Changes in protein structural characteristics upon processing of gluten-free millet pasta

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Abstract

Proso millet exhibits favorable agronomic and nutritional properties but is currently under-utilized in the northern hemisphere. This study compared processing-induced changes in protein characteristics of commercial pasta to fresh gluten-free pasta from proso millet varieties differing in prolamin profile. Protein solubility, accessible thiols and secondary structures were measured in dough, sheeted and cooked pasta. Relationships between protein conformation and characteristics related to pasta quality were determined. Cooking significantly lowered protein solubility and induced exposure of thiol groups as well as a shift in secondary structure distribution, while sheeting only had a minor effect. Random structures positively and significantly ($P<0.05$) correlated with solubility, cooking loss and protein digestibility. In contrast, β-sheets, the main secondary structure in cooked pasta, negatively correlated with these properties. The utilization of proso millet in gluten-free pasta is promising, however, processing optimization to elicit targeted protein modifications to balance quality and nutritional attributes requires further investigation.

1. Introduction

Millet has been a staple food around the world for millennia, has a short growing period, and is suited even for harsh and relatively dry climates (Habiyaremye et al. 2016). However, millet is an underutilized food source in North America and Europe, which presents an opportunity for introducing it into these markets by capitalizing on its nutritional benefits, gluten-free (GF) aspect, and low glycemic index (Annor, Tyl, Marcone, Ragae, & Marti, 2017). Identification of nutritional and functional features of millet is a prerequisite for the development of millet-based products. Based on the functional properties of Minnesota-grown proso millet (*Panicum miliaceum*), Tyl, Marti, Hayek, Anderson, & Ismail (2018) proposed the use of millet varieties with low amylose content (≤ 10%) for applications where retrogradation needs to be prevented, as in bread applications. On the other hand, proso millet varieties with high amylose content (i.e. >20%) were proposed for products that require higher cold paste viscosity or starch gel-forming abilities, desired for applications such as GF dried pasta. Beside amylose contents, differences in prolamins might also play a role in product quality. Notably, Cordelino, Tyl, Inamdar, Vickers, Marti & Ismail (2019) showed that varieties with more amylose and high-molecular weight prolamins produced a GF fresh pasta with low cooking loss and low stickiness scores. On the other hand, the millet sample with the lowest amylose and prolamin content yielded pasta of the lowest quality (Cordelino et al., 2019). These reported observations indicate that interactions among proteins in proso millet during the pasta-making process may result in a product with improved cooking quality, compared to GF fresh pasta currently available on the market.

Addressing the gap between compositional and functional traits of raw material and pasta cooking behavior is necessary to improve the quality of millet pasta as well as consumer perceptions. Only a few studies have been published linking molecular structure to the textural

characteristics of pasta, and all of them focused on wheat pasta (Bonomi et al., 2012; Bruneel, Pareyt, Brijs, & Delcour, 2010; Bock, West, Iametti, Bonomi, Marengo, & Seetharaman, 2015; Stuknyte et al., 2014). The quality and sensory attributes of cooked wheat pasta are dictated by a complex network of protein and starch interactions. In particular, protein aggregation and cross-linking into a network able to entrap and anchor starch granules during cooking have been suggested as key for optimal quality (Resmini and Pagani, 1983). However, protein networks in GF pasta differ from the gluten networks in wheat-based products. As a follow-up to the work of Cordelino et al. (2019), which focused on identifying the cooking behavior and sensory properties of millet pasta, there is a need to elucidate the relation between the processing/cooking behavior and protein structural organization in the same millet pasta.

Therefore, the objectives of this study were to: 1) understand the effect of processing (i.e. mixing, sheeting, and cooking) on the structural characteristic of proteins in GF fresh pasta from proso millet; and 2) elucidate the relation between cooking behavior and protein structural organization in the produced pasta.

2. Materials and Methods

2.1 Materials

Millet samples used represented a subset of proso millet varieties grown in two locations, Lamberton and Waseca, in Minnesota in 2015. The grains were decorticated and milled to particle size < 0.25 mm (Bunge Limited, St. Louis, MO, US). Compositional attributes of flours were described previously (Tyl et al. 2018), and led to the selection of four proso millet types

that represented contrasting patterns in their starch and prolamin make-up: While the raw millet pasta samples did not differ in protein content based on the variety used (10.9 – 11.6%), the flours differed in their profile of high-molecular weight prolamins (Cordelino et al. 2019). Horizon cv. (H-L) and Sunrise cv. (Sr-L) both grown at Lamberton displayed a richer profile in high-molecular weight (HMW) prolamins (50–150 kDa) than Earlybird cv. grown also at Lamberton (E-L) and Sunrise cv. grown at Waseca (Sr-W), which were deficient in HMW prolamins. Sr-L and Sr-W were characterized by high contents of amylose, E-L had significantly lower values, and H-L fell in between. Therefore, the chosen four samples represented a spectrum of prolamin make-up, with one variety (Sunrise) also representing the influence of growing location on the prolamin profile. The influence of these compositional traits on pasta attributes was reported previously and compared with those of commercial wheat and GF pasta (Cordelino et al. 2019).

All reagents (5,5'-dithiobis-(2-nitrobenzoate), dithiothreitol, sodium chloride, sodium phosphate mono- and dibasic, urea) used in solubility experiments or for determination of thiol groups were of reagent grade or higher and were purchased from Fisher (Waltham, MA) or Sigma (St. Lois, MO).

2.2 Pasta Production

Fresh GF pasta was produced as reported by Tyl, Marti, Hayek, Anderson, & Ismail (2018). Briefly, dry ingredients (millet flour, potato starch, guar gum and salt) were combined, followed by addition of liquid eggs and water. The dough was kneaded by hand for 5 min and sheeted to 1 mm thickness using a KitchenAid Classicplus (KitchenAid, St. Joseph, MI, USA). Pasta sheets were cut into fettuccine and cooked in distilled water (1:20 ratio of pasta to water) for their

optimum cooking time (which ranged from 1.83 – 2.73 min for the different millet pasta types) following the AACCI method 66-50.01 (AACCI, 2011). Two batches of each millet pasta type were prepared. Samples were collected at three processing stages: after mixing (i.e., dough), after sheeting (i.e., raw pasta) and after cooking (i.e., cooked pasta). Samples were used as is for Fourier-transformation infrared spectroscopy (section 2.5), whereas they were lyophilized and ground (< 0.5 mm) prior to protein solubility testing (section 2.3) and thiol assessment (section 2.4). Wheat (Buitoni Pasta Company North America, Solon, OH, US) and a GF (RP's pasta company, Madison, WI, US) commercially available fresh pasta were used as references. The optimum cooking times were 3.6 min and 2.3 min for GF and wheat pasta, respectively, and they were analyzed at the raw and cooked processing stage. In addition to being characterized via descriptive sensory analysis, all pasta samples were also analyzed for firmness, cooking loss, water absorption as well as starch and protein digestibility (Cordelino et al. 2019).

2.3 Protein solubility

Protein solubility in three solvents was assessed based on the method described by Marengo, Bonomi, Marti, Pagani, Elkhalifa, & Iametti (2015) with slight modifications in sample weights and solvent volumes, which were downscaled by a factor of 10. The solvents were: a) 0.1 M phosphate buffer at pH 7 containing 0.1 M sodium chloride, b) the same buffer containing 4 M urea, and c) the same buffer containing 4 M urea and 0.01 M dithiothreitol (DTT). The ratio of sample to solvent was 1:20 and extraction time was 1 h. After extraction, samples were centrifuged for 5 min at 13,000 x g and solubilized protein was quantified ($n \geq 2$ per batch) by reacting 10 µL of sample with 1 mL Bradford reagent (Bradford, 1976) against a bovine serum

albumin standard curve. All results were expressed as percent protein solubility to account for the fact that total protein contents differed between wheat, commercial GF and millet pasta.

2.4 Accessible thiols

Based on the protocol reported by Marengo et al. (2015), accessible thiols were assessed ($n \geq 2$ per batch) by derivatization with dithiobis(2-nitrobenzoate) and spectrophotometric quantification at 412 nm using a Shimadzu UV-1800 (Torrance, CA) spectrophotometer. Compared to the reported method, sample weights and solvent volume were downscaled by a factor of 10. Samples were passed through a 0.2 µm syringe filter before measuring absorbance to correct for cloudiness.

2.5 Fourier-transformation infrared spectroscopy

After mixing of the millet dough, sheeting, or cooking, sample spectra were immediately recorded. A Bruker Tensor 37ATR-FTIR spectrophotometer (Bruker Optics, Inc., Billerica, MA, USA) equipped with a horizontal multi-reflectance zinc selenide crystal was used. Spectra were collected and analyzed based on the method reported by Marti, Bock, Pagani, Ismail & Seetharaman (2016). Specifically, spectra were collected in the 4000–600 cm^{-1} infrared spectral range at room temperature. Each spectrum was an average of 32 scans at 4 cm^{-1} resolution. A background spectrum of the empty trough sampling plate was collected before each sample run. Reference spectra of water (H_2O), corresponding to sample's moisture content, were obtained by mixing H_2O with deuterium oxide (D_2O) and scanning the mixture in the 4000–600 cm^{-1} spectral range. The spectrum was used to digitally subtract contribution of H_2O to the absorption in the amide I region

(1600–1700 cm^{-1}). D_2O showed no absorption either in the 3000–3800 cm^{-1} or in the amide I regions (Bock & Damodaran, 2013). Sample spectra were collected within 10 min of sample preparation in order to minimize post-processing structural changes. Spectral analysis was performed by using OPUS software v. 7.0, following the method of Bock and Damodaran (2013), developed for cereal dough systems. The quantitative estimation of protein secondary structure in the amide I region of dough was based on second-derivative spectra using a five-point Savitsky–Golay function as described by Bock and Damodaran (2013). The spectral regions were assigned as 1620–1644 for β-sheets, 1644–1652 for random, 1652–1660 for α-helix, and 1660–1685 cm^{-1} for β-turn structures. The area for each secondary structural region was divided by the total area of the amide I region.

Two batches (i.e., individual dough samples) were prepared from each pasta type and analyzed at every processing stage. At least 3 spectra were collected for each sample.

2.6 Statistical analyses

Millet pasta dough from each variety was prepared in duplicate. Raw millet pasta and commercial pasta from two packages were cooked in duplicate. Each replicate was analyzed at least in duplicate in the tests described in 2.3, 2.4 and 2.5. Commercial pasta samples were analyzed with the same replication as the millet pasta. One-way analysis of variance (ANOVA) was carried out using R 3.1.0 (R Core Team, 2013) to assess differences among pasta types of the same processing stage, followed by Tukey's Honestly Significant Difference test when $P \leq 0.05$. Two- and three-way ANOVA was also performed in R. For accessible thiols and protein secondary structures, the factors were pasta type and processing stage, while for protein

166 solubility the solvent was included as the third factor. Differences between raw and cooked
167 commercial pasta were assessed for significance via a paired t-test ($P \leq 0.05$) in Excel 2010. The
168 data set was also subjected to a Principle Component Analysis (PCA) using R.
169
170 **3. Results and Discussion**
171
172 **3.1 Impact of millet type, processing stage and solvent used on protein solubility**
173 Interactions among proteins in the dough, sheeted raw pasta, and cooked fresh pasta were
174 evaluated by measuring protein solubility in various solvents. Since wheat and GF pasta were
175 commercially obtained, protein solubility of their dough samples could not be evaluated.
176 Sample type, processing stage, and solvent all significantly affected protein solubility (Figures
177 1A-C; Supplementary Figure 1). Interaction plots (Supplementary Figure 1) illustrate the effect
178 of each factor: at all processing stages, the successive addition of urea and DTT maintained or
179 increased solubility, (Supplementary figure 1A) whereas cooking severely decreased it for all
180 pasta types (Supplementary figure 1A and 1B). The solubility of millet dough samples in
181 phosphate buffer ranged from 18.4-20.1% (Figure 1A). This low solubility indicated potential
182 aggregation via various interactions. Partial solubilization observed could be due to the
183 dissociation of the proteins held together by ionic interactions, as the presence of salt in the
184 buffer increases its ionic strength. Addition of chaotropes (such as urea) to the buffer facilitates
185 the breakdown of hydrophobic interactions and solubilizes protein complexes held together
186 exclusively by this type of interaction (Bonomi, Iametti, Mamone & Ferranti, 2013). In the
187 present study, protein solubility remained essentially unchanged in phosphate buffer plus urea.

188 This observation could be partially attributed to possibly weak hydrophobic interactions, except
189 for H-L dough, where solubility significantly increased after urea addition, though not by a great
190 numerical value. Alternatively, it is possible that the urea concentration used (4 M) was
191 insufficient to completely break up strong hydrophobic protein interactions in the samples. Other
192 studies (Bonomi et al. 2012; Bock et al. 2015) used higher concentrations of urea, typically 8 M.
193 When evaluated, this concentration (8 M urea) resulted in a moderate, but significant increase in
194 solubility of the dough proteins in 8 M urea + phosphate buffer as well as DTT + 8M urea +
195 phosphate buffer (data not shown). However, this concentration was incompatible with the
196 cooked pasta as incorporation of 8 M urea caused the formation of a gel that entrapped almost all
197 of the liquid. Thus, an 8 M urea concentration was deemed unsuitable for our samples.

198 The incorporation of DTT into the buffer resulted in significant ($P \leq 0.05$), but moderate,
199 increases in solubility up to 26% for some samples, indicating the presence of covalent
200 interactions. Notably, adding reducing agents (such as DTT) to buffered aqueous urea aids in
201 solubilizing proteins that form insoluble polymeric aggregates stabilized by disulfide bonds
202 (Bonomi et al. 2013). The behavior of E-L dough was uniquely different than the other dough
203 samples, where the protein solubility was not different regardless of solvent. This observation
204 could be attributed to the deficiency of HMW prolamins in the E-L millet variety. Comparing
205 protein solubility in dough made by Sr-W with that of the dough by Sr-L (i.e. same millet variety
206 grown in two locations) similarly highlighted the potential role of HMW prolamins. Dough from
207 Sr-L (variety rich in HMW prolamins) appeared to be stabilized by protein disulfide linkages,
208 whereas dough from Sr-W (variety deficient in HMW prolamins) was most likely stabilized by
209 non-covalent interactions. Predominance of disulfide interactions was also evident in dough from
210 H-L, a millet variety also characterized by the presence of HMW prolamins.

211 The role of HMW prolamins in the formation of disulfide linkages was reduced by dough
212 sheeting since no significant differences in proteins soluble in urea and in DTT-containing buffer
213 were detected among most of the millet samples (Figure 1B). After sheeting, protein solubility
214 was not significantly different in buffer containing 4 M urea and that containing DTT among
215 most millet pasta types, indicating that upon sheeting protein hydrophobic interactions
216 dominated. E-L raw pasta had significantly ($P \leq 0.01$) lower protein solubility in phosphate
217 buffer alone than the other three millet samples, highlighting the presence of aggregates
218 stabilized by hydrophobic interactions and/or proteins trapped within those aggregates.

219 In contrast to the millet samples, the commercial GF raw sheeted pasta showed significantly ($P \leq$
220 0.05) higher solubility than all other pasta types in all three solvents (Figure 1B). Its solubility in
221 phosphate buffer was not significantly ($P > 0.05$) different from its solubility in buffer + urea,
222 but significantly increased by the addition of DTT. This observation suggests that protein
223 aggregates were less in abundance than in the millet samples, and that hydrophobic interactions
224 played a minor role while disulfide linkages were responsible for maintaining some sort of a
225 protein network. In wheat pasta however, solubility in pure buffer was significantly ($P \leq 0.01$)
226 lower than that of all other samples, indicating a much stronger protein network, as expected.
227 Both urea alone and urea + DTT addition successively increased protein solubility compared to
228 phosphate buffer alone ($P \leq 0.01$), thus hydrophobic interactions as well as disulfide linkages
229 were crucial for protein network stability. In the case of wheat pasta, it has been shown that the
230 sheeting step allows for a better alignment of the gluten strands (Pagani et al., 1989).

231 After cooking, the solubility considerably ($P \leq 0.01$) declined for all samples, in all solvents
232 (Supplementary Figure 1 A and B), specifically, the solubility of all cooked samples in all
233 solvents was significantly ($P < 0.01$) lower than for all dough or raw samples. This is

presumably due to protein aggregation as shown in other studies on proteins undergoing heat treatments (Georget and Belton, 2006; Lambrecht, Rombouts, Nivelle & Delcour, 2017; Xu, Obielodan, Sismour, Arnett, Alzahrani, & Zhang. 2017). Moreover, the increase in solubility in the presence of DTT suggested that protein disulfide interactions are predominant in the cooked samples (Figure 1C).

While addition of both urea and urea + DTT significantly ($P \leq 0.05$) increased cooked millet pasta protein solubility compared to buffer, the values remained very low (< 2.5% for urea, ≤ 7.2% for urea + DTT), with hardly any differences among the millet pasta types. The low solubility of cooked pasta proteins in phosphate buffer indicates that tight protein aggregates were formed during cooking, corresponding to observations made by other researchers (Bock et al., 2015). The significantly lower solubility of raw millet compared to GF pasta ties well into the cooking loss observed in our previous study (Cordelino et al., 2019), which had been significantly lower for millet than for GF as will be further discussed in section 3.4.

3.2 Effect of processing stage on the accessible thiols in the different pasta samples

Thiol groups of the cysteine residues have a profound impact on protein interactions in cereal products. Processing can induce thiol-disulfide interchange that impact the protein network formation (Veraverbeke & Delcour, 2002). A change in accessible thiols can also indicate a change in protein structure as a result of denaturation (buried in the core of a protein vs being located on the surface due to unfolding). Sample type, processing stage, and their interaction all significantly ($P < 0.05$) affected accessible thiol concentrations in the sample set. In millet pasta, sheeting did not significantly change the concentration of accessible thiols, while cooking did (Figure 2). This observation indicates that protein denaturation due to cooking opened up the

protein structures, which in turn exposed previously inaccessible thiol groups. E-L had significantly more accessible thiols than H-L at the dough stage, which could be a consequence of E-L dough exhibiting a slightly but significantly ($P < 0.05$) higher protein content than other millet pasta dough, with the difference being the greatest between E-L (10.43 g/100 g) and H-L (9.73 g/100 g). However, E-L had the lowest protein solubility among millet dough in buffer + urea + DTT (Figure 1A), which suggests that thiols played a lesser role in holding proteins together via disulfide linkages than in the other millet pasta doughs. Aside from that, the accessible thiols did not significantly ($P > 0.05$) differ among the millet pasta samples at any processing stage. However, accessible thiol concentrations were increased upon cooking in both millet and commercial GF pasta, and to a similar degree. On the other hand, wheat pasta differed in its pattern of accessible thiols from millet and commercial GF pasta. The accessible thiol concentration was significantly higher in raw wheat pasta than in all other pasta types, while there was no significant difference ($P > 0.05$) between raw and cooked W pasta. Accessible thiol contents are typically expressed per g flour, and wheat pasta contained significantly ($P < 0.01$) more protein than all other samples. The thiol contents in raw pasta may have been influenced by the protein content as well as the cysteine contents. More notably though, these results indicate that in wheat pasta, disulfide linkages were formed mostly during dough formation and were not altered during cooking (at least not at the concentration of DTT employed in the assay), thus most likely played an integral part in establishing and maintaining the gluten network, in addition to possible linkages between egg and wheat proteins (Lambrecht et al. 2017).

3.3 Effect of processing stage on the protein secondary structure in the different pasta samples

The protein secondary structure distribution estimated for all samples – regardless of the millet variety and the processing stage followed the order β-sheet > random coil> α-helix > β-turn (Table 1). In contrast, in common wheat dough prepared at 30% hydration level, which is typical for dried pasta, β-turn structures are higher than α-helix (Bock et al., 2015). The higher α-helix structure content compared to β-turn found in the present study could be related to differences between millet and wheat and to the presence of eggs in all of our pasta samples, which is a crucial structuring ingredient in fresh pasta in general, and in GF pasta in particular. Notably, liquid eggs contain more α-helix than β-turn structures (about 14 vs 3%, respectively) (Hesso et al., 2015).

There is not much reported on the protein secondary structure in raw fresh pasta and the effect of sheeting; however, for the millet samples in this study the effect was marginal. On the contrary, the partial conversion of β-turns to β-sheets due to application of force has been demonstrated in gluten, adding to the matrix's stability and elasticity (Wellner et al., 2005). Gluten forming proteins in wheat are very different than the prolamins found in millet, so differences in behavior are expected.

Even after sheeting, raw pasta from H-L exhibited a significantly lower content in β-sheets than Sr-L and Sr-W. For α-helices, the only difference for α-helices was that wheat pasta had a significantly higher content ($P < 0.01$) than all millet samples, which coincided with a significantly lower β-sheet content than the other pasta samples.

Cooking had a significant ($P < 0.05$) effect on protein conformation. Proteins were almost exclusively (≥ 93.9%) aligned in β-sheets, and their content did not differ among the millet pasta types. This increase of β-sheets compared to raw pasta was in line with other studies that found such increases are due to heat-induced denaturation and aggregation (Bock et al., 2015; Bruun,

303 Sonderaard, & Jacobsen, 2007; Georget and Belton, 2006), and came at the expenses of α-helix,
304 random, and β-turn structures (Table 1). The β-turn structure completely disappeared after
305 cooking, and only ≤ 1.1% α-helices remained. However, a decrease of β-sheets content was
306 observed upon sheeting and cooking of gluten isolated from extruded wheat pasta (Li, Chen, Li,
307 Gao & Dong, 2017). The authors observed a more equal distribution among the secondary
308 structure types, with contents of α-helices, β-sheets, β-turns and random structures between 20
309 and 30% in mixed dough, sheeted and cooked noodles. These differences may be related to the
310 differences in preparation of both pasta (for example, the recipe did not contain eggs) and the
311 sample used for FT-IR analysis (starch washed out from gluten, which was then freeze-dried and
312 pressed with KBr).
313 In addition to cooking, sample type significantly ($P < 0.01$) affected α-helices, β-sheets, and
314 random coils. Just like for millet pasta, β-sheets were also dominant in the commercial pasta
315 samples (> 80%); however, random structures still represented ≈ ≥10% of the protein secondary
316 structures, while being almost absent in cooked millet pasta. Cooked commercial GF and W
317 pasta had a significantly ($P < 0.05$) lower β-sheet, and significantly ($P < 0.05$) higher random
318 structure content than most millet pastas (except for E-L). This was the most notable difference
319 between cooked millet-based and commercial samples. A 2-way ANOVA analysis showed a
320 significant ($P < 0.01$) interaction between processing stage and sample type for random
321 structures because millet samples retained fewer of them. This observation indicates that millet
322 proteins had a higher degree of order than the proteins in the commercial pasta samples, which
323 may have influenced other properties as will be further discussed below.
324

325 **3.4 Correlating protein conformation, solubility, cooking loss and digestibility**

In our previous work (Cordelino et al., 2019), we reported that millet pasta had lower cooking loss (ranging from 1.64 to 2.36 g/100 g raw pasta) than the commercial GF and wheat samples that were used for comparison purposes (4.82 g/100 g raw pasta for GF vs 3.48 g/100 g raw pasta for wheat). The two commercial pastas also had longer optimum cooking times (3.60 min for GF, 2.32 min for wheat vs 1.83-2.73 min for millet pastas). The significantly higher cooking loss of the commercial GF fettuccine observed previously (Cordelino et al., 2019) is a likely consequence of its significantly higher protein solubility in phosphate buffer, i.e. likely more proteins (as well as starch) were able to leach into the cooking water. The proportion of random coil structures in cooked pasta strongly and significantly correlated with protein solubility of cooked pasta in all three solvents with r=0.966 and $P < 0.01$, r=0.823 and $P< 0.05$, r=0.913 and $P<0.05$ for solubility in buffer, buffer + urea, and buffer + urea and buffer + urea + DTT, respectively. To the best of our knowledge, correlations between secondary structure distribution and digestibility have not yet been reported for pasta; however, work on legumes (Mune Mune, Sogi, & Minka, 2017) and commonly used feed protein preparations (Bai, Qin, Sund & Long, 2016) also displayed a positive correlation between random structural arrangements and protein digestibility. There were also significant positive correlations between the α-helix content in cooked pasta and their solubility in phosphate buffer (r=0.88, $P = 0.02$) and phosphate buffer containing urea and DTT (r=0.92, $P < 0.01$). In contrast, the β-sheets correlated negatively with solubility in these two media. In gluten networks, β-sheets are associated with regions where glutenins predominantly interact with each other via hydrogen bonds (i.e. train regions), whereas β-turn structures are related to regions where interactions with water are dominant (i.e. loops) (Belton, 1999). Thus, β-sheets represent areas of tightly associated proteins where the protein-water interactions are outweighed by protein-protein interactions, limiting the solubility.

Interestingly, Bock et al. (2015) and West et al. (2013) observed a different association of β-sheets and cooking loss. Specifically, whole wheat pasta dried at a high temperature exhibited higher cooking loss than pasta dried at a low temperature even though the β-sheet content of the pasta dried at the high temperature appeared higher. In contrast, for our samples, cooking loss correlated positively with helix ($r=0.959$, $P< 0.01$) and random coil contents ($r=0.822$, $P<0.05$) but negatively with β-sheet content ($r=-0.834$, $P<0.05$) in cooked pasta.

In our previous work we noticed that protein digestibility of cooked millet flour (Tyl et al., 2018) as well as the pasta prepared from it was significantly lower than that of wheat flour, with values for pasta falling between 41 and 50% (Cordelino et al, 2019). Another study also reported low protein digestibility of heat-treated proso millet flour (Gulati, Li, Holding, Santra, Zhang, & Rose, 2017) and related it to aggregate formation via hydrophobic interactions, with possible involvement of tryptophan residues.

The detrimental effect of β-sheets on protein digestibility (Cordelino et al., 2019) observed in our samples ($r = -0.856$, $p < 0.05$) has also been shown in a range of foods and feeds (Bai et al. 2016), and was especially prominent in certain legumes (Carbonaro, Maselli, & Nucara, 2012). A significant positive correlation was found between digestibility and the ratio of α-helices to β-sheets. With more helices and fewer sheets, digestibility was higher in soy bean flour (de la Rosa-Millan, Chuck-Hernzndez, & Serna-Saldivar, 2015) as well as in gluten isolated from wheat (Rahaman, Vasiljevic, & Ramchandran, 2016). In another study that contrasted storage effects on properties of corn tortillas produced via different processing methods, a decrease in protein digestibility over storage coincided with an increase in β-structures (Martinez-Velasco, Alvarez-Ramirez, Rodriguez-Huezo, Meraz-Rodriguez, Vernon-Carter, & Lobato-Calleros, 2018). In wheat bread slices, regions with higher β-turn content, mainly found in the crumb,

were associated with higher digestibility (Alvarez-Ramirez et al., 2018). However, very few studies have analyzed this relationship in detail, and to the best of our knowledge, studies on pasta and many other cereal-based foods are lacking. While disulfide-mediated protein polymerization in sorghum contributes to low digestibility, millet proteins have not been studied as thoroughly (Taylor, Taylor, Campanella, & Hamaker, 2016; Annor et al., 2017). In plant proteins, low solubility can contribute to poor digestibility (Becker & Yu, 2013), and it is intriguing that aside from known anti-nutritional factors such as tannins (which have mostly been reported to occur in pigmented varieties (Gabazza, Shumoy, Muchuweti, Vandamme, & Raes, 2016)), the alignment of proteins may be a contributing factor.

3.5 Characterizing variations in the different pasta types using principal component analysis

Results were further evaluated by conducting an explorative multivariate analysis via PCA to provide additional discriminatory power. The two principal components provided a good summary of the data, accounting for ≈86% of the overall variance (PC1 = 54.5%; PC2 = 32%) (Figure 3 A and B). Millet pasta samples were separated from both commercial pastas through component 1, while component 2 separated commercial GF from wheat pasta. Moreover, Figure 3B easily distinguishes the variables affecting sample distributions the most, which are the ones more distant from the origin of the plot. Specifically, PCA highlighted the relationship between protein structural attributes and other characteristics. Textural attributes, evaluated through instrumental as well as sensory analysis, had been a focus of our previous work and were shown to be influenced by prolamin profile and amylose to amylopectin ratios (Cordelino et al. 2019). There was a significant correlation between α-helix content and firmness of cooked pasta (r =

0.953, $p < 0.05$). To the best of our knowledge, the relationship between pasta texture and protein secondary structure among samples made from different cereal flours has not yet been systematically evaluated. However, in fresh wheat pasta supplemented with tea powder, an increase in hardness induced by higher contents of tea polyphenols also corresponded to higher contents in α-helices (Han, Ma, Zhang, Li, & Sun, 2020). For our samples, cooked pasta's firmness, random structures and helix content, along with protein solubility in all the buffers had a strong positive correlation with PC 1 (Figure 3B). In contrast, the β-sheets in raw and cooked pasta had a strong negative correlation with PC 1. As discussed in section 3.3, the high β-sheet content of cooked millet pasta was one of its characteristic features, thus differentiating it from the commercial samples along PC 1. On the other hand, protein solubility in cooked millet pasta was very low, and together with low protein digestibility resulted in their negative scores for PC 1, underlining the adverse effect of β-sheets on solubility and digestibility. Factors strongly and positively associated with PC 2 were the solubility of raw pasta in buffer, which had been high for GF and low for wheat, and the accessible thiols in cooked pasta. In contrast, accessible thiols in raw pasta were negatively associated with PC 2. GF pasta was also significantly firmer than other samples (Cordelino et al. 2019), while raw wheat pasta had more accessible thiols than other pasta samples; these factors further contributed to separation between GF and wheat pasta via PC 2.

4. Conclusions

This study was the first to report on protein secondary structure distribution in millet-based pasta. Cooking induced major changes in all pasta types, while the sheeting process had comparatively minor effects. Millet-based pasta displayed key differences to commercial GF and

418 wheat pasta; however, different millet samples exhibited minor variations. The presence of
419 HMW prolamins exerted some influence on solubility in dough and raw pasta. Depending on
420 their presence, different protein interactions appeared dominant. Moreover, this study adds to the
421 growing body of research that protein characteristics and secondary structure distributions affect
422 the cooking quality as well as digestibility of fresh pasta, though potentially in opposite ways.
423 Cooking quality may be improved due to less leaching, whereas extensively aggregated proteins
424 may not be sufficiently accessible to digestive enzymes. Especially when considering the role of
425 millet as nutrition source in places with high food insecurity and possible supply shortages, a
426 thorough understanding of reasons for low protein digestibility is needed to find strategies for its
427 improvement. Overall, proso millet is a promising raw material for the preparation of gluten-free
428 products such as pasta. However, further research is needed to find formulation and processing
429 strategies to better balance its functional and nutritional properties.

430

431 **Declaration of competing interests**

432 The authors have no competing interests to report that would have influenced this manuscript.

433

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Figure Captions

Figure 1A-C. Solubility of pasta proteins in A) 0.1 M sodium phosphate buffer containing 0.1M sodium chloride, B) the same buffer supplemented with 4 M urea, C) the same buffer supplemented with 4 M urea and 0.01 M dithiothreitol. Significant ($P < 0.05$) differences according to Tukey's HSD test are indicated with different lowercase letters for different pasta types extracted with the same solvent, and different uppercase letters for the same pasta type extracted with different solvents. E-L, Earlybird cv. grown at Lamberton; H-L, Horizon cv. grown at Lamberton; S_r-L, Sunrise cv. grown at Lamberton; S_r-W, Sunrise cv. grown at Waseca, GF, commercial gluten-free pasta, W, commercial wheat pasta.

Figure 2. Accessible thiols in pasta at dough, raw and cooked stage. Different lowercase letters indicate significant ($P < 0.05$) differences among means according to Tukey's HSD test among pasta types at the same processing stage. Different upper case letters denote differences within a pasta type at different processing stages (assessed according to Tukey's HSD test for millet pasta, and paired t-test for GF and W). E-L, Earlybird cv. grown at Lamberton; H-L, Horizon cv. grown at Lamberton; S_r-L, Sunrise cv. grown at Lamberton; S_r-W, Sunrise cv. grown at Waseca, GF, commercial gluten-free pasta, W, commercial wheat pasta.

Figure 3. Principal component (PC) analysis score (A) and loading plot (B) displaying the first two principal components that together accounted for 86.5% of the variability among pasta attributes. B: buffer; F: firmness; OCT: optimum cooking time; SH: thiol groups; Sol: solubility; WA: water absorption