Fall 12-4-2014

Improving the Health Impacts of Whole Grains through Processing: Resistant Starch, Dietary Fiber Solubility, and Mineral Bioaccessibility

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By

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska-Lincoln

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Food Science & Technology

Under the Supervision of Professor Devin J. Rose

Lincoln, Nebraska

December,2014

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University of Nebraska-Lincoln 2014

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Whole grains are good sources of starch, dietary fiber, and mineral elements. Starch is an important source of energy but also can be transformed to resistant starch to impart lower caloric value and increase total dietary fiber. Dietary fibers impart physiological benefits to human body once they are fermented by the gut microbiota. Mineral elements are important co-factors of a wide range of enzymes involved for instance in glucose and lipid metabolism. In general human body is capable of absorbing only 21-28% of most mineral elements, and gut bacteria are only able to ferment 34% of the dietary fiber from grain-based foods. The objective of this study was to improve the health impact of whole grains using strategies that either increase or modify the accessibility of dietary fiber and mineral elements. First, whole wheat flour was processed with cooking-freezing cycles to convert part of the native starch into resistant starch. Resistant starch is known to be a highly fermentable substrate for beneficial gut bacteria. After processing, resistant starch increased up to 8-fold in whole wheat flour and total beneficial bacterial metabolites increased up to 31% after *in vitro* fermentation. The second strategy was to extrude wheat bran to increase dietary fiber accessibility to gut microbiota. Combinations of moisture (15% and 30% wb) and screw speed (120 and 250 rpm) were chosen to generate severe and mild extrusion conditions. Severe

extrusion conditions of 250 rpm, 15% moisture and 120°C in the barrel using a single screw extruder led to 3-fold increase in dietary fiber solubility and a 1.4-fold increase in beneficial metabolites after in vitro fermentation with human fecal microbiota. Finally, the same experimental design of extrusion of wheat bran was used to conduct a preliminary study on the effect of processing conditions on mineral solubility and phytate content. Wheat bran extrusion reduced phytate content up to 11.3% using high speed high moisture processing conditions; however, element solubility results were inconclusive. In conclusion, thermal processing of whole wheat flour and wheat bran enhanced *in vitro* fermentation properties associated with benefits to human health.

In memory of Amalia Castillo de Arcila

for her bravery and unconditional love.

ACKNOWLEDGMENTS

I wish to thank my adviser, Dr. Devin Rose for his patience, guidance and support. I wish to thank the members of my committee Dr. Bob Hutkins, Dr. Brian Waters and Dr. Bob Graybosch for their advice during the completion of this thesis.

Thanks to my parents Luis Eduardo Arcila Castillo and Deifan Edith Castillo Arroyo for your infinite love, for each lesson and for all your efforts that allow me to achieve each of my dreams.

Thanks to my family who always believe in me, you are my reason to reach each goal. To my all my friends in Lincoln, Nebraska who made my stay a memorable moment. To my friends in Colombia that although in the distance were always supportive.

Special thanks to Camilo Tamayo your songs always make me happy, I will be always grateful for all your support.

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PREFACE

Whole grains are characterized as having greater quantities of nutrients per serving than refined grains. Whole grains are good sources of trace elements, starch and dietary fiber. Dietary fiber in whole grains is composed of resistant starch, cellulose, arabinoxylans and other polysaccharides. Dietary fiber is well known to be a selective modifier of growth and metabolic activity of beneficial bacteria in the colon. Products of bacterial fiber fermentation, short chain fatty acids (SCFA), have tremendous beneficial influence on colon health. Homeostasis of the colonic cells is related to the entire health state of the body (Walker & Lawley, 2013). On the other hand, trace elements play several roles in normal functions in the body, such as carbohydrate metabolism, redox homeostasis, and cellular signal transduction (Fairweather-Tait & Hurrell, 1996).

A large portion of the trace elements and dietary fibers is not available in whole grains for use or absorption in the body. Trace element deficiency has become a public health problem. For instance, around 50 to 80% of the United States population has an inadequate intake of magnesium (Chaudhary, Sharma, & Bansal, 2010). Low magnesium intake has been implicated in the development of hypertension, type 2 diabetes and coronary heart disease (Combs & Nielsen, 2009). In order to meet the daily reference intake (men 38g per day and women 25g per day) (US Department of Agriculture, 2010) for dietary fiber, it is necessary to have a high consumption of whole grain products. High consumption of whole grains also entails high carbohydrate intake. Therefore, strategies to enhance the health effects of whole grains, focused on increasing the availability of mineral elements and dietary fibers, are essential to exploit this nutrient-rich food.

In this work thermal processing, such as extrusion and cooking-freezing cycles, of either whole wheat flour or wheat bran, were investigated as tools to improve the health impact of whole grains. Extrusion processing combines shearing action, temperature and moisture that may disrupt cell wall components and fiber polysaccharides structure, increasing element and dietary fiber the solubility and bioavailability of whole grains fiber (Gualberto, Bergman, Kazemzadeh, & Weber, 1997; Robin, Schuchmann, & Palzer, 2012). Cooking-freezing cycles are effective for converting native starch to resistant starch (RS) (Sajilata, Singhal, & Kulkarni, 2006). RS consumption has several physiological benefits in humans. In healthy humans RS consumption may decrease postprandial plasma glucose and insulin, while increasing insulin sensitivity (Robertson, Currie, Morgan, Jewell, & Frayn, 2003).

Objectives

- To subject whole wheat flour to repeated cooking freezing cycles to increase the RS content and then determine the impact of increased RS content on *in vitro* fecal fermentation properties.
- To determine the changes in dietary fiber fermentation of wheat bran following extrusion, with an emphasis on the percentage of non-starch polysaccharides available for fermentation by the microbiota and the quantity of short chain fatty acids generated by such fermentation. Furthermore, to use extruded bran in a whole wheat bread formulation to determine if the changes in non-starch polysaccharide solubility and fermentation are maintained in the bread.
- To determine the effect of extrusion processing variables on phytic acid concentration and solubility of the most abundant elements in wheat bran.

Hypothesis

It is possible to use thermal processing to manipulate several components of whole wheat in order to enhance its health benefits. Thermal processing such as extrusion or cooking-freezing cycles can modify carbohydrate structures and increase trace element bioavailability. Cooking-freezing cycles have been effective in transforming native starch to resistant starch, and thus may be a good strategy to increase resistant starch in whole wheat flour. Heat treatment may degrade naturally occurring structures such as phytic acid in whole grains, increasing mineral bioavailability. Modifying the carbohydrates already present in whole grains, through thermal processing, may lead to increases in soluble dietary fiber and bacterial fiber fermentation.

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CHAPTER 1. Repeated cooking and freezing of whole wheat flour increases resistant starch with beneficial impacts on *in vitro* faecal fermentation properties

Abstract

Resistant starch (RS) has shown benefits to gastrointestinal health, but it is present in only small amounts in most grain-based foods. The purpose of this study was to increase RS in whole wheat flour to improve its potential health benefits. Zero to 7 cycles of cooking (20 min, boiling water) and freezing (-18 oC, 23 h) of whole wheat flour in water (1:15 %w/v) were performed. Increasing cooking-freezing cycles increased RS from 1.03 to 8.07% during *in vitro* starch digestion. During *in vitro* faecal fermentation, increasing cooking-freezing cycles increased short chain fatty acids, mainly propionate. Increases in butyrate were also noted during the first 8 h of fermentation. All flours resulted in significant increases in *Bifidobacterium* of >0.5 log during fermentation compared to baseline. Thus, even modest increases in the RS content of whole wheat flour modulated the metabolic activity of gut microbiota to increase production of beneficial metabolites.

Keywords: Short chain fatty acids; propionate; butyrate; starch retrogradation; digestion; gut health

Submitted to *Journal of Functional Foods*

1. Introduction

The large intestine is home to a vast number of microorganisms. This gut microbiota is involved in many factors associated with host health, including immune system regulation, inhibition of pathogen proliferation, gut motility, and energy recovery (Flint, Scott, Louis, & Duncan, 2012). Imbalance in the proportions of gut microbes or in production of microbial metabolites in the intestine may lead to numerous diseases such as inflammatory bowel disease, colorectal cancer, diabetes and metabolic syndrome (de Vos & de Vos, 2012).

The gut microbiota requires undigested substrates from the host diet for its growth and survival. The gut microbiota is sensitive to dietary intervention with consequences on host health (Delzenne, Neyrinck, & Cani, 2011). Products of carbohydrate bacterial metabolism have been proposed as one mechanism of protection that the gut microbiota imparts to the host (Delzenne & Cani, 2011; Flint et al., 2012). Therefore, there is interest in the types of bacteria that are selected by different dietary fibres and the types of metabolites that are produced upon fermentation of these substrates.

End products of dietary fibre fermentation by the gut microbiota are short chain fatty acids (SCFA), principally acetate, propionate, and butyrate. SCFA production is associated with a decreased pH in the gut, which can result in inhibition of pathogens and increased mineral bioavailability (Hopkins & Macfarlane, 2003; Y. Wang et al., 2010). Acetate and propionate are also absorbed and become substrates in glucose and lipid metabolic pathways in the human body (Tremaroli & Bäckhed, 2012). Propionate may also possess additional health benefits by preventing cholesterol synthesis in the liver (Kaczmarczyk, Miller, & Freund, 2012). Butyrate has the most diverse role in human metabolism. It is the principal energy source for colonic cells, acts as immune modulator,

it is involved in cell differentiation and proliferation, and plays a role in gut barrier function (Peng, Li, Green, Holzman, & Lin, 2009).

Whole wheat flour is a rich source of dietary fibres, containing 9-17% total dietary fibre (principally arabinoxylans and cellulose). Unfortunately only about 34% is fermentable by gut bacteria (van Dokkum, Pikaar, & Thissen, 1983). In contrast, resistant starch (RS), which is relatively low in whole grain foods (0-5%) (Englyst, Liu, & Englyst, 2007), is a highly fermentable dietary fibre that promotes growth of beneficial bacteria in the gut (Lesmes, Beards, Gibson, Tuohy, & Shimoni, 2008). RS has been classified as a functional food ingredient since may have positive effects on preventing colon cancer, diabetes and obesity (Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010). Increased RS consumption has shown several physiological benefits in humans. In healthy humans RS consumption may decrease postprandial plasma glucose and insulin, while increasing insulin sensitivity (Robertson, Currie, Morgan, Jewell, & Frayn, 2003).

Thus, strategies to convert a portion of digestible starch to RS may be used to improve health input of the dietary fibre fractions of whole wheat flour and create a complete functional ingredient. One strategy to produce RS is to subject pure starch to thermal processing in the presence of water with controlled cooling to induce retrogradation (Sajilata, Singhal, & Kulkarni, 2006). Following cooking and during cooling, a small portion of starch retrogrades. The new arrangement of starch polymers resist enzymatic activity during human digestion (Eerlingen, Crombez, & Delcour, 1993). Cooking and cooling of starch can be conducted several times to further increase the RS portion (Silverio, Fredriksson, Andersson, Eliasson, & Åman, 2000). Repeating the cooking and cooling procedure increases RS because the amorphous or digestible starch fractions are re-dispersed during re-cooking and can then form more crystallites

during subsequent cooling (Yadav, Sharma, & Yadav, 2009). Newly formed crystallites are not degraded by subsequent cooking steps (in boiling water) since the melting point of retrograded starch is 110-120°C (Eerlingen & Delcour, 1995).

The objective of this contribution was to subject whole wheat flour to repeated cooking freezing cycles to increase the RS content and then to determinate the impact of increased RS content on *in vitro* faecal fermentation properties.

2. Materials and methods

2.1. Starting material

Hard red winter wheat (Triticum aestivum 'McGill') was obtained from Husker Genetics, the University of Nebraska-Lincoln Foundation Seed Division, Ithaca, NE, USA (2011-2012 crop year). Whole wheat flour was produced by milling on a Quadrumat Jr laboratory mill (CW Brabender, South Hackensack, NJ, USA) without tempering (Doblado-Maldonado, Flores, & Rose, 2013). Total dietary fibre (11.9 ± 1.3% db), protein (16.0 ± 0.1% db), and total starch (52.8 ± 0.7% db) were measured in the whole wheat flour following approved methods 32-25, 46-09, and 76-13, respectively (AACC International, 2013). A kit was used for the total starch assay (K-TSTA, Megazyme, Wicklow, Ireland).

2.2. Cooking-freezing treatment

One gram of whole wheat flour was suspended in 15 mL of water in a 50 mL plastic centrifuge tube. The tube was then immersed in a boiling water bath for 20 min. During the first 5 min of cooking, the slurry was mixed by vortex and the cap was vented several times to relieve steam pressure buildup. After cooking, the samples were cooled for 5 min at room temperature and then stored at -20°C for 23 h. The above steps were defined as one cooking-freezing cycle. Samples were subjected to cooking-freezing for

0, 1, 3, 5, and 7 cycles. Between cycles the samples were thawed by immersing the plastic tubes in water at room temperature for 5 min with vortex mixing several times.

Finally the samples were placed in a water bath at 37°C for *in vitro* digestion (see section 2.3). For 0 cycles (control treatment), the samples were cooked in the boiling water cooled for 5 min and then immediately transferred to the 37 °C water bath for *in vitro* digestion. All cooking-freezing treatments were performed in triplicate using whole wheat flour from the same wheat batch.

2.3 *In vitro* digestion and quantification of starch digestible fractions

In vitro digestion was performed according to a procedure described previously (K. N. Englyst, Englyst, Hudson, Cole, & Cummings, 1999) with some modifications (Mkandawire et al., 2013). Briefly, 5 mL of 1% (w/v) freshly prepared pepsin (P7000, Sigma, St. Louis, MO, USA) in 0.1 M HCl were added to the samples, the tubes were capped, mixed by vortexing and placed horizontally in a water bath at 37°C with shaking at 250 rpm for 30 min. Then 5 mL of 0.5 M sodium acetate buffer (pH 5.2) were added to each tube with vortex mixing to equilibrate. At 1 min intervals, 5 mL of freshly prepared enzyme solution containing 9 mg of pancreatin (P7545, Sigma) and 40 µL of amyloglucosidase (E-AMGDF, Megazyme,) per millilitre in water were added to each tube. At exactly 20 and 120 min a 0.2 mL aliquot was removed from each tube and mixed with 4 mL of absolute ethanol. The samples were centrifuged at 4,000g for 5 min, and the glucose content was measured in the supernatant by the glucose oxidase-peroxidase method (K-GLUC, Megazyme) and converted to starch multiplying by a correction factor of 0.9 to account for the water added during hydrolysis of starch to glucose (162 g/mol anhydroglucose unit/ 180 g/mol glucose).

Following 120 min of digestion, the samples were transferred to dialysis tubes (3,500 MWCO, Spectrum Laboratories, Rancho Dominguez, CA, USA), and dialyzed against water at 4°C. At the end of the dialysis glucose content was measured in the retentate by the glucose oxidase-peroxidase method (K-GLUC, Megazyme) to confirm removal of free glucose and then the retentate was freeze dried. The freeze dried samples were analyzed for total neutral sugars with a sample size of 20 mg (Rose & Inglett, 2010). Residual starch in the freeze dried material was measured using a total starch assay kit (K-TSTA, Megazyme).

Starch digestibility was categorized into three starch digestible fractions: rapidly digestible starch (RDS), slowly digestible starch (SDS), and RS (K. N. Englyst et al., 1999). RDS was the quantity of starch that was converted to glucose in the first 20 min of *in vitro* digestion; RS was the amount of starch remaining in the freeze dried material after dialysis; SDS was the remainder of starch that was not categorized as RDS or RS. Data were expressed as percentages of the total starch content of the starting material.

2.4 *In vitro* faecal fermentation and analysis

In vitro batch faecal fermentation was performed according to Hartzell, Maldonado-Gómez, Hutkins, and Rose (2013). In short, 100 mg of digested, freeze-dried material were suspended in 9 mL sterile fermentation medium consisting of (per L): peptone (2 g; BP1420-100, Fisher Scientific, Pittsburgh, PA, USA), yeast extract (2 g; CAS8013-01-2; Alfa Aesar, Ward Hill, MA, USA), bile salts (0.5 g; S71919-1; Fisher Science; Hannover Park, IL, USA), NaHCO3 (2 g), NaCl (0.1 g), K2HPO4 (0.08 g), MgSO4.7H2O (0.01 g), CaCl2.6H2O (0.01 g), L-cysteine hydrochloride (0.5 g; Sigma), hemin (50 mg; Sigma), Tween 80 (2 mL), vitamin K (10 µL; Sigma), and 0.025% (w/v) resazurin solution (4 mL), and hydrated overnight on ice. In the morning, fresh faecal

samples were collected from 3 healthy adults with no history of gastrointestinal abnormalities and having not taken antibiotics in the last 6 months. The faecal slurry was prepared by blending the faecal sample with sterile phosphate buffered saline (1:9 w/v) using a hand blender for 1 min and then filtering through 4 layers of cheesecloth. Tubes were then inoculated with 1 mL of faecal slurry, capped, and incubated at 37 °C with shaking (125 rpm). During fermentation, 0.4 mL of fermentation slurry were removed after 0, 4, 8, 12, and 24 h of fermentation and immediately transferred to a microfuge tube containing 0.1 mL of 7 mM 2-ethylbutyric acid (internal standard) in 2M sodium hydroxide. An additional 1 mL sample was withdrawn at 0 and 24 h and placed in an empty tube in ice for analysis of bifidobacteria. All steps for fermentation were conducted in an anaerobic hood (Bactron IV, Sheldon manufacturing, Cornelius, OR, USA) containing 5% H2, 5% CO2, and 90% N2. As soon as all samples were taken at each time point, microfuge tubes were removed from the anaerobic cabinet and immediately stored at -80 °C.

SCFA were quantified by gas chromatography (Hartzell et al., 2013). Samples were vortex mixed with ~0.4 g of NaCl and 0.2 mL of 9 M H2SO4. Then 0.5 mL of diethyl ether were added to the tubes which were capped and shaken vigorously. Tubes were centrifuged (10,000 × g, 5 min), and then the diethyl ether layer was transferred to a new tube. One microlitre of the diethyl ether extract was injected onto a gas chromatograph (Clarus 580, PerkinElmer, MA, USA) equipped with a capillary column (Elite-FFAP, 15 m × 0.25 mm inner diameter × 0.25 μm film thickness, PerkinElmer) and detected with a flame ionization detector. SCFA were quantified by calculating response factors for each SCFA relative to 2-ethylbutyric acid using injections of pure standards. Data for SCFA were expressed as a function of the weight of whole wheat flour used as starting material for digestion.

Bifidobacterium were quantified by quantitative real time PCR (qPCR) as described previously (Hartzell et al., 2013). Briefly, DNA was extracted by the phenol/chloroform method (Martínez et al., 2009) from samples at 0 hours (baseline) and 24 h of fermentation. qPCR was performed using Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany), SYBR Green (Real master Mix, 5 PRIME Inc., Gaithersburg, MD, USA) with specific primers for *Bifidobacterium* (F:5'TCGCGTC(C/T)GGTGTGAAAG'3 and R:5'CCACATCCAGC(A/G)TCCAC'3) and an annealing temperature of 58 °C. Standard curve for total quantification of *Bifidobacterium* with a correlation coefficient above 0.95 after running qPCR was prepared using overnight cultures of *Bifidobacterium* longum ssp. longum AH1206 (Alimentary Health Ltd., Cork, Ireland). Data for *Bifidobacterium* were expressed as log cells/mL of fermentation slurry.

2.5 Data analysis

All treatments were performed in triplicate. For detecting significant differences in starch digestible fractions and *Bifidobacterium*, data were analyzed using one way analysis of variance (ANOVA) with the number of cook-freeze cycles as the factor. For SCFA analysis, a repeated measures ANOVA with sample and time as the factors was used. Significant differences among samples were determined using Fisher's least significant difference (LSD) test with $\alpha=0.05$. All data were analyzed using SAS statistical software (version 9.2, SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. *In vitro* digestion

Repeated cooking and freezing of an aqueous slurry of whole wheat flour (6.67%, w/v) significantly increased the RS with each repeated cycle (Fig. 1). In the

control, RS was 1.03% of the total starch, but the RS increased significantly with each cooking-freezing cycle to 8.07% with 7 cycles. This was compensated for by a decrease in SDS.

The impact of cycled retrogradation on wheat starch digestion fractions in whole wheat flour has not been report before. The increase in RS was likely a result of retrogradation of the starch, as has previously been demonstrated in pure starch systems (Sievert & Pomeranz, 1989; Silverio et al., 2000) and other flours (Yadav et al., 2009). Park et al. (2009) showed that waxy maize starch subjected to gelatinization and subsequently stored at 4°C for several days, underwent changes principally from RDS to SDS. However, if samples were stored under cycled temperature changes (4°C for 2 days and 30°C for 2 days), the principal changes were from SDS to RS. Results from Zhang et al. (2011) confirmed that storage conditions affect the shift in SDS fraction. They subjected native and waxy starch from rice to gelatinization and further storage at 4°C, 25°C and 4/25°C cycles during several days. They reported an increase in SDS during isothermal storage conditions for both types of starch. Unfortunately, they did not report data on RS or RDS and it is not clear what changes between the three fractions occurred. The above mentioned studies show similar trends to the changes that we found in digestion fractions with the cycled thermal processing.

The digestion fractions correspond to selected time points along the enzymatic digestion curve for starch under certain *in vitro* conditions. Therefore, these points may not reflect exactly what happens in vivo. Starch digestion in the human body is a dynamic process governed by many biological and physical factors such as pH fluctuation, enzyme expression in the individual, starch transit time, starch accessibility, food matrix among others (Englyst, 1992). However, specific conditions had been chosen and starch digestion had been classified in to RDS, SDS and RS in order to

allow comparisons among samples to assess their potential to be digested in the human body.

After the *in vitro* digestion process, the remaining material was dialyzed to remove glucose and other small molecules and then freeze dried in preparation for *in vitro* faecal fermentation. Confirming the increase in RS with repeated cooking-freezing cycles, neutral sugars in this digested whole wheat flour exhibited an increase in the percentage of glucan (sum of all polymeric glucose residues in the sample) as cooking-freezing cycles increased with concomitant decrease in arabinan and xylan (Fig. 2).

3.2. *In vitro* fermentation

During the *in vitro* faecal fermentation of treated whole wheat flour, total SCFA increased as cooking-freezing cycles increased (Fig. 3a). The increased carbohydrate content in the fermentation substrate probably led to the higher SCFA production (Fig. 2). This would imply enhanced beneficial effects after consumption of equivalent amounts of whole wheat flour that had been treated with cooking-freezing cycles compared with flour not treated with such cycles due to higher SCFA production and associated health-related effects, including potential inhibition of pathogens and increase in mineral bioavailability (Hopkins & Macfarlane, 2003; Y. Wang et al., 2010).

Acetate increased as cooking-freezing cycles increased (Fig 3b). The difference in the amount of acetate produced at 24 h in samples treated with 7 cycles of cooking-freezing compared with zero cycles was 37%. Acetate is commonly produce in a greater ratio than propionate or butyrate during dietary fibre fermentation (Bourquin, Titgemeyer, & Fahey, 1993). Therefore, the increase in acetate is consistent with a higher amount of SCFA production as cooking-freezing cycles increased.

Propionate increased 42% after 7 cooking-freezing cycles at 24 h of fermentation compared with the control (Fig. 3c). This was the highest percentage increase compared with the other SCFA. Experiments in humans on high and low RS diets show differences in butyrate and acetate production but not in propionate (Phillips et al., 1995). Therefore, it was somewhat surprising to see such a marked increase in propionate in the present study. This discrepancy could be due to differences in faecal microbiota composition among studies. Interestingly, *in vitro* faecal fermentation of arabinoxylans and other cell wall polysaccharides from wheat has shown a propensity toward elevated propionate production in many studies (Rose, Patterson, & Hamaker, 2010; Williams, Mikkelsen, le Paih, & Gidley, 2011; Wood, Arrigoni, Miller, & Amadò, 2002). Perhaps the increase in cooking-freezing cycles improved the fermentation of the other dietary fibres in wheat. Future studies could explore this possibility by measuring the residual dietary fibres in the samples following fermentation.

Butyrate increased as cooking-freezing cycles increased during first half (12 h) of fermentation (Fig. 3d). RS has been shown to be a highly fermentable and particularly butyrogenic substrate (Lesmes, Beards, Gibson, Tuohy, & Shimoni, 2008). Thus, the increase in RS with progressive cooking-freezing cycles could be a reason for the increased butyrate production during the early stages of fermentation.

In the second half of fermentation (12-24 h), there was a continued trend toward an increase in butyrate for up to 3 cooking-freezing cycles (Fig. 3b). However, for 5 and 7 cycles, the butyrate production decreased. This may be due to the limitations of the batch fermentation model. Total SCFA production increase dramatically before 12 h of fermentation for samples treated with 5 and 7 cycles (Fig 3a). During batch fermentation, pH drop can affect the types of bacteria that can grow and their metabolic pathways (Duncan, Louis, Thomson, & Flint, 2009). Perhaps changes in the fermentation

environment, due to the fast production of SCFA in the first half of fermentation in samples corresponding to 5 and 7 cooking-freezing cycles reduced butyrate production. Another explanation could be that there was not sufficient RS to maintain the high numbers of butyrate producers that grew during the first half of fermentation in the samples treated with 5 and 7 cooking-freezing cycles. Butyrate producers are very dependent of the presence of fermentable carbohydrate substrate and decrease rapidly with inadequate fermentable carbohydrate supply (Russell et al., 2011; David et al., 2014).

Bifidobacterium increased in all treatments compared to baseline, even with the whole wheat flour that had only been cooked and not subjected to any cooking-freezing cycles (Fig. 4). No significant differences among treatments were observed (when analyzed as a categorical variable), although there was a trend toward an increase in *Bifidobacterium* with increased cooking-freezing cycles (number of cycles treated as a continuous variable; p=0.08).

Although other genera of bacteria were no doubt affected by the different whole wheat substrates, *Bifidobacterium* was of special interest because some species have shown beneficial effects on host health such as anti-inflammatory properties and anti-microbial activity against enterophatogens (Walker & Lawley, 2013); thus an increase the population of *Bifidobacterium* has been widely accepted as a criterion for a prebiotic. Both whole grains and RS have been shown to result in increases in *Bifidobacterium* in clinical trials (Costabile et al., 2008; Martínez, Kim, Duffy, Schlegel, & Walter, 2010). The increase of about 0.5 log cells/mL in the present study is consistent with increases in faecal *Bifidobacterium* in human trials with whole grain products (Costabile et al., 2008) and with purified RS (Martínez et al., 2010). The trend toward an increase in *Bifidobacterium* with increase cooking-freezing cycles suggests that if whole wheat flour

could be further manipulated to increase the RS content even further, perhaps significantly enhanced bifidogenic effects could then be realized.

4. Conclusions

Cooking and freezing cycles of aqueous suspensions of whole wheat flour resulted in enhanced RS content that increased with each cycle. The increase in RS modulated the growth and metabolic activity of gut microbiota, which resulted in an increase in total SCFA and was particularly propiogenic. Our results demonstrate that it is possible to manipulate the native starch fractions in whole wheat flour to produce changes that are consistent with improved gut health. Thus, this flour could be treated as a functional ingredient due to the elevated RS and associated potential benefits to gut health. Future experiments should determine the enhanced health benefits associated with using this ingredient in foods.

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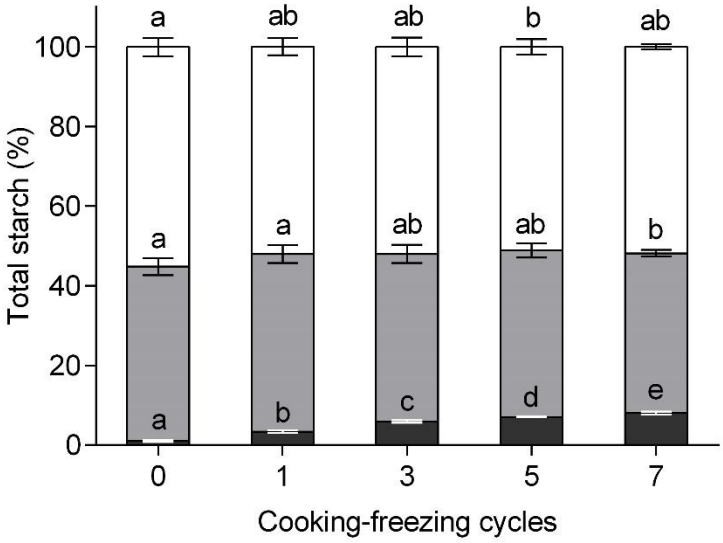

Figure 1: Rapidly digested starch (white bars), slowly digested starch (gray bars), and resistant starch (black bars) in whole wheat flour after subjecting the flour to different numbers of cooking-freezing cycles; different letters represent significant difference between cycles within each starch digestible fraction; error bars show standard deviation; $n = 3$; $p < 0.05$.

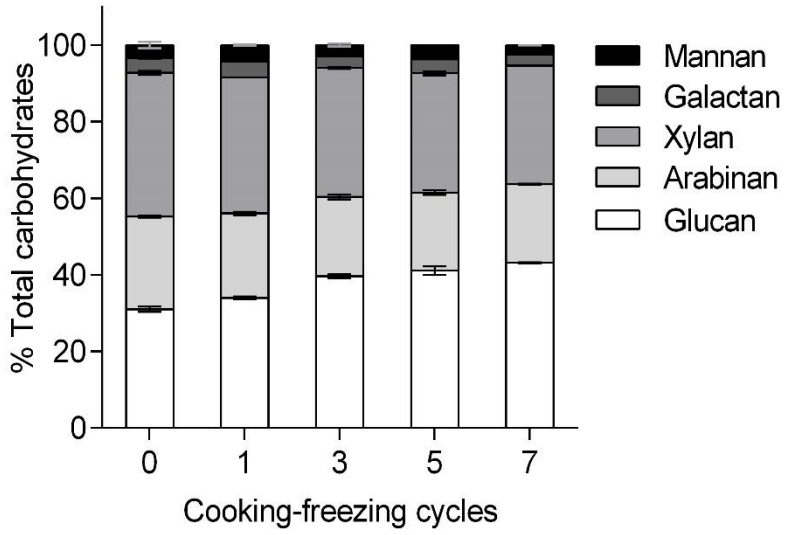

Figure 2: Non-digestible polysaccharide residues present in the dialyzed residues after in vitro digestion of whole wheat flour; error bars show standard deviation; n=3.

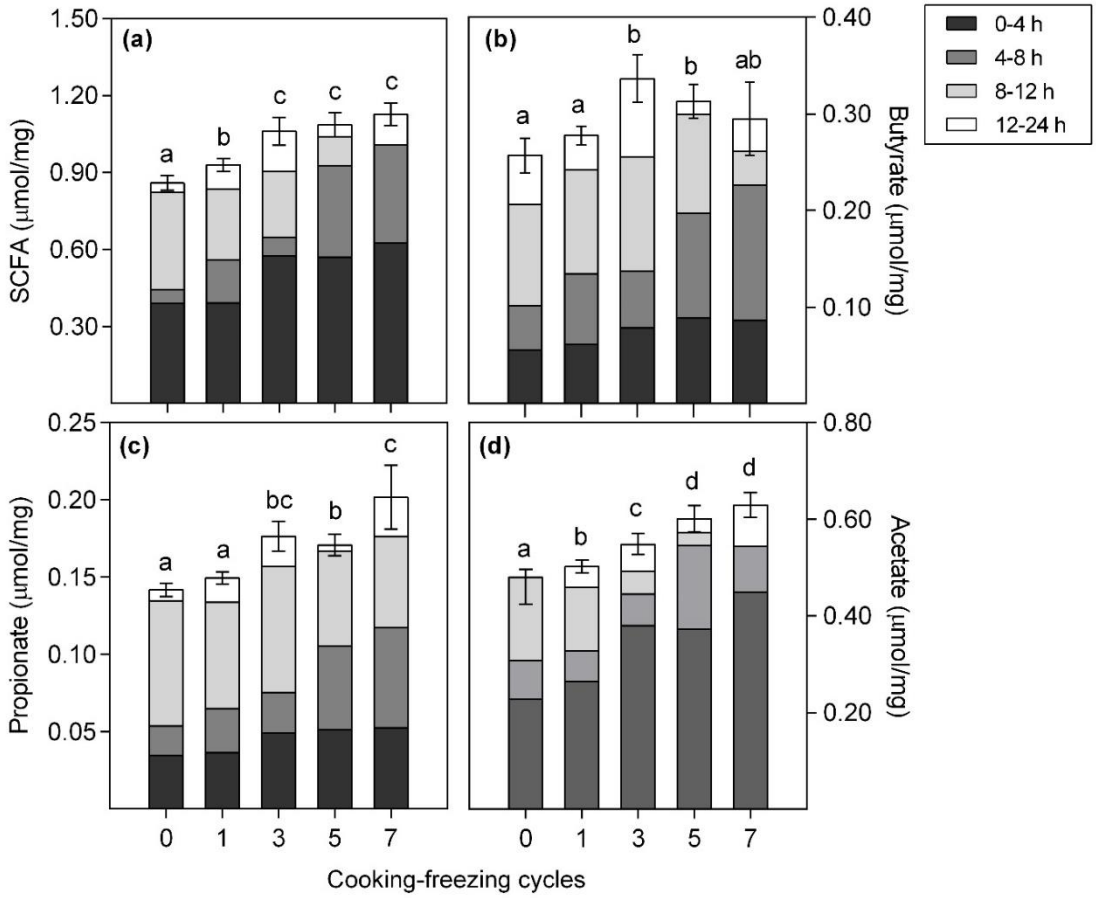

Figure 3: Total short chain fatty acids (SCFAs; a) and individual SCFAs (b, butyrate; c, propionate; d, acetate) produced during fermentation of whole wheat flour that had been subjected to different cooking-freezing cycles and *in vitro* digestion; units are expressed as µmol produced by the microbiota per mg of starting material; error bars show standard deviation, different letters represent significant differences between cycles at 24 h of fermentation; $p < 0.05$; $n = 3$.

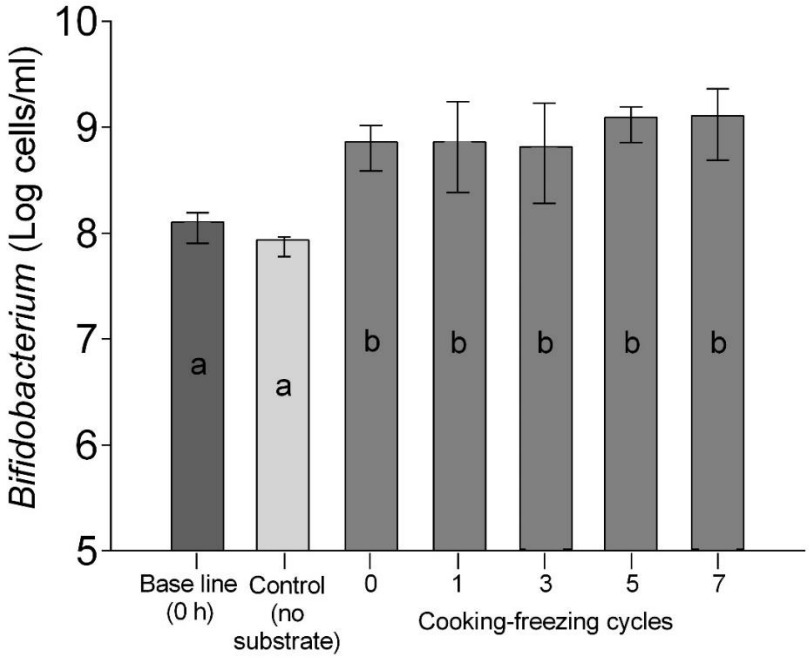

Figure 4: *Bifidobacterium* concentration in fecal inoculum and after 24 h of *in vitro* fermentation of pre-digested whole wheat flour; error bars show standard deviation; different letters represent significant difference between treatments; n=3; $p < 0.05$

CHAPTER 2. Changes in dietary fiber solubility and gut microbial fermentation of wheat bran after extrusion and bread making

Abstract

Dietary fiber fermentation in the human colon stimulates growth and metabolic activity of the microbiota that in turn produce beneficial metabolites for human health. Soluble dietary fiber tends to be more easily fermentable than insoluble dietary fiber. Wheat bran is known to be a rich source of dietary fiber; however, the dietary fiber in wheat bran is mostly insoluble and is poorly utilized by human gut microbiota. The purpose of this study was to use extrusion to increase dietary fiber solubility and at the same time induce greater production of short chain fatty acids (SCFA) during *in vitro* fecal fermentation. Furthermore, to use extruded bran into a product formulation to see if the possible changes in fiber fermentability are maintained. Combinations of high or low moisture (15% and 30% wb) and high or low screw speed (120 and 250 rpm) were chosen to generate mild and severe conditions during extrusion. All extrusion conditions resulted in improvements in both solubility and SCFA production compared with unextruded bran. More severe extrusion conditions of low screw speed and low moisture, resulted in the highest increase in soluble dietary fiber (3-fold) as well as the highest production of SCFA during fermentation (1.4-fold) compared with unextruded bran. Bread making increased fermentation of arabinan and galactan mainly. However, whole wheat breads containing extruded bran did not showed increases of either fiber solubility or SCFA production compared to the control. In conclusion, extrusion of wheat bran increased dietary fiber solubility, which enabled greater fermentability by human

fecal microbiota. However, once extruded bran was used in a whole wheat bread formulation these effects were no longer evident.

Key words: Butyrate; short chain fatty acids; screw speed; moisture; arabinoxylans; non-starch polysaccharides.

1. Introduction

Dietary fibers can be classified based on their solubility. Dietary fiber solubility is related to its chemical and physical structure and can define its physiological functionality. For instance, although wheat bran is a rich source of fiber, its fermentation in the human colon it is not complete; carbohydrate consumption after 24 h of fermentation ranges from 15 – 25% (Bourquin, Titgemeyer, & Fahey, 1996; Karppinen, Liukkonen, Aura, Forssell, & Poutanen, 2000). However, when the insoluble fiber from wheat is treated with enzymes to make it soluble, it becomes a highly fermentable dietary fiber with prebiotic properties (Napolitano et al., 2009).

Final products of dietary fiber fermentation in the human intestine are short chain fatty acids (SCFA). These metabolites in the colon are essential to human health because they are the primary energy source for colonic cells and serve as modulators in the immune response (Flint, Scott, Louis, & Duncan, 2012; Peng, Li, Green, Holzman, & Lin, 2009).

Extrusion combines mechanical shear, moisture, high pressure and high temperature that disrupts cell wall structures (Robin, Dubois, Pineau, Schuchmann, & Palzer, 2011). Improvements in dietary fiber solubility during extrusion of wheat bran has been reported before with assorted results. Ralet, Thibault, & Valle (1990) showed that processing wheat bran at 100°C, 240 rpm and 6.1% moisture conditions increase soluble dietary fiber up to 16%. A less marked effect was reported by Kahlon, Berrios, Smith, & Pan (2006), who obtained a maximum of 1% increase in soluble fiber in wheat bran using a twin-screw extruder operated at 400 rpm and barrel temperatures of 80 to 130°C. Gajula, Alavi, Adhikari, & Herald (2008) obtained increases in soluble fiber from 22% to 73% in reconstituted flours with 0-30% bran when the whole flour was extruded using a twin-screw extruder at 200 rpm, 14% moisture and barrel temperatures from 30

to 40°C. The same authors reported that cookies made with extruded and reconstituted whole wheat flour did not decrease in costumer acceptance but increased in their nutritional value when compared to the unextruded control.

Although an increase in dietary fiber solubility upon extrusion has been reported, the possible positive impact on human nutrition associated with fiber fermentability by human fecal microbiota has not been described. Furthermore, possible additional changes of fiber fractions during wheat bran-enrich foods manufacturing have not been investigated before. Thus, there were two objectives to this study. First, we determined the changes in dietary fiber fermentation of wheat bran following extrusion with an emphasis on the percentage of non-starch polysaccharides available for fermentation by the microbiota as well as the quantity of short chain fatty acids generated by such fermentation. Second, we added extruded wheat bran to straight grade flour to make reconstituted whole wheat flour and then made bread from this flour. Differences in non-starch polysaccharide fermentation were then assessed on the digested whole wheat breads to determine if the changes in non-starch polysaccharide solubility and fermentation were maintained.

2. Materials and methods

2.1 Wheat bran samples

Hard red winter wheat (Triticum aestivum ‘Wesley’) was obtained from Husker Genetics, the University of Nebraska-Lincoln’s Foundation Seed Division. Wheat kernels were tempered overnight to 15% moisture followed by milling on laboratory pneumatic Bühler mill (Bühler model MLU-202, Uzwil, Switzerland) according to the approved method 26-21A (AACC International, 2013). Dial settings for break rolls were 10 left, 8.5 right and for reduction rolls were 7 left, 3 right. Milling fractions obtained were 74.7%

straight-grade flour, 2.3% shorts and 23.0% bran. The shorts and bran were combined and used as ‘bran’ in this study.

2.2 Extrusion

A 2x2 factorial design was used to test the effect of screw speed and bran moisture on water extractability of wheat bran non-starch polysaccharides (NSP). High screw speed (HS, 250 rpm) and low screw speed (LS; 120 rpm) and high moisture (HM; 30% wet basis) and low moisture (LM; 30% wet basis) were chosen to generate different processing conditions. Bran moisture was adjusted 24 h before the experiment by the addition of water using a spray while mixing. Samples were stored in closed containers at 4 °C until extrusion. The experiment was conducted in a single screw KE 19 Brabender laboratory extruder with a single stage mixing zone, 3:1 compression ratio, 3 mm die diameter, and a 20:1 L/D ratio (CW Brabender Instruments, NJ, USA). Barrel temperature was set at a constant 120°C. All treatment combinations were performed in duplicate. Following extrusion, extrudates were ground in a cyclone mill (UDY Corporation, Co, USA) equipped with a 1 mm mesh screen. Control bran was also milled using the same equipment.

2.3 Bran composition analysis

The milled extruded and unextruded bran were assayed for protein (approved method 46-19), lipids (approved method 30-25) and ash (approved method 08-01; AACC International, 2013). Total starch was measured using a total starch assay kit (K-TSTA, Megazyme, Bray, Ireland) following the procedure for samples that contain resistant starch but do not contain free glucose (KOH format). Total dietary fiber, was determined as the sum of neutral sugars residues, uronic acid residues and Klason

lignin following approved method 32-25 (AACC International, 2013) with some modifications. Soluble and insoluble NSP were separated as described in section 2.6.

2.4 Bread making

Whole wheat flour was obtained by combining, in their original proportions, extruded or unextruded bran and straight-grade flour collected after milling hard red winter wheat (as in section 2.1). Whole wheat bread was preparing using approved method 10-13A (AACC International, 2013). Briefly, straight dough formula for 100 g loaves contained: 100 g whole wheat flour (14% moisture content basis), 1 g active dry yeast (Fleischmann's, ACH Food companies, Memphis, TN), 3 g unemulsified shortening (BBS shortening, Stratas food, Memphis, TN) , 1.5 g salt, 6 g sucrose, 0.2 g malt powder (Malt products corporation, Saddle Brook, NJ), 4 g whey powder (Darigold Caldwell, Caldwell, ID), 0.1 g ammonium phosphate (Innophos, Cranbury, NJ), 2 mg ascorbic acid (Sigma, St. Louis, MO) and water according to water absorption. Water absorption and mixing time, were estimated from Mixograph data (Appendix A, Table 1) (National Manufacturing, Lincoln, NE, USA; approved method 54-40A; AACC International, 2013). All dry ingredients were mixed well in a bowl and then water, shortening, and ascorbic acid (dissolved in some of the water) were added. The dough was fermented for 180 min at 30°C in a covered container, previously greased with shortening. Fermented dough was sheeted at gaps of 0.87, 0.47 and 0.32 cm before rolling and placing in a greased pan 5.1 x 7.6 x 15.2 cm. Proofing was performed at 37.5°C and 85% rh for about 1 h. Proofed dough was baked in a 204°C oven for 25 min and then the loaves were allowed to cool in a rack for 1 h. Four loaves per treatment, 2 per replicate were baked. Baking quality parameters were evaluate after cooling process (Appendix A, Table 1). No significant differences were detected in the baking quality

parameters of whole wheat breads loaves made with extruded wheat bran compared with the control. Bread was sliced using an electric knife (Black and Decker, 9'' EK700, Maryland, USA) and a cutting guide to obtain slices of about 12.5 mm thick. The slices were frozen (-20°C) overnight and then freeze dried. Freeze dried bread slides where reduced in particle size using a sample cyclone mill (UDY Corporation, Co, USA) equipped with a 1 mm mesh screen.

2.5 *In vitro* digestion

In vitro digestion was performed as described previously (Mishra & Monro, 2009) with some modifications. Briefly, the milled bran, extruded bran, or freeze dried milled bread with unextruded or extruded bran (10 g) were mixed with 120 ml distilled water in a 250 mL Erlenmeyer flask. To start the gastric digestion, 3.2 mL of 1 M HCl was added to reduce the pH to 2.5 and then 4 mL of pepsin (100 mg/mL in 50 mM HCl, P-700, Sigma, St Louis, MO, USA) was added and the flasks were placed on an orbital shaking water bath (150 rpm, MaxQ 7000, Dubuque, IA, USA) at 37°C for 30 min. Intestinal digestion was simulated by adding 20 mL of 0.1 M sodium maleate buffer (pH 6, containing 1 mM CaCl2) and 8 mL of 1 M NaHCO3 to raise the pH to 6.9, followed by 20 mL of pancreatin (125 mg/mL, P-7545, Sigma, St Louis, MO, USA) in sodium malate buffer and 0.8 mL of amyloglucosidase (3260 U/mL, E-AMG, Megazyme). The flasks were kept in the orbital shaker water bath at 37°C and 150 rpm for 6 h. Digested contents were then poured into dialysis tubing (molecular weight cutoff 12,000-14,000) and dialyzed for 3 days against distilled water, changing the water every 12 h. The retentates were frozen (-20°C) overnight and then freeze dried. The starch concentration in the retentates was less than 0.2% (K-TSTA, KOH format, Megazyme).

2.6 Extractability of NSP in digested wheat bran before fermentation

Total NSP were analyzed before *in vitro* fermentation of digested extruded, unextruded bran and whole wheat bread following approved method 32-25 (AACC International, 2013) with some modifications. Briefly, 300 mg of sample, 3 ml of acetate buffer (0.1 M, pH 5, containing 5 mM CaCl2) and 25 µL thermostable amylase (Sigma A-9505 or Megazyme) were combined into 15 ml test tube and then incubated at 60 °C overnight. Tubes were then centrifuge at 1000g, for 10 min, the supernatant was transferred to a 50 ml tube and the pellet was washed by suspending and re-centrifuging with water (2 × 3 ml). The collected supernatants contained the water-extractable NSP (WE-NSP), which was precipitated by adding 35 ml absolute ethanol while mixing. The tubes were allowed to stand for 1 h in an ice bath, centrifuged at 1000g for 10 min, and the supernatant was discarded. The pellet was washed by suspending and re-centrifuging with 80% ethanol (2 × 3 mL). The pellet obtained after washing with water contained the water-unextractable NSP (WU-NSP), which were washed by suspending and re-centrifuging with 80% ethanol (2 × 3 mL). Acetone (3 mL) was used to dry both pellets and again tubes were mixed, centrifuged and the supernatant was discarded. The WU-NSP pellet was subjected to acid hydrolysis with 0.3 mL of 12 M sulfuric acid at 30°C for 1 h. One mL of myo-inositol (3 mg/mL dissolved in water) was added as an internal standard and then additional water was added to make a final concentration of 0.4 mol H2SO4/L. The tubes were then autoclaved at 125°C for 1 h. The sugars in the hydrolysate following autoclaving were converted to alditol acetates as described (approved method 35-25, AACC International 2000). The alditol acetates were separated on a gas chromatograph (Clarus 580, PerkinElmer, MA USA) equipped with a fused silica capillary column (SPTM-2380, 30 m × 0.32 mm inner diameter × 0.2 µm film thickness, PerkinElmer) and detected with a flame ionization detector. Quantification

was accomplished by calculating correction factors for pure sugar standards relative to myo-insolitol. WE-NSP were quantified in a manner analogous to the WU-NSP portion, except the hydrolysis in 12 M H2SO4 for 1 h at 30 °C step was skipped. Cellulose and β-glucans were considered as non-starch glucans in this study. Arabinoxylans (AX) and arabinose/xylose (A/X) ratio was calculated as described by Maes & Delcour (2002).

Water extractable:

$$AX = 0.88 * (\%Xylose + \%Arabinose – 0.7 * \%Galactose)$$

Water Unextractable

$$AX = 0.88 * (\%Xylose + \%Arabinose)$$

2.7 *In vitro* fermentation using human fecal inoculum

In vitro batch fecal fermentation was performed using 100 mg of freeze dried retentate obtained after *in vitro* digestion of either wheat bran or bread, as described (Hartzell, Maldonado-Gómez, Hutkins, & Rose, 2013). In a 50 mL screw-cap Erlenmeyer flask, the freeze dried retentate was suspended in 9 mL sterile fermentation medium containing (per L): peptone (2 g; BP1420-100, Fisher Scientific, Pittsburgh, PA USA), yeast extract (2 g; CAS8013-01-2; Alfa Aesar, Ward Hill, MA USA), bile salts (0.5 g; LP0055; Oxoid), NaHCO3 (2 g), NaCl (0.1 g), K2HPO4 (0.08 g), MgSO4.7H2O (0.01 g), CaCl2.6H2O (0.01 g), L-cysteine hydrochloride (0.5 g; Sigma), hemin (50 mg; Sigma), Tween 80 (2 mL), vitamin K (10 µL; Sigma), and 0.025% (w/v) resazurin solution (4 mL), capped, and hydrated overnight on ice. Fresh fecal samples from 3 healthy adults with no history of gastrointestinal abnormalities or antibiotic consumption within the past 6 months were collected and pooled to be used as the fecal inoculum. The fecal slurry was prepared by blending the fecal samples with sterile phosphate buffered saline pH 7.0 (1:9 w/v) using a hand blender for 1 min and then filtering through 4 layers of

cheesecloth. Tubes were then inoculated with 1 mL of fecal slurry, capped, and incubated at 37 °C with orbital shaking (125 rpm). During fermentation, 0.4 mL of fermentation slurry were removed after 0, 4, 8, 12, and 24 h of fermentation and immediately transferred to a microfuge tube containing 0.1 mL of 7 mM 2-ethylbutyric acid (internal standard) in 2M sodium hydroxide (to halt microbial metabolism and enzyme activity and prevent volatilization of SCFAs). All steps for fermentation were conducted in an anaerobic hood (Bactron IV, Sheldon manufacturing, Cornelius, OR USA) containing 5% H2, 5% CO2, and 90% N2. As soon as all samples were taken at each time point, microfuge tubes were removed from the anaerobic cabinet and immediately stored at -80 °C. After 24 h, the remaining fermentation slurry was frozen (-80°C) overnight and then freeze dried. All the recovered freeze dried material was used to quantify total neutral sugars residues after *in vitro* fecal fermentation of bran and whole wheat bread as described in the section 2.6, starting at the hydrolysis in 12 M H2SO4 step and following the procedure for water unextractable fraction. Fecal fermentations of the digested bran were performed on different days with fecal samples from different donors compared with the fermentation of the whole wheat breads. Thus, because of differences in fermentation profiles among individuals, fermentations from bran versus bread were not quantitatively comparable.

2.8 Short chain fatty acids determination

SCFA were quantified by gas chromatography (Hartzell et al., 2013). Samples were vortex mixed with ~0.4 g of NaCl and 0.2 mL of 9 M H2SO4. Then 0.5 mL of diethyl ether was added to the tubes which were capped and shaken vigorously. Tubes were centrifuged (10,000 x g, 5 min) and then the diethyl ether layer was transferred to a new tube. One µL of the diethyl ether layer was injected onto a gas chromatograph (Clarus

580, PerkinElmer, MA USA) equipped with a capillary column (Elite-FFAP, 15 m × 0.25 mm inner diameter × 0.25 μm film thickness, PerkinElmer) and detected with a flame ionization detector. SCFA were quantified by calculating response factors for each SCFA relative to 2-ethyl butyric acid using injections of pure standards.

2.9 Data analysis

All treatments were performed in duplicate and measured twice. Two ANOVA models were used to analyze the data. For detecting significant differences in extruded bran or whole wheat bread non-starch polysaccharide solubility compared with the unextruded controls, data were analyzed using a one factor ANOVA followed by Dunnett's multiple comparison procedure, where the appropriate unextruded sample was used as the control. For analysis of differences among extruded samples, data were analyzed using a two factor ANOVA with screw speed and moisture as factors. For SCFA analysis, a repeated measures ANOVA with sample and time as the factors was used. Significant differences among samples were determined using Fisher's least significant difference (LSD) test with $\alpha=0.05$. All data were analyzed using SAS software (version 9.2, SAS Institute, Cary, NC USA).

3. Results and discussion

3.1 Composition of wheat bran and extrudates

The wheat bran used in this study contained 52.9 ± 0.9% total dietary fiber (1.1 ± <0.1% soluble fiber and 51.7 ± 0.8% insoluble dietary fiber), 13.0 ± 0.1% starch, 19.6 ± <0.1% protein, 4.24 ± 0.1% lipid, and 7.24 ± <0.1% ash. These are typical of previous reports on wheat bran (Long, Ye, & Zhao, 2014; Maes & Delcour, 2002). Extrusion did not affect the values for any of these analytes except for a minor (<0.5%) but significant decrease in total starch under the conditions used in this study. This is consistent with

Gualberto, Bergman, Kazemzadeh, & Weber (1997) and Dust et al. (2004), who reported that extrusion processing does not greatly affect either protein, ash, total dietary fiber or total starch. In contrast, some authors have reported increase of total dietary fiber during extrusion processing of wheat bran due to formation of resistant starch (Kahlon et al., 2006; Reyes-Pérez, Salazar-García, Romero-Baranzini, Islas-Rubio, & Ramírez-Wong, 2013).

3.2 Effect of extrusion on non-starch polysaccharide extractability

Although the total dietary fiber of wheat bran did not change with extrusion, extrusion did affect the partitioning of WE-NSP and WU-NSP (Fig. 1). Compared with the control, all treatments increased WE-NSP in wheat bran at least 2-fold. In consequence, there was a reduction in WU-NSP. The highest WE-NSP was obtained in samples that were processed at low screw speed (120 rpm) and low moisture (15%). In contrast, samples with high moisture and high screw speed had the lowest increase in WE-NSP. Low screw speed may increase water-extractability of wheat bran polysaccharides since residence time in the barrel is extended, thus translating into more mechanical shear experienced by the cell wall structures of wheat bran (Lamsal, Yoo, Brijwani, & Alavi, 2010). Additionally, moisture acts as a plasticizer in the extruder barrel; thus lower moisture indicates the sample experienced more abrasion and mechanical disruption (Akdogan, 1999).

Under the processing conditions studied in this research the interaction between moisture and screw speed was not significant ($p=0.26$). Therefore, each of the factors could be interpreted separately. Moisture and screw speed seemed to have similar effects on increasing NSP extractability: low moisture combined with either level of

screw speed, or low screw speed combined with either level of moisture led to equivalent estimates of WE-NSP (30.5 mg/g bran).

Increased NSP extractability in wheat bran dietary fiber due to extrusion has been showed previously. Ralet, Thibault, and Valle (1990) reported a 2-fold increase in soluble fiber obtained when samples were extruded using a treatment with high mechanical energy per unit of mass (240 rpm and 6.1% of added water). The same tendency, but with slightly higher WE-NSP, was observed by Kahlon, Berrios, Smith, and Pan (2006). In contrast to these results, Wang, Klopfenstein, and Ponte (1993) reported maximum fiber solubility increase of 2.5-fold after processing wheat bran at high moisture (28.8%) and high screw speed (400 rpm).

In terms of extrusion processing, it is not accurate to define universal processing conditions to obtain the maximum increases in WE-NSP. The effects of extrusion on dietary fiber fractions are characteristic of the equipment configuration, scale, and other variables like temperature, feed rate, screw configuration, and flour particle size. However, in general, it is possible to establish that the more mechanical stress on the food material the more soluble fiber is obtained. Changes in solubility may be due to disruption of polysaccharides associations with other cell wall components or due to a decrease on the polysaccharide molecular weight itself (Robin, Schuchmann, & Palzer, 2012).

Extrusion processing primary affected arabinoxylans, increasing extractability at least 3.5-fold (Fig. 2). Non-starch glucans (cellulose and mixed-linkage β-glucan) were less affected with about a 1-fold increase. Extrusion is effective at converting unextractable hemicellulosic fractions to extractable and less effective at breaking cellulose to smaller fractions (Singkhornart, Lee, & Ryu, 2013). Curiously, the greatest increase in arabinoxylan extractability was obtained under low moisture conditions, while

greater extractability of non-starch glucans was obtained with high moisture combined with low screw speed. Increased extractability of non-starch glucans as well as total soluble fiber using extrusion may depends of processing conditions combination and the natural source.

Extruded and unextruded bran were added to straight-grade flour in the proper proportions to make whole wheat flour and then made into bread. Once the extruded wheat bran was used in bread, the increase in NSP extractability was no longer evident (Fig. 3). Surprisingly, all treatments decreased WE-NSP, except the low screw speed low moisture condition, which resulted in the highest WE-NSP in bran. Total concentration of soluble fiber per gram of freeze dried bread after digestion in all treatments (32.1 ± 2.8 mg/g) was equal the soluble fiber concentration of bran treated with LSLM conditions 32.1 mg/g (Fig. 1).

Breadmaking may mask the effect of extrusion on increasing wheat bran fiber solubility. During breadmaking several changes in the food matrix structure may occur due to mixing, fermentation and baking processes. For instance, Cleemput, Booij, Hessing, Gruppen, & Delcour (1997) reported increases on non-starch polysaccharides solubility during mixing phase (7 to 12%) and baking phase (14-15%) during bread making. The authors explain the increased solubility as possible action of endogenous arabinoxylans hydrolytic enzymes but did not discard that changes in the protein network along the process also contribute with the increased water extractability of the non-starch polysaccharides. β-glucans can change during bread making. Andersson, Rüegg, & Åman (2008) reported an increase in water extractable β-glucans during rye dough fermentation due to endogenous β-glucanase present in the flour.

3.3 Effect of extrusion on non-starch polysaccharide fermentation

During bran fermentation, all treatments showed an increase in carbohydrate consumption compared with the control (Fig. 4). Extrusion conditions that resulted in greater WE-NSP showed higher NSP fermentation than the other conditions. In bran samples processed with low screw speed, consumption of arabinoxylans increase around 38% whereas non-starch glucan consumption increase 25% compared with the control. The greater increase in arabinoxylan consumption compared with glucan consumption in the extruded samples as well in the control (Fig. 4) suggests that hemicelluloses are more prone to be fermented than cellulose or β-glucans in wheat bran. Amrein, Gränicher, Arrigoni, & Amadò (2003) observed almost the same consumption of glucose and xylose (20%) during fecal fermentation of wheat bran. Glucose was consumed in the first hours of fermentation whereas xylose and arabinose were degraded along 24 hours. In contrast, Karppinen et al. (2000) reported that *in vitro* fermentation of wheat bran by human fecal bacteria leads to 52% consumption of glucans followed by 45% of xylans and only 9% of arabinans. Carbohydrate consumption during fermentation may differ due to high diversity and variability of the microbial community between donors (Eckburg et al., 2005).

Although significant differences in NSP utilization during fermentation of whole wheat bread were detected (Fig. 4), the results were not in agreement with the results of bran alone (Fig. 5) or of WE-NSP in bread (Fig. 3). During fermentation of digested whole wheat bread there was a marked arabinan and galactan consumption that was not observed during wheat bran fermentation. Arabinan and galactan were consumed up to 73.09% and 47.45% respectively during fecal fermentation, whereas xylans were consumed up to 59.53% and glucans up to 47.0.9 %. Wheat endosperm contains soluble arabinogalactan peptides, about 0.27-0.38% db of refine flour (Tryfona et al.,

2010), which may have been more degraded during fermentation than bran arabinoxylans, thus increasing arabinan and galactan consumption.

Consumption of NSP in both wheat bran and whole wheat bread fermentation were always more than total soluble non-starch carbohydrate, suggesting that not only solubility but also accessibility of wheat bran polysaccharides to microbial fermentation should be considered as a parameter in predicting NSP fermentability.

3.4 Effect of extrusion on short chain fatty acid production

SCFA production was measured during 24 h of wheat bran fermentation with human fecal microbiota (Fig. 6). After 24 h, all extrusion conditions tested increased production of SCFA compared to the control. The trends in SCFA production were in agreement with the data from WE-NSP (Fig. 1) and NSP utilization during fermentation (Fig. 4). During the first half of fermentation, no differences among extruded and unextruded wheat bran were found. However, during the second half of fermentation, significant increases in SCFA production were noted in the extruded samples, particularly for butyrate. Slow fermentation of NSP may represent advantages in terms of host health. For instance, dietary fibers that are slowly degraded may ensure a better distribution of carbon source for microbiota along the entire length of colon, leading to increased SCFA production in the last part of the human colon which it has been shown to have higher risk of colon cancer and chronic inflammation (Araki & Mukaisyo, 2010; Cummings, 1997)

Fermentation of control wheat bran during this study showed a SCFA production profile that has been reported by other authors: wheat bran fecal fermentation leads to higher production of acetate and nearly equal amounts of butyrate and propionate (Amrein et al., 2003; Crittenden et al., 2002). After wheat bran extrusion using LSLM

conditions, acetate production increased 37.9%, propionate production increased 21.7% and butyrate production increased 59.5% compared with the control. Increases in acetate and propionate may be attributed to the higher availability of arabinoxylans to fermentation after extrusion, since fecal fermentation of this arabinoxylyans has been reported to increase production of these metabolites more so than butyrate (Hopkins et al., 2003; Pollet et al., 2012). On the other hand, greater butyrate production during fermentation of extruded bran was possibly because an increased solubility of glucans. For instance, fermentation of β-glucans leads to higher production of acetate and butyrate than propionate (Kaur, Rose, Rumpagaporn, Patterson, & Hamaker, 2011). Butyrate is especially important because is the primary energy source for the epithelial colonic cells. Additionally, butyrate helps to maintain colonic cells barrier properties preventing pathogens migration inside to the lamina propia, thus reducing chronic inflammation that may lead to colon cancer (Peng et al., 2009).

SCFA production during fermentation of digested whole wheat bread did not follow the same trend as for the extruded bran (Fig. 7). Only significant differences in total acetate were found but it was not possible to establish any relation with pre-extrusion of the bran. Propionate production during whole wheat bread fermentation was higher than butyrate production in all treatments. Non-starch carbohydrates in whole wheat bread were mostly degraded during the first half of fermentation. This suggest that breadmaking itself could increase the fermentability of the dietary fibers present in whole wheat flour, masking the effects of bran pre-extrusion.

4. Conclusions

Slow screw speed (long residence time) and low moisture (high mechanical shear) led to greater WE-NSP in wheat bran. This translated into increased NSP

utilization and SCFA production during fermentation by human fecal microbiota. Differences in SCFA production among unextruded and extruded samples was only evident during the later stages of fermentation, suggesting that extrusion may be effective at providing fermentable carbohydrate in more distal regions of the colon. Butyrate production during fermentation with human fecal microbiota was enhanced by extrusion in all treatments. When extruded bran was added to flour in the process of making whole wheat bread formulation, the effect of extrusion on increasing fiber solubility was no longer evident. Changes in NSP extractability during mixing, fermentation, and baking may be responsible for masking extrusion effects. These results indicate that extrusion may be use as and strategy to enhance gut microbial fermentation of wheat bran dietary fibers. However, these effects may be negated if the bran is incorporated into bread. Additional studies should determine if this is also the case for other food products made from wheat flour.

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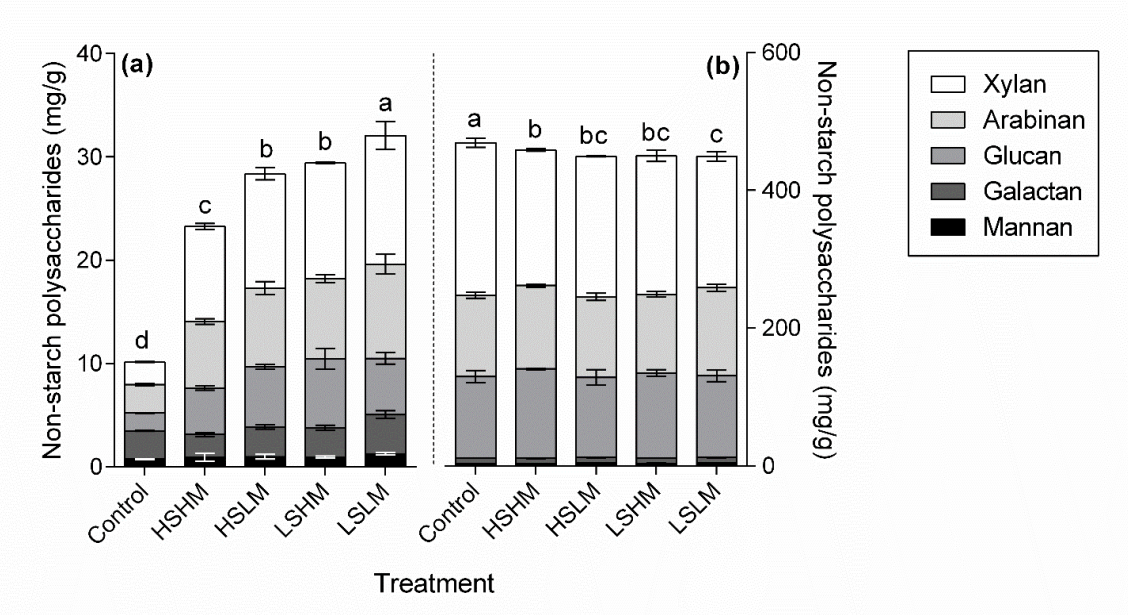

Figure 1. Water extractable (a) and water unextractable (b) non-starch polysaccharides of extruded and unextruded (Control) bran before *in vitro* fecal fermentation; note the different scales on the y-axes for water extractable and water unextractable non-starch polysaccharides; concentration in milligrams per gram of freeze dried sample; error bars show standard deviation of each non-starch polysaccharides; different letter represent significant differences of total non-starch polysaccharides between treatments; $p < 0.05$; $n = 2$.

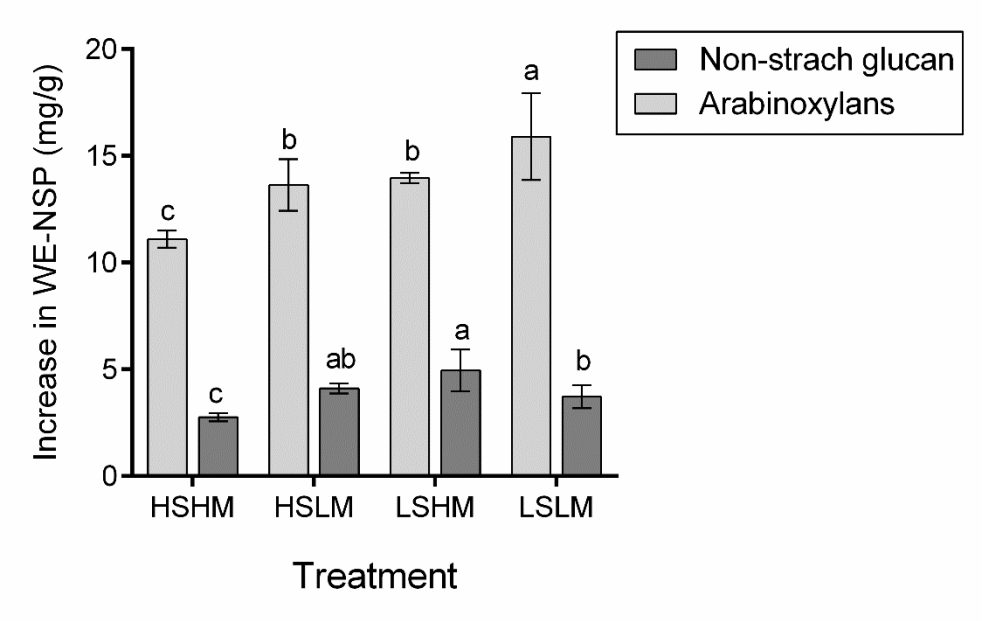

Figure 2. Increase in water extractable non-starch polysaccharides (WE-NSP) of wheat bran compared to the same polysaccharides in the control; Values expressed as increased milligrams of each polysaccharide compared to the control per gram of bran; error bars show standard deviation; different letters represent significant differences between treatments; $p < 0.05$; $n = 2$;

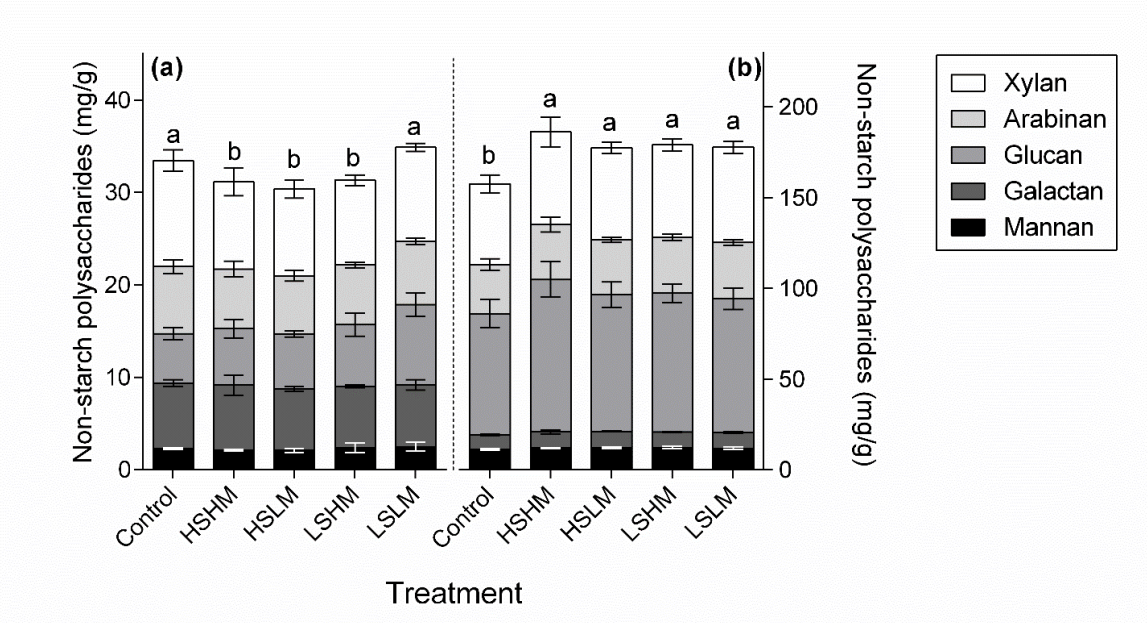

Figure 3. Water extractable (a) and water unextractable (b) non-starch polysaccharides before *in vitro* fecal fermentation of whole wheat bread containing extruded and unextruded (Control) wheat bran; note the different scales on the y-axes for water extractable and water unextractable non-starch polysaccharides; concentration in milligrams per gram of freeze dried sample; error bars show standard deviation of each non-starch polysaccharides; different letter represent significant differences of total non-starch polysaccharides between treatments; $p < 0.05$; $n = 2$.

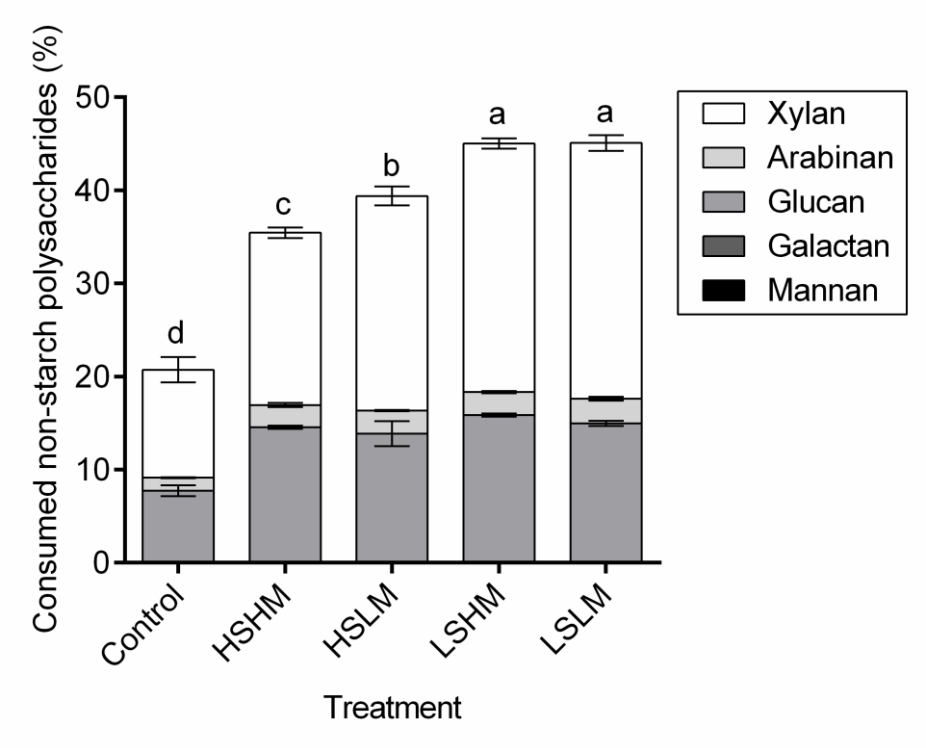

Figure 4. Consumed non-starch polysaccharides during *in vitro* fermentation of extruded and unextruded wheat bran (Control); Percentage base on total non-starch polysaccharides before fermentation; Consumed mannan and galactan were less than 0.1%; Error bars show standard deviation of each non-starch polysaccharide; different letter on top of the bars represent significant differences of total consumed non-starch polysaccharides between treatments; $p < 0.05$; $n = 2$; *A/X = %Arabinose / %Xylose; *different letter represent significant differences of A/X ratio of each treatment SD < 0.07.

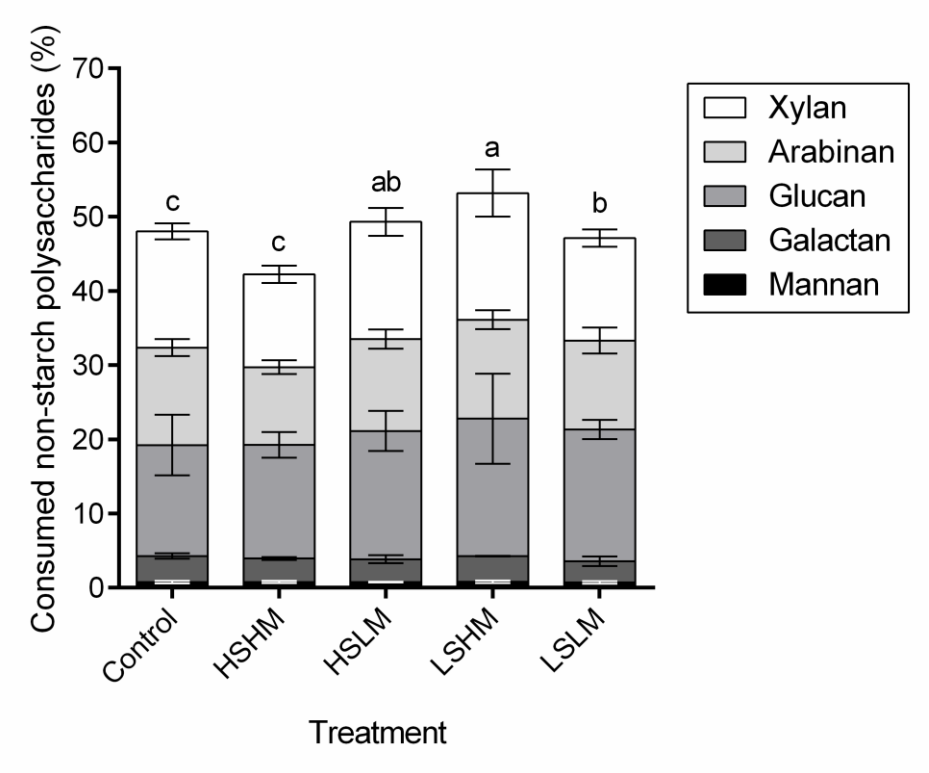

Figure 5. Consumed non-starch polysaccharides during *in vitro* fermentation of extruded and unextruded whole wheat bread (Control); Percentage base on total non-starch polysaccharides before fermentation; Error bars show standard deviation of each non-starch polysaccharide; different letter on top of the bars represent significant differences of total consumed non-starch polysaccharides between treatments; $p < 0.05$; $n = 2$; *A/X = %Arabinose / %Xylose; *different letter represent significant differences of A/X ratio of each treatment SD < 0.07.

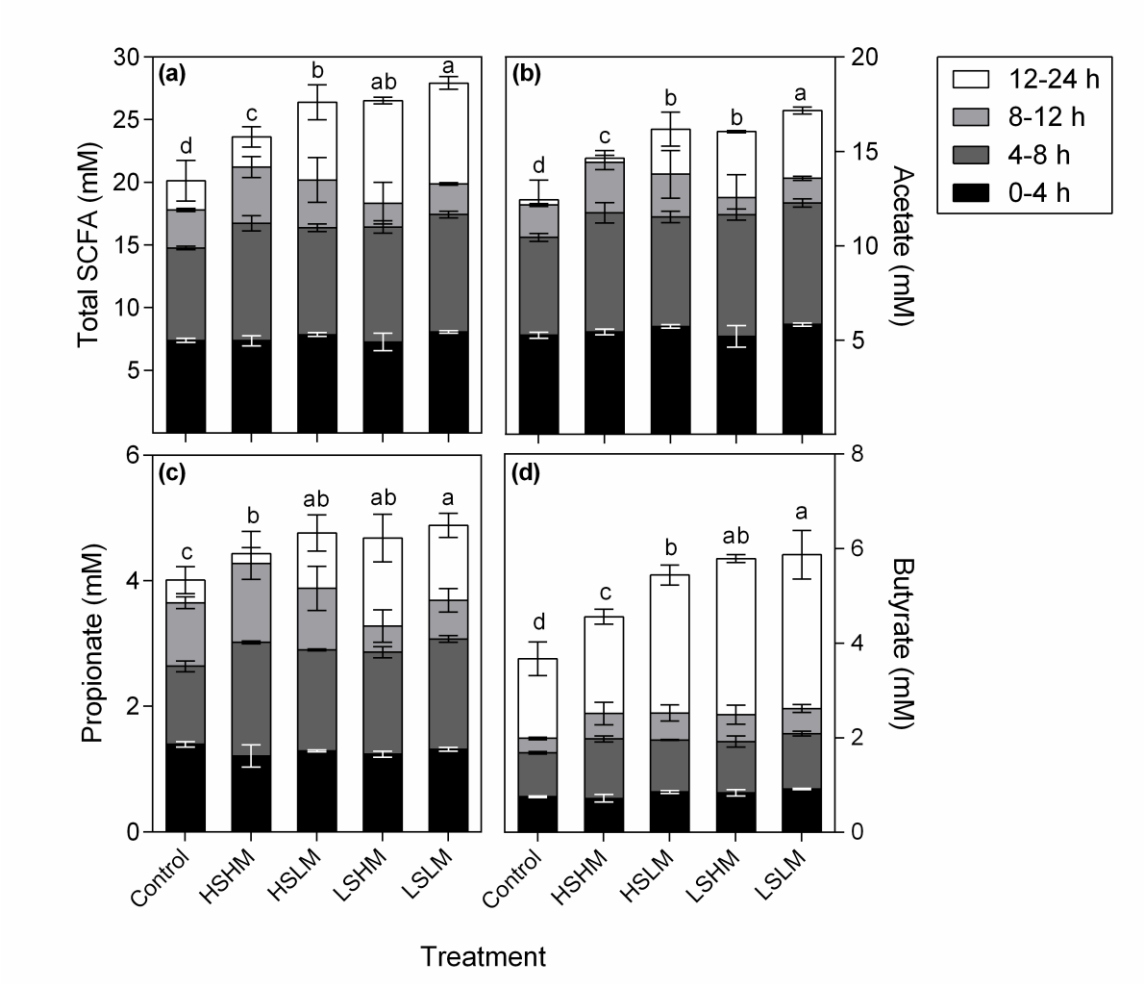

Figure 6. Short chain fatty acids production, (a) total, (b) acetate, (c) propionate and (d) butyrate during *in vitro* human fecal fermentation of extruded and unextruded (Control) wheat bran; units are expressed in millimolar concentration of SCFA produced by microbiota in the fecal slurry; error bars show standard deviation, different letters represent significant differences between treatments at 24 h of fermentation; $p < 0.05$; $n = 2$.

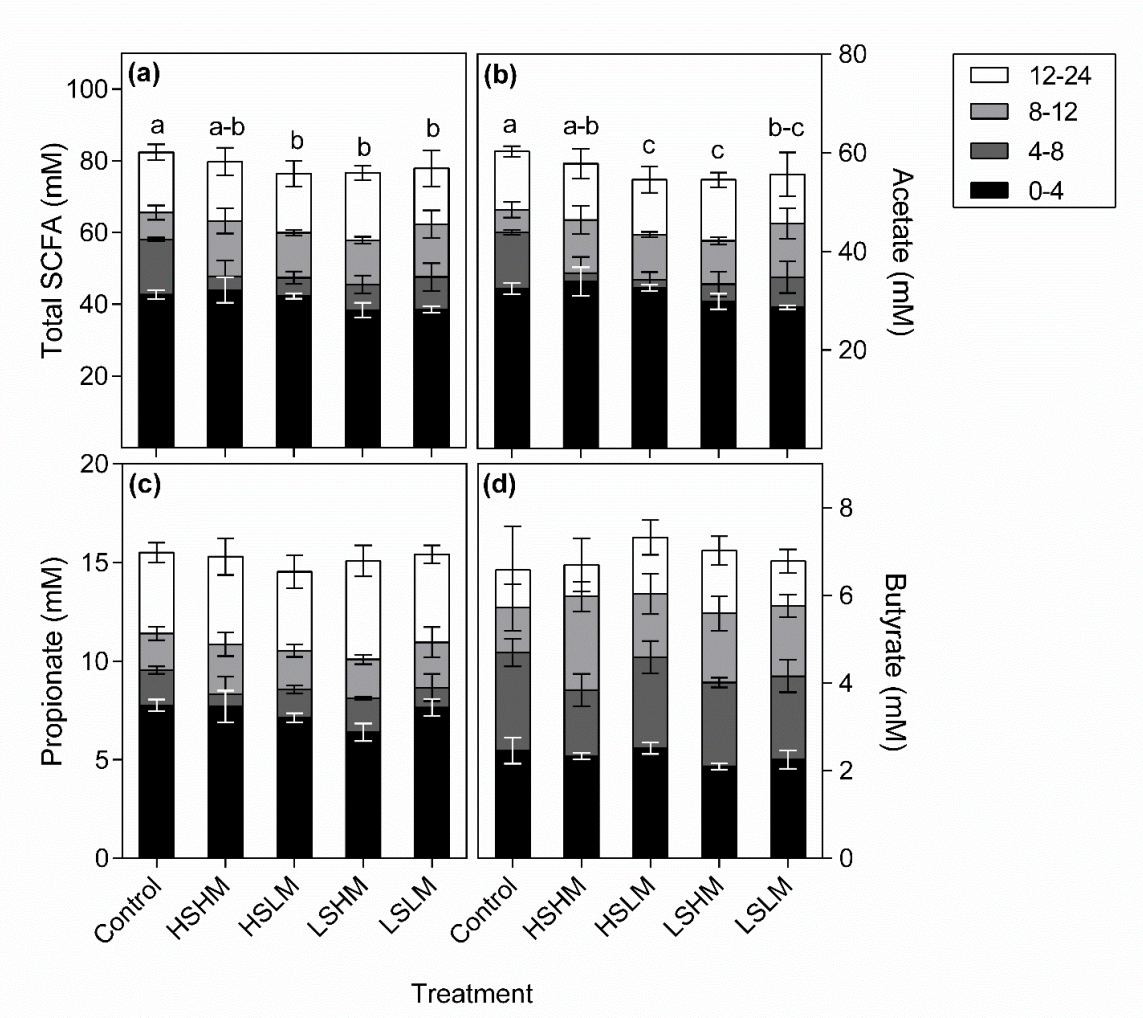

Figure 7. Short chain fatty acids production, (a) total, (b) acetate, (c) propionate and (d) butyrate during *in vitro* human fecal fermentation of whole wheat bread containing extruded and unextruded (Control) wheat bran; units are expressed in millimolar concentration of SCFA produced by microbiota in the fecal slurry; error bars show standard deviation, different letters represent significant differences between treatments at 24 h of fermentation; $p < 0.05$; $n = 2$.

CHAPTER 3. Preliminary study on element solubility of wheat bran after extrusion cooking

Abstract

Documentation of processing effects on elemental bioaccessibility from cereal sources is particularly lacking. A preliminary study on how extrusion affects phytate content and mineral elements (Cu, Fe, K, Mg, Mn, P, and Zn) solubility in wheat bran was conducted as an estimation of mineral elements available for absorption in the gastrointestinal tract. Wheat bran was extruded using combinations of low or high moisture (15% and 30% wb) and low or high screw speed (120 and 250 rpm). All extrusion conditions resulted in decreased phytic acid content. High screw speed and high moisture extrusion condition had the greatest phytic acid reduction (11.3%). Increased solubility of iron (3.51%) and magnesium (22.4%) compared with the control, were only observed in wheat bran extruded using low screw speed and low moisture. Other processing conditions resulted in decreased solubility of all mineral elements studied. A possible interaction between dietary fiber and mineral elements in wheat bran was proposed as a possible explanation for the reduced element solubility, even though phytic acid degradation during extrusion decreased. Usage of Caco-2 cell model as an estimation of element bioavailability of cereals was considered. Protease inactivation and pH control were propose as critical factors to control during experimentation with Caco-2 cells.

Key words: Bioavailability, phytic acid, element deficiency, Caco-2 cells

1. Introduction

Mineral elements accomplish essential roles in human metabolism as enzyme cofactors. Deficiency in certain mineral elements leads to an increase in reactive oxygen species, increasing the possibility of DNA damage, oxidative stress and lipid and protein oxidation. Imbalance in reactive oxygen species increase risk factors of major diseases such as cancer, atherosclerosis and cardiovascular diseases (Fang, Yang, & Wu, 2002). Magnesium and calcium deficiencies are the most common nutritional disorders in the world. Low magnesium intake has been implicated in the development of hypertension, type 2 diabetes and coronary heart disease (Combs & Nielsen, 2009) and it is estimated that 50 to 85% of the Unites States population have an inadequate intake of this element (Chaudhary, Sharma, & Bansal, 2010). Whereas, iron deficiency is estimate in 25% of the global population, around 1.6 billion people, being the first cause of anemia (Stein, 2009).

Although cereals are good sources of magnesium, potassium, phosphorus, copper, and manganese (Fardet, 2010), these mineral elements from cereal sources are poorly absorbed by the gastrointestinal tract because their bioavailability depend upon the breakdown of poorly digestible structures in which they are bound (Erdman, 1981). Mineral elements from cereals are principally located in the outer layers of the kernel and bound to cell wall components, such as lignin or phytate (Fairweather-Tait & Hurrell, 1996). In addition, the outer layers of cereal kernels are usually removed to produce refine flours and in consequence their element content is reduced (Slavin, Jacobs, & Marquart, 2001).

Degradation of phytate has been proposed to increase the element solubility in cereals, since phytate is a powerful inhibitor or iron, zinc, magnesium and manganese uptake in the body (Hurrell, 2003). Several approaches have been investigated in order to reduce phytate content in cereals. Leenhardt, Levrat-Verny, Chanliaud, & Rémésy (2005) showed that fermentation during bread making favors phytase activity when the pH drops

to 5.5, reducing phytate content. Thermal processing of cereals has also been shown reduction of phytate content in wheat bran. Kaur, Sharma, Dar, & Singh (2012) reported a decrease of 54% and 53% when wheat bran was treated using microwave (2450 MHz, 2.5 min) and steaming (110°C, 20min) respectively. Nwabueze (2007) investigated the effect of extrusion on phytate content in corn flour finding a reduction of about 33 to 44%. Furthermore, an alternative emerging technology, gamma irradiation, has been shown to be effective at reducing phytate up to 90% in aqueous solutions (Ahn, Kim, Jo, Kim, & Byun, 2004). Gamma irradiation has also shown complete degradation of phytate in velvet bean seeds treated with 15 kGy at room temperature and dose rate of 6.5 kGy/h (Bhat, Sridhar, & Tomita-Yokotani, 2007), and a reduction of up to 40% of phytate in sorghum porridge was reported using a dose of 10kGy (0.5 kGyh-1 for 20h) (Duodu, Minnaar, & Taylor, 1999).

Although, element solubility may be increased due to degradation of phytate, information about element bioavailability in food is particularly lacking. Element bio-availability depends not only on the solubility of the element but also on the potential of be absorbed in presence of a food component and/or other mineral elements (Etcheverry, Grusak, & Fleige, 2012). Mineral elements bioavailability can be estimated using Caco-2 cell models. CaCo-2 cells are human colonic adenocarcinoma cells, but behave as intestinal cells upon culture. CaCo-2 cell models allow researchers to study translocation of mineral elements and food components competition at the site of absorption as well as evaluate the speed of absorption (Etcheverry et al., 2012). However, most of the available literature is on element bio-availability in drinking water. Thus, more studies are needed to evaluate the applicability of this model for element bio-availability of foods.

In this preliminary study, the effects of extrusion on phytate content and element solubility in wheat bran were investigated. The main objective was to determine the effect

of extrusion processing variables on solubility of the most abundant mineral elements in wheat bran and phytate content.

2. Materials and Methods

2.1 Wheat bran samples and experimental design

Wheat bran samples were obtained and extruded as described before on chapter 2. Along the text extrusion conditions where denominate as low or high screw speed (LS=120 and HS=250 rpm) low or high moisture (LM=15% and HM=30% wb). Combined extrusion conditions were named first screw speed and then moisture.

2.2 Phytate content

Phytate in wheat bran was quantified as described (Haug & Lantzsch, 1983) with some modifications (Guttieri, Bowen, Dorsch, Raboy, & Souza, 2004). In a 15 mL centrifuge tubes, 100 mg of extruded or unextruded bran were mixed with 10 mL of 0.2M HCl, the tubes were then mixed in a vortex and placed on ice in a shaker water bath overnight. The next morning the tubes were centrifuge at 4000g and 4°C for 15min. The supernatants were collected into 50 mL plastic centrifuge tubes, diluted to 25 mL with 0.2M HCl and finally mixed. An aliquot of 250 µL of the diluted sample was mixed with 750 µL of 0.2M HCl into and 1 mL of ferric ammonium sulfate solution (415 µM in 0.2M HCl) into a new 15 mL centrifuge tube. The tubes were then placed into a boiling water bath for 30 min. Once the tubes reach approximately room temperature they were mixed and centrifuged at 3000 g for 30 min. One mL of the supernatant was transferred to a 15 ml glass tubes and 1.5 mL of 2,2’ bipyridine solution (10g 2,2’-bypiridine combined with 10 ml thioglycolic acid and dilute to 1 L with distilled water) was added. The absorbance of the formed color complex was measured at 530 nm. A standard curve was made using sodium phytate dodecahydrate with concentrations between 1.5 to 24 µg/mL.

2.3 Elemental analysis

Elemental analysis was performed using inductive coupled plasma mass spectrometry (ICP-MS) after wet acid hydrolysis of ~100mg of extruded or unextruded samples with 0.5 mL of nitric acid spiked with Gallium as internal standard. After overnight hydrolysis at 75°C, clear solutions were diluted 20-fold using deionized water and ran in replicate.

For soluble mineral elements an *in vitro* digestion of wheat bran was performed as described (Vitali, Radić, Cetina-Čižmek, & Vedrina Dragojević, 2010) with some modifications. In a 50 ml plastic centrifuge tube, 1 g of extruded or unextruded bran was dispersed in 7.5 mL of nanopure water, the pH was adjusted to 2 and 2.5 mL of pepsin solution (P-700, Sigma, St Louis, MO, USA; 20 gL-1 in 0.1M HCL) were added to the suspension. The tubes were placed capped in a shaker water bath at 37°C, 150rpm for 2 h. pH was then adjusted to 7.2, 12.5 mL of pancreatin-bile salt solution (8 gL-1 of pancreatin P-7545, Sigma, St Louis, MO, USA; and 10 mgL-1 bile salts S71919-1, Fisher Science, Hanover Park, IL in 0.1M NaHCO3) were added to the tubes and the incubation in the water bath continued for next 2 h. To reduce external element interferences no buffers were used during the *in vitro* digestion. The samples were centrifuged at 4,100g, 4°C for 20 min. An acid digestion was conducted mixing 500 µL of the clear supernatant and 500 µL of nitric acid spiked with Gallium as internal standard followed by overnight incubation at 75°C before running the samples for ICP-MS.

2.4 Element bio-availability using CaCo-2 Cells

Element bio-availability assays were conducted as reported before (Vitali et al., 2010) using CaCo-2 cells and the clear supernatant obtained after bran digestion in the elemental analysis procedure. Caco-2 cells were obtained from American Type Culture

Collection (Rockville, MD, USA) and used between passages 30-32. The cells were maintained in 175 cm2 culture flasks in Minimal Essential Medium with non-essential amino acids (MEM/NEAA) pH 7.4 (Fisher scientific, SH3005003, Hyclone Laboratories, Inc. South Logan, Utah), with 20% v/v fetal bovine serum (FBS) (SH3007003, Thermo Scientific Hyclone,Waltham, Massachusetts) and 1% v/v antibiotic / antimycotic solution (SV30079, Fisher Scientific,Hyclone Laboratories, Inc. South Logan, Utah). For the experiments the cells were collected from the flasks and seeded onto cell culture inserts with 0.4 µm transparent polyethylene terephthalate (PET) membrane (353090, Falcon, Corning, NY) at a density of 3x105 cells per filter. The filters were then carefully placed into the 6 well companion plates (353502, Falcon, Corning, NY) dividing the experiments in 2 compartments. The *In vitro* digested sample was placed inside the filter also called the donor compartment. The space below the filter is the second compartment also called acceptor where the mineral elements are concentrated after being transported by the cells from the sample. The experiments were performed with differentiated cells after 20 days post seeding. Before starting the experiment the clear supernatant from *In vitro* digestion was mixed with 1% v/v fetal bovine serum and 10mmol/L HEPES to inhibit the proteases. The proteases were not inactivated by heating since the effect of extrusion, a thermal treatment, was tested during this experiment. Subsequently the osmolality and the pH were adjusted to 290 ± 5 mOsm/kg and 7.4 respectively (Ekmekcioglu, Pomazal, Steffan, Schweiger, & Marktl, 1999). To start the experiment, 2.6 mL of the acceptor solution composed of 135mmol/L NaCl, 5mmol/L glucose and 10mmol/L HEPES, pH 7.4 was added to the acceptor compartment and 1.5 mL of the clear supernatant from bran digestion was added to the donor compartment. The wells were then placed in the tissue culture incubator for 2 h at culture conditions (5% CO2, 95% RH, 37°C). The integrity of the cell monolayer was determined before the experiment by determining the

transepithelial passage of phenol red as follows: 1.5 mL of Minimal Essential Medium, containing ~ 42 µM of phenol-red, was added to the donor compartment with the seed cells and 2.6 mL of phenol red-free acceptor solution was placed in the acceptor compartment. Afterward, the absorbance at 479nm of the solution in the acceptor compartment was measured in well-filter systems with and without cells. A phenol red transport rate of <0.25%/h compared to the transport without cells indicate the monolayer integrity (Ekmekcioglu et al., 1999). Unfortunately, the experiment had to be stopped and the element content of the acceptor and donor compartment was not measured due to visible disruption of the cell monolayer after 2 hour of incubation.

2.5 Data analysis

All treatments were performed in duplicate. For detecting significant differences in extruded bran phytate content compared with the control, data were analyzed using Dunnett's multiple comparison procedure. For interactions between processing conditions, data were analyzed using two factor analysis of variance (ANOVA) with speed and moisture as factors. Significant differences among samples were determined using Fisher's least significant difference (LSD) test with $\alpha=0.05$. All data were analyzed using SAS software (version 9.2, SAS Institute, Cary, NC USA).

3. Results and discussion

3.1 Phytate content

Phytate content of unextruded wheat bran (5.33% ± db) was in agreement with previous reported values (Gualberto, Bergman, Kazemzadeh, & Weber, 1997; Majzoobi, Pashangeh, Farahnaky, Eskandari, & Jamalian, 2014). Extrusion processing led to a significant decrease in phytate content in wheat bran compared to the control ($p < 0.01$). For different extrusion conditions the following values were obtained, HSHM = 4.73% ±

0.20 db, HSLM = 4.88% ± 0.11 db, LSHM = 4.94% ± 0.01 db and LSLM =5.05% ± 0.11 db. There was no significant interaction between screw speed and moisture, and only screw speed had a significant effect ($p = 0.03$) on phytate content in wheat bran. Wheat bran processed upon high screw speed condition resulted in highest reduction of phytate (11.26%) compared to the control.

Published literature about the effect of wheat bran extrusion on phytate content is divergent. Kaur, Sharma, Singh, & Dar (2013) studied the effect of temperature and moisture on phytate, reporting greatest decease (64.40%) when processing wheat bran at 115°C, 20% moisture and 400rpm in a single screw extruder. Kraler et al. (2014) reported a decrease of 7% of phytate after extrusion using a twin-screw extruder at 321 rpm with a barrel temperature from 99 to 150°C and 10.3% moisture. However, in disagreement with our results and with the results of the above mentioned studies, an early study showed no phytate degradation after extrusion of rice, oats and wheat brans when using a twin-screw extruder at 450 rpm, 163°C and 15% moisture (D G Gualberto et al., 1997). Different findings on the degree of phytate breakdown using extrusion may be due to high variability of the processing conditions. Reduction of phytate has been attributed to a thermal/chemical breakdown of the myoinositol hexa-hydrogen phosphate compound Nwabueze (2007).

3.2 Elemental analysis

Total content of mineral elements of unextruded wheat bran (Table 1) in this study were similar to results previously reported (Fardet, 2010). Variation in mineral elements content are often a result of differences in production environments and genetic factors (Zhao et al., 2009). Extrusion processing seemed not to affect mineral elements content in wheat bran (Table 1), except samples processed at high speed high moisture.

Considerable variation between replicates were detected in all studied mineral elements. Increased element content after extrusion is likely since mineral elements like iron can be released from the barrel or screw due to abrasion (Camire, 2001). However, mineral elements such as phosphorus and potassium are not expected to be release from a stainless steel equipment. The more likely explanation to the high variability between samples is a non-complete hydrolysis with nitric before sample injection in the ICP-MS. Therefore the nitric acid hydrolysis period could be extend to achieve complete disruption of wheat bran structures. Another possible explanation is the potential element contamination that could happen during extrusion processing of wheat bran, since the inside of the barrel cannot be acid washed to ensure complete material removal from previous trials. As expected, the most abundant elements in wheat bran found in this study were phosphorus, potassium and magnesium.

Extrusion of wheat bran affect solubility of all studied mineral elements but not zinc which remain insoluble even after processing (Table 2). There was a significant interaction ($p<0.05$) between moisture and screw speed on changing solubility of mineral elements in wheat bran. Low speed low moisture conditions resulted in increased solubility in all mineral elements, but only magnesium and iron were significantly different compared to the control. Using LSLM, solubility of magnesium and iron was increased to 22.36% and 3.51% respectively. Surprisingly, when HSHM, HSLM and LSHM condition were used during wheat bran extrusion, the solubility of all the studied mineral elements decreased compared to the control (Table 2). Effect of extrusion on element solubility is not well documented, to the best of our knowledge only few studies have been reported and the results cannot be compared since processing condition and food material are not similar. Hazell & Johnson (1989) reported an increase on iron solubility of 17% due to extrusion cooking during production of corn snacks. Drago, Velasco-González, Torres, González, &

Valencia, (2007) reported no significant increases on iron or zinc dializability after extrusion of bean grits compared whit the control. In animal models, Alonso, Rubio, Muzquiz, & Marzo (2001) showed that extrusion cooking of kidney beans and peas meals increase absorption of magnesium, iron, calcium, copper and phosphorus in rats, possibly because of reduction in total phytate after extrusion.

In our study the greatest phytate degradation did not occurred at the same conditions as the higher element solubility. Our results showed that high screw speed and high moisture extrusion conditions resulted in greater phytate degradation, whereas low speed and low moisture resulted in greater element solubility. Furthermore, element solubility decreased with the other extrusion conditions, whereas phytate always decrease during processing. A possible explanation to our results could be the presences of other chelating agents in wheat bran that can interfere with element solubility. For instance, Claye, Idouraine, & Weber (1998) showed that hemicellulose, lignocellulose, cellulose and lignin extracted from wheat bran have the capability of binding magnesium and calcium reducing their extractability. The same authors indicated that hemicellulose had the higher element binding capacity, compared with the rest of the fibers. Gualberto, Bergman, & Weber (1997) also described element binding capacity for copper, calcium and zinc of insoluble fiber form wheat bran in absence of phytate. They showed that dephytinized insoluble fiber from wheat bran increased its element binding capacity once it was extruded. It is possible that during our experiment we reduced phytate content but at the same time increased element binding affinity of the fibers present in wheat bran. In chapter 2 was showed that extrusion increase soluble dietary fiber but also may change the availability of wheat bran insoluble fibers. We hypothesize that mild extrusion conditions may enhance element binding capacity of the insoluble fibers without destroying their structure as might happen with harsher processing conditions.

3.3 Element bio-availability using CaCo-2 Cells

Disruption of the CaCo-cells monolayer during element bioavailability experiments may be due to different reasons. During the digestion step, proteases are added to simulate human gastric phase. Although fetal bovine serum was used to inactivate the enzymes, the concentration of the serum was calculated from an experiment for element bio-availability in drinking water. Incomplete inactivation of proteases by fetal bovine serum might occur since wheat bran also contain endogenous protease (Rani, Prasada Rao, Leelavathi, & Haridas Rao, 2001). Protease concentration may limit the enzyme-inactivation power of the fetal bovine serum. Therefore, fetal bovine serum needs to be re-calculated for protease-containing samples. Other enzymes could also be active during the incubation time with the CaCo-2 cells changing the pH of the system and resulting in detachment of the monolayer. Different configurations of the experiment with CaCo-2 cells may be useful to protect the cells during cereals element bio-availability assay. Co-culture of CaCo-2 cells and HT29-MTX human mucus-producing cells might be a solution since the generated mucus layer would protect CaCo-2 cells form changes in the environment as well form the enzymes present in the sample (Mahler, Shuler, & Glahn, 2009). Another approach that has been used consists of placing an additional dialysis membrane secured to the inside of the donor chamber just on top of the cell monolayer. The samples are then placed inside the donor chamber and the dialysis membrane protects the cells from the enzyme activity (Etcheverry et al., 2012). Any of the above mention approaches have been used to determine element bio-availability in cereals, thus more research is needed to validate the methods.

4. Conclusion

Increased total element content in wheat bran after extrusion cooking is likely due to a possible element release from the barrel or screw due to abrasion or cross contamination from past trials. Future experiments of extrusion effect on element solubility should minimize element contamination by stablishing a cleaning procedure between samples or through the use of an internal standard using the salt of the element on interest. The internal standard will be incorporated to a specific mass of sample into the water using to fix moisture content. Contamination due to processing will be calculated at the end of the extrusion subtracting from the total element content the initial content plus internal standard content.

Phytate content was reduced in wheat bran during extrusion cooking at every level of the studied conditions. Greatest phytate reduction resulted after extrusion of wheat bran at high screw speed and high moisture. Reduction of phytate did not correlate with increased solubility of mineral elements in wheat bran after extrusion. Extrusion may or may not increase element solidity in wheat bran depending on the extrusion conditions. Low screw speed and low moisture conditions increase mainly solubility of magnesium and iron, whereas, LSHM, HSHM and HSLM extrusion conditions decrease solubility of all studied mineral elements compared to the control. Extrusion decreased phytate a chelating agent, but may improve element binding of others wheat bran components such as fiber. More research is need in order to establish a possible relationship between fiber structure and element binding capacity. In order to have a better approximation to physiological conditions, Caco-2 cells models, animal or human trials are need to estimate element bio-availability in cereals and cereal-based products. More documentation is needed to assess the applicability of CaCo-2 cells model.

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Table 1. Total elemental analysis of extruded and unextruded wheat bran

	Mg (mg/g)	K (mg/g)	P (mg/g)	Cu (µg/g)	Mn (µg/g)	Fe (µg/g)	Zn (µg/g)
Control	5.12 ± 0.36 b	13.09 ± 0.80 b	13.76 ± 0.99 b	27.44 ± 1.56 b	137.48 ± 8.77 b	248.46 ± 14.90 c	229.81 ± 13.57 b
HSHM	11.75 ± 5.80 a	30.28 ± 14.97 a	31.93 ± 15.90 a	71.68 ± 36.42 a	330.32 ± 161.44 a	489.56 ± 221.95 a	520.83 ± 244.30 a
HSLM	8.27 ± 1.11 ab	20.90 ± 2.89 ab	22.31 ± 3.22 ab	47.46 ± 5.93 ab	222.65 ± 28.70 ab	434.94 ± 30.02 ab	351.70 ± 42.22 ab
LSHM	6.79 ± 3.52 b	17.37 ± 9.21 b	18.63 ± 9.80 b	38.86 ± 15.84 b	185.55 ± 94.12 b	288.77 ± 146.76 bc	303.22 ± 154.76 b
LSLM	4.86 ± 0.23 b	12.11 ± 0.58 b	13.09 ± 0.10 b	28.89 ± 3.00 b	130.67 ± 6.04 b	243.98 ± 15.39 c	210.16 ± 12.34 b

Means of duplicates ± standard deviation; means by column with different letters are significantly different ($p < 0.05$). Values in element milligram per gram of wheat bran

Table 2. Soluble mineral elements in extruded and unextruded wheat bran (%)

	Mg	K	P	Cu	Mn	Fe
Control	27.72 ± 5.44 b	81.36 ± 9.78 ab	49.81 ± 6.85 a	78.23 ± 7.78 a	4.23 ± 2.99 a	5.11 ± 1.80 b
HSHM	13.03 ± 7.12 b	45.59 ± 22.35 c	25.95 ± 13.53 b	40.66 ± 18.63 b	0.97 ± 0.10 c	0.02 ± 0.04 d
HSLM	26.58 ± 8.75 b	75.94 ± 21.97 b	48.15 ± 16.09 a	70.86 ± 23.79 a	1.79 ± 0.59 bc	2.08 ± 2.41 c
LSHM	13.78 ± 0.13 b	43.88 ± 0.02 c	25.93 ± 0.12 b	49.29 ± 1.07 b	0.88 ± 0.01 c	NS
LSLM	50.08 ± 21.17 a	99.60 ± 1.47 a	58.44 ± 0.68 a	85.35 ± 1.59 a	3.31 ± 1.56 ab	8.62 ± 0.31 a

Means of duplicates ± standard deviation; means by column with different letters are significantly different ($p < 0.05$); values in soluble element percentage base on total element present in wheat bran; NS = non soluble mineral element

GENERAL CONCLUSIONS

The present document evaluated different thermal processing strategies, either extrusion or cooking-freezing cycles, to improve health impact of whole grains. It was possible to manipulate native components in whole wheat flour and wheat bran to increase resistant starch (RS), dietary fiber solubility and beneficial metabolites form fiber bacterial fermentation. However, the effect of thermal processing on mineral elements bioavailability remains unclear.

Fulfilling the first objective, increasing cooking-freezing cycles increased RS from 1.03% to 8.07% during *in vitro* starch digestion. All short chain fatty acids (SCFA), especially propionate, increased during human fecal fermentation as result of the thermal treatment, although butyrate, the SCFA with more diverse role in the human metabolism, was increased only during the first 8 h of fermentation. A possible exhaustion of resistant starch, particularly butyrogenic, during the first half of fermentation could be the reason of limited butyrate production later in fermentation. Modest increases in the RS content of whole wheat flour were effective modulating the metabolic activity of gut microbiota increasing production of beneficial metabolites.

Extrusion of wheat bran increased dietary fiber solubility, which enabled greater fermentability by human fecal microbiota. More severe extrusion conditions, low screw speed and low moisture, resulted in a 3-fold increase in soluble dietary fiber as well as a 1.4-fold increase in SCFA production during fermentation compared with unextruded bran. Bread making increased fermentation of mainly arabinan and galactan. However, whole wheat breads containing extruded bran did not show increases of either fiber solubility or SCFA production compared to the control. Further experiments were

proposed to evaluate changes in dietary fiber solubility during breadmaking when the percentage of extruded bran is increase in the formulation.

The extrusion effect on element solubility and phytate content was evaluated. Extrusion may or may not increase element solubility in wheat bran depending on the extrusion conditions. All extrusion conditions resulted in decreased phytate content. High screw speed and high moisture extrusion condition had the greatest phytate reduction (11.3%). When wheat bran was extruded using low screw speed and low moisture, only iron and magnesium solubility increased (3.51% and 22.4% respectively) compared with the control,. Other processing conditions resulted in decreased solubility of all elements studied. A possible interaction between dietary fiber and elements in wheat bran was proposed as a possible explanation for the reduced element solubility, even though phytate degradation during extrusion decreased. More research is need in order to establish a possible relationship between fiber structure and element binding capacity.

APPENDICES

Appendix A. Evaluation of baking quality of whole wheat flour containing extruded and unextruded wheat bran.

1. Mixing properties of dough

Protein (LECO system, N factor: 5.85) and moisture content of whole wheat flour containing extruded and unextruded wheat bran were measured. The moisture content were then adjusted to 14%. The preliminary water absorption was determined following the approved method 54-40 (AACC International, 2013) using the equation:

$$Y = 1.5X + 43.6$$

Where X is the percent of flour protein content (14% moisture basis) 13.13% and Y is the water absorption 63.30%. To the obtained preliminary water absorption 1.8% was added to adjust water absorption for whole wheat flour (65.09%) (Bruinsma, Anderson, & Rubenthaler, 1978). The adjusted water absorption was used to calculate mixing time in the mixograph for all samples. Each sample was run in triplicate and the average of the peak time was used as the mixing time during baking (Table 1).

2. Evaluation of baking quality

All quality test of bread loaves were made right after one hour of cooling process. Loaf volume of each bread was measure using rapeseed displacement test. Then each loaf was sliced using an electric knife and a cutting guide to obtain slices of about 12.5 mm tick. Appearance analysis was perform using a C-Cell instrument. Three slices per loaf were analyzed and the means were recorded (Table 1). Bread firmness were measured following the approved method 74-09 (AACC International, 2013).

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Table 1. Analysis of whole wheat dough and bread loaves made with extruded or unextruded wheat bran

		Treatment				
	Analysis parameter	Control	HSHM	HSLM	LSHM	LSLM
Dough	Mixing time (min)	5.30 ± 0.20	5.31 ± 0.19	5.42 ± 0.72	5.45 ± 0.36	4.94 ± 0.38
	Weight loss during proofing (g)	3.11 ± 0.11	3.11 ± 0.39	3.06 ± 0.37	2.86 ± 0.80	3.05 ± 0.12
	Weight loss during baking (g)	28.77 ± 2.32	25.34 ± 2.37	25.87 ± 1.08	23.29 ± 4.25	26.84 ± 0.97
Bread	Loaf volume (cc)	528 ± 47	480 ± 45	486 ± 34	498 ± 39	520 ± 26
	Bread firmeness (N)	3.38 ± 0.68	4.44 ± 0.46	4.43 ± 0.99	4.51 ± 1.04	3.49 ± 1.09
	Slice area (mm2)	4191 ± 350	4242 ± 462	3846 ± 210	4242 ± 147	4314 ± 260
	Slice height (mm)	75.9 ± 6.8	76.7 ± 6.8	70.7 ± 4.8	79.5 ±2.5	80.1 ± 3.1
	Slice brightness	81.5 ± 1.87	79.3 ± 3.6	75.9 ± 0.64*	74.9 ± 2.6*	75.2 ± 2.9*
	Number of cells	2616 ± 250	2698 ± 324	2468 ± 208	2535 ± 98	2356 ± 132
	Cell diameter (mm)	1.94 ± 0.05	1.85 ± 0.06	1.91 ± 0.10	2.07 ± 0.10	2.07 ± 0.27
	Average cell elongation (mm)	1.72 ± 0.08	1.74 ± 0.04	1.73 ± 0.13	1.80 ± 0.10	1.80 ± 0.04

Values represent mean ± standard error; * significantly different from the corresponding control for the same analysis parameter ($p < 0.05$) adjustment for multiple comparison Dunnett; HSHM, HSLM, LSHM and LSLM dough or bread loaves made with wheat bran extruded using combinations of high (HS) or low (LS) speed and high (HM) or low (LM) moisture. Control was whole wheat dough or bread made with unextruded wheat bran; flour protein content (14% moisture basis) 13.13%.