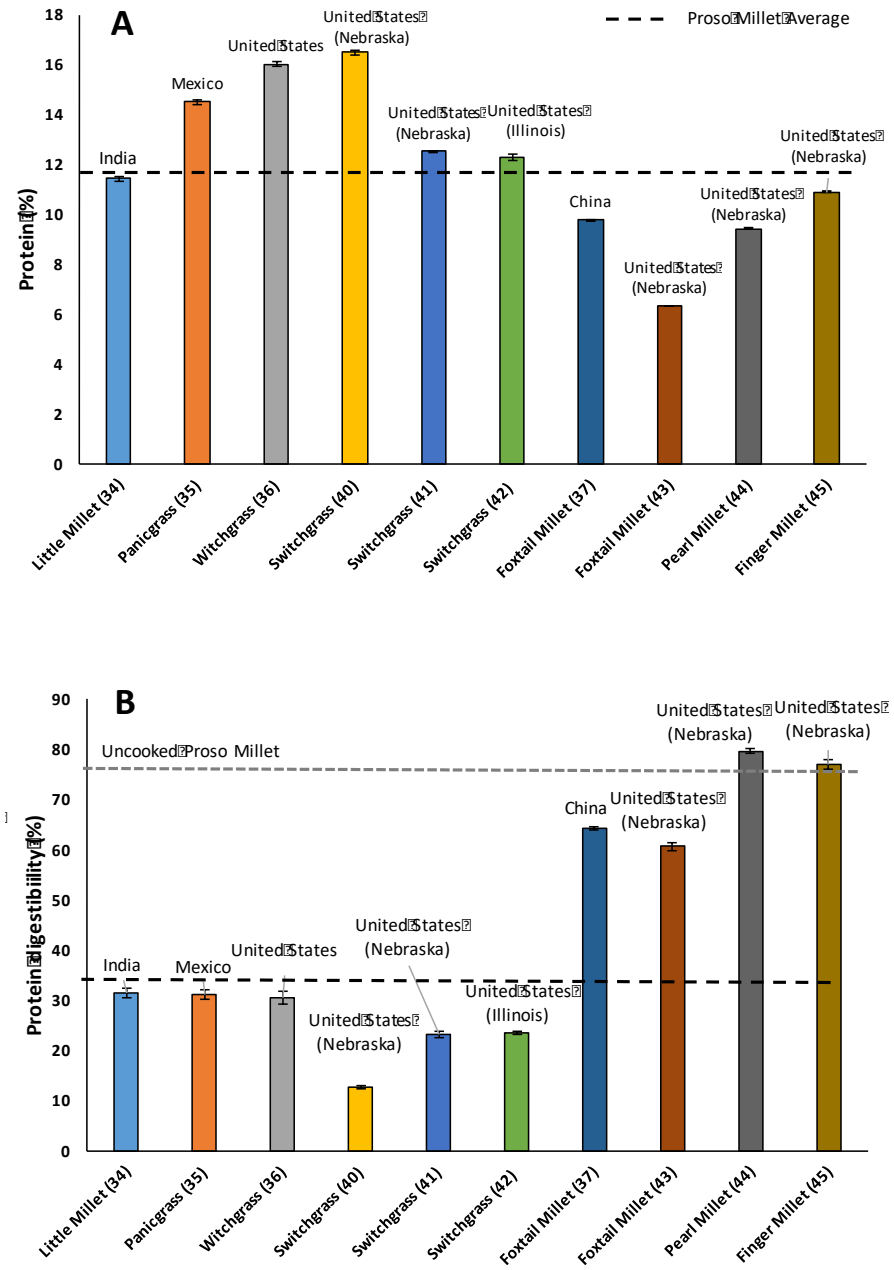

Figure 5.4. Average protein content (A), digestibility after cooking (B) of proso related species and other millet varieties.[22]

[22] Error bars show standard deviation (n=3); some error bars were too small to plot; dotted line represents average protein content and protein digestibility of proso millet samples. Numbers in parenthesis of horizontal axis labels are the sample # from Table 5.1.

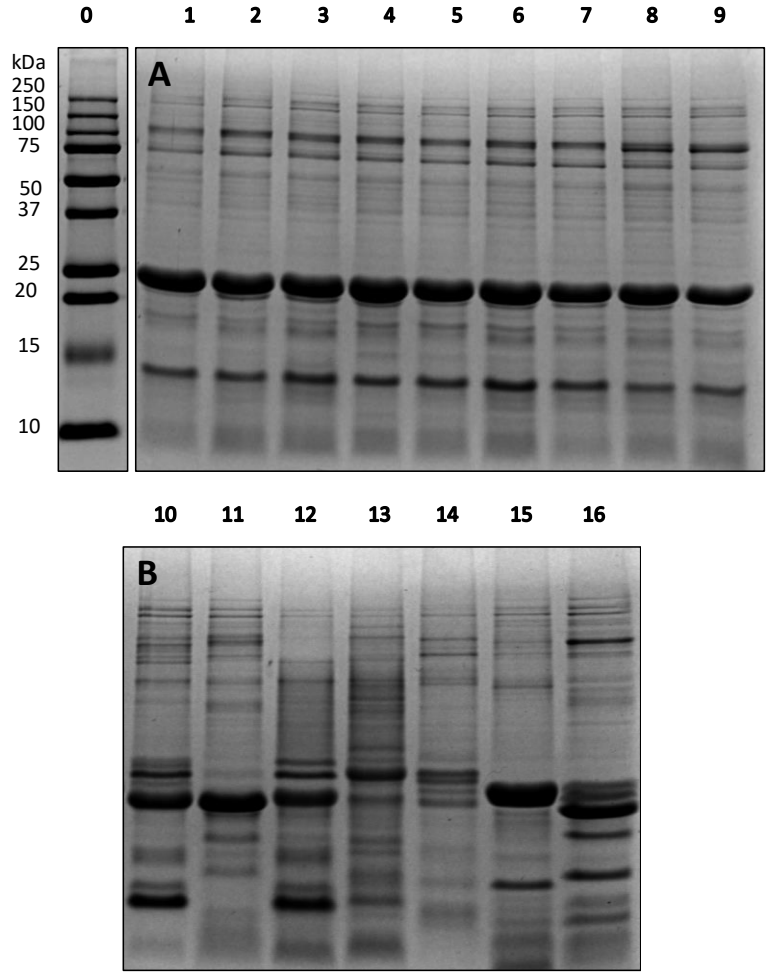

Figure 5.5. SDS-PAGE gels of different proso millet samples (A), and non-proso samples (B) used in the study.[23]

[23] Lane 0, MW marker; 1, commercial proso millet (#38); 2, #6 original seed flour (lowest digestibility); 3, #6 greenhouse seed flour (lowest digestibility); 4, #30 original seed flour (intermediate digestibility); 5, #30 greenhouse seed flour (intermediate digestibility); 6, #19 original seed flour (intermediate digestibility); 7, #19 greenhouse seed flour (highest digestibility); 8, #20 original seed flour (highest digestibility); 9, #20 greenhouse seed flour (highest digestibility); 10, #37 (commercial foxtail millet); 11, #34 (little millet); 12, #42 (foxtail millet); 13, #43 (pearl millet); 14, #44 (finger millet); 15, #36 (witchgrass); 16, #35 (panicgrass).

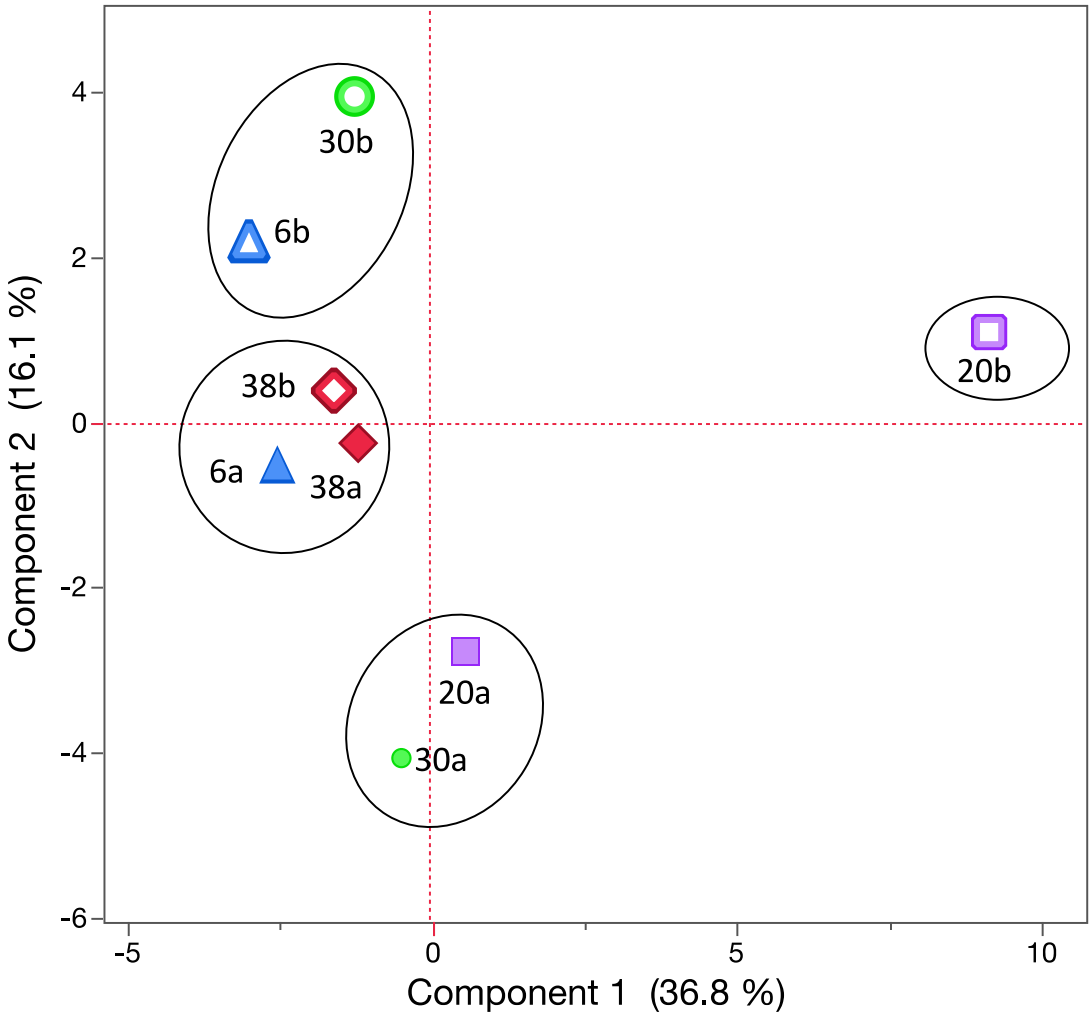

Figure 5.6. PCA score plot based upon correlations between identified proteins and digestibility of samples.

	Original seed protein	Original seed protein digestibility	Green house seed protein	Green house seed protein digestibility	Change in protein (%)	Change in protein digestibility (%)
Original seed protein					-0.74	0.51
Original seed protein digestibility				0.78		
Green house seed protein					0.51	
Green house seed protein digestibility		0.78				0.58
Change in protein (%)	-0.74		0.51			
Change in protein digestibility (%)	0.50			0.58		

Negatively correlated		No correlation	Positively correlated	
p < 0.01	p < 0.05		p < 0.05	p < 0.01

N=26

Figure 5.7. (Supplementary) Correlations among variables.

CHAPTER 6: UNDERSTANDING THE EFFECT OF EXTRUSION ON PHYSICAL PROPERTIES AND DIGESTIBILITY OF GREAT NORTHERN BEANS USING A MODIFIED CENTRAL COMPOSITE SPLIT PLOT DESIGN

6.1. ABSTRACT

Great Northern beans (*Phaseolus Vulgaris* L.) (GNB) are a protein- and fiber-rich food with substantial functional components such as resistant starch and antioxidants. The consumption of GNB is limited due to the presence of anti-nutritional factors and complex polysaccharides and is mostly consumed in canned form. Extrusion is a versatile processing technique with known effects in lowering anti-nutritional factors and improvement in digestibility of several grains. Often extrusion experiments are conducted as completely randomized designs when in fact some variables, especially barrel temperature (T), are not actually randomized due to operating and time constraints. Thus, the objective of the present study was to evaluate the effect of extrusion as a more rapid bean processing technique using a split plot analysis. GNB were extruded in a laboratory scale twin screw extruder using a modified central composite design structure with T as a whole plot factor and MC and SS as split plot factors. Effect of extrusion on physical (expansion, density, WAI, WSI) properties, trypsin inhibitor activity and protein digestibility of GNB was analyzed. Bean extrudates had lower expansion when compared with cereal extrudates due to high amount of fiber and proteins. Extrusion was effective in significantly reducing up to 97% of trypsin inhibitor activity from bean flour. This with the combination of barrel temperature significantly improved the protein digestibility of GNB flour. MC significantly affected all the responses for bean extrusion

which was followed by SS. T had the most significant effect on protein digestibility of bean extrudates.

6.2. INTRODUCTION

Great Northern beans (GNB) are the principal white colored dry bean (*Phaseolus Vulgaris* L.) variety grown in the US with major production in the state of Nebraska (USDA crop production database, 2017). Like other dry bean varieties, GNB are a protein and fiber-rich food together with other non-nutritive functional compounds such as polyphenols and resistant starch, which are known for their low glycemic index, anti-oxidant, anti-cancer, prebiotic, cholesterol lowering and other health promoting properties (Xu et al., 2007; Wang et al., 2010; Siddiq et al., 2010; Messina, 2014,). However, dry beans take considerable time to cook, have a complex oligosaccharide profile (raffinose, stachyose), and contain anti-nutritional components such as trypsin inhibitors, phytates. which interfere with utilization of proteins and carbohydrates and cause intestinal discomfort (Reddy et al., 1984; Chavan and Kadam, 1989). All these undesirable properties overshadow the goodness of beans and confines its consumption to canned form to limited group of consumers.

Several traditional processing techniques like soaking, germination, fermentation etc. can reduce these anti-nutritional compounds in beans (Sathe et al., 1981, Oboh et al., 2000) but they are time consuming with limited versatility and mass appeal. Extrusion processing is a resourceful technique that has been used extensively to either modify food ingredients or develop convenient, cost-effective, and nutritious food products like breakfast cereals or ready to eat snacks. The technique involves a combination of high temperature, shear and pressure to gelatinize starches (and modify other components) which produces expanded, low density products with crunchy texture (Meng et al., 2010).

Further, extrusion has been shown to significantly reduce anti-nutritional compounds and improve digestibility of various legumes (Alonso et al., 2000; Steel et al., 1995).

GNB has nutritional and functional advantages similar to other dry bean varieties but being light in color they lack bitterness causing components (tannins) and can be used to make food products with neutral appearance and taste, thus, making them more acceptable. There is limited research available on extrusion of GNB, the only available report on GNB extrusion is from Sutivisedsak et al., (2012) where they used defatted GNB flour for extrusion and observed slight improvement in water absorption capacity and loss in oil absorption capacity. Other works on GNB are focused mostly on nutraceutical properties of bean components but with simple cooking or canning techniques (Mojica et al., 2014). There is no literature available with emphasis on the extrusion performance of whole GNB flour with the aim of developing a snack product.

Extrusion processing has been extensively used to understand and develop cereal and legume based products. Among the many variables studied in extrusion based experiments, feed moisture content (MC), screw speed (SS) and barrel temperature (T) are the most commonly varied parameters (Gulati et al., 2016; Singh et al., 2007; Brennan et al., 2013). Among these, T is usually a hard to change (HTC) factor due to time and machine constraints, making randomization difficult. In contrast, MC and SS are comparatively easier to change (ETC) and can be randomized. Thus, most of the extrusion based experiments either take considerable time to conduct, or are done without temperature randomization. Lack of randomization for the temperature variable gives rise to a split plot structure, although experiments are often inappropriately analyzed as completely randomized designs under either fractional or full factorial experimental

setup. This approach can create undesirable confounding among predictor variables and result in biased conclusions, especially for the HTC factors like temperature and its interaction with other variables. Very few researchers (Ramos Diaz et al., 2013, 2015) have addressed this issue and used alternative data analysis techniques for food extrusion based experiments.

Thus, the main objectives of this research were to 1) understand the effect of extrusion on anti-nutritional factor and digestibility of whole great northern bean (GNB) flour, and 2) investigate the feasibility of a modified central composite split plot design and analysis for extrusion based experiments.

6.3. MATERIALS AND METHODS

6.3.1. Preparation of bean flour and proximate analysis

GNB were obtained from FNJ Inc. (Alta Loma, CA, USA) and milled using a pilot scale hammer mill (20SSHMBD, C.S. Bell, Tiffin, OH, USA) with screen size of 0.7 mm. The flour was analyzed for moisture, fat, and ash content using approved methods (AACC International, 2013). Protein content was analyzed using a nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25. Total starch content was analyzed using total starch assay kit (K-TSTA, Megazyme, Bray, Ireland) following the KOH format. Bean flour was stored at 4 °C until extrusion.

6.3.2. Experimental design

The effects of three extrusion factors: barrel temperature (T) (90-140°C), feed moisture content (MC) (17-25%), and screw speed (SS) (170-250 rpm), on GNB extrusion were studied while keeping other factors such as feed rate and screw configuration constant. The levels of these factors were determined based on preliminary

trials. Barrel temperature in extrusion based experiments is HTC due to machine stabilization which leads to time and money constraints and makes it difficult to randomize. In order to address this issue a modified central composite split plot design was used in this study (Fig 6.1). The changes to the common CCD was as described by Draper and John (1998). Mainly, the axial points for temperature were removed and the effect of temperature was tested at three fixed levels, -1, 0, and +1 (i.e., 90, 115 and 140 °C, respectively). The design space was rotated by 45° which resulted in new axial (±a = 1.43) and factorial (±b = 0.70) points for MC and SS. The new values for MC and SS were calculated based on preliminary experiments and calculating the ratio of block error variance and experimental error (Draper and John, 1998). Based upon the new design, extrusion was carried under 18 experimental conditions with 2 replicates at (MC, SS, T): 2 runs at ±a, 0, 0 levels; 2 runs at 0, ±a, 0; 8 runs at ±b, ±b, ±1; and 6 center points (i.e. 0,0,0). All experimental conditions were replicated on a separate day resulting in total of 36 data points. The experimental runs with coded and un-coded values for extrusion factors is shown in Table 6.1.

6.3.3. Extrusion process

A laboratory scale co-rotating conical twin-screw extruder with mixing zones was used for extrusion (CTSE-V, C.W. Brabender, Hackensack, NJ, USA). The specifications of extruder and operating conditions used were described previously (Gulati et al., 2016).

To adjust the moisture content of bean flour, batches (2 kg) representing each experimental run were mixed in an upright blender (H-600-D, Hobart, Troy, Ohio, USA) at medium speed with the required water to obtain the target moisture content as per the experimental design. The moistened samples were sealed in polyethylene bags and

tempered for 16 h at 4°C. The flour was then fed into the extruder barrel using a single screw volumetric feeder (FW 40 Plus, C. W. Brabender) set at a constant delivery rate of 76 g/min.

The extrudate sample for each experimental condition was collected after a stable temperature and torque reading was observed. The collected samples were dried in a belt drier (4800 series Wenger, Sabetha, KS, USA) at 100°C for 10 min. Half of the samples were sealed in plastic bags for measuring expansion index bulk density, whereas the other half was ground using cyclone sample mill (UDY, Fort Collins, CO, USA) with a screen size of 1 mm and used for measuring water absorption index (WAI), water solubility index (WSI), trypsin inhibitors (TI), and protein digestibility (PD).

6.3.4. Physical and Chemical Responses

The volume of the extrudates was measured using rapeseed displacement, which was used to calculate the apparent bulk density (BD; g/cm^3) of the extrudates. Five measurements were taken for each experimental condition.

Radial expansion ratio (RER) was calculated by dividing the cross-sectional diameter of each extrudate, measured using a Vernier caliper (Mitutoyo Co., Kawasaki, Japan), by the extruder die diameter (3mm). Ten measurements were taken for each experimental condition.

Water absorption index (WAI) and water solubility index (WSI) were measured after Anderson et al. (1969). Ground extrudate (2.5 g) was suspended in 30 mL water at 30 °C for 30 min with intermittent stirring, centrifuged at 3000 × g for 10 min. The supernatant was decanted into a tared evaporating dish. The WSI was recorded as percent weight of dry solids in the supernatant to the original weight of sample. The WAI was the

weight of gel obtained after removal of the supernatant per unit weight of original dry solids. Three measurements were taken for each experimental condition.

Trypsin inhibitors (TI) in bean extrudates were quantified spectrophotometrically using benzoyl-DL-arginine-*p*-nitroanalide hydrochloride (BAPA) and trypsin as substrate (Kakade et al., 1969). Briefly, trypsin inhibitors were extracted from 0.5 g extrudate sample using 0.01 N NaOH for 3 h under gentle stirring. The sample extract was diluted with NaOH (1:30) and 0.4 mL of diluted extract was used for analysis. Diluted extract was mixed with 0.4 mL of trypsin solution (12 µg/mL) and incubated for 10 min at 37 °C, whereupon 1 mL of BAPA solution in tris-buffer (pH:8.2) containing 0.01% dimethyl sulfoxide was added and incubated for 10 min. The reaction was stopped using acetic acid and the absorbance was measured at 410 nm against reagent blank. TI were calculated in mg/g sample with a conversion factor of 0.019 (i.e., absorbance of 1µg of pure trypsin).

In vitro protein digestibility (PD) was measured as described in Gulati et al. (2017) with slight modifications. Extruded flour (150 mg) was suspended in simulated gastric fluid (SGF; 0.5 M NaCl, pH:2.5) to achieve a pH of 2.5 (approximately 6 mL) and incubated at 37 °C for 10 min. The contents were then mixed with pepsin dissolved in SGF to give an activity of 200 U of pepsin/mg of protein in sample and incubated for 2 h. Gastric digestion was stopped by raising the pH to 7 using 0.5 M sodium bicarbonate. For intestinal digestion, pepsin hydrolyzed samples were mixed with 1 mL simulated intestinal fluid (SIF) (0.05 M KH_2PO_4, pH: 7.0) and warmed at 37 °C. Three mL of pancreatin solution in SIF (3 mg/mL) was then added to the flour mixture and incubated for 4 h. Intestinal digestion was stopped by plunging the tubes into a boiling water bath

for 5 min and immediately cooling. The digested samples were centrifuged and the supernatants were used to measure degree of hydrolysis (DH) by the reaction of free amine groups with TNBS using leucine as the standard. DH was then calculated as described in Gulati et al., 2017 with h_{total} of 7.77 mmol/g protein based upon concentrations of amino acids in GNB flour, obtained by preliminary amino acid analysis. Digestion for each experimental run was performed in duplicate and DH for each digested sample was further analyzed in duplicate.

6.3.5. Data analysis

The data were analyzed using a split plot analysis with T being the whole plot factor and MC and SS as split plot factors, combined for both replicates (Fig 6.2). The following second order model was fitted for each response variable:

$$Y_{ijkl} = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2j} + \beta_3 x_{3k} + \beta_{11} x_{1i}^2 + \beta_{22} x_{2j}^2 + \beta_{33} x_{3k}^2 + \beta_{12} x_{1i} x_{2j} + \beta_{13} x_{1i} x_{3k} + \beta_{23} x_{2j} x_{3k} + \delta_{l(i)} + \varepsilon_{ijkl}$$

where, Y is the response (RER, BD, WAI, WSI, TI or PD) for i^{th} level of x_1 (T); j^{th} level of x_2 (MC) and k^{th} level of x_3 (SS). $\delta_{l(i)}$ is the whole plot error, rep(T) which is independent and identically distributed as $N(0,\sigma_{wp}^2)$ and ε_{ijkl} is the split plot error which is independent and identically distributed as $N(0,\sigma_{sp}^2)$. The variance for $\mathrm{Var}(Y_{ijkl}) = \delta_{l(i)} + \varepsilon_{ijkl}$.

Data analysis was performed using mixed model approach with SAS software (version 9.4. SAS Institute, Cary, NC, USA) with rep(T) as random error which was used to test for the effect of whole plot factor T and its quadratic term. The remaining model parameters were tested using the residual error. Given two error variances in the model, the degrees of freedom for the pooled variance was calculated using Satterthwaite's

approximation (Sahai and Ojeda, 2004). The regression coefficients in the model were estimated and tested using the F-test at significance level of 0.05.

The validity of fitted models were checked using R^2 values, a lack-of-fit test (LOF), and model-fit-error (MFE), which is the ratio of difference between measured and predicted values of a response to the measured value. A MFE range of ±5% was desirable. Further, the distribution of MFE for each response was also checked to make sure there that the error was not biased.

Based on the effects of each variable on responses studied, a profiler illustrating the effect of each variable was constructed using JMP statistical software (JMP version 10.0.0, SAS institute).

The efficiency of the current split plot analysis was evaluated relative to a randomized complete block design (RCBD) (usual way of extrusion analysis with reps in model), assuming that the total error variance of the experiment would be equal to the combined whole plot and split plot error variance (Mugabi et al., 2017). In order to compute individual errors a generalized linear model (GLM) procedure was used in SAS. The efficiencies were calculated as the ratio of combined mean square error (MSE) if the study has been conducted as RCBD (i.e. sum of whole plot and split plot mean square) to MSE for split plot or whole plot tests in a split plot design (Federer and King, 2007; Hinkelmann and Kempthorne, 2007). The following formulae were used:

$$P_{sp} = \frac{(a-1)MSE_{wp}+a(b-1)\ MSE_{sp}}{(ab-1)\ MSE_{sp}} \text{ and } P_{wp} = \frac{(a-1)MSE_{wp}+a(b-1)\ MSE_{sp}}{(ab-1)MSE_{wp}}$$

where, P_{sp} and P_{wp} are the precisions of split plot and whole plot factors; MSE_{wp} is the mean square whole plot error; MSE_{sp} is the mean square split plot error, a=3 is the number of level of whole plot factor i.e. barrel temperature; b=25 is the combined

number of level of both split plot factors MC and SS (5*5). A P_{sp} or P_{wp} value of 1 suggests the precision is no different from a RCBD.

Correlations among response variables were calculated using Pearson's method and further used to conduct principal component analysis (PCA).

6.4. RESULTS AND DISCUSSION

6.4.1. Great Northern bean flour composition

The GNB flour used for extrusion had the following proximate composition (mean of 3 replicates ± standard deviation, wet basis): 14.1±0.9% moisture; 21.6±0.2% protein; 1.15±0.1% crude fat; 4.89±0.01% ash, and 40.1±0.9% starch. The proximate composition of GNB was found comparable to that reported previously by Wang and Ratnayake (2014).

6.4.2. Model diagnostics

The fitted models explained almost 90% of the variance for almost all the responses except for protein digestibility, which explained 70% of the variance in the data and was still considered acceptable (Table 6.2). LOF for all models was non-significant except BD. A cubic or higher model might be more appropriate for fitting BD, but this could not be calculated in the present study due to the limited data points.

MFE can be considered as the deviation in our observed data from the ideal fit, lower the deviation the better. For all responses except BD and TI, MFE was under ±5% indicating a good fit for the model (Table 6.2). Further, the distribution of MFE for all responses were 'near' normal except BD. The higher range and slightly skewed distribution for BD suggests more variation in the data (potentially due to some significant outliers) which could not be captured by fitted second order model. The high

MFE for TI was because extrusion had such a strong effect on reducing TI activity that some of the samples had no recorded value. This is further discussed in a subsequent section.

Based on the above diagnostic parameters, all models fit well except BD. However, the second order model still explained the general trends for BD, and thus was used to make conclusions regarding effect of extrusion parameters on BD.

Apart from the above observations, if the whole plot error variance [rep(T)] was estimated to be 0 using standard residual likelihood methodology (REML), the denominator for the F-test was based on the residual error with pooled degrees of freedom (df). If whole plot error variance was not estimated to be 0, the whole plot error was used in the denominator of the F-test and denominator degree of freedom was computed using Satterthwaite's approximation. In our study, the whole plot error variance was non-zero for all variables; thus, new df were calculated and used for analysis in the F-test. The numerator df for all responses was 1, but the denominator df (25) was different for some variables (BD, WSI, and TI) for whole plot and split plot terms (Table 2) suggesting a significant whole plot variance for these variables and reasonable to analyze them as split plot design and not a CCRD.

6.4.3. Effect of extrusion variables on expansion and density of GNB extrudates

The RER of GNB extrudates ranged from 1.20 to 1.98 with low MC resulting in greater expansion. All extrusion parameters significantly affected GNB expansion, with SS having a positive linear effect, T having a negative linear effect and MC having a negative quadratic effect on expansion. The importance of MC on expansion was also evident by significant interactions of feed moisture with both SS and T.

Expansion is an important quality parameter for extruded snack products with greater expansion resulting in a lighter, crunchier and more acceptable product. The expansion values obtained for GNB extrudates were lower and had a narrower range compared with cereal based extrudates (1.5 to 3) (Bhattacharya, 1996) but was similar to legume based extrudates (Lobato et al., 2011). The high amount of protein and fiber and low amount of starch in GNB flour can increase the dough viscosity and resist the flow in extruder barrel which would result in a less expanded product when compared with cereal based extrudates (Pitts et al., 2014). MC and T have been recognized as important extrusion variables that affect expansion (Thymi et al., 2005). Increasing moisture content can reduce melt elasticity by changing amylopectin molecular structure, thereby decreasing expansion (Ilo et al., 1996). Based on regression results and profiler (Fig 6.2), low barrel temperatures (between 100 to 120°C) resulted in maximum expansion while higher temperatures decreased the expansion of extrudates. The negative effect of temperature on expansion mainly attributed to starch dextrinization at higher temperature has been reported by other researchers also (Kokini et al., 1992; Colonna et al., 1989) but in our study the temperature range was lower than reported by others (Balandran-Quintana et al., 1998; Gujska and Khan, 1990). The difference in temperatures could be either due to difference in machine parameters or the fiber and protein in GNB flour increased the viscosity and residence time of flour in the barrel which resulted in greater starch damage and lower expansion.

Bulk density had an inverse relationship with expansion, with higher MC resulting in a denser and less expanded product (Table 6.3; Fig 6.2). SS also had a pronounced negative quadratic effect on bulk density. Low to medium SS increases

barrel residence time, which would be expected to give the GNB flour additional time for gelatinization to occur, resulting in increased expansion and less density. However, very high SS can result in a shearing effect which can cause structural breakdown of starch molecules thereby limiting expansion and resulting in a denser product (Filli, 2009).

6.4.4. Effect of extrusion variables on WAI and WSI

WAI and WSI are usually related to changes in starch with opposite effects which could be associated with the structure of extrudates. WAI, the measure of the amount of water absorbed by the intact starch (and other water absorbing components) and can be linked with gelatinization, ranged from 3.3 to 4.3 g/g for GNB extrudates, while the un-extruded flour had a WAI of 2.1 ± 0.08 g/g. The observed values were similar to earlier report for extruded GNB (Sutivisedsal et al., 2013) but lower than extruded pinto beans (Rocha-Guzman et al., 2006). Among different extrusion parameters, MC and SS were identified as the important variables affecting WAI but with opposite effects: higher MC and lower SS resulted in larger WAI. T alone did not significantly impact on WAI, but its interaction with MC was significant.

WSI is an indicator of starch degradation (and other soluble flour components) and is measured in terms of soluble starch released from whole starch component (Kirby et al., 1988). The WSI values obtained for extruded GNB flour ranged from 21.1 to 37.1%. Only linear effects of MC and SS were found to be significant with contrasting effects on WAI. Similar effects of MC and SS have been reported by other researchers for dry beans (Sutivisedsal et al., 2013) and bean-cereal blends (Sumargo et al., 2016).

Feed MC had opposite effects on WAI and WSI, which can be explained by the plasticizer effect of high moisture that prevents degradation of starch granules resulting in

increased water absorption but lower solubility (Hegenimina et al., 2006). On the other hand, high SS could enhance damage to starch granules due to increased shear, which would increase WSI but reduce absorption (Ding et al., 2006).

6.4.5. Effect of extrusion variables on trypsin inhibitors and in vitro protein digestibility of GNB extrudates

Un-extruded GNB flour had a trypsin inhibitor (TI) activity of 23.3 ± 0.6 mg/g sample, whereas, depending on extrusion condition, was either completely inactivated (not detected) or was reduced to 0.91 mg/g sample (almost 97% reduction). Feed MC had the most significant impact on TI activity with lower MC resulting in maximum reduction in TI activity (Tables 6.2 and 6.3). The interaction between MC and T was also significant in lowering the TI in GNB extrudates. The interaction between T and SS was also found to be significant in reducing the TI activity. In particular, the lower SS the more residence time in the extruder barrel and thus more exposure to high T and more destruction of TI. Similar reduction in TI activity on extrusion and role of moisture in TI inactivation has been reported by other researchers (Martin-Cabrejas et al., 1999; Balandran-Quintana et al., 1998).

In our study, raw GNB flour had PD of 60.6 ± 1.7% which, depending on extrusion condition, increased to 68.9 to 78.6 %. The overall digestion value was slightly lower but the percentage increase upon extrusion in our study was consistent with other reports (Balandran-Quintana et al.,1998). SS and T had the most significant impacts on protein digestibility, with low SS and high T being most impactful (Table 6.2 and 6.3). The observed increase in digestibility in GNB could be due to individual effects or a combination of TI inactivation and protein denaturation, which are both temperature

dependent phenomenon. The observed increase in PD upon extrusion is similar to other reports (Steel et al, 1995).

6.4.6. Precision analysis

Split plot designs are most useful when the experimental units are of different sizes, as in extrusion based experiments, where, experimental unit for HTC, whole plot factor T is the extruder barrel while batch of bean flour is the experimental unit for ETC, split plot factors MC and SS. But this type of design can also lead to lower precision in analyzing whole plot factor and increased artificial precision for split plot factor and their interactions when compared to a completely randomized or a randomized block design (Mead, 1990). This discrepancy is mostly due to greater heterogeneity in larger whole plot units when compared to small split plot units. Based on the efficiency analysis in our study, we found that for BD, WSI and TI, analyzing the data as a split plot design resulted in a huge loss in precision for T, while the precision in estimating effects of MC and SS increased to varying degrees (Table 6.4; Pwp $<<1$ and Psp >1). For the remaining responses (RER, WAI and PD), split plot analysis was highly effective as it not only increased the precision in estimation of T but did not lose on the precision for MC and SS (Pwp$>>1$; Psp~1). This suggests for RER, WAI and PD, the data may be analyzed using a split pot design or RCBD with similar conclusions, but for the other variables analyzing the data as RCBD could result in confounding and biased results. Also, based upon our approximated df (Table 6.2), the whole plot denominator df for BD, WSI, and TI were different and lower than the split plot denominator df which suggests more variation in whole plot factors for these responses resulting in lower precision for their whole plot factors. Thus, it is important to consider

that split plot analyses like these should only be conducted if the researcher is willing to sacrifice information for some variables in the study while gaining for others.

6.4.7. Multivariate analysis

BD was positively correlated with WAI and TI, while it has a strong negative correlation with RER and WSI (Fig 6.3A). Similar results have been reported before (Gulati et al., 2016; Altan et al., 2008). Higher bulk density of the product suggests limited expansion with greater ability to absorb water as the starch molecules are intact. Further, the more intact the starch molecules, lesser is their solubility as shown by negative correlation between WAI and WSI. Also, conditions like high MC and low SS which promote higher density are less severe which can have a preventive effect on TI, resulting in high TI activity. PD had a negative correlation with RER.

The correlations among response variables were used to conduct principal component analysis and the bi-plot showed a clear distinction between response variables (Fig 6.3B). Since MC had a significant effect on all the variables, the PC plot was marked with runs corresponding to different levels of MC to demonstrate the effect of MC on these responses. The first two PCs explained 74% of the variation in the data and showed grouping of WAI, BD and TI which showed greater values under high feed MC conditions while WSI and RER were mostly affected by extrusion under low to medium MC, respectively.

6.5. CONCLUSION

Extrusion had a positive impact in reducing the anti-nutritional component, trypsin inhibitor in GNB while increasing protein digestibility. Feed MC was recognized as an important extrusion parameter affecting almost all the responses.

The other objective of this study was to understand if split plot analysis would be more beneficial than the usually conducted RCBD analysis for extrusion based experiments. Split plot analysis helped gain more precision for whole plot factors like barrel temperature for responses like RER, WAI and PD but without any loss in precision for split plot factors. This suggest for these responses split plot analysis or RCBD analysis won't make much difference but for other variables there was an increased precision in estimating split plot effects, this indicates split plot should be the ideal way to draw conclusions on these variables to avoid confounding and bias. Overall, split plot analysis should be considered the ideal way of extrusion analysis. While it's no better than RCBD for some responses, for others it can be a more accurate way of predicting effect of extrusion variables.

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Table 6.1. Experimental design with combination of predictor variables*

Run order	Coded values			Actual values					
	T (°C)	SS (rpm)	MC (%)	T (°C)	SS (rpm)	MC (%)	T (°C)	SS (rpm)	MC (%)
1	-1	b	b	-1	0.7	0.7	90.0	225	23.1
2	-1	-b	-b	-1	-0.7	-0.7	90.0	180	18.9
3	-1	-b	b	-1	-0.7	0.7	90.0	180	23.1
4	-1	b	-b	-1	0.7	-0.7	90.0	225	18.9
5	0	0	0	0	0.0	0.0	115	203	21.0
6	0	0	0	0	0.0	0.0	115	203	21.0
7	0	-a	0	0	-1.43	0.0	115	156	21.0
8	0	0	0	0	0.0	0.0	115	203	21.0
9	0	a	0	0	1.43	0.0	115	250	21.0
10	0	0	0	0	0.0	0.0	115	203	21.0
11	0	0	0	0	0.0	0.0	115	203	21.0
12	0	0	a	0	0.0	1.43	115	203	25.3
13	0	0	0	0	0.0	0.0	115	203	21.0
14	0	0	-a	0	0.0	-1.43	115	203	16.7
15	1	b	b	1	0.7	0.7	140	225	23.1
16	1	-b	-b	1	-0.7	0.7	140	180	23.1
17	1	-b	b	1	-0.7	-0.7	140	180	18.9
18	1	-b	-b	1	0.7	-0.7	140	225	18.9

* MC: Feed moisture content, T: barrel temperature, SS: Screw speed, a and b are new axial and factorial points identified after CCD modification.

Table 6.2. Model diagnostics and tests for fixed effects of MC, SS and Temp on physical and chemical properties of Great northern bean extrudates

Effect	Physical responses								Chemical responses			
	RER		BD		WAI		WSI		TI		PD	
	Den df	F value	Den df	F value	Den df	F value	Den df	F value	Den df	F value	Den df	F value
Rep	25.0	113**	1.94	1.76	25.0	11.4*	1.63	26.7*	2.04	0.17	25.0	4.21*
T	25.0	62.9**	2.02	1.98	25.0	1.03	2.42	0.96	2.35	4.53	25.0	3.04
T*T	25.0	3.09	1.86	0.73	25.0	0.27	1.48	1.02	1.87	0.44	25.0	8.02*
MC	25.0	21.8**	22.9	106**	25.0	102**	22.9	103**	22.1	43.9**	25.0	0.01
SS	25.0	4.12*	22.9	19.4**	25.0	56.1**	22.9	15.3**	22.1	0.38	25.0	0.99
MC*SS	25.0	5.12*	22.9	3.54	25.0	9.05*	22.9	0.06	22.1	0.63	25.0	0.48
MC*MC	25.0	51.5**	22.9	1.57	25.0	54.0**	22.9	0.18	22.2	6.95*	25.0	1.48
SS*SS	25.0	0.00	22.9	6.66*	25.0	5.86*	22.9	0.59	22.1	0.05	25.0	10.1**
MC*T	25.0	17.2**	22.9	8.18*	25.0	10.7*	22.9	0.15	22.1	8.61**	25.0	0.15
SS*T	25.0	1.24	22.9	1.25	25.0	0.20	22.9	0.00	22.1	5.34*	25.0	0.74
R^2	0.93		0.94		0.92		0.90		0.87		0.70	
LOF	NS		S		NS		NS		NS		NS	
MFE	(-3.62, 4.20)		(-8.82, 10.89)		(-3.08, 2.76)		(-4.36, 5.08)		-		(-1.54, 3.90)	

T, Barrel temperature; SS, Screw Speed; MC, Moisture content, RER, Radial Expansion Ratio; BD, Bulk Density; WAI, Water Absorption Index; WSI, Water Solubility Index; TI, Trypsin Inhibitors; PD, Protein digestibility. *indicates significant effect at $p<0.05$; **significant effect at $p<0.01$. df: Degrees of freedom, calculated using Satterthwaite's approximation, numerator df for all responses was 1, Den df: Denominator degrees of freedom varied and mentioned above. R^2: coefficient of determination, LOF: Lack of fit, MFE: model fit error.

Table 6.3. Regression coefficients for each response surface equation fitted

Parameter[a]	Physical responses				Chemical responses	
	RER	BD	WAI	WSI	TI	PD
Intercept	1.61*	0.30*	4.03*	24.94*	0.17	74.95*
T	-0.14*	0.02	-0.018	-0.66	0.15	0.59
T*T	-0.03	0.03	-0.007	0.88	0.04	2.19*
MC	-0.07*	0.05*	0.147*	-3.06*	0.24*	-0.05
SS	0.029*	-0.02*	-0.013*	1.15*	-0.005	-0.26
MC*MC	-0.07*	-0.002	-0.07*	0.14	0.067*	0.11
SS*SS	-0.0005	0.009*	0.026*	0.18	0.005	-0.86*
SS*T	0.02	-0.008	0.008	-0.03	0.09*	0.42
MC*T	-0.07*	0.02*	0.063*	-0.16	0.12*	-0.19
MC*SS	0.04*	0.01	0.059*	-0.11	0.03	0.35

[a] SS, screw speed; MC, moisture content; T, temperature; RER, Radial expansion ratio; BD, Bulk density; WAI, Water absorption index; WSI, Water solubility index; TI, Trypsin inhibitors; PD, Protein digestibility.
*indicates significant effect at $p<0.05$

Table 6.4. Precision analysis for whole plot and split plot factors for each variable

Response variable	Whole plot factor precision	Split plot factors precision
RER^	2.09	0.98
BD	0.10	1.38
WAI^	1.87	0.99
WSI	0.57	1.03
TI	0.16	1.17
PD^	2.18	0.98

RER, Radial expansion ratio; BD, Bulk density; WAI, Water absorption index; WSI, Water solubility index; TI, Trypsin inhibitors; PD, Protein digestibility. ^Precision analysis for current design can be considered same as for RCBD.

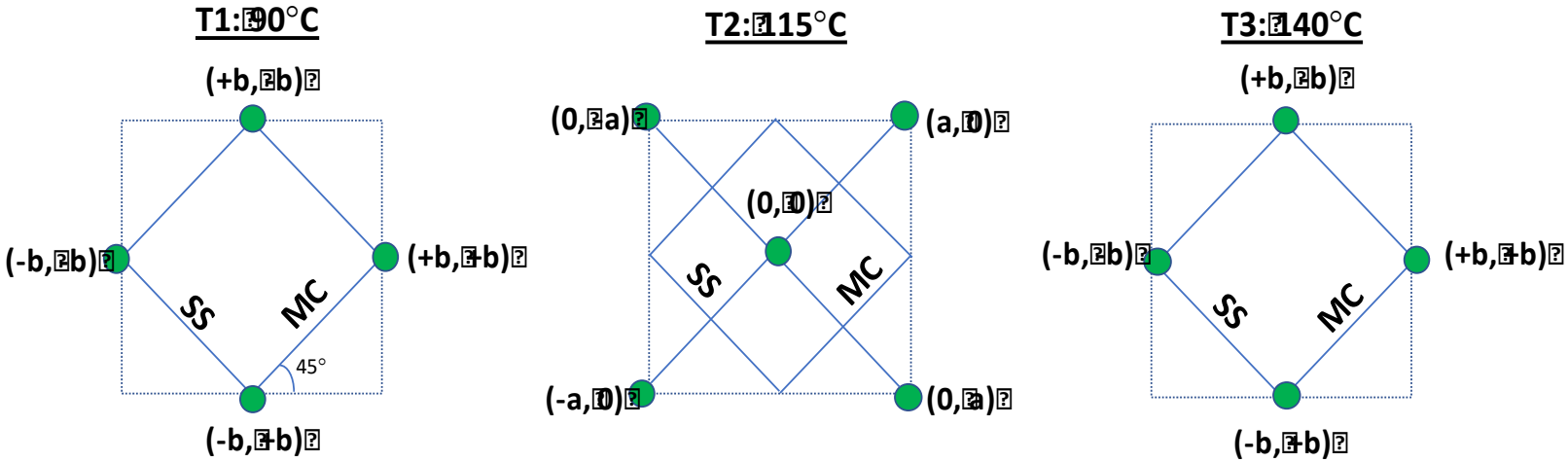

Figure 6.1. Illustration of the design of experiments at three levels of temperature (T) (the whole plot factor) with different combinations of moisture content (MC) and screw speed (SS) (split plot factors).[24]

[24] Squares represent the design space (dotted square: original design space; solid square: original design space rotated by 45°, each green circle represents an experimental condition); a = ±1.43; b = ±0.7, 0=center point.

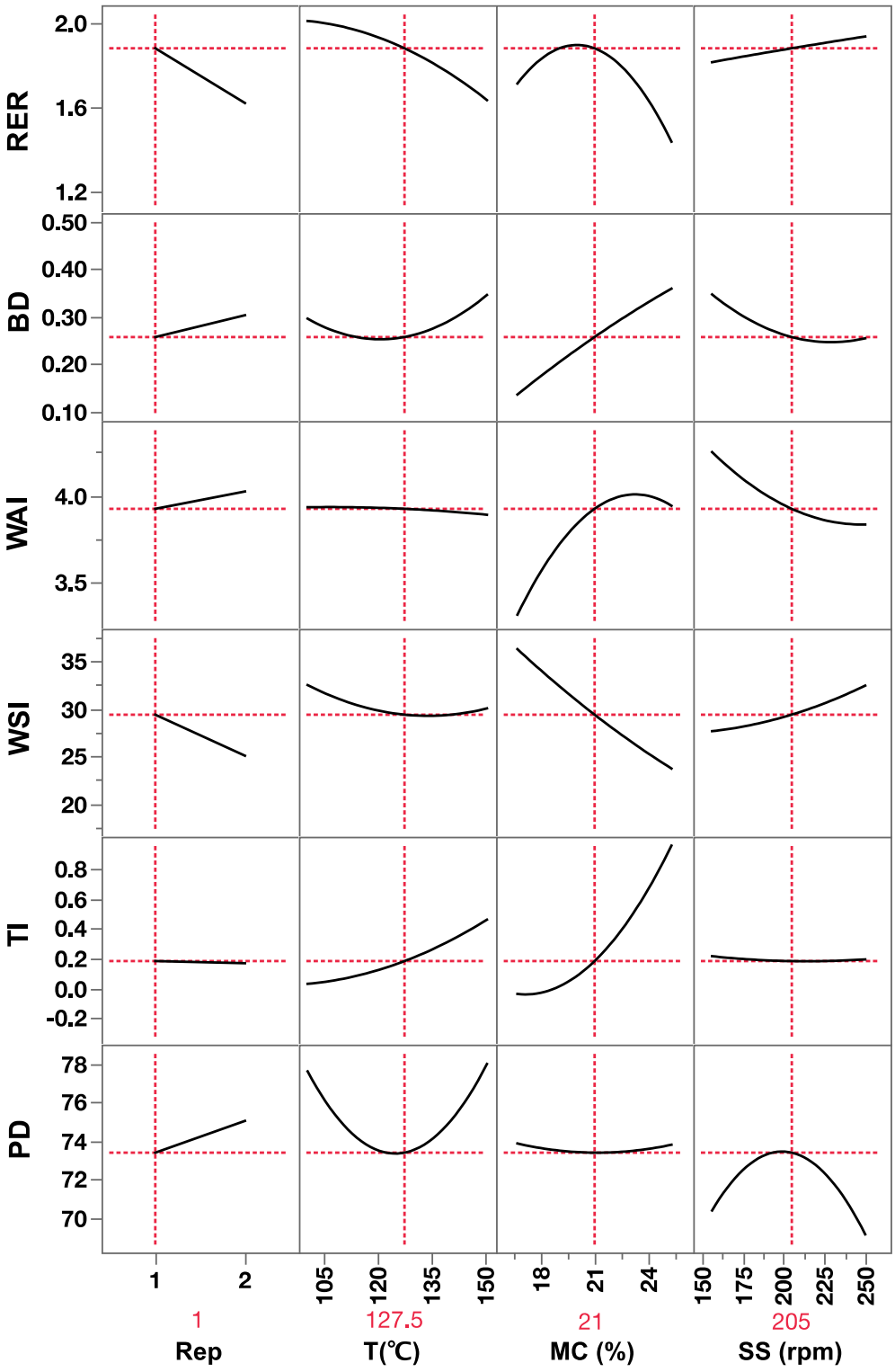

Figure 6.2. Influence of each processing variable on response variables when other processing variables are held constant

A

	RER	BD	WAI	WSI	TI	PD
RER		-0.50	-0.18	0.58	-0.38	-0.42
BD	-0.50		0.72	-0.73	0.67	0.18
WAI	-0.18	0.72		-0.72	0.35	-0.05
WSI	0.58	-0.73	-0.72		-0.41	-0.20
TI	-0.38	0.67	0.35	-0.41		0.07
PD	-0.42	0.18	-0.05	-0.20	0.07	

Negatively correlated		No correlation	Positively correlated	
p < 0.01	p < 0.05		p < 0.05	p < 0.01

B

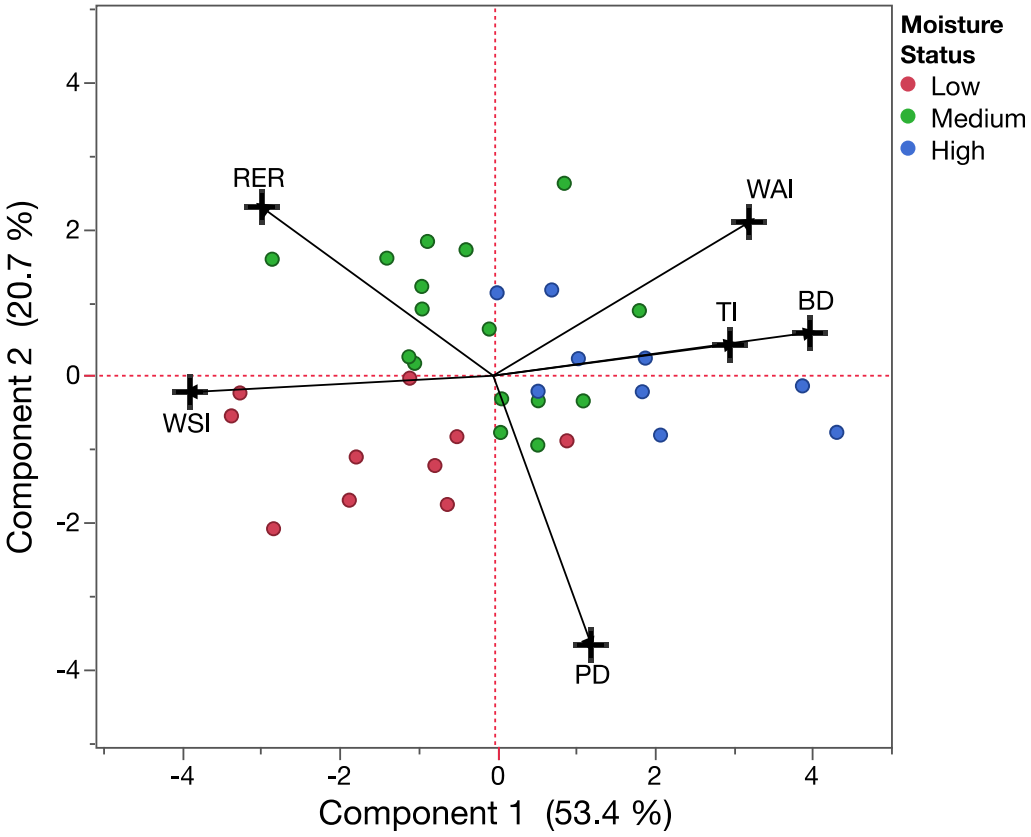

Figure 6.3. Multivariate analysis on responses, A: Pearson's correlation among response variables; B: Principal component analysis on correlations

CHAPTER 7: EFFECT OF EXTRUSION ON FOLIC ACID CONCENTRATION AND MINERAL ELEMENT DIALYZABILITY IN GREAT NORTHERN BEAN (*Phaseolus Vulgaris L.*)

7.1. ABSTRACT

Great Northern beans (GNB) contain appreciable Mg, K, P, and Fe, together with the labile B vitamin, folate. However, GNB also contain phytate and can be a source of the heavy metal Cd. Extrusion was used as a processing technique to degrade phytate and increase dialyzability of essential mineral elements while minimizing destruction of folate and dializability of Cd. Extrusion resulted in increases of as much as 56%, 50%, 25%, and 84% in dialyzability of Mg, P, K, and Fe, respectively, while, remarkably, generally decreasing Cd dialyzability. Screw speed (SS) had a significant quadratic effect on dialyzability of all elements, with high SS resulting in more dialyzable elements. This was followed by feed moisture, which had a significant impact on dialyzability of Fe and Mg, mostly in interaction with SS. Low moisture and low SS had the maximum impact in reducing Cd dialyzability. Extrusion resulted in up to 85% reduction in phytate compared with GNB flour, while there was a significant loss in folic acid content too (up to 40%). Low moisture conditions resulted in maximum degradation of phytate and folate. Low barrel temperature, medium feed moisture content and high SS was identified as the best extrusion condition to maximize essential mineral element dialyzability and folate retention while minimizing phytate and dialyzable Cd.

7.2. INTRODUCTION

Despite the availability of food sources and various approaches like fortification, supplementation, and enrichment, there are several micronutrients, including Mg, K, choline, Ca, Fe, and vitamins A, D, E, and C, that are underconsumed in the US. Additionally, folate deficiency, which was prevalent in the early 20th century, is nearly non-existent today; however, the major source of folate in the US is fortified, processed food which can have a negative connotation (Odewole et al., 2013). While not manifesting overt deficiency diseases, individuals with chronic moderate deficiency can develop conditions like hypertension, coronary heart disease, diabetes, metabolic syndromes that are common in the US (Long and Romani, 2015). Thus, it is important to identify food sources that are naturally rich in micronutrients and promote their consumption in efforts to tackle nutrient deficiencies and encourage healthy eating habits.

Typically, dry beans (*Phaseolus vulgaris* L.) are valued from a nutritional standpoint for their high protein and dietary fiber contents. However, they are also good sources (i.e., >10% of the US daily recommendation) of folate and the essential minerals Mg, Fe, K, and P (USDA nutrient database 2017; NIH RDA values), making it an excellent source of many underconsumed nutrients in US diet.

However, the availability of these nutrients for absorption and utilization is not only dependent on their concentrations, but also on factors like anti-nutritional compounds, processing techniques, and physicochemical state of the nutrient (Fairweather-Tait, 1987). For example, phytate is a naturally occurring compound present in grains and legumes, including GNB, and can form insoluble complexes with essential elements and reduce their absorption (Thomspon, 1993). Different processing techniques

like germination, pressure cooking, and extrusion have been shown to reduce phytate, but the extent of reduction depends upon the raw material and processing technique (Nergiz and Gokgoz, 2007).

The techniques adapted for reducing these anti-nutritional factors and increasing mineral element bioavailability can unfortunately adversely affect other important nutrients like the heat labile folates. They may also increase bioavailability of toxic heavy metals like Cd (Watzke et al., 1998). Thus, it is important to identify processing techniques that can enhance the bioavailability of essential mineral elements while minimizing loss in important labile vitamins and reducing bioavailability of heavy metals. One such processing technique may be extrusion.

Extrusion is a versatile food processing technique involving high-temperature short time cooking combined with high mechanical shear and pressure. This processing technique has been effective in reducing anti-nutritional compounds while improving digestibility of macro- and micronutrients in a variety of raw materials. Furthermore, by modifying parameters either in the raw material (e.g., moisture content, particle size) or during the extrusion process (e.g., screw speed, feed rate, temperature) or by combining extrusion with other processes one can achieve specific results depending upon product requirement or desired chemical changes (Adamidou et al., 2007). Extrusion has shown promising results in improving the bioavailability of mineral elements in legumes and cereal products (Hazell and Johnson, 1989, Galan et al, 2013; Alonso et al., 2001) but no research has simultaneously focused on the effect of extrusion on vitamins and bioavailability of essential mineral elements and heavy metals in dry beans.

Bioavailability is a complex process which is defined as the amount of an ingested nutrient that is absorbed and is available for physiological function while bio-accessibility is the amount of an ingested nutrient that has the potential to be absorbed and utilized for physiological functions (Etcheverry et al., 2012). In vitro dialyzability measures the proportion of the total elements that diffuse through a membrane during food matrix digestion (Miller et al., 1981) and is commonly employed to measure the bio-accessibility of mineral elements. This method can also be used as a screening tool to assess if a certain process would have an effect on bioavailability when moved to more complex models.

Thus, the objective of this study was to determine the effect of extrusion on degradation of folate and dialyzability of Mg, Fe, K, P, Fe, and Cd from GNB flour and identify conditions that achieve maximum dialyzability of the essential mineral elements while minimizing dialyzability of heavy metals and degradation of folate. The results from this study can serve as the basis for accessing bioavailability of essential nutrients from processed GNB flour to promote dry beans in the diet.

7.3. MATERIALS AND METHODS

7.3.1. Materials

Great Northern beans (GNB) were obtained from FNJ Inc. (Alta Loma, CA, USA) and milled using a pilot scale hammer mill (20SSHMBD, C.S. Bell, Tiffin, OH, USA) with screen size of 0.7 mm. The flour was analyzed for moisture, fat, and ash content using approved methods (AACC International, 2013). Protein content was analyzed using a nitrogen analyzer (FP 528, Leco, St. Joseph, MI, USA) with a protein factor of 6.25. Total starch content was analyzed using total starch assay kit (K-TSTA,

Megazyme, Bray, Ireland) following the KOH format. Bean flour was stored at 4°C until extrusion.

7.3.2. Experimental design

The effect of extrusion on GNB flour was studied by varying three extrusion factors: barrel temperature (Temp) (90-140°C), feed moisture content (MC) (17-25%), and screw speed (SS) (156-250 rpm), while keeping other factors such as feed rate and screw configuration constant. The levels of these factors were determined based on preliminary trials. The commonly used central composite rotatable design (CCRD) was modified slightly because barrel temperature was not randomized during the experiment. The experiments were conducted in increasing order of temperature while randomizing MC and SS. Temp was tested at three fixed levels: -1, 0, +1, i.e., 90, 115 and 140°C, respectively. In order to maintain orthogonality of the experiment, the design space was rotated by 45° which resulted in new axial (±a = 1.43) and factorial (±b = 0.70) points for MC (+a = 25%; -a=17%; +b = 22.5%; -b = 19%) and SS (+a = 250 rpm; -a=156rpm; +b = 225 rpm; -b = 180 rpm). The new values for MC and SS were calculated based on preliminary experiments and calculating the ratio of block error variance and experimental error (Draper and John, 1998). Based on the design, extrusion was carried under 18 experimental conditions (MC, SS, Temp): ±a, 0, 0 (2 runs); 0, ±a, 0 (2 runs); ±b, ±b, ±1 (8 runs); and 0,0,0 (6 runs). The complete experimental design used in the study is shown in Table 1.

7.3.3. Extrusion process

To adjust the moisture content of bean flour, batches (2 kg) representing each experimental run were blended in an upright blender (H-600-D, Hobart, Troy, Ohio,

USA) at medium speed with the required water to obtain the target moisture content according to the experimental design. The moist samples were sealed in polyethylene bags and tempered for 16 h at 4 °C. The flour was then fed into the extruder barrel using a single screw volumetric feeder (FW 40 Plus, C. W. Brabender) set at a constant delivery rate of 76 g/min.

A laboratory scale co-rotating conical twin screw extruder with mixing zones was used for extrusion (CTSE-V, C.W. Brabender, Hackensack, NJ, USA). The specifications of extruder and operating conditions used were the same as described in Gulati et al. (2016). The extrudate sample for each experimental condition was collected after a stable temperature and torque reading was observed. The collected samples were dried in a belt drier (4800 series Wenger, Sabetha, KS, USA) at 100°C for 10 min and ground using cyclone sample mill (UDY, Fort Collins, CO, USA) with a screen size of 1 mm. The ground extrudate samples were stored at 4°C until analysis.

7.3.4. Phytic acid content

Phytic acid in GNB flour and extrudates was quantified as phytate phosphorus using 2,2'- bipyridine as described (Haug & Lanstzsch, 1983), with slight modifications. Briefly, phytic acid was extracted from the sample (250 mg) using 0.2 N HCl (10 mL) overnight at 4 °C with gentle shaking. The contents were centrifuged and supernatant was used for analysis after dilution with distilled water (25 mL). For unprocessed flour, 0.25 mL of the diluted supernatant was mixed with 0.75 mL of 0.2 N HCl; for extrudates, 0.5 mL of the diluted supernatant was mixed 0.5 mL of 0.2 N HCl. One mL of 415 μM $Fe(NH_4)(SO_4)_2$ was then added to diluted extracts and tubes were placed a in boiling water for 30 min. The tubes were cooled immediately and contents centrifuged. One mL

of the supernatant was mixed with 1.5 mL of 2, 2'-bipyridine. The color developed was measured at 530 nm. The samples were quantified by means of external calibration using sodium phytate dodecahydrate (Sigma-Aldrich, 71649) which contained 19% phytate phosphorus as measured with inductive coupled plasma mass spectrometry (ICP-MS) as described later.

7.3.5. Folic acid content

Total folates were measured using the standard microbiological assay (*L. casei* subsp. Rhamnosus, ATCC no. 7469) with tri-enzyme extraction technique (AACC method 86-47).

7.3.6. In vitro digestion

In order to measure the dialyzability of elements, bean flour was digested *in vitro* under conditions described by Luten et al. (1996), with some modifications. The modifications were to reduce sample weights and volumes to fit in a 48-well plate format (Rapid Equilibrium Dialysis Plate, MWCO 8K Dalton, Thermoscientific, 90006). For digestion, 20 mg of sample was weighed in the sample chamber and mixed with 0.2 mL of pepsin solution (50 mg/mL in 50mM HCl). The plate was covered with a sealing tape (15036 ThermoScientific), and incubated at 37 °C for 2 h with gentle shaking at 125 rpm. Pepsin digestion was stopped by adding 0.25 mL of dialysis buffer (0.1 M $NaHCO_3$) in the buffer chamber and the mixture incubated for 55 min under previously described conditions. The amount of dialysis buffer added was pre-determined by titrating gastric mixture with dialysis buffer until the pH reached 6. Meanwhile a pancreatin-bile solution (0.4 g pancreatin; 2.5 g bile salts) was prepared in 10 mL of 0.1 M $NaHCO_3$. Given the low solubility of pancreatin, the solution was centrifuged and 0.05 mL of the supernatant

was added to each sample chamber, covered with sealing tape and incubated for 2 h. After digestion, the dialysis buffer (0.07-0.08 mL) was collected from the buffer chamber and used to quantify dialyzable elements. In order to avoid element contamination from enzymes, both pepsin and pancreatin enzyme solutions were dialyzed using centrifugal filter devices (Centriprep 10K, Merck Millipore, Burlington, MA) by centrifuging four times at 3000 g for 30 min each at 4 °C to remove contaminating elements. The concentrated enzyme solutions were collected from outer chamber of the tube and made up to the required volume and used for digestion. In preliminary testing, there was no significant reduction in protease activity of these enzymes before or after dialysis.

7.3.7. Total and dialyzable element concentrations

The total concentration of mineral elements in bean flour and extrudates were quantified after wet ashing. Briefly, 500 mg of sample was digested using 4 mL of concentrated nitric acid at 100°C for 1 h. The tubes were cooled to room temperature, mixed with 4 mL of hydrogen peroxide (30%) and heated for 1.5 h at 125°C. A second volume of hydrogen peroxide was then added and tubes were heated at 150 °C until the sample dried. The dried samples were re-suspended in 10mL of 1% nitric acid, mixed thoroughly and used for element analysis (Guttieri, 2014).

Element analysis in unprocessed wet ashed samples and dialysis buffer collected after digestion and dialysis from RED plate was performed using inductively coupled plasma mass spectrometry (7500cx, Agilent Technologies, Santa Clara, CA) operating in kinetic discrimination mode with helium gas at 5 mL/min. with 50 ppb Ga as internal standard Approximately 40 μL of sample was injected using a micro peripump, 2% nitric acid was used for rinsing between runs. Each sample was analyzed in duplicate. The

method was optimized for the analysis of Li, B, Na, Mg, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, and Cd. The results for Mg, Fe, P, K, and Cd are only discussed in this paper since GNB has greater than 20% DV of these elements and Cd is a heavy metal of concern. The results were calculated in mg/kg sample for model fitting. For presentation of the data, units were converted to 1) the percentage of total elements that were dialyzable and 2) the percent change in the dialyzability in the extruded sample compared to unprocessed bean flour.

7.3.8. Data analysis

The data were analyzed using JMP statistical software (JMP version 10.0.0, SAS institute). The following second order model was fitted for each response variable:

$$Y_{ijk} = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2j} + \beta_3 x_{3k} + \beta_{11} x_{1i}^2 + \beta_{22} x_{2j}^2 + \beta_{33} x_{3k}^2 + \beta_{12} x_{1i} x_{2j} + \beta_{13} x_{1i} x_{3k} + \beta_{23} x_{2j} x_{3k}$$

where, Y was the response (folic acid, phytic acid, total and dialyzable Mg, K, P, Ca, Fe and Cd) for i^{th} level of x_1 (Temp); j^{th} level of x_2 (MC) and k^{th} level of x_3 (SS). The models were checked using ANOVA (F test), lack of fit test, and adjusted R^2 values. Results of extruded samples were compared with unprocessed bean flour using Dunnett's multiple comparison test at significance level of 0.05 with extrudate results averaged over low, medium and high MC, SS and temp settings separately. Correlations among response variables were calculated using Pearson's method and further used to conduct principal component analysis (PCA).

To predict extrusion conditions that would result in the highest folic acid and dialyzable Mg, P, K, and Fe together with the lowest phytic acid and dialyzable Cd, a simultaneous optimization technique was used, which makes use of a desirability

function (Myers et al., 2009). All responses were given equal weight in the optimization process. The desirability function (d_i) ranges from 0 to 1 and was constructed for each response with targets to minimize phytic acid content and Cd dialyzability while maximizing folic acid and dialyzable Mg, P, K, and Fe. Overall desirability was calculated using the following equation:

$$D = (d_1 d_2 \ldots . d_m)^{1/m}$$

Where, m=number of responses (7).

7.4. RESULTS AND DISCUSSION

7.4.1. Composition of bean flour and changes upon extrusion

The GNB flour used for extrusion had the following composition (mean of 3 replicates ± standard deviation, wet basis): 14.1 ± 0.87% moisture; 21.6 ± 0.2% protein; 1.2 ± 0.10% crude fat; 40.1 ± 0.9% starch; 2546 ± 65 mg/kg Mg; 6379 ± 85 mg/kg P; 19178 ± 855 mg/kg K; 58.6 ± 3.4 mg/kg Fe; 0.029 ± 0.014 mg/kg Cd; 1430 µg/kg folic acid; 4.33 ± 0.07 mg/g phytic acid.

Extrusion reduced the phytic acid and folic acid concentrations when compared with the amounts present in unprocessed flour (Table 7.1). Depending on the extrusion condition, the anti-nutritional factor, phytic acid, decreased by up to 85% while there was a loss of up to 43% in the vitamin, folate. There was no change in the total concentrations of Mg, P, K, Ca, Fe and Cd upon extrusion when compared with unprocessed flour (data not shown) but there was a significant change in the dialyzable concentrations of extruded bean flour compared with unprocessed flour. When compared with unprocessed bean flour, extrusion resulted in as much as 56%, 50%, 25%, and 84% increase in dialyzability of Mg, P, K, and Fe respectively. The effect on Cd dialyzability was much

more varied, with some extrusion conditions increasing and other conditions decreasing dialyzability compared with unprocessed bean flour.

7.4.2. Effect of extrusion on phytates, folate and element dialyzability

Model diagnostics

A non-significant lack of fit for all the dependent variables indicated that fitted second order models were appropriate for all the responses (Table 7.2). Further, all responses, except dialyzable K, had an adjusted R^2 greater than 0.70, which suggested that the model could explain more than 70% variation in the data and a significant F ratio for these responses indicated that different extrusion conditions affected the response. For K, a non-significant F ratio showed that there was no effect of individual extrusion condition on K dialyzability but from the previous comparison with unprocessed flour there was an overall improvement in dialyzability of K by extrusion.

Phytic acid

Extrusion dramatically reduced the concentration of phytic acid in GNB flour (Fig. 7.1); however, among the extrusion variables linear effects of MC and Temp and their interaction significantly affected the extent of change during extrusion (Table 7.2). Higher values of both MC and Temp resulted in higher measured concentration of phytic acid and thus less degradation. On the other hand, lower MC conditions resulted in reduced phytic acid concentration (Fig. 7.1).

A similar effect of extrusion on reduction of phytic acid in legumes and other bean varieties has been reported by other researchers (Alonso et al., 2000; Batista et al., 2010a; Marzo et al., 2011). The effect of extrusion on phytate is mainly linked to thermal hydrolysis of reactive phosphate esters (Sandberg et al., 1987). The dephosphorylated

phytate no longer possesses the detrimental chelating effects of the native compound. Samples at high feed MC would be exposed to less severe conditions due to the lubricating action of water, resulting in less degradation of phytate, as seen in our study. By the same logic we would expect high barrel temperature to have a severe destructive effect on phytic acid; however, we found that an increase in barrel temperature resulted in less degradation. Similar results were shown when the phytic acid content was averaged over low, med and high conditions of MC, SS and Temp (Fig. 7.1), although high Temp runs also showed greater variability in the data. We observed a significant interaction between MC and Temp, with increasing values of both resulting in higher phytic acid. This suggests that high feed MC has a greater protective effect on phytate than the destructive effect of high temperature.

Folic acid

Feed MC had a significant quadratic effect on folic acid in GNB, while other extrusion variables had no effect (Table 7.2). Higher MC resulted in more folate (less loss) than low moisture conditions (Fig. 7.2). The observed range of folate in processed GNB were similar to previous reports for other beans (Hefni et al., 2014). As mentioned earlier, the observed folate concentrations in extrudates were significantly lower than the folate concentration in bean flour, which can be explained by the sensitivity of the vitamin to heat, pressure, and shear (Dozier, 2002). The loss in folate during extrusion has been reported by other researchers for different folate rich grains with thermal degradation and shear identified as the main cause (Broz et al., 1997; Ramos-Diaz, 2016). The significant effect of MC found in our study can be linked to protective effect of moisture inside the extruder barrel such that at low moisture conditions the exposure to

folate to high temperature was more, resulting in greater thermal degradation. Folate is sensitive to a wide range of processing techniques, but based on our study and previous reports the loss in folate is moderate during extrusion when compared to other techniques like roasting and autoclaving (Kariluoto et al., 2006) or just simple cooking (Xue et al., 2011). Charlton and Ewing (2007) showed that there is complete destruction of folate when exposed to temperatures over 95 °C in other processing techniques; however, in our study at the maximum temperature of 140 °C we observed only moderate loss in folate (except for 1 sample with lowest MC resulting in 40% loss). This suggests that extrusion is a much more complicated process and with optimized conditions one can achieve minimum loss in folate even after processing.

Mineral element dialyzability

Extrusion significantly increased the dialyzability of Mg, P, K, and Fe when compared with unprocessed flour (Fig 7.3). While only 19%, 15%, 54%, and 2% of Mg, P, K, and Fe was dialyzed respectively, from the flour, the percent increased up to 38%, 22%, 58%, and 9% for Mg, P, K, and Fe upon extrusion. The maximum improvement in dialyzability was observed for Mg and Fe upon extrusion. On the other hand, extrusion greatly reduced the dialyzability of the heavy metal Cd when compared to unprocessed flour with 4% Cd dialyzed from unprocessed flour and as low as 0.4% dialyzed from extruded flour. SS had a significant linear or quadratic effect on the dialyzability of all the elements except K which was not effected by any extrusion parameter (Table 7.2). After SS, feed MC had a significant impact on element dialyzability with a quadratic effect and negative interaction with SS for Fe, a negative interaction with SS for Mg, and

a linear impact on Cd dialyzability. Temp had a significant effect only on Mg dialyzability.

Several researchers have reported an increase in either apparent absorption, dialyzability or bio-accessibility of these elements after extrusion (Alonso et al., 2001; Mercier, 1993; Hazell and Johnson, 1989), while some reported no change (Drago et al., 2007). Most of these reports focused on limited extrusion conditions and linked the improvement in availability of these elements to thermal degradation of phytate that is known to form complexes with Fe. In our study, we found a significant effect of SS on dialyzability of elements, which suggests that apart from thermal degradation of interfering substances like phytates, shear during extrusion may facilitate other changes in bean flour that increase element dialyzability. Alonso et al, (2000) showed that there was a significant improvement in apparent absorption of Mg, P and Fe for an extruded sample enriched with amino acids due to enhanced function of enterocyte with respect to mineral intestinal uptake (Welters et al., 1999). Additionally, lignin and other fiber fractions are known to form insoluble complexes with divalent cations (Lestienne et al., 2005) and extrusion is known to reduce the lignin fraction in bean fiber while changing the distribution of soluble and insoluble dietary fibers resulting in an improved absorption of divalent cations like Mg and Ca (Martin-Cabrejas et al, 1999; Sumargo, 2016).

High MC and SS had a significant impact on the amount of dialyzable Cd with higher values increasing Cd dialyzability. Coupling high MC and high SS typically does not result in a desirable puffed product, suggesting an unlikely scenario for higher Cd dialyzability. The effects of processes like baking and milling on Cd concentration (Cubadda et al., 2002; Guttieri et al., 2015) and processing like microwave cooking on

Cd dialyzability (Wang et al., 2014) have been reported before, but there is no report on the effect of extrusion processing on dialyzability or bio-accessibility of Cd. In our study, we found that lower MC and SS would result in lower Cd dialyzability. This could be due to thermal degradation of phytates or increased soluble dietary fibre content during extrusion as reported by others. Several studies have shown that Cd binds more readily with insoluble fiber fractions than soluble fractions (Ou et al., 1999), and, although the effect of phytates on bio-availability of Cd is controversial, several researchers have shown a positive correlation between phytate content and Cd in sample (Persson et al.,1991; Rimbach et al., 1995).

7.4.3. Multivariate analysis

Fig 7.4A shows the pearson's correlations between phytic acid, folic acid, and total and dialyzable element concentrations in extrudates. Phytic acid was not correlated with any other variable, while folic acid was negatively correlated with dialyzable P. There was no association between the total concentration of a certain element and how much was dialyzed. However, there were correlations among the total amount of all serum elements (Mg, K and P) and among dialyzable concentrations of these elements, which suggests that changes during extrusion were consistent for these elements. Total and dialyzed fractions of the heavy metal Cd were not correlated with any variable.

Since there was no correlation between the total and dialyzable element concentrations, and the objective of our study was to have better understanding of factors that affect dialyzable element concentrations, all responses except total element concentrations were used for principal component analysis (PCA) (Fig 7.4B). The first two components explained 67% of the variation in the data. The essential elements (Mg,

K, P, and Fe) had high positive loadings on Component 1 and were grouped together, while phytic acid and folic acid were negative on Component 1 and positive on Component 2. Low MC conditions combined with either high or low SS seemed to have maximum effect on dialyzability of these elements, while folic acid and phytic acid were retained under high MC as discussed. Cd could be considered separate from all, with almost no loading on Component 1 and high positive loading on Component 2. The dialyzability of Cd was enhanced by high MC and low SS, which, as discussed, are unlikely extrusion conditions as it results in an unacceptable product from a physical standpoint.

7.4.4. Optimization

Based on the results of model fitting and analysis, extrusion parameters have differential effects on phytic acid, folic acid, and dialyzability of elements from bean flour. Because some of these components are desirable and others are undesirable, it is important to identify extrusion conditions that yield the best results in terms of measured responses. With the criteria of minimizing the phytic acid content and cadmium dialyzability while maximizing all other responses a desirability function was constructed using the simultaneous optimization technique which gave the maximum desirability of 0.87 for following extrusion conditions: Temp: 100°C; MC: 20.7%; SS: 245 rpm. This suggests a low barrel temperature, medium feed moisture content, and high screw speed are ideal conditions to achieve maximum destruction in phytic acid while achieving minimum loss in folate and high element dialyzability in GNB flour.

7.5. CONCLUSION

Extrusion resulted in up to 85% reduction in phytates in GNB flour while moderately affecting folate content. Low MC conditions resulted in maximum reduction of these compounds. Extrusion significantly improved the dialyzability of all the mineral elements (Mg, P, K and Fe) while significantly reducing the dialyzability of heavy metal Cd when compared with dialyzed amounts from unprocessed flour. SS was the major extrusion variable causing the changes in dialyzable elements which was followed by feed MC. Low barrel temperature, medium MC and high SS were identified as extrusion conditions that resulted in maximum dialyzability while minimum destruction to folate. The study suggests that extrusion had a marked influenced on availability of mineral elements in GNB and the results can form basis for bio-availability studies for GNB or other dry bean varieties using Caco-2-cell or other models.

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Table 7.1. Experimental design and percentage change in element dialysability, folic acid and phytic acid content from flour

	Extrusion Variables			Change from flour (%)*						
Run	Temp (°C)	MC (%)	SS (rpm)	Folic acid	Phytic acid	Dialysability				
						Mg	P	K	Fe	Cd
1	90.0	23.1	225	-18.2	-84.3	43.7	20.0	19.3	76.1	-331
2	90.0	18.9	180	-30.0	-79.7	49.7	45.7	25.3	81.3	-20.4
3	90.0	23.1	180	-22.2	-81.2	47.1	24.1	15.8	74.9	62.6
4	90.0	18.9	225	-25.4	-79.7	56.3	49.6	23.5	84.3	39.6
5	115	21.0	203	-18.2	-81.2	41.4	5.50	15.9	59.8	25.8
6	115	21.0	203	-20.2	-78.9	38.1	6.80	12.2	61.0	-395
7	115	21.0	156	-23.3	-75.3	51.5	33.3	17.7	78.7	18.1
8	115	21.0	203	-21.2	-81.2	45.7	29.4	19.7	73.3	41.5
9	115	21.0	250	-10.8	-78.2	53.0	46.8	22.5	80.7	-15.9
10	115	21.0	203	-23.3	-78.9	40.7	17.9	18.1	56.5	34.5
11	115	21.0	203	-17.2	-79.7	44.1	15.6	20.5	56.2	30.4
12	115	25.3	203	-20.2	-74.6	29.5	4.20	15.2	75.2	-13.7
13	115	21.0	203	-26.5	-76.7	43.7	23.9	21.7	73.1	17.7
14	115	16.7	203	-43.0	-84.3	51.1	46.4	16.7	78.9	-582
15	140	23.1	225	-12.6	-66.5	42.2	-16.5	13.8	41.3	-207
16	140	23.1	180	-22.2	-69.2	44.2	27.5	17.2	77.8	35.6
17	140	18.9	180	-31.2	-81.9	47.4	36.4	19.9	39.8	-795
18	140	18.9	225	-26.5	-85.1	49.0	32.3	25.4	72.1	10.9

Temp-Barrel temperature, MC-Feed Moisture content, SS-Screw speed. *there was a significant difference between concentration of these responses in unprocessed flour and extruded samples tested using Dunnett's multiple comparison test at α-0.05.

Table 7.2. Regression coefficients for each response surface equation and model fit parameters.

	Phytic acid	**Folic**	**Dialyzability**				
Parameter[a]		**acid**	Mg	P	K	Fe	Cd
Intercept	6.44	36.8	3235	12469	15574	47.6	-0.08
SS	-0.01	-1.14	-15.44	-35.8	-26.9	-0.03	0.0003*
MC	0.15*	16.5*	79.50	-451	959	-2.10	0.005*
Temp	0.03*	-0.24	-21.74	-25.9	-146	-0.28	-0.0001
SS^2	0.00016	0.002	0.08*	0.22*	0.15	0.001*	-9.6e-8
SS*MC	0.00	0.02	-0.71*	-1.67	-1.64	-0.02*	-1.6e-5
MC^2	0.00095	-0.47*	-0.13	13.3	-18.5	0.09*	-0.00004
SS*Temp	0.000085	0.001	-0.02	-0.15	0.06	-0.0005	3.4e-7
MC*Temp	0.001*	0.02	0.32	1.07	-0.02	0.01	5.8e-7
$Temp^2$	0.00012	-0.002	0.08*	0.13	0.56	0.0004	7.6e-8
LOF	NS	NS	NS	NS	NS	NS	NS
Adjusted R^2	0.86	0.83	0.85	0.75	0.20	0.75	0.79
F ratio	12.8*	10.3*	11.8*	6.58*	0.83	5.26*	3.46*

LOF: Lack of Fit; NS: Not significant, *significant at $p \leq 0.05$

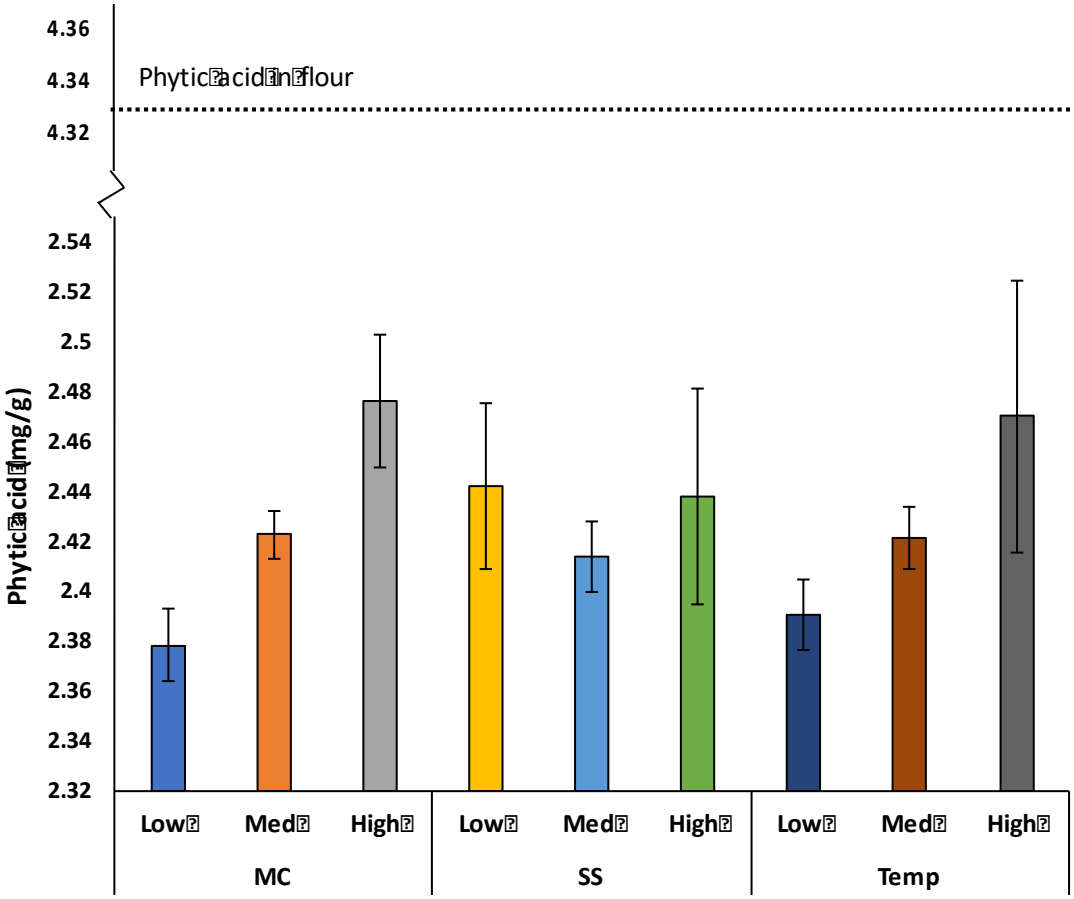

Figure 7.1. Phytic acid in extruded samples under low, medium and high conditions of moisture content (MC, %), screw speed (SS, rpm), and barrel temperature (Temp, °C)[25]

[25] Low and high conditions n=5, medium conditions n=8 with standard error bars plotted; the decrease in phytic acid was a significant for all extrusion conditions when compared with unprocessed bean flour (α=0.05).

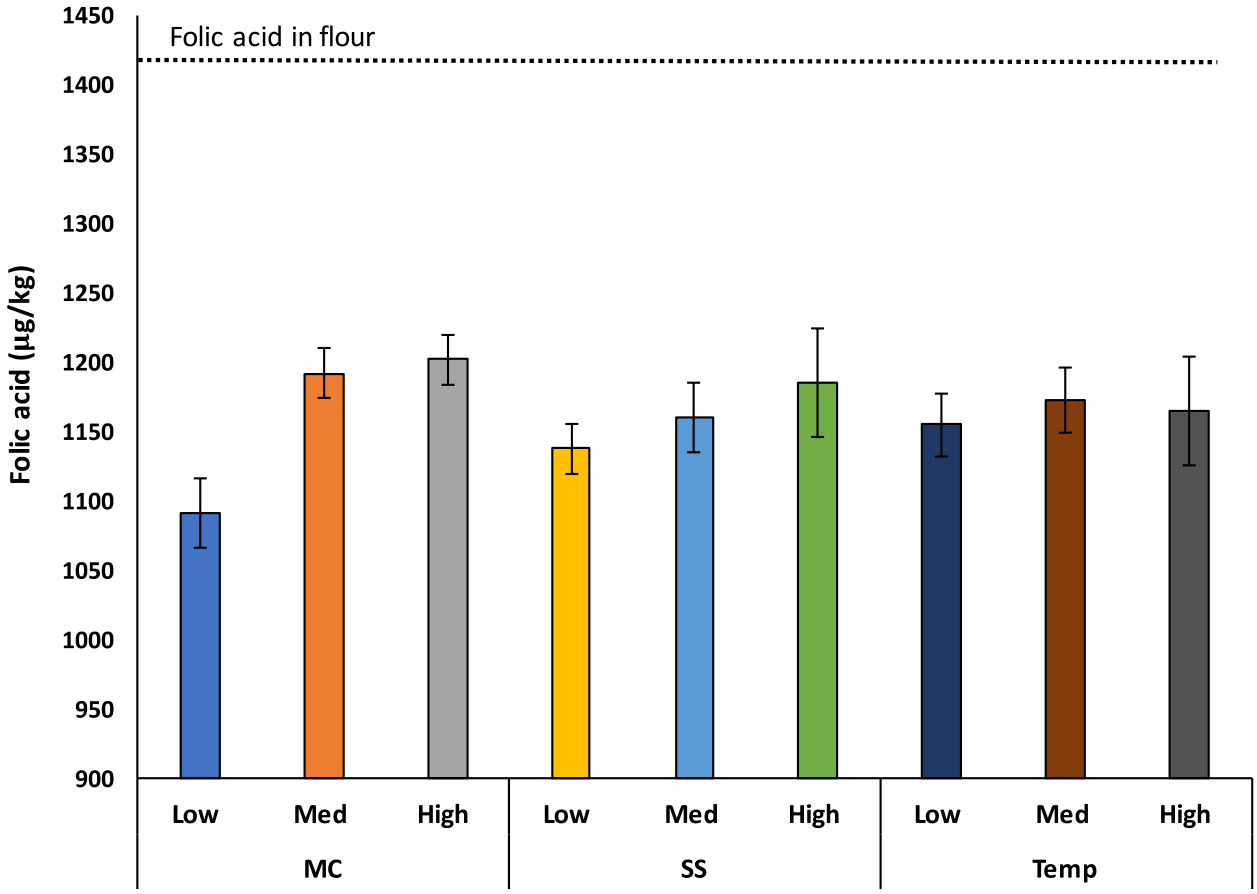

Figure 7.2. Folic acid in extruded samples averaged over low, medium and high conditions of moisture content (MC, %), screw speed (SS, rpm), and Barrel temperature (Temp, °C)[26]

[26] Low and high conditions n=5, medium conditions n=8, standard error bars plotted, *there was a significant change in all extruded conditions when compared with the amount in flour (α=0.05).

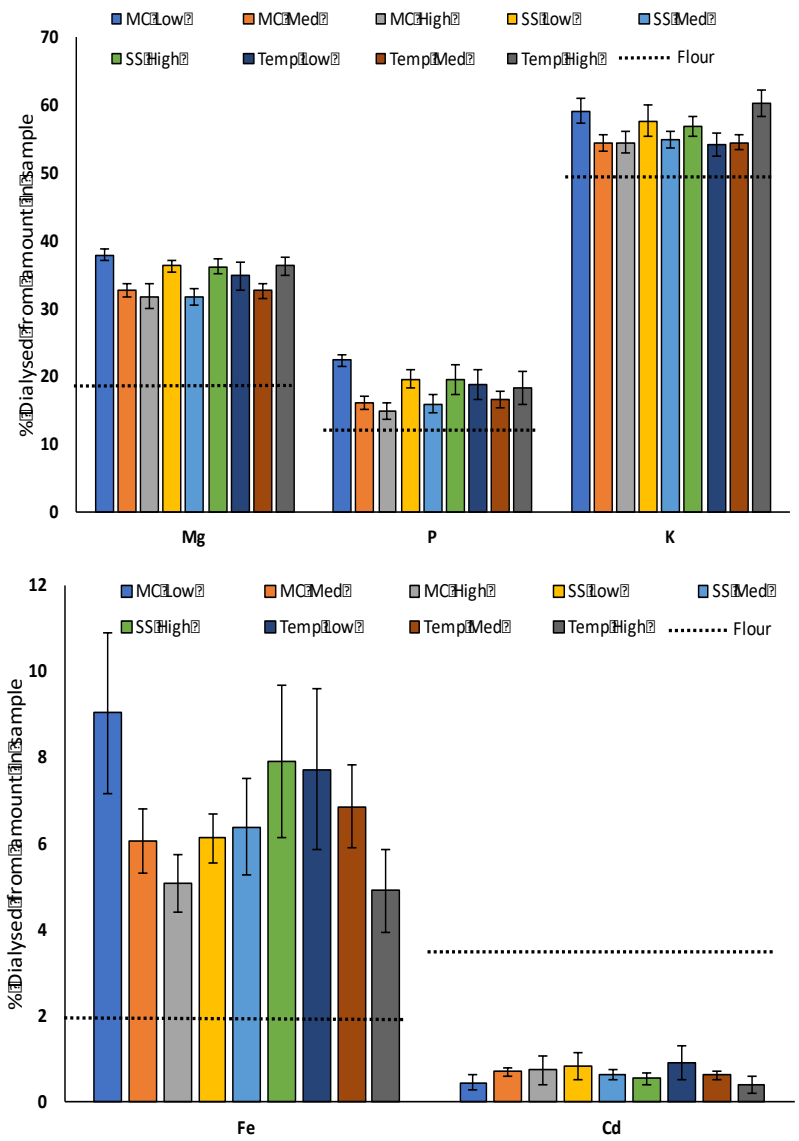

Figure 7.3. Percentage of total elements that were dialyzable under low, medium and high conditions of moisture content (MC, %), screw speed (SS, rpm), and barrel temperature (Temp, °C)[27]

[27] low and high conditions n=5, medium conditions n=8, standard error bars plotted, dotted lines represent the percent dialyzed from un-extruded flour, *there was a significant change in all extruded conditions when compared with the amount in flour (α=0.05).

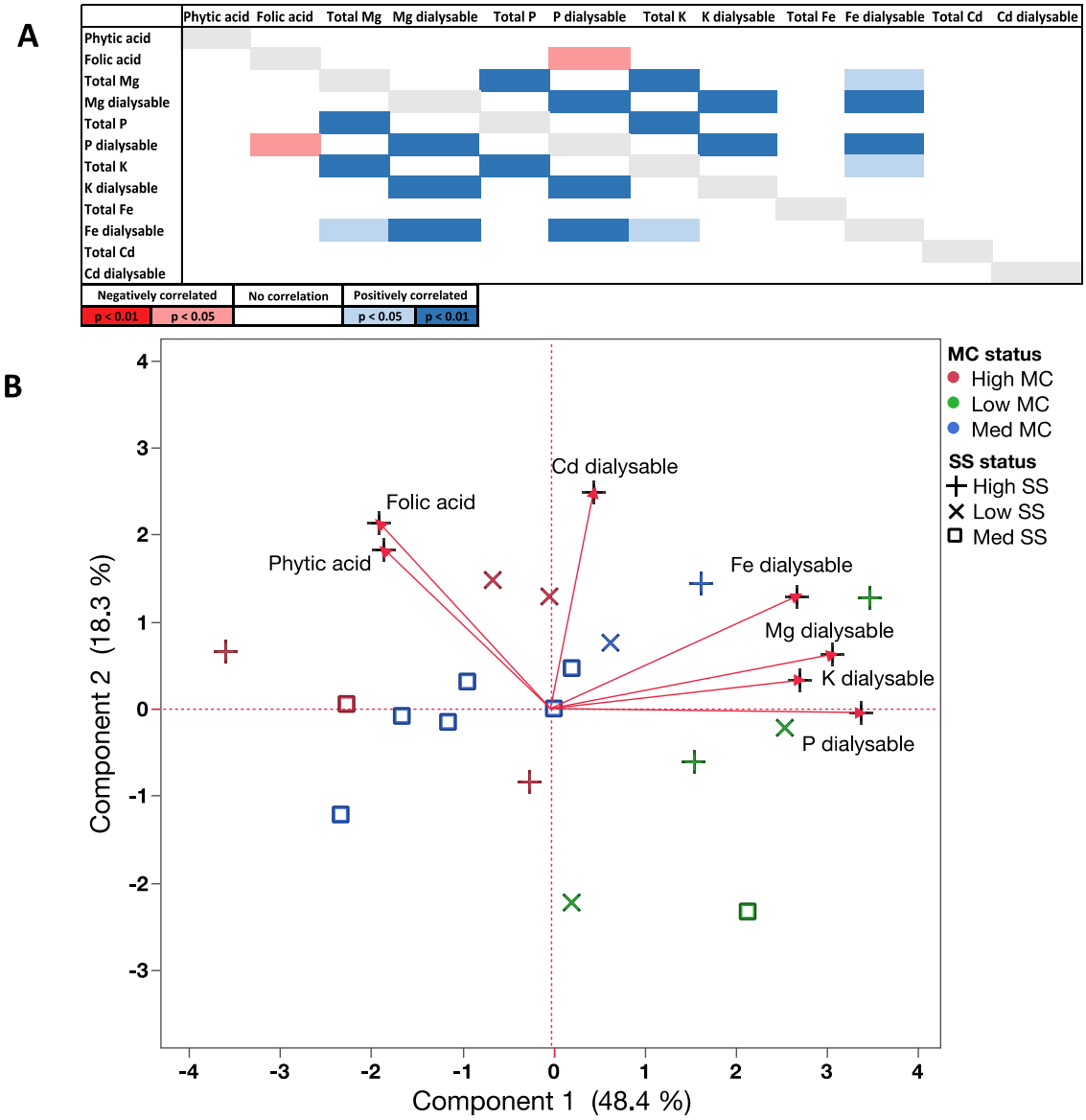

Figure 7.4. Multivariate analysis on extruded samples, A) Correlation matrix; B) Bi-plot using Principal Component Analysis (PCA) on correlations

CHAPTER 8: QUALITY CHARACTERISTICS AND *IN VITRO* PROTEIN DIGESTIBILITY OF BREADS MADE FROM HISTORICAL AND MODERN WHEAT CULTIVARS

8.1. ABSTRACT

Breads made with 21 cultivars of wheat introduced or released in US between 1870 and 2014 were evaluated for end-use quality attributes and *in-vitro* protein digestibility. All cultivars were planted over two harvest years with three field replicates. Grain yield was positively correlated with release year, with modern cultivars having highest yields with no effect of planting year. Grain yield was negatively (r^2=-0.9) correlated with flour protein content, with land races and old cultivars having high protein content and low yield. Flour absorption was positively correlated (r^2=0.74) with protein content while mixing time was not correlated with any observed variable. Year of planting had a significant effect on mixing time suggesting an environmental effect on other flour components than proteins. Breads made with Turkey, Kharkof and Red chief had lowest protein digestibility of 83, 79 and 82% when compared with other cultivars. All cultivars released after Cheyenne (1931) had digestibility of at least 90%. In our study, Cheyenne was the oldest wheat cultivar released from a modern wheat breeding program. Interestingly, Cheyenne was developed from Turkey and had the same protein concentration, yet Cheyenne had significantly higher protein digestibility. Further investigation is needed to identify the cause of improvement in protein digestibility of wheat with modern breeding.

8.2. INTRODUCTION

Bread wheat (*Triticum aestivum*), a hexaploid wheat species, is the second largest cereal grain produced in the US (USDA, crop production database, 2018). The production and processing of this wheat class in the US has increased by 37 and 55% respectively over the last 50 years (USDA, ERS database, 2018). The main cause of this increase is the wheat breeding programs initiated in the 1940s with the aim to release new cultivars of wheat with improved grain yield, disease tolerance, better bread making characteristics etc. (Braun et al., 1996). These breeding programs have ensured a continuous supply of high-quality wheat for the rapidly growing world population.

However, these breeding programs have faced severe public criticism in the past decade and modern wheat cultivars have been blamed for chronic conditions like obesity, increased celiac disease, and gluten intolerance, among others. A simple web search for 'modern wheat' generate articles, blogs and discussions labelling wheat as 'poison', 'toxic junk food', 'less nutritious', and 'harmful'. Despite the efforts of the wheat community to refute such claims by scientific evidence (Brouns et al., 2013), the image of wheat has been tarnished among many of the general population. This has resulted in a rapid boom in the gluten-free foods industry, which is further projected to increase by 25% by 2020 (Terazono, 2017). While a gluten-free diet is a necessity for some, for others it is unnecessary and can result in nutritional inadequacies (Hallert et al., 2002). Thus, it is important to break the myths surrounding wheat and human health and rebuild its place in our diets.

In an effort to do so, many researchers have evaluated old and new wheat varieties to determine how modern wheats have changed. Hucl et al., (2015) reported a

significant improvement in agronomic and end-use quality traits in modern wheat while Ribeiro et al., (2016) observed higher amounts of potential celiac disease's imunostimulatory epitopes in old cultivars when compared with modern wheat.

With a similar approach, the present study focused on protein digestibility. Because wheat forms a viscoelastic network upon mixing with water that appears almost like rubber, many people assume that wheat gluten is difficult to digest. Research, however, has shown that among cereal proteins, wheat has highly digestible protein. However, breeding efforts are focused on developing wheats with even stronger gluten that can withstand high speed mixers and industrial bread production. The process of selecting wheat with these 'modified gluten' characteristics may affect protein digestibility.

In the present research, we used an *in vitro* approach to compare the protein digestibility of 21 wheat cultivars. Of these, 2 were land-races, i.e., wheat varieties that maintain their original form and have not been manipulated by intentional breeding efforts, and 19 were wheat cultivars released in US between 1901 and 2014 either by natural selection or crossing between earlier cultivars as part of wheat improvement and breeding programs. The primary aim of this work was to determine if there was any difference in digestibility of wheat varieties released over the years. Apart from digestibility, grain yield, protein content, and dough characteristics of these cultivars were compared.

8.3. MATERIALS AND METHODS

8.3.1. Wheat samples growth and preparation

Field studies were seeded as the wheat (*Triticum aestivum* L.) component of a wheat/oats (*Avena sativa* L.) /soy (*Glycine max* L.) rotation. A collection of 21 wheat cultivars and land-races were planted for harvest years 2016 and 2017 (Table 8.1). All field studies were conducted at the University of Nebraska Agricultural Research and Development Center, located near Mead, NE, approximate GPS coordinates N 41° 08.782 W 096° 29.985. Materials were planted in randomized complete block designs with three replications (field reps) with the exceptions of 'Red Chief' in 2016 with only one replication and 'Anton' in 2 replications due to inadequate seed supplies. Field plots were machine planted and harvested. Harvested area of each plot was approximately 3 square meters. Grain yield was calculated in kg/ha. Plots were maintained disease free via applications of the fungicides metconazole (5-[(4-chlorophenyl) methyl]-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl) cyclopentanol; Caramba®, BASF) and pyraclostrobin (carbamic acid, [2-[[[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxy]methyl]phenyl]methoxy-,methyl ester) plus metconazole; Twinline®, BASF), applied alternately during growing, flowering, and grain filling. Wheat samples after harvesting were milled using a Buhler mill and stored under refrigerated conditions until further analysis.

The land-races Turkey and Kharkof, were both introduced to the United States before 1901. The remaining 19 entries were all U.S. released cultivars with varying release dates. Based on the date of release, wheat cultivars were divided into four groups: pre-1901, land-races; 1901-1950, 'very old'; 1951-2000, 'old'; 2000-2014, 'new' (Table 8.1).

8.3.2. Flour characteristics

Moisture (44-15A) and protein content (NIR analysis, 39-11) of flour samples were determined by AACC standard methods. The measured moisture and protein values were used to calculate flour absorption and mixing time (corrected) by standard mixograph method (AACC- 54-40A). Each test was done in duplicate.

8.3.3. Bread Making

For each flour sample, breads were baked in duplicate, resulting in 120 samples for planting year 2016 and 126 samples for planting year 2017. The order of bread making was randomized for each year. Breads were made by modification of the AACC straight dough bread making method (10-09). Thirty grams of flour was mixed with instant yeast (0.66%, flour basis); sugar (6%); salt (1.5%) and shortening (3%) in a mixograph bowl. Water (maintained at 25-30 °C) was added and the doughs were mixed based on the absorption and mixing requirements from the mixograph data. The dough was fermented for 60 min at 30 °C, whereupon the dough was shaped by hand, panned, and proofed for 70 min at 30 °C. Proofed doughs were baked for 20 min at 204 °C. The baked breads were cooled to room temperature and sliced. The bread slices were frozen at -80 °C and then freeze dried using a plate freeze drier for 24 h (Model 2400, Freeze Dry Company Inc., Pine River, MN). The dried bread pieces were crushed in to a fine powdered using blender. The powdered samples were kept at 4°C until further analysis.

8.3.4. In vitro protein digestibility

Gastrointestinal protein digestion was measured as described (Gulati et al. 2017) with slight modifications. Dried, ground bread (120 mg) was suspended in simulated gastric fluid (SGF; 0.5 M NaCl, pH:2.5) to achieve a pH of 2.5 (approximately 4 mL) and incubated at 37 °C for 10 min. The contents were then mixed with pepsin dissolved in

SGF to give an activity of 200 U of pepsin/mg of protein in sample and incubated for 2 h. Gastric digestion was stopped by raising the pH to 7 using 0.5 M sodium bicarbonate. For intestinal digestion, pepsin hydrolyzed samples were mixed with 1 mL simulated intestinal fluid (SIF) (0.05 M KH_2PO_4, pH: 7.0) and warmed at 37 °C. Three milliliters of pancreatin solution in SIF (2 mg/mL) containing 2 µL/mL amyloglucosidase was added to flour mixture and incubated for 4 h. Intestinal digestion was stopped by plunging the tubes into a boiling water bath for 5 min and immediately cooling. The digested samples were centrifuged and the supernatants were used to measure degree of hydrolysis (DH) by the reaction of free amine groups with TNBS using leucine as the standard. DH was then calculated as described in Gulati et al. (2017) with h_{total} of 7.67 mmol/g protein (weighted average molecular weight of the amino acids in wheat flour). Digestion for each sample was performed in duplicate and degree of hydrolysis for each digested sample was further analyzed in duplicate.

8.3.5. Statistical data analysis

Grain yield, flour protein content, absorption, mixing time, and bread in-vitro protein digestibility were the responses analyzed in the study. Data were initially analyzed using two factor ANOVA. Wheat cultivar, year of planting, and their interaction were fixed effects, and field replicates nested within year was a random effect. Differences among least square means by cultivar, by release year group, and by planting year, were tested by multiple comparison test with Tukey's adjustment (cultivar and release year group) and t-test (planting year) at α=0.05. Pearson's correlations on LS means (N=21) were calculated among responses. All statistical analyses and graphs were generated using JMP software (JMP version 10.0.0, SAS institute).

8.4. RESULTS

8.4.1. Grain yield and flour characteristics of different wheat cultivars

Cultivar type but not planting year or the interaction of planting year with cultivar significantly affected grain yield (Table 8.2). The average yield obtained was 4300 kg/ha. The maximum yield was obtained for Settler CL while minimum yield was obtained for Kharkof (Fig. 8.1; Table 8.3). When yields were compared based on year of release it was found that there was no statistical difference between very old and land-race cultivars, but there were differences among all other groups (Table 8.3). New cultivars (2000-2014) had the maximum yield, followed by old cultivars (1950-2000), and finally very old and land-race varieties. Further, there was a positive correlation between grain yield and release year (Fig 8.5).

The overall protein content of flour ranged from 9.5 to 13.1% with an average protein content of 11.4%. There was a significant effect of year of planting, cultivar, and their interaction on the protein content (Table 8.2). When comparing the protein content among cultivars, the highest protein content was obtained for Turkey, Kharkof, Cheyenne, Wichita and Triumph64 while Freeman had the lowest protein content (Table 8.3). When compared among the two planting years, wheat grown in 2016 had a slightly higher protein content (11.9%) than same wheat grown in 2017 (11.1%) (Table 8.3; Fig. 8.3). Furthermore, there was a significant difference between protein content of new and old wheat cultivars (Table 8.3) and also, other groups while very old and land-race ones had highest protein content and were statistically same.

There was a significant negative correlation between grain yield and flour protein content (r^2 = -0.90) which has been observed by other researchers (Simmonds, 1995;

Bogard, 2010). Further, there was a negative correlation between release year and flour protein content (Fig 8.4; 8.5).

Flour absorption and mixing time are measures of dough characteristics and associated with flour protein content (Roels et al., 1993). Usually, the higher the protein content the higher the water absorption, which increases the mixing time to an extent based on other flour characteristics (Baig and Hoseney, 1976).

Both flour absorption and mixing times were affected by cultivar and year of planting (Table 8.2). Mixing time also had a significant interaction between cultivar and year of planting. The maximum flour absorption was obtained for Mattern (Table 8.3; Fig. 8.2), a waxy wheat cultivar, which was expected to have high absorption as observed for other waxy wheat varieties (Takata et al., 2005). The significant year effect for flour absorption can be attributed to the high absorption of Mattern, especially in 2016. After, Mattern, Turkey and Cheyenne had the highest absorption, while Freeman had the lowest absorption, which can be due to its low protein content. A significant positive correlation was obtained between protein content and absorption (Table 8.4). There was a significant negative correlation between flour absorption and both release year and yield. Based on year of release, new cultivars had the lowest absorption and were significantly different from the other three categories while there was no difference between land-race, very old or old cultivars (Table 8.3). Mixing time was not correlated with any variable but there was a significant effect of planting year on mixing time of cultivars (Fig. 8.2), wheat planted in 2016 had lower mixing time than wheat planted in 2017. There was no effect of year of release on mixing time (Table 8.3).

8.4.2. In vitro digestibility of bread made with different wheat cultivars

Protein digestibility of bread made with different wheat cultivars was significantly affected by cultivar type and the interaction of planting year and cultivar (Table 8.2). The protein digestibility of all cultivars ranged from 89 to 94% except Red chief, Turkey, and Kharkof, which had significantly lower digestibility compared with all other cultivars (Table 8.3). When LS means for digestibility of samples were plotted by planting year, all samples grouped together except Red chief, Turkey and Kharkof, which had digestibilities that were noticeably lower than average. The separation between these cultivars and the rest of the samples was clearer in 2017, which explains the significant interaction between cultivar and year of planting. When digestibility of all samples was plotted against release year (Fig 8.4), the obtained trend was opposite to protein content, with newer samples having higher digestibility. Based on the year of release, all cultivars grouped well with their respective group except Red Chief, which grouped with the land-race cultivars.

8.5. DISCUSSION

Turkey and Kharkof are winter wheat varieties introduced in the Great Plains of the US in 1874 and 1900, respectively, by early Mennonite settlers (Ross, 1969). Both the cultivars had excellent adaptation to the Great Plains and were used as parents in early wheat breeding programs. Cultivars developed from Turkey and Kharkof had improved grain yield, disease and insect resistance, and baking quality (Auvochanon, 2010), such as Cheyenne, which was introduced in 1931. On the other hand, Red Chief was a cross between two early wheat cultivars Red Clawson and Red Arcadian, developed in Eastern US, in 1903. This cultivar was introduced to Nebraska in 1926 but was never grown commercially and was mostly used as parent variety for new cultivars (Clark, 1927). As

part of wheat breeding programs, modern cultivars were developed or selected to meet requirements other than yield. For instance, Anton was developed to meet low polyphenol oxidase requirement (Graybosch, 2011), while Clark's cream was developed as a pre-harvest sprouting resistant germplasm (Morris, 1982).

It has been observed by many researchers that land-race and older wheat cultivars like Turkey, Kharkof, Red Chief, and Cheyenne have lower yield when compared to new wheat cultivars due to changing climate resulting in winter injuries, while new cultivars have greater winter hardiness (Auvochanon, 2010; Fufa et al., 2005a). The lower yield results in higher protein content of these cultivars to overcome stress. The negative correlation between grain yield and protein content can be associated with a N dilution effect by carbon-based compounds under optimal yield (Acreche and Slafer, 2009). One of the major objective of wheat breeding programs is to increase grain yield, which is clearly evident in the present study where older wheat cultivars had higher protein content but low yield. Similar results were reported by Cox et al. (1989) for cultivars released up to 1988 and ancient cultivars.

Based on morphology and end use quality, Turkey, Kharkof, Red Chief have been grouped together by many researchers as having inferior end quality characteristics than modern cultivars (Fufa et al., 2005b; Auvochanon, 2010). In our study, we observed that these three cultivars also grouped together in terms of lower bread digestibility.

In our study, based upon the release year, the improvement in bread digestibility was first observed for Cheyenne, after Cheyenne (or 1930s) all cultivars had the same digestibility. Released in 1931, Cheyenne is one of the earliest cultivars originating from wheat breeding program and ancestor of many prominent cultivars like Warrior and

Sturdy. It has also been reported previously that Cheyenne is genetically most closely related to Kharkof and Turkey while other modern wheat cultivars (after 1960) have diverged from these ancestors due to breeding for adaptation and yield improvement (Auvochanon, 2010). Further, it has been shown that Turkey and Kharkof have similar seed storage proteins and Red Chief has unique storage proteins, while the proteins of Cheyenne are similar to modern cultivars (Fufa et al, 2005b). Further, Graybosch, (1992) showed that there is a significant difference in HMW glutenin subunits for Turkey, Red Chief and Cheyenne when compared with cultivars grown after 1946. Mainly, cultivars before 1946 had higher frequency of 2+12 subunits which results in weak dough strength, while frequency of the 5+10 subunits known for higher gluten strength increased with increasing release year.

Despite the genetic similarity of Cheyenne with ancestral wheat cultivars, its protein digestibility was different from ancient cultivars and rather similar to modern cultivars potentially due to similarity in seed storage proteins as reported. This suggests that initial breeding practices might have incurred useful changes in storage proteins of wheat which has been carried and preserved by modern wheat cultivars despite differences in their developmental objectives.

Given the evidence for genetic diversity, seed storage profiles, and glutenin subunit we can at least say that the protein makeup of ancestral wheat cultivars is different than the modern cultivars which resulted in improved digestibility but requires further investigation to explore the cause of observed effect.

Thus, the wheat breeding programs that have been criticized for destroying wheat has in fact made the quintessential wheat crop more adaptable to changing environments

resulting in better yield and improved protein from both nutritional and bread making stand point.

8.6. CONCLUSION

A significant positive correlation was observed between release year of wheat cultivars with both grain yield and bread *in vitro* digestibility. Improved yield is an indication of better adaptability and winter hardiness of modern wheat cultivar facilitated by the wheat breeding programs. Lower yield in old cultivars resulted in more accumulated protein. Ancestral cultivars like Turkey, Kharkof and Red Chief had highest protein but lowest digestibility among all cultivars. Prominent improvement in digestibility among cultivars was observed beginning with Cheyenne, one of the earlier cultivars originating from wheat breeding programs in Great Plains. There was no significant difference between digestibility of cultivars released after Cheyenne suggesting preliminary beneficial changes to wheat proteins which were carried on in the modern cultivars. Flour absorption was positively correlated with protein content with Mattern, a waxy wheat variety having the highest absorption. There was a significant effect of planting year on mixing time but it was not significantly correlated with any measured response in the study suggesting an environmental effect on other flour constituents. This study indicates that the protein digestibility of wheat cultivars has improved when compared with landrace varieties but needs further investigation to explore the cause of improvement and further comparison with more ancient wheat cultivars.

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Table 8.1. Wheat cultivars used in the study with their release year and year status

Wheat Cultivar	Year of Introduction in US or release	Year Status
Turkey	1874	Land Race
Kharkof	1900	Land Race
Red Chief	1926	Very Old
Cheyenne	1933	Very Old
Wichita	1944	Very Old
Warrior	1963	Old
Triumph64	1964	Old
Lancer	1965	Old
Sturdy	1966	Old
Scout66	1967	Old
Centurk 78	1978	Old
Clark's Cream	1982	Old
Centura	1983	Old
TAM107	1987	Old
Wesley	1998	Old
Jagalene	2001	New
Overland	2006	New
Anton	2007	New
Settler CL	2011	New
Mattern	2012	New
Freeman	2014	New

Table 8.2. Analysis of Variance (Mean squares) for all measured responses

Sources of variation	df	Grain Yield	Flour Protein content	Flour Absorption	Mixing Time	Protein digestibility
PY	1	3847833	44.3*	15.3*	49.3*	1.54
Error a = rep(PY)	4	540950	0.73	1.40	0.17	16.8
Cultivar	20	10297499*	7.04*	18.0*	3.86*	165*
Cultivar X PY	20	483337	0.86*	1.61	0.62*	27.3*
Residual Error	200	47877690	92.2	1.12	0.11	11.5

(*) Mean squares marked with asterisks represents significant effect on F-test at alpha 0.05; df = degrees of freedom; PY = Planting Year; rep -= field rep

Table 8.3. Multiple mean comparisons by year of planting, year status and cultivar for measured variables

Criterion of comparison	Category	Mean Yield (Kg/ha)	Mean flour protein (%)	Mean Absorption (%)	Mean Mixing Time (min)	Mean Protein Digestibility (%)
Year of Planting	2016	4206 a	11.9 a	62.6 a	2.8 b	90.2 a
	2017	4566 a	11.1 b	62.1 a	3.7 a	89.8 a
Release Year Status	Land Race	2785 c	12.6 a	63.3 a	3.4 a	80.8 c
	Very Old	3020 c	12.4 a	63.5 a	3.2 a	88.9 b
	Old	4209 b	11.6 b	62.4 ab	3.2 a	91.1 a
	New	5898 a	10.6 c	61.4 b	3.4 a	91.8 a
Cultivars	Turkey	3067 fg	12.6 a	63.5 ab	2.9 b-g	82.9 b
	Kharkof	2503 g	12.5 a	63.0 a-c	3.9 a	78.6 b
	Red Chief	2969 fg	12.2 ab	63.6 ab	2.8 c-g	81.9 b
	Cheyenne	3190 fg	12.4 a	63.6 ab	3.9 a	91.7 a
	Wichita	3193 fg	12.5a	63.2 ab	2.7 d-g	90.6 a
	Warrior	3963 d-g	11.6 bc	62.3 a-d	3.7 a-c	92.1 a
	Triumph64	3235 fg	12.6 a	63.3 ab	2.5 fg	92.0 a
	Lancer	3379 fg	11.5 b-d	63.0 a-c	2.8 c-g	89.7 a
	Sturdy	5128 a-e	11.5 b-e	62.1 a-d	2.6 e-g	91.6 a
	Scoutt66	3041 fg	11.7 bc	62.7 a-c	2.8 c-g	93.6 a
	Centurk 78	3814 e-g	11.5 b-d	62.1 a-d	2.8 c-g	91.0 a
	Clark's Cream	3778 e-g	12.0 ab	63.0 a-c	3.4 a-f	90.1 a
	Centura	4443 c-f	11.1b-e	61.6 b-e	3.8 ab	90.3 a
	TAM107	5374 a-e	11.2 b-e	61.5 b-e	2.7 d-g	90.7 a
	Wesley	5940 a-c	11.2 b-e	61.9 b-e	4.1 a	89.5 a
	Jagalene	6036 a-c	10.9 c-e	61.4 b-e	3.9 a	93.4 a
	Overland	6273 ab	10.3 de	60.0 de	2.4 g	92.7 a
	Anton	4781 b-f	11.0 b-e	61.7 b-e	4.2 a	92.5 a
	Settler CL	6795 a	10.5 c-e	60.7 c-e	3.5 a-e	92.2 a
	Mattern	5619 a-d	10.6 c-e	64.5 a	2.9 b-g	88.6 a
	Freeman	5823 a-c	10.3 e	59.6 e	3.6 a-d	91.5 a

Samples marked with different alphabets in each criterion of comparison are significantly different

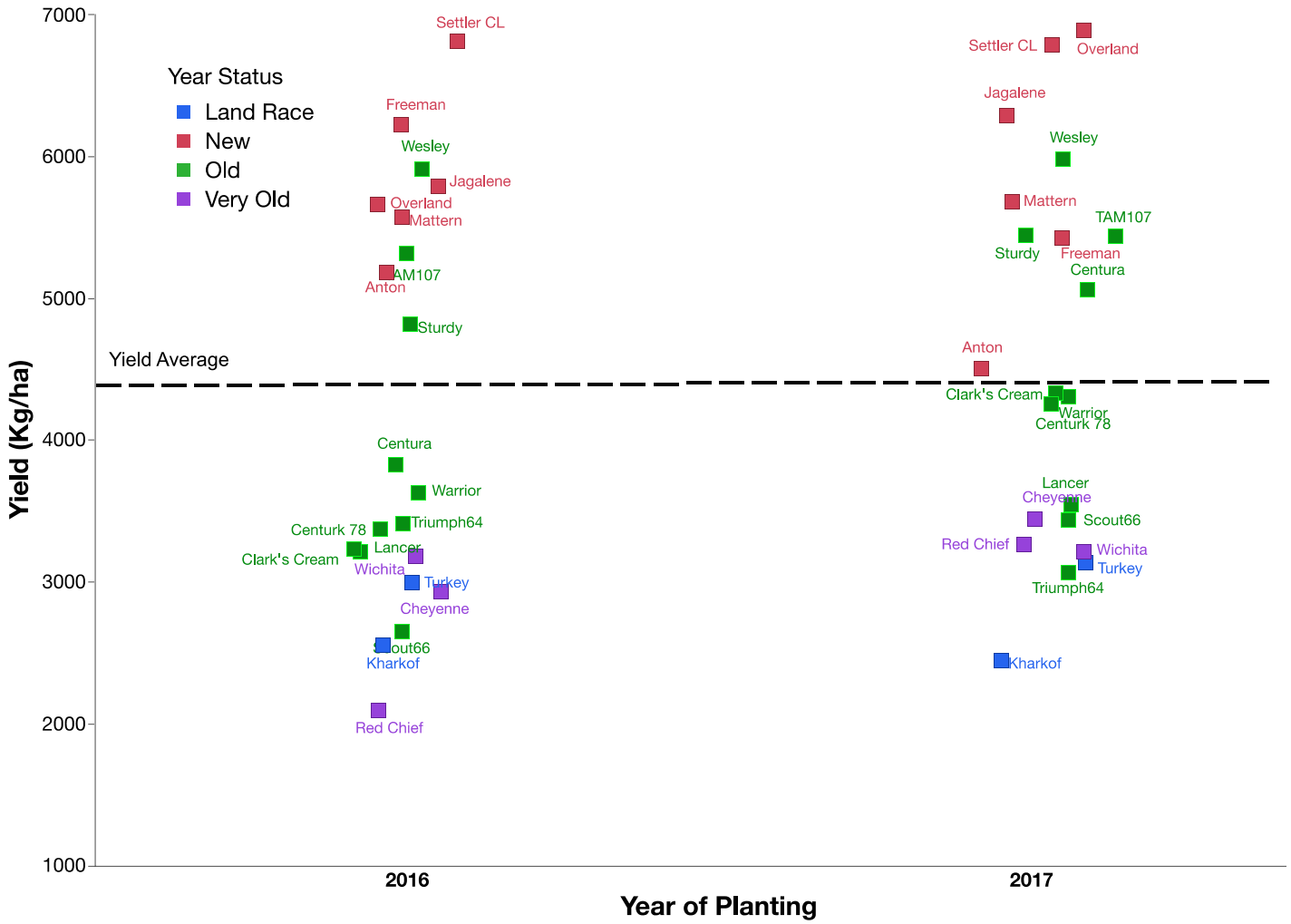

Figure 8.1. Grain yield of different cultivars for both planting years data is based upon LS means, N=21 for each year

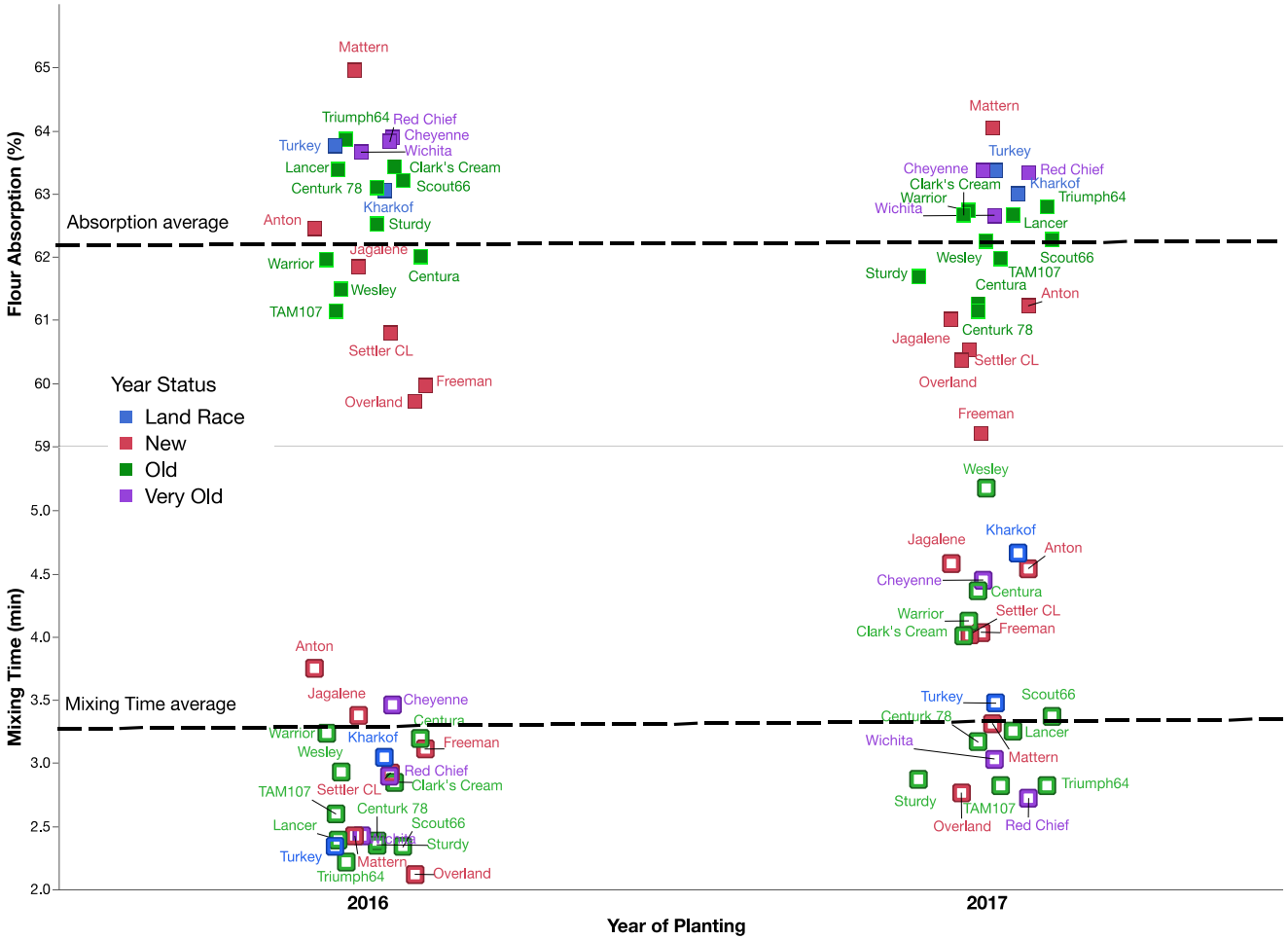

Figure 8.2. Flour absorption and mixing time of different cultivars for both planting years; data is based upon LS means, N=21 for each year

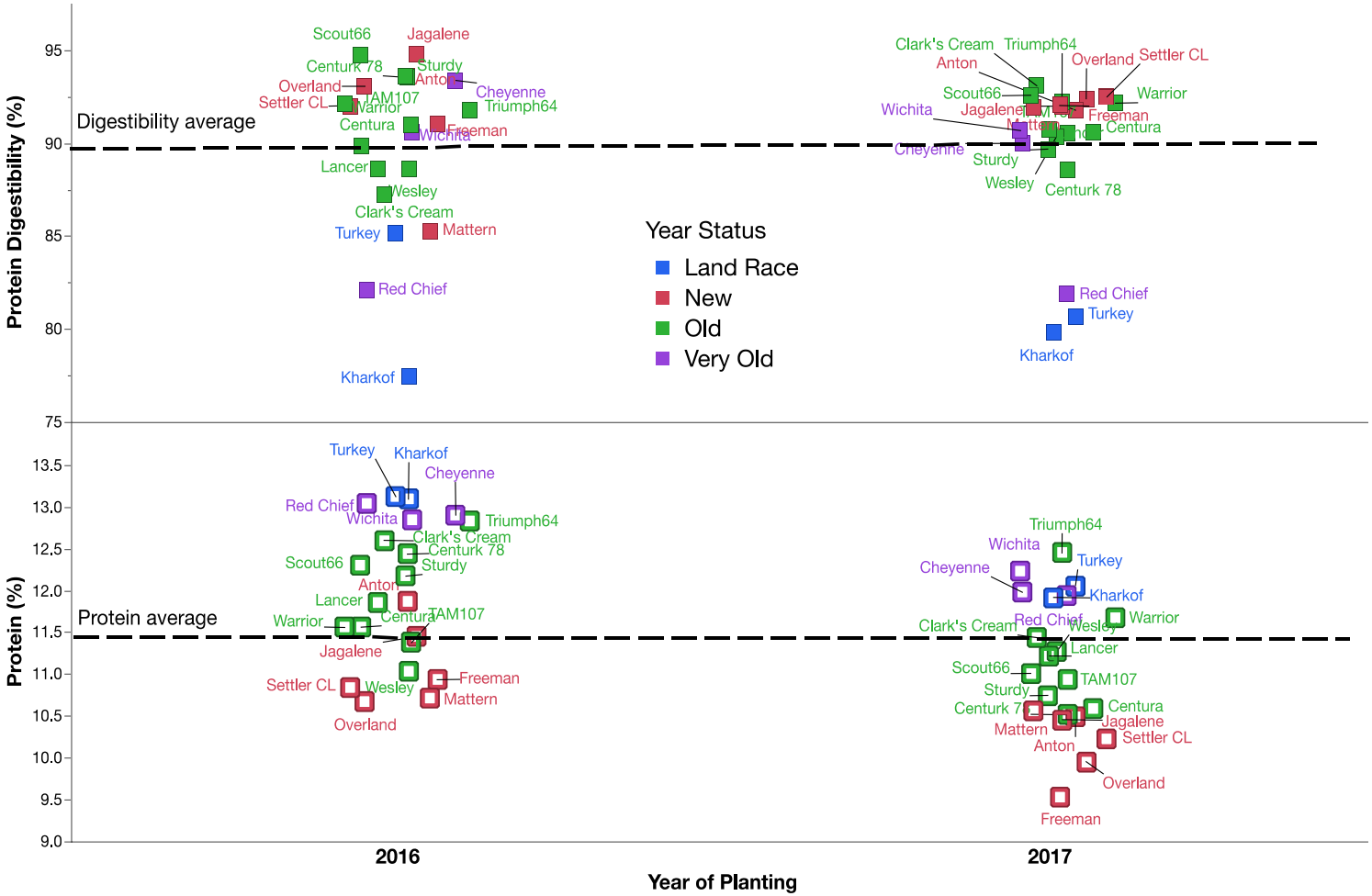

Figure 8.3. Flour protein content and bread in-vitro protein digestibility of different cultivars for both planting years; data is based upon LS means, N=21 for each year

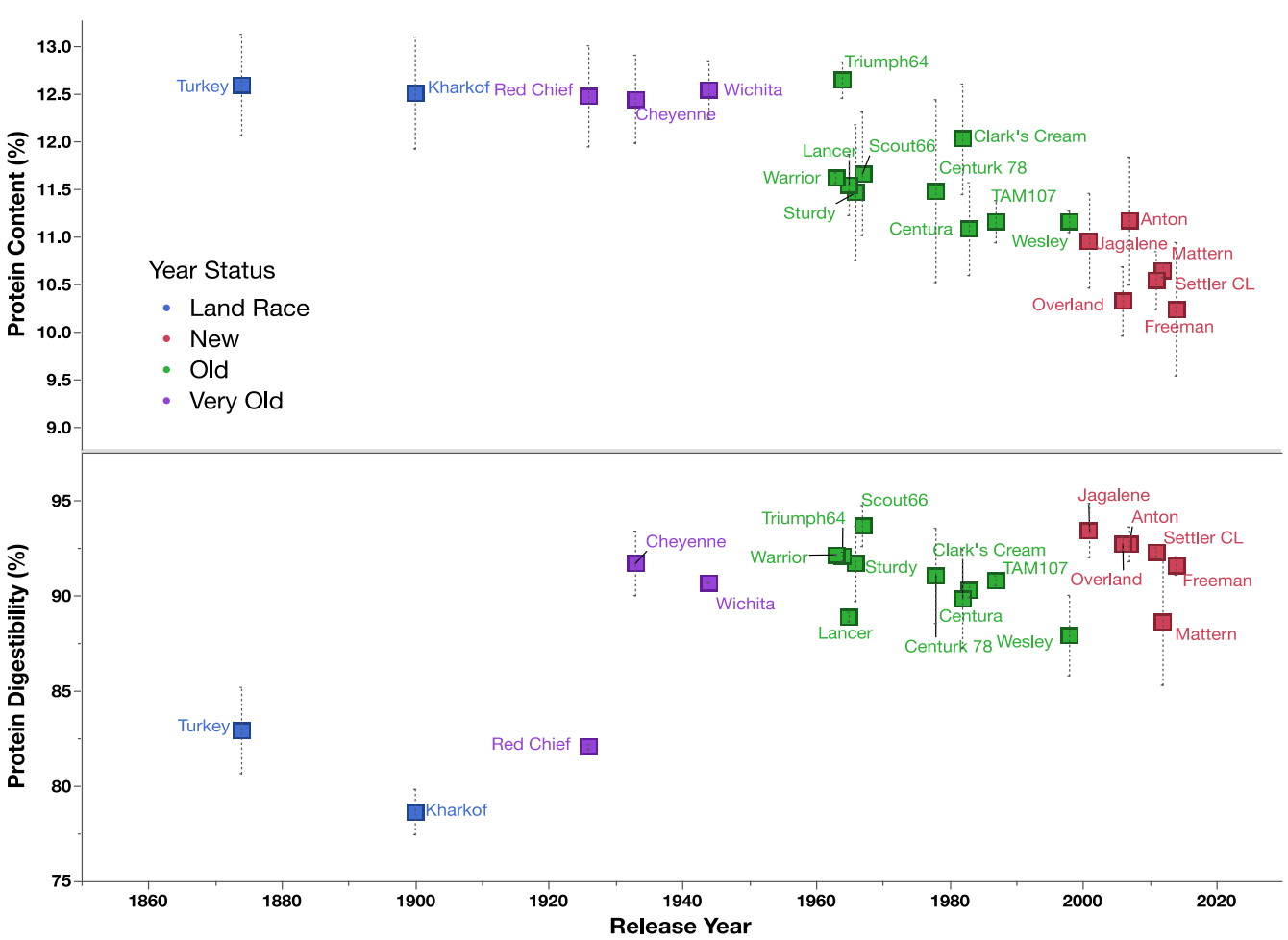

Figure 8.4. Mean Flour protein content and bread in-vitro protein digestibility of different cultivars plotted against their release year; error bars are plotted on standard deviation; n=6.

	Release Year	Protein Digestibility	Flour Protein	Flour Absorption	Mixing Time	Yield
Release Year		0.72	-0.86	-0.59		0.82
Protein digestibility	0.72		-0.50	-0.45		0.50
Flour Protein	-0.86	-0.50		0.74		-0.90
Flour Absorption	-0.59	-0.45	0.74			-0.71
Mixing Time						
Yield	0.82	0.50	-0.90	-0.71		

Negatively correlated		No correlation	Positively correlated	
p < 0.01	p < 0.05		p < 0.05	p < 0.01

Figure 8.5. Pearson's correlations among different variables[28]

[28] Correlations were calculated on LS means for N=21

CHAPTER 9: OVERALL CONCLUSIONS

The first objective of this research was focused on exploring the extrusion performance of proso millet and Great Northern bean flour to develop snack products that can promote the consumption of these crops and provide a gluten-free alternative for the growing market.

Upon extrusion, proso millet flour resulted in an expanded product with physical properties similar to commonly extruded rice flour but lower expansion than corn flour. Feed moisture content was the most important variable that affected the physical properties of proso millet extrudates, while barrel temperature had a limited effect. Antioxidant activity of proso millet extrudates was directly proportional to expansion and darkness of the product. The amount of phenolics detected in proso millet flour was very low due to the de-hulled sample used in the study.

In terms of physical properties, extrusion of GNB flour did not prove to be as promising as for proso millet. The GNB extrudates had less expansion and high bulk density mainly due to the high protein and fiber content in the bean flour. However, extrusion positively impacted the properties of bean starch like water absorption and solubility.

The proposed hypothesis of successful extrusion performance of proso millet proved to be true, while for GNB I was unable to get a decent expanded product even by manipulating extrusion conditions. This signifies the importance and contribution of raw materials in extrusion processing. Thus, based on the recorded physical properties I can conclude that proso millet has a potential to be used as extruded snack product but needs further investigation to establish its sensory acceptance by the general population. In

contrast, GNB flour itself cannot be used to develop extruded snack, although if combined with a starch-rich raw material like corn (or maybe proso millet!) may result in a desirable gluten-free extruded product.

The second objective of this research was to understand the effect of extrusion on *in-vitro* protein digestibility and other nutritional components of proso millet and GNB. Contrary to the proposed hypothesis, extrusion had a significantly negative impact on the *in-vitro* digestibility of proso millet proteins (about 50% reduction). The main cause of lower digestibility of proso millet proteins was identified as formation of hydrophobic aggregates. Further investigation revealed that the observed effect is not only because of extrusion but any processing technique with greater than 10% water and temperature higher than 55°C would result in the same effect in proso millet proteins. The identified property was unique to proso millet storage proteins and has not been reported previously for other cereal proteins. Moreover, a similar property of lower digestibility was identified in 33 accessions of proso millet with different countries of origin throughout the world and also in other edible and non-edible species belonging to the same genus (*Panicum)* as proso millet. While other millet varieties (foxtail, finger, pearl) did not have the same protein property. This suggests the uniqueness of proteins of genus *Panicum* to develop hydrophobic aggregates on cooking resulting in lower digestibility. In many publications, proso millet proteins have been proposed similar to sorghum proteins and sometimes also referred as kafirins but based on the new findings, proso millet prolamins are different from sorghum prolamins and should not be grouped together. Thus, a new name for proso millet prolamin, Panicin, was coined in my research publication.

Further, in order to prevent the observed effect in millet proteins, several mitigation techniques aimed at either breaking the formed hydrophobic aggregates or preventing their formation were explored. It was found that heating millet flour in naturally occurring low a_w substances like honey and maple syrup or modifying the structure of millet proteins with food grade enzymes like transglutaminase can substantially prevent the effect.

Unlike proso millet, the proposed hypothesis for GNB proved true and extrusion improved the digestibility of GNB proteins (20% improvement). Further, extrusion had a significant impact in reducing anti-nutritional factors like phytates and trypsin inhibitors in GNB flour. More than 90% reduction was observed in trypsin inhibitors in bean flour upon extrusion while phytates in bean flour reduced by 85%. The processing technique also resulted in only moderate losses in folate in GNB when compared with other processing techniques as reported in literature. The most significant impact of extrusion was observed on mineral dialyzability in GNB flour. Extrusion resulted in up to 56%, 50%, 25%, and 84% increase in dialyzability of Mg, P, K, and Fe respectively while significantly reducing dialyzability of the heavy metal Cd.

Thus, as hypothesized extrusion had a positive impact on nutritional components in GNB, while, unfortunately, it drastically decreased protein digestibility for proso millet. However, the new findings for proso millet proteins might help in development of novel modifications that might be useful in mitigating the low digestibility of proso millet proteins.

The final objective of this study was to compare the protein digestibility of legacy and modern wheat cultivars to understand if breeding programs have made it worse as

claimed. I hypothesized that there is no difference in digestibility of legacy and modern wheat cultivars but the obtained results were even better than predicted. The results showed that breeding programs did not negatively impact protein digestibility but instead improved it. On comparing the *in vitro* digestibility of breads made from wheat cultivars introduced or released in US from 1870 to 2014, it was found that for all cultivars released after 1930, originating from the wheat breeding programs, the protein digestibility improved by 10-15%. Old and land race cultivars like Kharkof, Turkey, and Red Chief, had digestibility of 70 to 80% while all modern cultivars had above 90% digestibility. This suggests, the changes incurred in the early stages of wheat breeding programs either by selection or crossing have been preserved and carried over to the new cultivars that resulted in improved digestibility and yield of wheat crops.

The thesis can be summarized as follows:

1) Extrusion had a significant impact on nutritional properties of GNB flour especially element mineral dialyzability but lacked physical appeal. Combining GNB flour with a high starch raw material like rice or even proso millet might prove highly beneficial on both nutritional and physical fronts.

2) Proso millet can be used to develop extruded snack products but with nutritional losses especially in protein digestibility. Loss in protein digestibility of proso millet can be prevented by combining different processing techniques with enzymatic protein modification and maintaining low a_w environment but requires detailed investigation.

3) Wheat breeding programs have not destroyed the nutritional quality of modern wheat, but instead have made it better especially in terms of digestibility of proteins.

Potential ideas for future work

- Investigating the fate of undigested proso millet protein in large intestine. Mainly, can the gut microbiota ferment millet proteins which are not digested by human proteolytic enzymes. If so, what are the by-products of millet protein gut fermentation. Are these by-products toxic?
- Developing puffed snack from proso millet-Great Northern bean flour mixture with optimum starch and protein contents. Comparing their physical and nutritional properties with focus on protein digestibility. Understanding if loss in protein digestibility as observed for proso millet would still be a matter of concern if we add additional proteins from a digestible protein rich source like Great Northern bean.
- Enzymatic modification (Transglutaminase) of germinated proso millet grains/flour as a means to prevent loss in digestibility and develop novel food product.
- Analyzing the changes in dietary fiber in GNB flour upon extrusion and correlating it with observed improvement in element dialyzability. In-depth analysis of loss in cadmium dialyzability with respect to changes in phytic acid content and changes in fiber profile.
- Comparing protein profiles of different wheat cultivars using electrophoresis. Using HPLC or other techniques to analyze changes in wheat gluten fractions in legacy and modern wheat cultivars and correlate them with changes in digestibility.
- Compare digestibility of breads made with ancient wheat cultivars (like emmer, kamut etc.) with the landrace cultivars used in the study. Also, comparing digestibility of modern cultivars obtained from other locations in US and not originally grown or developed in mid-western US.