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FOOD MICROBIOLOGY

Processing Has Differential Effects on Microbiota-Accessible Carbohydrates in Whole Grains during *In Vitro* Fermentation

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ABSTRACT Whole grains are generally low in nondigestible carbohydrates that are available for fermentation by the gut microbiota, or microbiota-accessible carbohydrates (MAC). However, there is potential to increase MAC in whole grains through food processing. Five processing methods: boiling, extrusion, sourdough bread, unleavened bread, and yeast bread, were applied to whole wheat flour and then subjected to *in vitro* digestion followed by fermentation using fecal microbiomes from 10 subjects. The microbiomes separated into 2 groups: those that showed high carbohydrate utilization (CU) and those that exhibited low CU. The former exhibited not only enhanced CU but also increased butyrate production (MAC, 31.1 ± 1.1% versus 19.3 ± 1.2%, $P < 0.001$; butyrate, 5.26 ± 0.26 mM versus 3.17 ± 0.27 mM, $P < 0.001$). Only the microbiomes in the high-CU group showed significant differences among processing methods: extrusion and sourdough bread led to dichotomous results for MAC and short-chain fatty acid production, where extrusion resulted in high MAC but low butyrate production while sourdough bread resulted in low MAC but high butyrate production. Extrusion led to a noticeable decrease in α-diversity and some members of the families *Ruminococcaceae* and *Lachnospiraceae*, with increases in *Acinetobacter*, *Enterococcus*, and *Staphylococcaceae*. This study demonstrated that only microbiomes that exhibited high CU responded to the effects of processing by showing significant differences among processing methods. In these microbiomes, extrusion was able to increase accessibility of the cell wall polysaccharides but did not increase butyrate production. In contrast, sourdough bread led to high butyrate production by supporting important butyrate-producers in the families *Lachnospiraceae* and *Ruminococcaceae*.

IMPORTANCE Dietary nondigestible carbohydrates, or dietary fiber, have long been recognized for their beneficial health effects. However, recent studies have revealed that fermentation of nondigestible carbohydrates by gut bacteria is critical in mediating many of the health-promoting properties of dietary fibers. Whole grains are excellent candidates to supply the microbiome with a plentiful source of nondigestible carbohydrates, although unfortunately a majority of these carbohydrates in whole grains are not available to gut bacteria for fermentation. Processing is known to alter the structural characteristics of nondigestible carbohydrates in whole grains, yet the relationship between these effects and gut microbial fermentation is unknown. This research aimed to address this important research gap by identifying interactions between whole-grain processing and gut bacteria, with the ultimate goal of increasing the availability of nondigestible carbohydrates for fermentation to enhance host health.

KEYWORDS *Lachnospiraceae*, *Ruminococcaceae*, butyrate, dietary fiber, wheat

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Dietary, nondigestible carbohydrates, mainly from dietary fiber, are the major energy sources for the microbiome (1). Fermentation of these carbohydrates by the microbiota results in numerous metabolites, including short-chain fatty acids (SCFA), that are absorbed by the host and have pleiotropic local effects on intestinal function as well as systemic roles in insulin secretion, lipid metabolism, and inflammation, among others (2–4).

Unfortunately, a large proportion of nondigestible carbohydrates in whole grains are not metabolically available to gut microbes for fermentation (5–7). Human feeding trials have suggested that only about one-third of the nondigestible carbohydrates in grains are accessible for fermentation by the microbiota, compared with 75 to 90% for fruit and vegetable fibers (6). Therefore, although whole grains are rich sources of nondigestible carbohydrates, they may not be good sources of microbiota-accessible carbohydrates (MAC) (8).

Processing has been proposed as a way to manipulate carbohydrate utilization (CU) by the microbiome (9). For example, boiling is a minimal form of grain processing that involves moderate temperatures (100°C), atmospheric pressure, minimal shear, and an abundance of water. During boiling, water swells the starch granules, resulting in gelatinization, but the structure of the principal nondigestible carbohydrates, β-glucan and arabinoxylan, appears to be unaffected (10). In contrast, extrusion is a severe form of processing that uses high heat (>120°C), pressure, and mechanical shear at low moisture contents to process grains. This process results in not only gelatinization but also partial depolymerization of the starch and nondigestible carbohydrates (10). Furthermore, extrusion enables the conversion of some insoluble fibers to soluble fibers, which exhibit increased fermentability (11). Accordingly, extrusion has been shown to increase the metabolism of dietary fibers from grains by gut bacteria both *in vitro* using human fecal microbiota (12) and *in vivo* in rat feeding studies (13).

Another form of grain processing, bread making, is the most common processing technique for wheat (14). The breadmaking process has been shown to increase soluble dietary fiber at the expense of insoluble fiber (15). Spontaneous fermentation of wheat bran increased solubility of arabinoxylan with a lower average degree of arabinose substitution than native bran, which is thought to be more easily degradable by intestinal microbiota (16). Bread making techniques were compared in whole-grain rye bread, yeast-fermented crispbread, unfermented crispbread, and sourdough bread (10). In all breads, β-glucan and arabinoxylan were distributed as small fragments throughout the starch matrix. The unfermented crispbread had larger pieces of bran and intact cell structures compared with the other breads. For sourdough, starch granules were less swollen and surrounded by more leached amylose, which was proposed to act as a protective layer against starch hydrolysis during digestion. Furthermore, prefermentation of wheat bran with yeast and enzymes in bread making resulted in increased SCFA production during *in vitro* fermentation compared with no prefermentation (17).

Clearly, the variety of processing methods of grains affect the chemical and physical structures of nondigestible carbohydrates in differential ways. While most of these chemical and physical changes would likely increase MAC, no studies have compared processing methods to determine which method might result in the greatest improvement in CU by the microbiome. Thus, the purpose of this study was to evaluate how different common food processing techniques change MAC in whole grains and shift the microbiota during *in vitro* fermentation.

(This research was conducted by Caroline Smith in partial fulfillment of the requirements for a Master of Science degree from the University of Nebraska–Lincoln [18].)

RESULTS

Whole-wheat processing and digestion. Because grain processing is known to affect the physical and, in some cases, the chemical composition of the nondigestible carbohydrates in whole grains, whole-wheat flour was processed using five common grain processing methods—boiling, extrusion, and making sourdough bread, unleavened bread, and yeast bread—to determine how these processes may affect MAC and

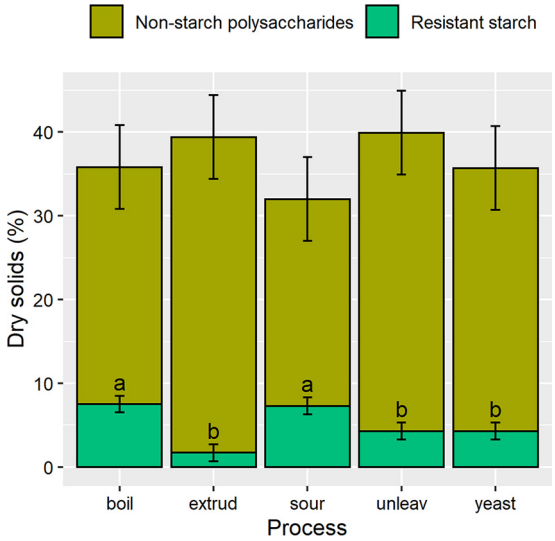

FIG 1 Carbohydrates remaining after *in vitro* digestion of processed whole-wheat flour. Boil, boiled wheat; extrud, extruded wheat; sour, sourdough bread; unleav, unleavened bread; yeast, yeast-leavened bread. Different letters (a, b) indicate significant differences among processing methods.

thus change CU by the microbiome during fermentation. As expected, processing did not significantly affect the total carbohydrate concentration among samples. All samples contained a mean of 61.6% total carbohydrate (range, 56.5% to 66.7%), of which 83% was starch and 17% was nonstarch polysaccharides. Following processing, whole-wheat samples were subject to *in vitro* digestion and dialysis to remove digestible sugars, starch, and protein. After digestion, the freeze-dried, processed wheat samples contained similar concentrations of total nondigestible carbohydrates, but with significant differences in resistant starch due to processing: boiled wheat and sourdough bread had more residual starch than the other products (Fig. 1).

Microbiota-accessible carbohydrates in processed whole-wheat samples. Different grain processing methods resulted in significant differences in MAC in processed whole-wheat samples during *in vitro* fermentation using microbiomes collected from 10 subjects (Fig. 2A). Extrusion resulted in higher levels of MAC than boiling wheat and making sourdough bread.

Examining the results by microbiome, it was evident that CU across 5 microbiomes (1, 3, 4, 9, 10) was higher than that of the other 5 microbiomes (2, 5–8) (Fig. 2B). Indeed, the microbiomes exhibiting high CU fermented 31.1 $\pm$ 1.1% of the total carbohydrates during fermentation, compared with only 19.3 $\pm$ 1.2% for the low-CU microbiomes ($P < 0.001$). Thus, microbiomes 1, 3, 4, 9, and 10 were termed "high-CU" and 2, 5, 6, 7, and 8 were termed "low-CU" microbiomes in some subsequent analyses.

An analysis of the proportion of carbohydrates fermented by CU group revealed that the significant differences among processing methods were driven by the high-CU microbiomes; the microbiomes with low CU showed no significant differences among processed samples (Fig. 2C). Notably, however, the differently processed samples ranked the same across CU groups (i.e., extrusion > yeast > unleavened > boiling > sourdough). Therefore, although both CU groups responded similarly to the processed samples, the high-CU microbiomes responded to a greater magnitude than the low-CU microbiomes did.

Comparison of fecal microbiota composition between high- and low-carbohydrate-utilization groups. Because MAC utilization was the primary outcome of this research, it was relevant to identify features of the fecal microbiota that were associated with high CU during *in vitro* fermentation of the processed grains. First, the relationship between diversity metrics and CU group was examined. Principal-coordinate analysis based on the Bray-Curtis β-diversity distance matrix did not group, cluster, or separate

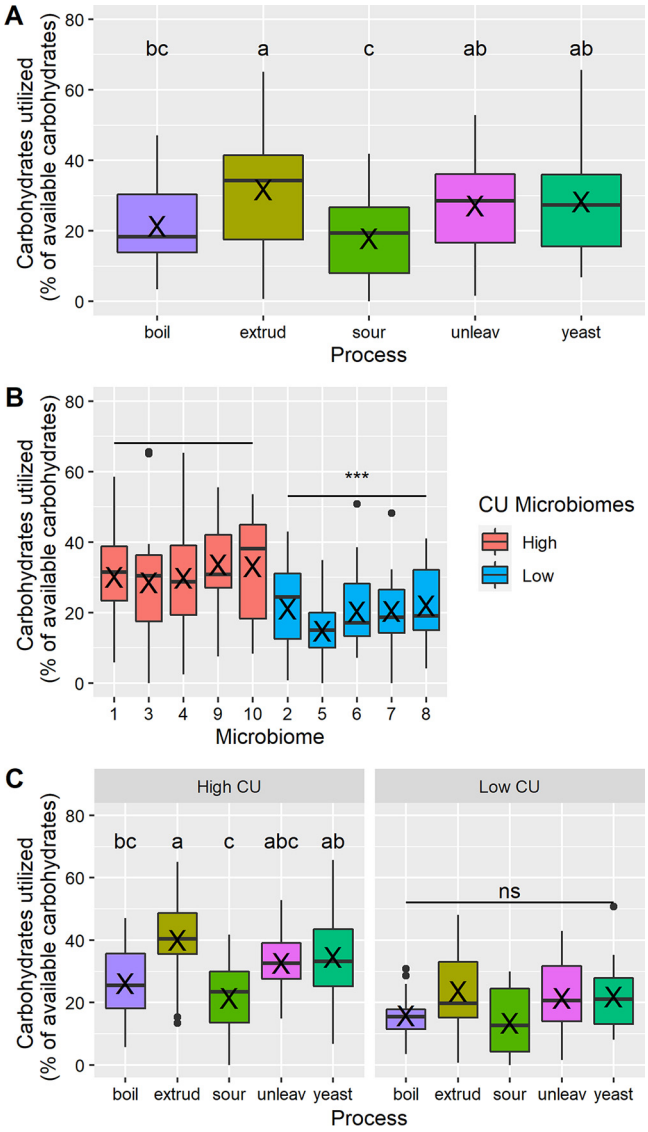

FIG 2 Microbiota-accessible carbohydrates in processed whole-wheat samples. Results are presented according to processing method (A), microbiome (B), and processing method and carbohydrate utilization (CU) group (C). Boil, boiled wheat; extrud, extruded wheat; sour, sourdough bread; unleav, unleavened bread; yeast, yeast-leavened bread. Within box plots, X indicates the mean. Different letters (a, b, c) indicate significant differences among processing methods. ns, no significant differences among processing methods; ***, $P < 0.001$ for high-CU group versus low-CU group.

microbiomes (0 h fermentation) based on CU group (Fig. S1A). However, α-diversity was different among microbiome groups: high-CU microbiomes had significantly more amplicon sequence variants (ASVs) and were more phylogenetically diverse than the low-CU microbiomes (Fig. 3A). Shannon diversity was not significantly different between high- and low-CU microbiomes.

Microbiota composition was compared between the high- and low-CU groups. There were 154 differential ASVs between the high- and low-CU microbiomes, with 97 ASVs being significantly associated with the high-CU microbiomes and 57 associated with the low-CU microbiomes (Fig. 3C). There was one ASV in each of the three low-abundance phyla, *Cyanobacteria*, *Synergistetes*, and *Verrucomicrobia*, that was associated with the high-CU microbiomes. Most of the differentially abundant ASVs were from the phyla *Bacteroidetes* and *Firmicutes*.

Within the phylum *Bacteroidetes*, ASVs assigned to *Butyricimonas*, *Odoribacter*, and *Alistipes* and several unassigned genera were associated with the high-CU microbiomes,

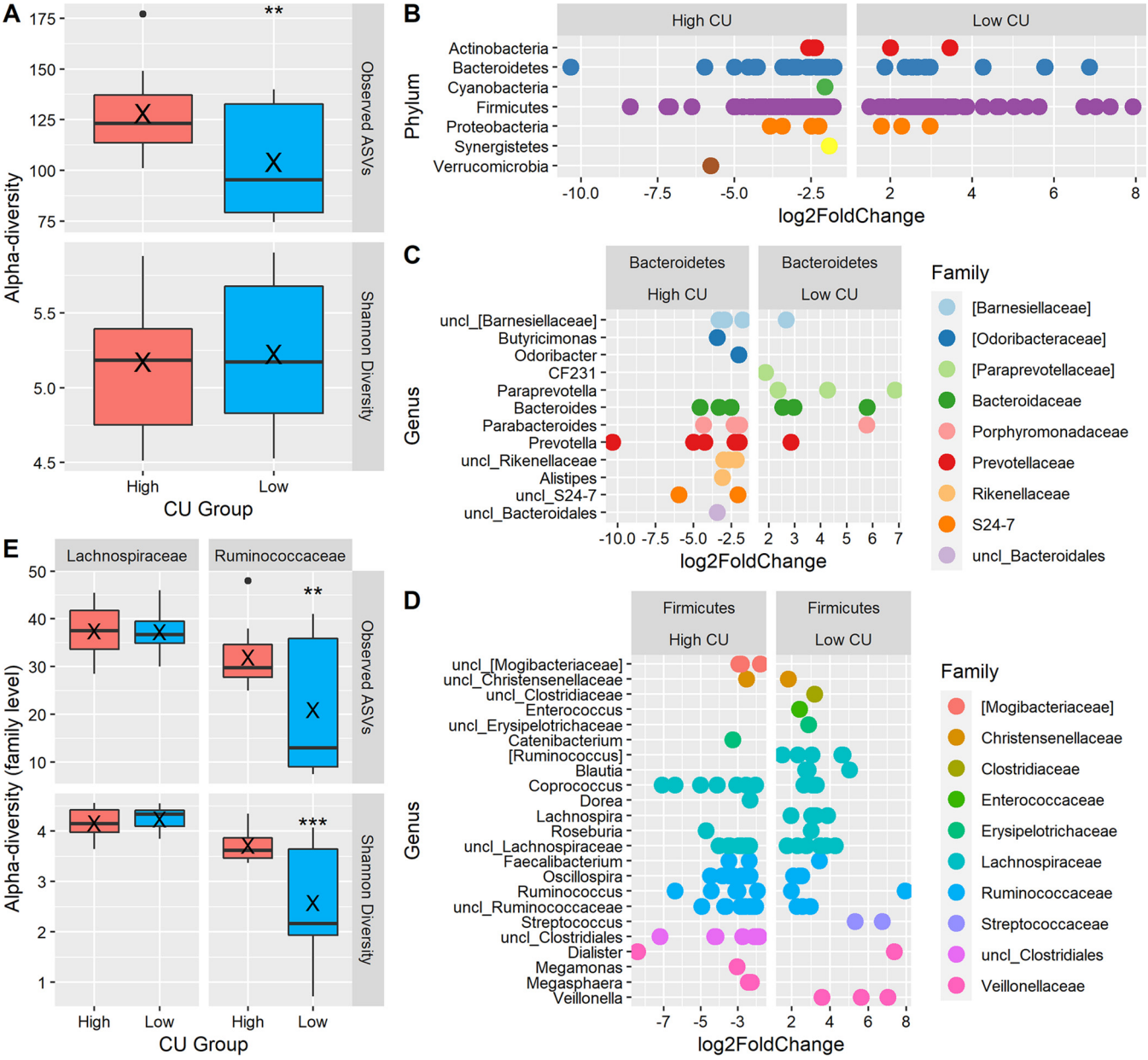

FIG 3 Comparison of fecal microbiota composition between high- and low-carbohydrate-utilization (CU) groups. (A) α-diversity; (B to D) differentially abundant ASVs by phylum (B) and within the phyla *Bacteroidetes* (C) and *Firmicutes* (D); (E) α-diversity of the *Lachnospiraceae* and *Ruminococcaceae* families (E). Boil, boiled wheat; extrud, extruded wheat; sour, sourdough bread; unleav, unleavened bread; yeast, yeast-leavened bread. Within box plots, X indicates the mean. **, $P < 0.01$, and ***, $P < 0.001$, for high-CU group versus low-CU group.

while CF231 (a member of the *Paraprevotellaceae*) and *Paraprevotella* were associated with the low-CU microbiomes (Fig. 3D). The high-CU microbiomes also had five differentially abundant ASVs in the genus *Prevotella*, while the low-CU microbiomes had only one differentially abundant ASV in this genus. Indeed, at the genus level, the high-CU group had significantly higher relative abundance of *Prevotella*, while the low-CU group had higher *Bacteroides* abundance (*Prevotella*: high CU, 17.5% $\pm$ 0.9%, versus low CU, 0.9% $\pm$ 0.5%, Wilcoxon $P = 0.002$; *Bacteroides*: high CU, 12.0% $\pm$ 2.1%, versus low CU, 24.2% $\pm$ 3.8%, Wilcoxon $P = 0.03$).

Within the phylum *Firmicutes*, the composition within the most abundant families, *Lachnospiraceae* and *Ruminococcaceae*, was very different between the high- and low-CU microbiomes (Fig. 3E). Within the family *Lachnospiraceae*, the high-CU micro-

biome had one differential ASV in the genus *Dorea* but also had seven differentially abundant ASVs in the genus *Coprococcus*, compared with three in the low-CU group. The low-CU microbiomes had differentially abundant ASVs in *Ruminococcus*, *Blautia*, and *Lachnospira*. Each CU group had seven unassigned *Lachnospiraceae* that differed between the two groups. Within the family *Ruminococcaceae*, the high-CU microbiomes had 30 differentially abundant ASVs, compared to only 9 in the low-CU group. The differential ASVs in the high-CU microbiomes were mainly from *Oscillospira*, *Ruminococcus*, and unassigned genera. Apart from the families *Lachnospiraceae* and *Ruminococcaceae*, ASVs assigned to *Catenibacterium*, *Megamonas*, and *Megasphaera* and an unassigned member of the *Mogibacteriaceae* were associated with high-CU microbiomes, while ASVs from *Enterococcus*, *Streptococcus*, *Veillonella*, and a few unassigned genera were associated with low-CU microbiomes.

Since there were evident differences at the ASV level between CU groups within the families *Lachnospiraceae* and *Ruminococcaceae*, we examined the diversity and abundance of each family. The relative abundances of these two families were not significantly different between the high-CU and low-CU microbiomes (*Lachnospiraceae*: high CU, 22.5% $\pm$ 2.5%, versus low CU, 28.1% $\pm$ 1.3%, Wilcoxon $P = 0.1$; *Ruminococcaceae*: high CU, 21.0% $\pm$ 2.1%, versus low CU, 21.2% $\pm$ 3.7%, Wilcoxon $P = 0.9$). Likewise, the α-diversity within the family *Lachnospiraceae* was not different between the two CU groups; however, *Ruminococcaceae* was significantly more diverse in the high-CU group than the low-CU group by all α-diversity metrics analyzed (Fig. 3B).

Relationships among grain processing method, carbohydrate utilization microbiome group, and microbiome diversity after fermentation. As seen for the fecal samples, principal-coordinate analysis based on the Bray-Curtis β-diversity distance matrix did not group, cluster, or separate microbiomes based on grain processing method or CU group after fermentation (Fig. S1B and C). However, to determine if there were differences in the magnitude of shift in microbiota composition during fermentation among processing methods or CU groups, we examined Bray-Curtis distance between the fecal samples and the fermented samples. Extrusion of wheat caused the greatest shift in microbiota composition, measured in terms of Bray-Curtis distance from the fecal sample, among processing methods, and this shift was significantly greater than that seen with boiled wheat and unleavened bread (Fig. 4A). Between CU groups, the low-CU microbiomes had greater shifts in composition during fermentation than the high-CU microbiomes (Fig. 4B).

Extrusion resulted in lower α-diversity after fermentation than unleavened bread (Fig. 4C). The other processing methods fell between these two processing methods. The high-CU microbiomes were more diverse than the low-CU microbiomes by all diversity metrics analyzed (Fig. 4D), including Shannon diversity, which was not different between the fecal microbiomes (Fig. 3A).

Diversity within the families *Lachnospiraceae* and *Ruminococcaceae* was also examined across processing methods and between CU groups. Within the family *Lachnospiraceae*, grain processing resulted in significant differences in observed ASVs and Shannon diversity, with extrusion supporting generally lower diversity than the other samples (Fig. 4E). Extrusion also supported lower diversity in the family *Ruminococcaceae* using observed ASVs. The high-CU microbiomes were more diverse than the low-CU microbiomes by all diversity metrics analyzed, including *Lachnospiraceae* diversity (Fig. 4F), which was not different between the fecal microbiomes (Fig. 3B).

Changes in microbiota composition during fermentation. Fermentation resulted in 31 differentially abundant ASVs across the five grain processing methods. In a clustered heat map generated using these 31 differential ASVs, the minimally processed samples of boiled wheat and unleavened bread clustered together and the fermented breads (yeast and sourdough) clustered together, with extruded wheat being separated from the other samples (Fig. 5A). The 31 differential ASVs could be grouped into five clusters, each representing ASVs that distinguish the five processing methods. The first 2 clusters contained the ASVs associated with the minimally processed samples. These

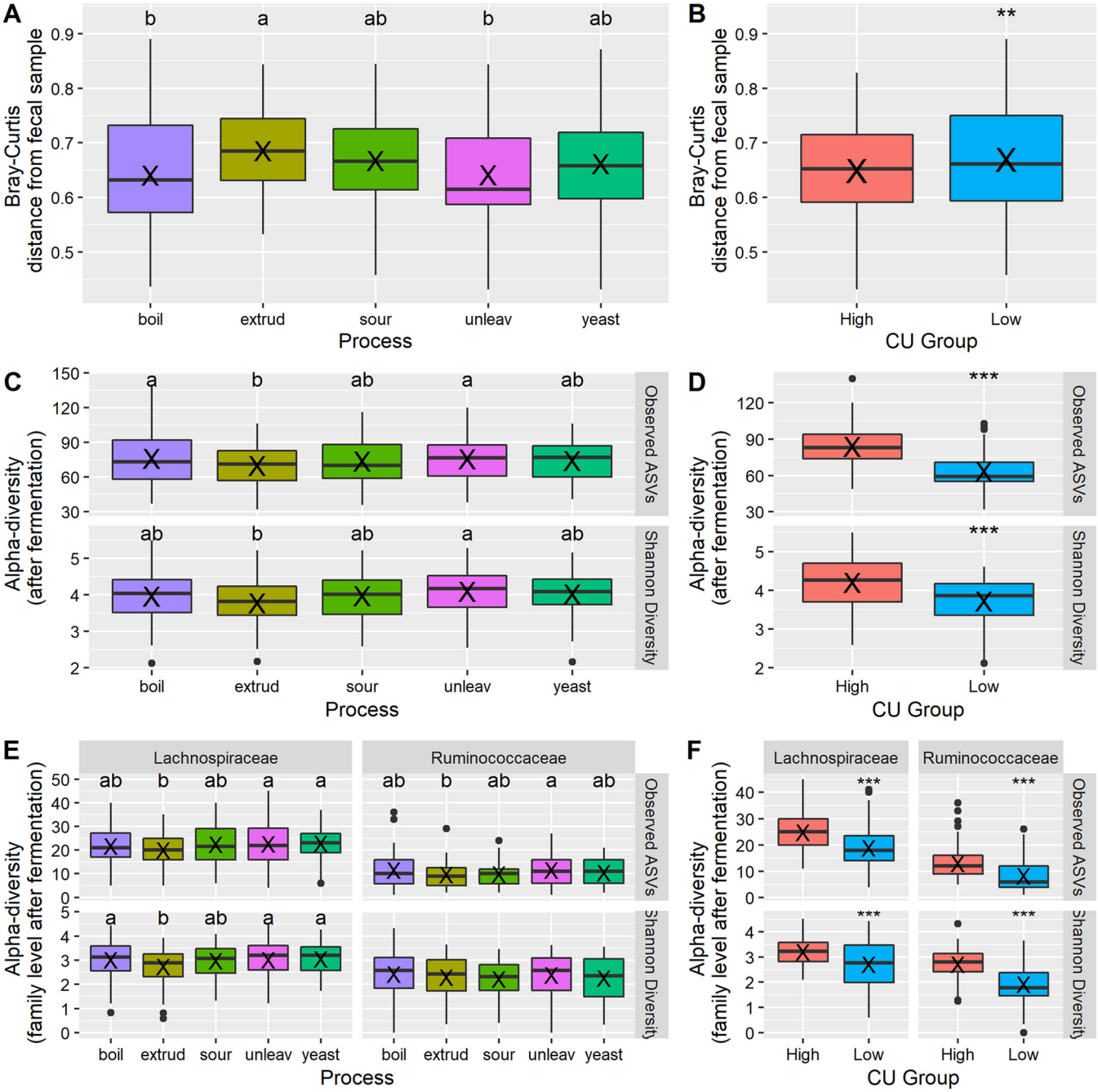

FIG 4 Microbiome diversity after fermentation. (A and B) Bray-Curtis distance from fecal sample; (C and D) α-diversity; (E and F) α-diversity within the families *Lachnospiraceae* and *Ruminococcaceae*. Boil, boiled wheat; extrud, extruded wheat; sour, sourdough bread; unleav, unleavened bread; yeast, yeast-leavened bread. Within box plots, X indicates the mean. Different letters (a, b) indicate significant difference among processing methods. **, $P < 0.01$, and ***, $P < 0.001$, for high-CU group versus low-CU group.

clusters included all of the ASVs belonging to the phylum *Bacteroidetes* as well as a few from other phyla. The next cluster represented ASVs that were associated with the extruded wheat. ASVs that were increased on extruded wheat included one from the phylum *Proteobacteria* (*Acinetobacter*) as well as two in the phylum *Firmicutes* not normally associated with complex carbohydrate degradation (*Enterococcus* and an unassigned member of the *Staphylococcaceae*). The extruded wheat also resulted in a noticeable dearth of ASVs in the other clusters, namely, from the genus *Prevotella* in the phylum *Bacteroidetes* and, in the phylum *Firmicutes*, several ASVs from *Lachnospiraceae*

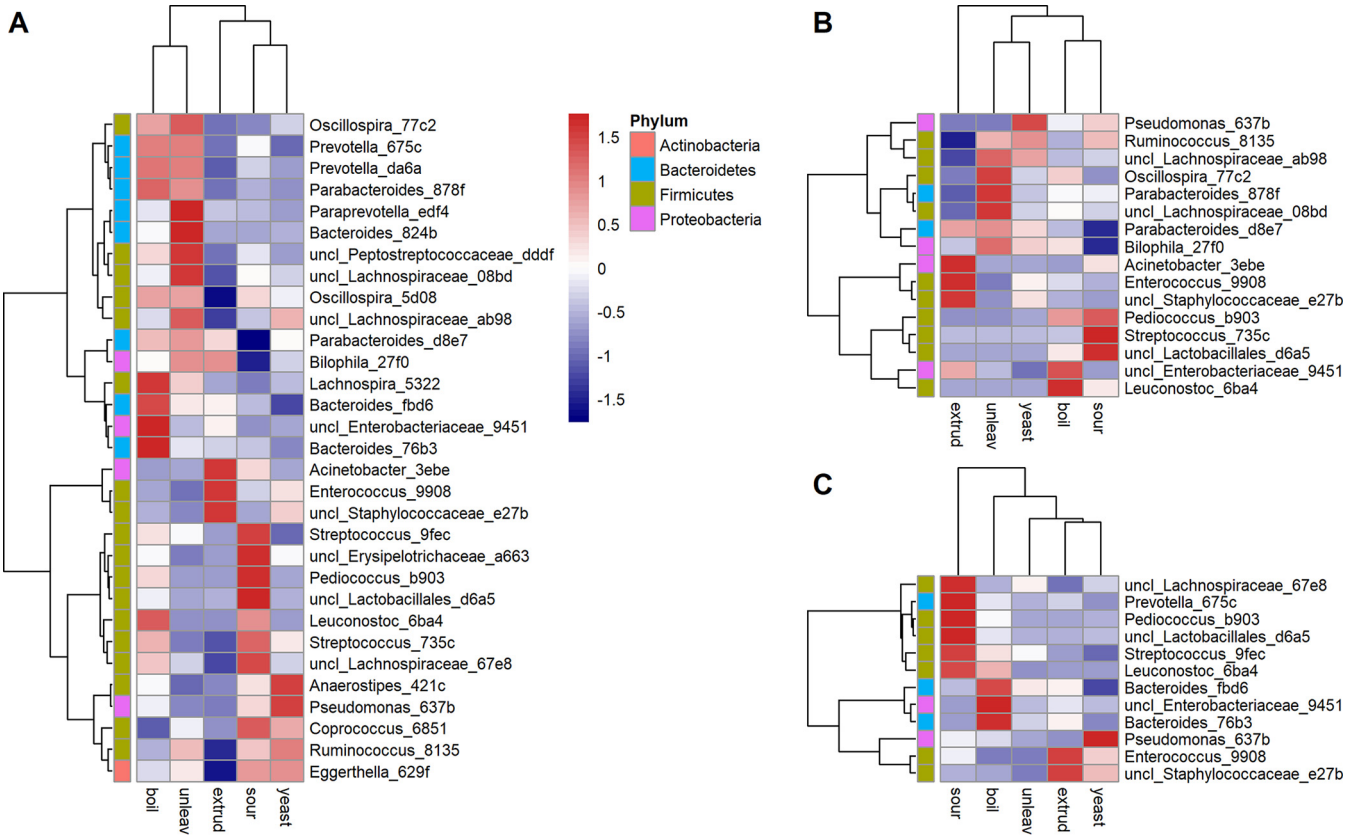

FIG 5 Clustered heat maps of differentially abundant ASVs among processing methods after fermentation. (A) All microbiomes; (B) high-carbohydrate-utilization (CU) microbiomes; (C) low-CU microbiomes. Centered and scaled data were clustered by Euclidian distance using Ward's method; ASVs were named by genus (or lowest taxonomic rank available) followed by the first 4 characters of the feature ID generated from QIIME2.

(*Coprococcus*, *Anaerostipes*, *Lachnospira*, *Pediococcus*, and unassigned) and *Ruminococcaceae* (*Oscillospira* and *Ruminococcus*). The fourth cluster consisted of ASVs belonging mostly to the order *Lactobacillales* that were associated with the sourdough bread. The last cluster, representing ASVs associated with the yeast-leavened bread, contained the single ASV from *Actinobacteria*, a member of the genus *Eggerthella*, and a number of carbohydrate-degrading *Firmicutes*, including *Coprococcus*, *Ruminococcus*, and an unassigned member of the *Lachnospiraceae*.

When data were analyzed by CU group, 16 of the 31 differential ASVs remained significantly different across processing methods in the high-CU microbiomes, while 12 were significant in the low-CU group. A heat map of the high-CU microbiomes showed that extrusion resulted in a distinct composition compared with the other samples (Fig. 5B). Selectivity for *Acinetobacter*, *Enterococcus*, and an unassigned member of the *Staphylococcaceae* was evident, which was similar to the full data set. In contrast, the clustering of the processed samples was very different from the full data set when only the low-CU microbiomes were used (Fig. 5C). In this case, sourdough was separated from the other processed samples and led to increases in several ASVs assigned to the order *Lactobacillales*.

Relationships among grain processing method, carbohydrate utilization microbiome group, and short-chain fatty acid production during fermentation. Even though processing method resulted in significant differences in MAC and microbiota composition during fermentation, no differences in SCFA production were evident when data were analyzed across all 10 microbiomes (Fig. 6A). However, between the high and low-CU microbiomes, the high-CU microbiomes produced more butyrate and the low-CU microbiomes produced more propionate (Fig. 6B). When the differences among processing methods were analyzed within CU groups, the high-CU microbiomes

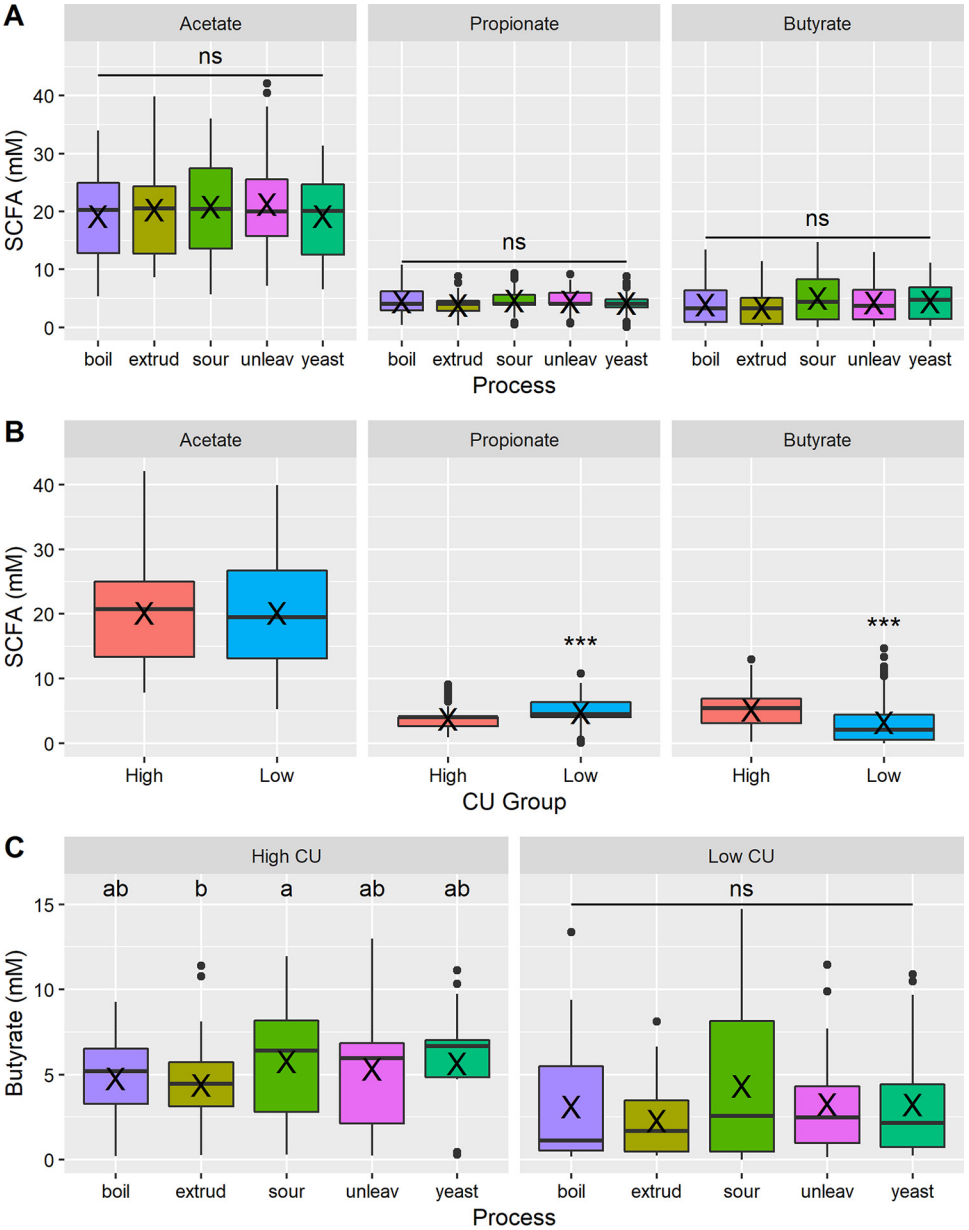

FIG 6 Short-chain fatty acid (SCFA) production during 12 h of *in vitro* fermentation of processed whole-wheat samples. (A) Processing method; (B) carbohydrate utilization (CU) group; (C) processing method within CU group. Boil, boiled wheat; extrud, extruded wheat; sour, sourdough bread; unleav, unleavened bread; yeast, yeast-leavened bread. Within box plots, X is the mean. Different letters (a, b) indicate significant difference among processing methods. ns, no significant differences among processing methods; ***, $P < 0.001$ for high-CU group versus low-CU group.

were able to distinguish among processed samples, with sourdough bread resulting in higher butyrate production than extruded wheat (Fig. 6C). The low-CU microbiomes showed no significant differences among processed wheat samples.

DISCUSSION

Due to the effect of processing on the physicochemical composition of the nondigestible carbohydrates in whole grains, five common processing methods for whole wheat were evaluated for their influence on MAC. Across the 10 microbiomes, extrusion resulted in the highest level of MAC and sourdough bread the lowest. However, microbiomes differed significantly in the magnitude of CU, where high-CU microbiomes

exhibited, on average, 37.5% more carbohydrate fermentation than those in the low-CU group. It was only these high-CU microbiomes that were able to distinguish (significantly) among the differently processed samples in terms of both MAC and butyrate production; the low-CU microbiomes showed no significant differences between processed samples. Although there were no significant differences in MAC among processed samples when they were fermented with the low-CU microbiomes, the numeric ranking of the samples was the same as for the high-CU microbiomes (i.e., extruded wheat > yeast-leavened bread > unleavened bread > boiled wheat > sourdough bread). Therefore, the processing methods induced similar but diminished responses in the low-CU microbiomes compared to the high-CU microbiomes.

These results are similar to those of a previous study performed in our laboratory (using different fecal donors) where only microbiomes in the high-CU group showed significant differences in MAC and butyrate production among different types of grain (19). This provides some possible insight into why some individuals "respond" in terms of improvements in host health, while others are "nonresponders" (20). Since fermentation by the microbiome is considered critical for bringing about the health benefits of many dietary fibers (8), diminished fiber fermentation may attenuate these effects. Others have noted that food science and food processing will play a central role in the development of foods that target the microbiome (21). However, judging from the present results, it may be that only people who harbor microbiomes that are capable of high CU will be able to realize the benefits of such foods derived from whole wheat.

The microbiomes in the high-CU group exhibited some important differences compared to the low-CU microbiomes in the fecal samples that help explain why the high-CU microbiomes were able to ferment more carbohydrates. First, the high-CU microbiomes were significantly more diverse in terms of observed ASVs, but not Shannon diversity, than the low-CU microbiomes. Since observed ASVs takes into account only presence or absence of ASVs without considering abundance, while Shannon diversity takes into account the abundance (evenness), this suggests that the high-CU microbiomes had more low-abundance ASVs than low-CU microbiomes. Indeed, when differentially abundant ASVs were analyzed between the two CU groups, many more ASVs were significantly associated with the high-CU microbiomes than the low-CU microbiomes. In general, having a diverse microbial community results in a broader range of carbohydrate fermenters that are able to hydrolyze a wider range of glycosidic bonds and carbohydrate configurations (22). Upon examination of the differentially abundant ASVs between CU groups, it was immediately evident that the composition within the families *Lachnospiraceae* and *Ruminococcaceae* in particular was very different (not in terms of overall relative abundance but in terms of types of ASVs present). This was especially relevant because members of these families have been identified as important complex-fiber degraders (23).

Although we did not collect dietary data from the subjects that donated fecal samples for this study, the differences in microbiota composition between the high- and low-CU microbiomes suggest that the high-CU microbiomes may have come from fecal donors with higher diet quality, including higher fiber intakes. Several previous studies have shown associations between host dietary fiber intake and abundance of *Prevotella* and certain fiber-degrading members of the families *Lachnospiraceae* and *Ruminococcaceae* (24–26), which were notable distinguishing differences between the high- and low-CU microbiomes in this study. Furthermore, in a previous study, we did differentiate microbiomes on the basis of fiber intake and found that only the microbiomes from donors with high fiber intake showed significant differences between different types of grain, which is analogous to different processing methods in the present study (19).

Although important differences in microbiome diversity and composition between the high- and low-CU microbiomes were noted in fecal samples, the changes that occurred during fermentation exaggerated these differences. For instance, in the fecal samples, Shannon diversity across the entire microbiota was not different between the high- and low-CU microbiomes, nor were any of the measures of α-diversity within the

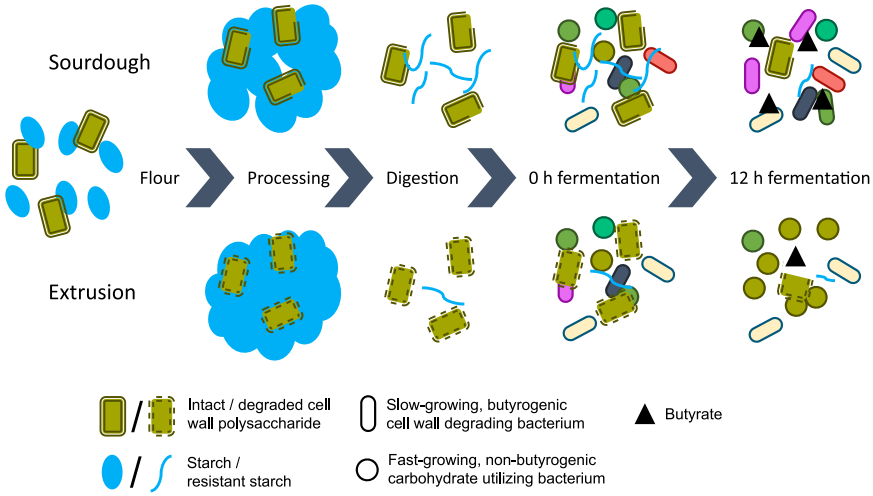

FIG 7 Schematic of proposed fermentation of nondigestible carbohydrates from whole grains processed through sourdough versus extrusion.

family *Lachnospiraceae*. However, after fermentation, all of these metrics were significantly different. The high-CU microbiomes not only were more diverse before fermentation but also maintained that diversity even during fermentation. In contrast, the low-CU microbiomes started with less diversity and ended with even less diversity. This was evident in the β-diversity results, where the high-CU microbiomes exhibited a smaller shift during fermentation than the low-CU microbiomes.

The smaller shift could be framed as microbiota "resilience." Previous research has shown that when the microbiome undergoes a severe perturbation, such as an antibiotic regimen, a fiber-rich diet increases resilience; i.e., it reduces loss of taxa and speeds the time until the microbiota recovers (27). Using a stool sample in an *in vitro* fermentation experiment could certainly be classified as a severe perturbation to the microbiota, where it is taken from its normal environment and moved to a test tube.

Within the high-CU group, some important significant differences were exhibited between processing methods. At the outset of the study we hypothesized that extrusion would result in the highest MAC, since previous researchers have shown that extrusion leads to modifications in the carbohydrates, such as increased porosity and fineness of cell wall structures and the conversion of a portion of the insoluble fiber to soluble fiber, which is generally considered more fermentable (22). This is indeed what we found. Surprisingly, however, this was not accompanied by higher butyrate production. This may be explained by the decreases in α-diversity and carbohydrate-degrading bacteria from the families *Ruminococcaceae* and *Lachnospiraceae*, which are important butyrate producers in the gut (28). Instead, extrusion resulted in increases in *Acinetobacter*, *Enterococcus*, and *Staphylococcaceae*, some members of which grow rapidly and prolifically in fermentation media but are not butyrate producers (29).

Therefore, the disruption of the cell wall matrix by extrusion may change the competition dynamics among bacteria such that the microbiota is less reliant on specialized complex carbohydrate-degrading microorganisms to initiate cell wall metabolism; i.e., cell wall breakdown is already initiated by the extrusion process. This would mean that fast-growing microbes could outcompete other bacteria for the newly available MAC, leading to decreased diversity and decreases in butyrate producers (Fig. 7).

The unexpected dichotomy between high carbohydrate fermentation and low butyrate production introduces the importance of microbiota-accessible-carbohydrate quality rather than just quantity. There are several papers and reviews that discuss the importance of fiber structure for fermentation properties (30). In these papers, authors argue that the structure of the fiber, rather than simply the quantity, should be

considered when selecting a dietary fiber to promote gut health. Here, extrusion was effective in increasing fermentable fiber, but the fiber that was released was not associated with positive outcomes. Therefore, food processing operations that are designed to increase carbohydrate fermentation should increase accessibility of "quality" carbohydrates, i.e., carbohydrates that will support beneficial microbes (such as butyrate producers) in the gut, rather than just increasing carbohydrate fermentation in general.

The high CU and low butyrate production during fermentation of the extruded wheat are different from results of a previous study conducted in our laboratory using wheat bran instead of whole grain (12). In our previous study, extrusion led to increased CU but also showed increases in butyrate production compared to boiling wheat bran. The contrasting results could be due to the different substrates (whole wheat versus wheat bran). Wheat bran, which primarily consists of nonstarch polysaccharides, has an increased concentration of cell wall components. Thus, the effect of extrusion on MAC and SCFA production could have been diminished in the present study due to the dilution of the cell wall components in whole grains compared to what bran. Indeed, in the previous study, the extruded bran was reconstituted to whole-wheat flour to make whole-grain breads and there were no significant differences in MAC or SCFA production between the whole-grain breads after fermentation.

Importantly, the extrusion conditions selected in this study were very severe. These were chosen based on our previous study with wheat bran to maximize the effects of the processing method (12). However, the combination of very low moisture and high temperature and screw speed would be unlikely to be used in a typical food product. Therefore, the effect of extrusion on gut microbial fermentation in real food products may not be as pronounced as shown in this study.

In contrast to extrusion, the sourdough bread making process resulted in lower CU yet resulted in higher butyrate production in the high-CU microbiomes. Sourdough bread, along with the other breads, resulted in increased abundance of beneficial microbes from the families *Ruminococcaceae* and *Lachnospiraceae* that ferment complex carbohydrates to SCFA (23). Sourdough-like fermentation of wheat bran with enzyme mixtures and yeast has been previously shown to promote SCFA production *in vitro* compared to native wheat bran (17).

The especially high butyrate production from fermentation of the sourdough samples could be a result of the changes in the starch that occur during the sourdough process. Sourdough has been shown to increase the leaching of amylose during bread making, which is rapidly retrograded during cooling and is associated with resistant starch (10, 31). Indeed, together with the boiled wheat, the sourdough bread samples had the most resistant starch among samples from all of the processing methods.

Although resistant starch may explain some of the positive effects noted during fermentation of the sourdough samples, it cannot explain everything. As mentioned, the boiled samples contained concentrations of resistant starch similar to those in the sourdough samples, yet boiling resulted in only moderate butyrate production, maintenance of microbial diversity, and increases in *Lachnospiraceae* and *Ruminococcaceae*. Therefore, the sourdough process must induce some additional effects, likely on the cell wall polysaccharides (10), that help maintain microbial diversity and enable high butyrate production (Fig. 7).

In summary, it is hypothesized that severe extrusion changes the microstructure of carbohydrates in whole grains by breaking down the cell wall components. These changes in structure result in easier access to the cell wall polysaccharides by microbiotas with high capacity for CU. This changes the competition dynamics among the microbes such that the fastest-growing taxa can outcompete specialized cell wall-degrading bacteria that typically break down cell wall polysaccharides but may grow more slowly. This leads to decreased diversity and production of beneficial metabolites such as butyrate. In contrast, during sourdough bread making, increased resistant starch together with minor modifications to the cell wall matrix creates higher-quality MAC in terms of both variety and complexity. These phenomena lead to a more diverse

microbiome that has increased capacity to produce SCFA, especially butyrate, in microbiomes with high CU. Similar trends can be seen in microbiomes with low CU, but the differences are diminished. This information can be used to better understand how to use food processing to create microbiota-directed foods that promote a healthy microbiome.

MATERIALS AND METHODS

Whole wheat processing. Hard red wheat was obtained from Bay State Milling (Quincy, MA). Wheat kernels were dried at 40°C for 16 h before milling as described previously (32). All milled fractions obtained from the mill were mixed together to obtain whole-wheat flour.

For boiling, water (530 g) was brought to a boil on a gas range. Once the water was boiling, the heat was reduced to a simmer, and 1 g of salt and 100 g of whole-wheat flour were added with rapid manual stirring for 5 min. The boiled wheat porridge was then cooled to room temperature before being frozen at −80°C and then freeze-dried. This procedure was performed in triplicate.

For extrusion, whole-wheat flour (1 kg) was mixed for 10 min with 1% salt (wt/wt) and water to adjust to a 20% moisture content (dry weight basis) in a stand mixer (c-100; Hobart, Troy, OH). The mixtures were equilibrated in closed containers at 4°C overnight. The moisture-adjusted whole-wheat flours were then extruded using a benchtop-scale twin-screw extruder (C.W. Brabender Instruments, NJ, USA) equipped with a single-stage mixing zone and a 3-mm outlet die at 250 rpm, a 3:1 compression ratio, and a 20:1 length/diameter (L/D) ratio. The extruder was operated by a direct current drive unit (Intelli-Torque Plasti-Corder lab station; C.W. Brabender) with a 5.6-kW motor. The flour was fed into the extruder using a volumetric feeder (FW 40 Plus; C.W. Brabender) set at a constant flow rate of ~50 g/min. Barrel temperatures were set at 60°C (zone 1; inlet), 70°C (zone 2), 120°C (zone 3), and 120°C (zone 4; die assembly). Samples were collected from the extruder die once steady state had been reached. Extrudates were then dried in a convection oven overnight at 70°C to complete the extrusion processing. Although the product contained very low moisture at this stage (~3%), the dried extrudates were subject to freeze-drying (model 3600 freeze dryer; Freeze Dry Co., Pine River, MN) because the other processed wheat products were freeze-dried following processing. The whole-wheat flour was extruded in triplicate.

For bread making, this study employed an in-house method for bread making that involved production of flatbreads. Flatbread-type breads were selected because they can be made using any of the three selected fermentation procedures: unleavened, baker's yeast fermentation, or sourdough fermentation. The procedures were planned to unify the essential bread making procedures and vary only the steps that were unique to each fermentation procedure. For the baker's yeast fermentation, instant dry yeast was used (Saf Instant Red; Lesaffre, Milwaukee, WI). For the sourdough fermentation, a type 1 spontaneous sourdough starter culture was used. To prepare the culture, 50 g of whole-wheat flour and 50 g of water (100% absorption) were incubated at 30°C for 24 h, whereupon 50 g of that mixture was mixed with 50 g of fresh whole-wheat flour and 50 g of water and then allowed to ferment for another 24 h at 30°C. After 5 days, the starter culture was sampled for yeast and lactic acid bacteria using standard methods (33, 34). At this point, the culture contained 10^7 CFU/g of lactic acid bacteria and 10^6 CFU/g of yeast, measured using standard methods. During the analysis, daily feeding of the starter culture was continued. Once sufficient numbers of yeast and lactic acid bacteria were confirmed, the starter culture was used to make breads, which was on day 9.

All breads were prepared on the same day in triplicate 500-g batches with 70% absorption (70 g water/100 g whole-wheat flour on a 14% moisture basis). The doughs were prepared by kneading for 10 min in an electric stand mixer with a dough hook at speed 2 (KitchenAid; Whirlpool, Benton Harbor, MI, USA). The dough was then rounded by hand, covered, and allowed to rest for 20 min (unleavened) or ferment for 90 min (baker's yeast) or 240 min (sourdough). The dough was then divided into five 100-g pieces, rounded by hand, covered, and allowed to rest for 20 min. The dough was then sheeted by passing through a dough sheeting device (KitchenAid) to a thickness of 6 mm. A fork was then used to poke holes in the top of the dough piece uniformly 5 cm apart (docking) to prevent bubbling up during baking. The flatbreads were then baked for 4 min at 232°C. The baked flatbread was cooled overnight at room temperature inside a plastic bag once the flatbreads were at room temperature and then freeze-dried.

***In vitro* digestion.** Samples were digested following established procedures (35). Briefly, freeze-dried processed whole-wheat products were broken into small pieces by hand. Twenty-five grams of sample were then stomached with 300 ml of water for 1 min. Then, the pH was adjusted to 2.5 with 1 M HCl followed by the addition of 10 ml of 10% (wt/vol) pepsin (P7000; Sigma, St. Louis, MO) in 50 mM HCl. The slurry was then incubated for 30 min at 37°C with orbital shaking at 150 rpm. Following pepsinolysis, 50 ml of 0.1 M sodium maleate buffer (pH 6, containing 1 mM $CaCl_2$) was added, and the pH was adjusted to 6.9 with 1 M $NaHCO_3$. Next, 50 ml of 12.5% (wt/vol) pancreatin (P7545; Sigma) in sodium maleate buffer was added followed by 2 ml of amyloglucosidase (3,260 U/ml; Megazyme, Bray, Ireland). The slurry was then incubated for 6 h 37°C with orbital shaking at 150 rpm. Following digestion, the material was transferred to dialysis tubing (molecular weight cutoff, 12,000 to 14,000) and dialyzed against distilled water for 4 days at 4°C with a water change at least every 12 h. The retentate from dialysis was collected and freeze-dried.

***In vitro* fermentation.** Fresh fecal samples from 10 healthy adults with no history of gastrointestinal abnormalities and no prebiotic, probiotic, or antibiotic consumption within the past 6 months were collected. All procedures involving human subjects were approved by the Institutional Review Board of the University of Nebraska–Lincoln before initiating the study (approval number 20160816311EP). A fecal

slurry was prepared in an anaerobic hood (Bactron X [Sheldon Manufacturing, Cornelius, OR, USA]; containing 5% H_2, 5% CO_2, and 90% N_2) within 2 h of defecation by blending (model 2774 blender; Sunbeam) each fresh fecal sample separately with sterile phosphate-buffered saline, pH 7.0 (1:9 wt/vol), containing 10% glycerol as a cryoprotectant for 1 min. The slurry was then filtered through 4 layers of sterile cheesecloth and then frozen at −80°C until fermentation was performed. Two fecal slurries from fecal samples collected on different days (from 1 day to 2 weeks apart) were prepared from each individual.

In vitro batch fecal fermentation was performed as described previously (35). Briefly, inside the anaerobic hood, 15 mg of the freeze-dried material obtained after *in vitro* digestion and dialysis of the processed whole-wheat products was suspended in 1 ml of sterile fermentation medium containing (per liter) peptone (2 g; Fisher Scientific, Waltham, MA), yeast extract (2 g; Fisher Scientific, Waltham, MA), bile salts (0.5 g; Oxoid, Cheshire, England), $NaHCO_3$ (2 g), NaCl (0.1 g), K_2HPO_4 (0.08 g), $MgSO_4{\cdot}7H_2O$ (0.01 g), $CaCl_2{\cdot}6H_2O$ (0.01 g), L-cysteine hydrochloride (0.5 g; Fisher Scientific, Waltham, MA), hemin (50 mg dissolved in dimethyl sulfoxide [DMSO]), Tween 80 (2 ml, Fisher Scientific, Waltham, MA), vitamin K (10 μl, dissolved in ethanol; Alfa Aesar, Haverhill, MA), and 0.025% (wt/vol) resazurin solution (4 ml, dissolved in water; Alfa Aesar, Haverhill, MA), capped, and hydrated for 1 h. Tubes were then inoculated with 0.1 ml of fecal slurry, capped, and incubated at 37°C with orbital shaking (140 rpm) for 12 h before being flash frozen in liquid nitrogen. Samples that were immediately flash frozen in liquid nitrogen following inoculation were used as controls (0 h fermentation). After freezing in liquid nitrogen, tubes were stored at −80°C until analysis. Because most of the dietary fibers in whole grains are not soluble, separate fermentation tubes were prepared for analysis of carbohydrates versus microbiota composition and SCFA.

A 12-h fermentation time was selected because previous *in vivo* carbohydrate balance studies have reported that (on average) one third of the nondigestible carbohydrates in grains are fermented by gut bacteria (6, 7). Therefore, we performed preliminary studies to determine how long fermentation should proceed to reach fermentation of one-third of the carbohydrates. Based on fermentation using 49 separate microbial communities, we found that this was 12 h (Fig. S2). Therefore, even though this represents a shorter time than typical transit time, it represents the time required to ferment approximately the proportion of carbohydrates that would be fermented *in vivo*, which was our primary objective.

Carbohydrate analysis. For analysis of the proportion of nondigestible carbohydrates fermented by the microbiota, a modified version of the procedure used by Arcila et al. (12). The entire contents of the tubes designated for carbohydrate analysis were freeze-dried. The freeze-dried samples were then treated with 0.3 ml of 12 M sulfuric acid for 1 h at 30°C with periodic vortex mixing to begin the hydrolysis. One milliliter of a fucose solution (5 mg/ml) was then added as an internal standard, and 7.7 ml of water was added to bring the final sulfuric acid concentration to 0.4 M. The samples were then autoclaved at 121°C for 1 h, cooled, and filtered through a 0.45-μm membrane filter. Ten microliters was injected into a high-performance anion-exchange chromatograph (ICS-5000+ SP; Dionex) equipped with an anion-exchange column (CarboPac PA1; Dionex) operating at 30°C with a 1-ml/min flow rate. The eluent was 200 mM NaOH for column cleaning followed by 10 min of equilibration of 3 mM NaOH before injection of the sample. The sugars were detected over 22 min with pulsed amperometry waveform (ICS-5000+ SP chromatograph; Dionex). Sugar residues were quantified by calculating response factors for each sugar relative to fucose using injections of pure standards. The concentration of each sugar residue was corrected for its weight as it occurs in a polysaccharide (0.88 × weight for pentose sugars and 0.9 × weight for hexose sugars). The percent change in the amount of carbohydrates measured was calculated to determine the proportion of MAC. Total carbohydrates were measured with the same procedure using 100 mg of raw, processed, or digested whole wheat. Total starch in the processed whole-grain samples and after digestion was measured using a kit according to the manufacturer's directions (K-TSTA; Megazyme, Bray, Ireland). The starch remaining in the processed samples after digestion was considered resistant starch.

Microbiota composition. Changes in microbial population were observed using 16S rRNA sequencing. A kit from Biovet (BioSprint 96 One-For-All Vet kit; Biovet, Quebec, Canada) was used for bacterial DNA extraction. Microbiome characterization was performed by amplicon sequencing of the V4 region of the 16S rRNA gene on the Illumina MiSeq platform using the MiSeq reagent kit v2 (2 × 250 bp) following the protocol of Kozich et al. (36). Sequences were demultiplexed and barcodes were removed prior to sequence analysis with the QIIME 2 platform (37). Sequence quality control, trimming, chimera removal, and denoising were performed with DADA2 (38). Forward and reverse reads were truncated to 245 and 160 bp, respectively, to maintain sequence qualities above a phred score of 30. Using DADA2, sequences were dereplicated into 100% ASVs for exact sequence matching. Taxonomy was assigned using the Greengenes database (39). Reads were rarefied to a sampling depth of 9,000 prior to statistical and diversity analyses. Diversity metrics of the fecal and fermented samples were calculated using QIIME2.

Short-chain fatty acid analysis. SCFA were extracted and measured by gas chromatography as described previously (40). In brief, 0.4 ml of fermented sample, 0.1 ml of 7 mM 2-ethylbutyric acid in 2 M potassium hydroxide, 0.2 ml of 9 M sulfuric acid, and ~0.1 g of sodium chloride were mixed together. Then, 0.5 ml of diethyl ether was added and mixed. The top layer was injected into a gas chromatograph (Clarus 580; PerkinElmer, MA, USA) equipped with a capillary column (Nukol; 30 m by 0.25 mm [inner diameter] by 0.25 μm [film thickness]; Supelco, Bellefonte, PA) and flame ionization detector. SCFA were quantified by calculating response factors for each SCFA relative to 2-ethylbutyric acid using injections of pure standards.

Statistical analysis. Two-factor analysis of variance (ANOVA) was used to determine the effect of process and microbiome (or CU microbiome group) and their interaction on MAC, SCFA, and α-diversity of the microbiota (SAS version 9.4; SAS Institute, Cary, NC, USA). Tukey's honestly significant difference (HSD) test was used to determine significant differences among samples within a factor, where a P value of <0.05 was considered significant. To determine differential ASVs between CU groups or among processing methods, the DESeq2 package in R (version 4) was used (41). To plot the differential ASVs among processing methods, a clustered heat map was generated where the centered and scaled data were clustered by Euclidian distance using Ward's method (pheatmap package in R).

Data availability. Raw sequence reads and metadata from fecal samples and *in vitro* fermentations are available in the SRA under accession no. PRJNA603983.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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