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

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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 °C, 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* fecal 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.

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 fibers and the types of metabolites that are produced upon fermentation of these substrates.

End products of dietary fiber 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; 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,

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Abbreviations: RS, resistant starch; SCFA, short chain fatty acids
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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 fibers, containing 9–17% total dietary fiber (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 fiber 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 fiber 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 resists 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* fecal 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 fiber (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. Those 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 (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 was 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) was 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 milliliter 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 4000 *g* 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 (3500 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 (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 *fecal fermentation and analysis*

In vitro batch fecal fermentation was performed according to Hartzell, Maldonado-Gómez, Hutkins, and Rose (2013). In short, 100 mg of digested, freeze-dried material was suspended in 9 mL sterile fermentation medium consisting of (per liter) 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), $NaHCO_3$ (2 g), NaCl (0.1 g), K_2HPO_4 (0.08 g), $MgSO_4.7H_2O$ (0.01 g), $CaCl_2.6H_2O$ (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 fecal samples were collected from 3 healthy adults with no history of gastrointestinal abnormalities and having not taken antibiotics in the last 6 months. The fecal slurry was prepared by blending the fecal 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 fecal 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 microcentrifuge tube containing 0.1 mL of 7 mM 2-ethylbutyric acid (internal standard) in 2 M 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% H_2, 5% CO_2, and 90% N_2. As soon as all samples were taken at each time point, microcentrifuge 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 H_2SO_4. Then 0.5 mL of diethyl ether was 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 microliter 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 h (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 measure 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

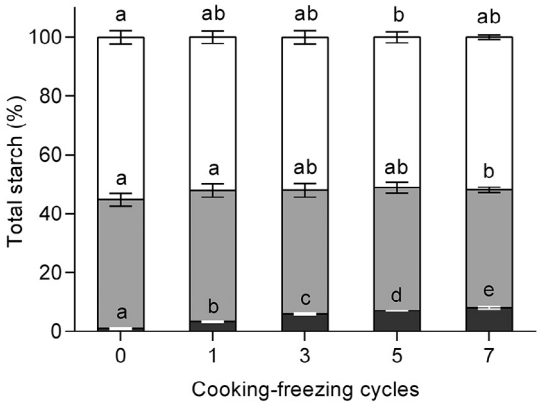

Fig. 1 – Rapidly digested starch (white bars), slowly digested starch (gray bars), and resistant starch (black bars) in whole wheat flour after subjecting the flour to different numbers of cooking–freezing cycles; different letters represent significant difference between cycles within each starch digestible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

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 reported 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, Baik, and Lim (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, Hu, Xu, Jin, and Tian (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 studies mentioned earlier 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* fecal 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).

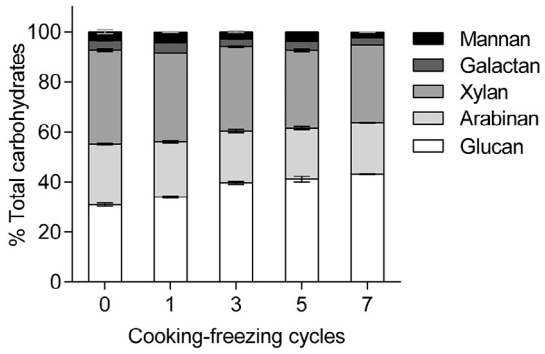

Fig. 2 – Non-digestible polysaccharide residues present in the dialyzed residues after *in vitro* digestion of whole wheat flour; error bars show standard deviation; *n* = 3.

3.2. In vitro *fermentation*

During the *in vitro* fecal 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; 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 produced in a greater ratio than propionate or butyrate during dietary fiber 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 fecal microbiota composition among studies. Interestingly, *in vitro* fecal 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 fibers in wheat. Future studies could explore this possibility by measuring the residual dietary fibers 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 et al., 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 increased 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

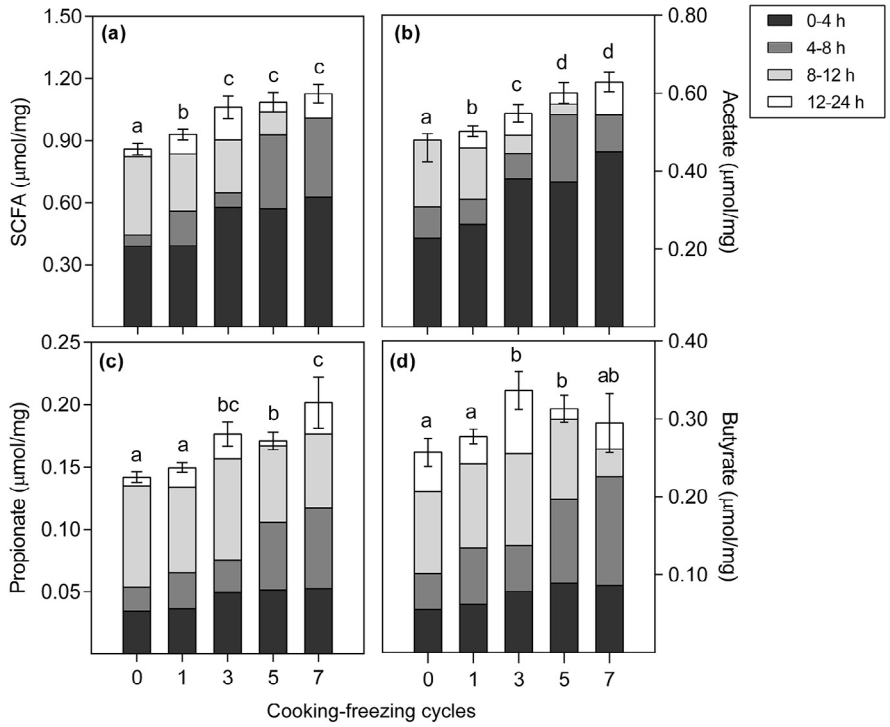

Fig. 3 – Total short chain fatty acids (SCFA; a) and individual SCFA (b, acetate; c, propionate; d, butyrate) produced during fermentation of whole wheat flour that had been subjected to different cooking–freezing cycles and *in vitro* digestion; units are expressed as μmol produced by the microbiota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

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 on the presence of fermentable carbohydrate substrate and decrease rapidly with inadequate fermentable carbohydrate supply (David et al., 2014; Russell et al., 2011).

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 in 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 fecal *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

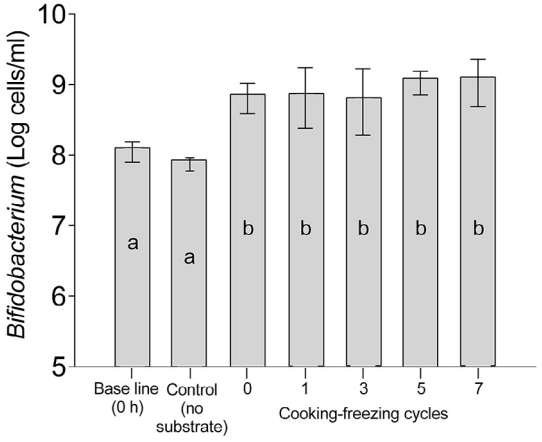

Fig. 4 – *Bifidobacterium* concentration in fecal inoculum and after 24 h of *in vitro* fermentation of pre-digested whole wheat flour; error bars show standard deviation; different letters represent significant differences among samples; $n = 3$; $p < 0.05$.

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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