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# Computer-controlled simulated gastrointestinal digestion changes in vitro fermentation kinetics, microbial community, and short-chain fatty acid production of fiber-rich ingredients

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**ABSTRACT:** Dietary fibers (DF) are well-established prebiotics that modulate gut microbiota and enhance host health. Nevertheless, how previous gastric-small intestinal digestion of DF impacts microbiota and metabolites during fermentation remains poorly understood. This study investigated the effects of pre-digestion, using a computer-controlled simulated digestion system (CCSDS) that mimics in vivo gastrointestinal conditions, on the fermentation kinetics, microbial community, and short-chain fatty acid (SCFA) production of six fiber-rich ingredients [sugar beet pulp (SBP), wheat bran (WB), corn DDGS (DDGS), soybean hull (SH), rice bran (RB), and alfalfa meal (AM)], followed an *in vitro* fecal fermentation was employed. Results showed that simulated digestion reshaped DF fermentation kinetics, with gas production decreased for WB, but increased for SH, SBP, DDGS, and AM ($P < 0.05$). SCFA profiles diverged significantly, reflected by SBP and DDGS residues increased butyrate production, whereas WB and RB residues reduced acetate and propionate yields ($P < 0.05$). α-diversity (Sobs, Chao1, and Ace indexes) increased in residues of WB, SBP, and AM ($P < 0.05$). PCoA analysis confirmed distinct clustering between ingredients and residues ($P < 0.01$), with residues increasing the abundance of fiber-degrading and SCFA-producing genera (e.g., *Prevotellaceae_NK3B31_group* in WB residues, *Treponema* in DDGS residues, *Lysinibacillus* in RB, SBP, and AM residues). These findings demonstrate that pre-digestion critically modifies DF fermentation and microbiota interactions, providing valuable insights for the selection of DF in diet formulations and enhancing our understanding of their beneficial effects on gut health.

**Keywords:** Dietary fibers; Fermentation properties; Gut microbiota; In vitro fermentation; Simulated gastric-small intestinal digestion

## 1. Introduction

Dietary fibers (DF) are recognized for their significant prebiotic potential in regulating gut microbiota and improving metabolic functions [1, 2]. Short-chain fatty acids (SCFAs), the primary metabolites of DF fermentation, mediate critical host-microbiota interactions, which contributing to preserve the gut barrier

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function, enhance immune function, anti-inflammatory and anti-oxidant responses [3-5]. It is worth noting that the physiological effects of DF depend largely on their fermentation in the colon. However, before their eventually reaches the hindgut, DF are subjected to the gastro-intestinal digestion with enzymatic activity, pH fluctuations, water activity, and interactions with bile acids minerals. Although resistant to gastrointestinal digestion, DF can be altered in the physical and chemical properties, including the chemical composition, monosaccharide composition, and molecular weight [6], which critically influence the rate and extent of their fermentation and subsequent microbial metabolism in the colon. In particular, the fiber-rich ingredients, as main source of DF, are often directly utilized in animal and human diets, which has great health potential for human health and wellbeing [7, 8]. The fiber-rich ingredients also contain some digestible nutrients (such as starch and protein), which would be partly removed during gastrointestinal digestion, thereby modifying the fiber matrix and contents in ingredients and affecting microbial fermentation. Previously *in vitro* fermentation without pre-gastrointestinal digestion study reported that higher gas production and SCFA production for wheat bran (WB) and oat bran (OB) compared to sugar beet pulp (SBP), corn bran (CB) and soybean hull (SH) [9]. Inconsistently, the results in an *in vivo* study indicated that SH, SBP, and OB had higher fermentability and SCFA production than and WB and CB in pigs [10]. This discrepancy highlights the potential impact of gastrointestinal digestion on DF structure and composition, which is often overlooked in conventional in vitro models. Therefore, it is meaningful to investigate how the prior gastric-small intestinal digestion treatments affect fermentation properties of DF and its subsequent effects on gut microbiota.

In recent years, a critical debate in *in vitro* fermentation models is whether a previous simulated gastro-intestinal digestion steps should be incorporated [11]. Proponents argue that to perform an *in vitro* digestion previously is necessary, which can simulate the physiological digestion process in vivo, such as injects into digestive enzymes, removes digestible nutrients (e.g., starch, protein), concentrates fiber components, and mimics structural breakdown for microbial accessibility [12, 13]. The changes of nutrient content and chemical structure of fiber-rich ingredients may impact the fermentation properties and microbial composition regulation [14]. A previous study has indicated that the enzymatic hydrolysis prior to fermentation affected the kinetics parameters, final gas production, and the ranking order of the fermented substrates [15]. For instance, the study on the digestion and fermentation characteristics of polysaccharides from lotus root residue highlights how simulated gastric and intestinal juices can slightly alter the molecular weight and sugar content, which subsequently affects fermentation outcomes [16]. Additionally, the enzymatic hydrolysis prior to fermentation yield the residues may not have similar fermentability to ileal digesta. In previous *in vitro* gastrointestinal digestion study, the hydrolysis residues of DF mostly obtained from suction filtration method with filter paper, resulting in the disappearance of the part of soluble fiber, which cannot be taken into account during *in vitro* fermentation [13]. Whereas, it rarely reported whether and how those alterations affect microbiota and related metabolites during *in vitro* fermentation. Therefore, a more effectively pre-digestion method is required to simulate the digestion of stomach and small intestine for accurately mimicking the physiological conditions that DF encounter.

In our previous study, we invented a computer-controlled simulated digestion system (CCSDS) to simulate the digestion of stomach-small intestine and the clearance of byproducts according to in vivo physiological conditions, which can preserve soluble fiber fractions via molecular weight-based dialysis, enabling accurate simulation of ileal digesta [17, 18]. In this study, six fiber-rich ingredients, containing sugar beet pulp (SBP), wheat bran (WB), corn DDGS (DDGS), soybean hull (SH), rice bran (RB), and alfalfa meal (AM) were selected as fiber source, and performed the simulated stomach and small intestinal digestion using CCSDS. The ingredients and corresponding hydrolysis residues were used as fermentation substrates for *in vitro* fermentation. The gas production, *in vitro* dry matter fermentability (IVDMF), microbial composition and SCFAs production were investigated. This study can provide data support for the selection of fiber-rich ingredients and design of high fiber diets, which are beneficial for gut health.

## 2. Materials and methods

### *2.1 Fiber samples and chemical analysis*

Six fiber-rich ingredients with SDF/TDF ratio ranged from 1.99%-36.13% were used in this study, including sugar beet pulp (SBP), wheat bran (WB), corn DDGS (DDGS), soybean hull (SH), rice bran (RB), and alfalfa meal (AM). The chemical composition of six ingredients were analyzed and are presented in Table 1.

**Table 1** Dietary fiber Content of fiber-rich ingredients and corresponding residues (air-dry basis).

| Item | DM, % | NDF, % | ADF, % | TDF, % | IDF, % | SDF, % |
|---|---|---|---|---|---|---|
| Ingredients | | | | | | |
| RB | 92.26 | 51.65 | 37.12 | 58.99 | 57.84 | 1.15 |
| DDGS | 92.86 | 33.92 | 8.26 | 33.04 | 30.37 | 2.67 |
| SH | 92.62 | 61.31 | 44.71 | 78.57 | 70.76 | 7.81 |
| WB | 92.37 | 39.98 | 14.16 | 45.71 | 40.76 | 4.95 |
| AM | 93.82 | 44.06 | 31.52 | 52.52 | 44.84 | 7.69 |
| SBP | 89.67 | 39.35 | 22.55 | 64.36 | 47.27 | 17.08 |
| Residues | | | | | | |
| RB | 91.39 | 54.22 | 38.52 | 62.01 | 60.72 | 1.29 |
| DDGS | 91.11 | 38.18 | 14.15 | 40.92 | 37.65 | 3.27 |
| SH | 91.02 | 63.14 | 45.77 | 83.46 | 75.15 | 8.31 |
| WB | 90.50 | 43.52 | 17.48 | 49.85 | 44.51 | 5.34 |
| AM | 91.22 | 48.32 | 34.86 | 58.48 | 50.05 | 8.43 |
| SBP | 90.17 | 42.15 | 24.14 | 66.43 | 49.05 | 17.38 |

All data are analyzed in duplicate.
ADF=acid detergent fiber; DM = dry matter; IDF = insoluble dietary fiber; NDF = neutral detergent fiber; SDF = soluble dietary fiber; TDF = total dietary fiber.

All samples were analyzed in duplicate for dry matter (DM) according to AOAC procedures [19]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured according to previous method [20] with small modified. Total dietary fiber (TDF) and insoluble dietary fiber (IDF) were analyzed using the Dietary Fiber Kit according to the manufacturer's instructions.

### *2.2 In vitro simulated stomach-small intestine digestion*

The ingredients were subjected to pre-digestion using a computer-controlled simulated digestion system (CCSDS), which mimics the physiological conditions of the gastrointestinal tract of pigs (Fig. 1). The system

was set to simulate gastric digestion at pH 2.0 for 4 hours, followed by small intestinal digestion at pH 6.8 for 8 hours, with enzyme concentrations adjusted to match in vivo conditions. The simulated gastric fluid, simulated small intestinal fluid, gastric buffer solution, and small intestinal buffer solution were prepared as previous report [17].

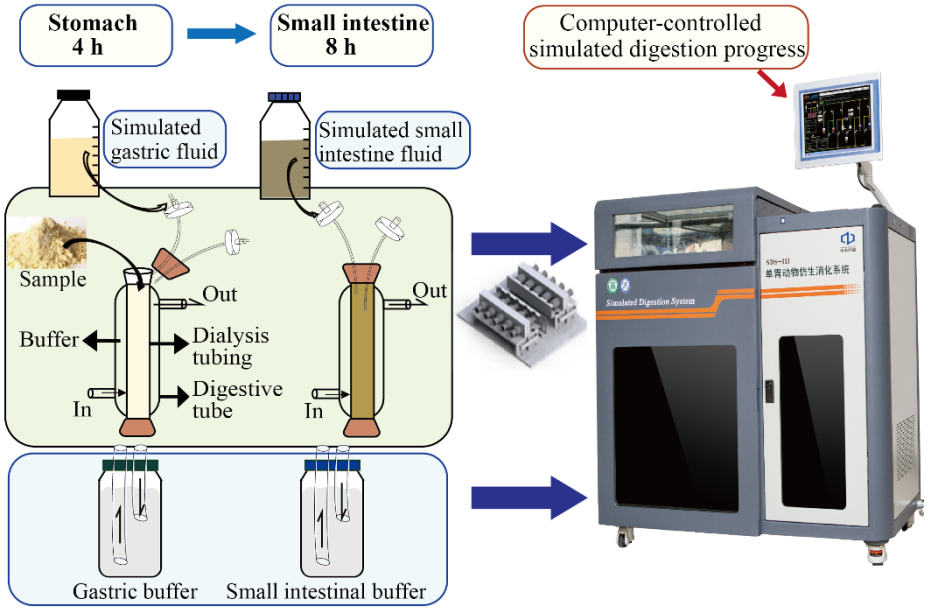


**Figure 1** Schematic representation of computer-controlled simulated digestion system (CCSDS) to simulate the gastrointestinal digestion.

The automatic control process was performed as previous study [17], with small modified. In brief, the simulated *in vitro* gastric digestion was conducted with 2 g of each sample and 20 mL of simulated gastric enzyme added to the dialysis tubing in digestion chambers. Each sample was analyzed in 5 replicates for obtain sufficient substrates for *in vitro* fermentation. The 5 replicated digestive chambers were fixed on a platform and connected in series with middle silicone tubes, and then fixed in a shaking bath (180 r/min at 39℃) of CCSDS. The 1,000 mL of gastric buffer solution was pumped and circulated outside the dialysis tubing in the connected chambers (120 mL/min) by a peristaltic pump. After simulated gastric digestion, an emptying procedure of buffer solution was conducted. Next, the intestinal buffer solution (1000 mL) was pumped and circulated outside the dialysis tubing in the connected chambers for 30 min. Then, 2 mL of stimulated small intestinal digestion fluid was added into each dialysis tubing. The incubation time was 4 h for gastric digestion and 8 h for small intestine digestion. After the digestion was finished, the buffer was automatically emptied. Six replicated clearance procedures followed by an emptying procedure were automatically progressed to clear the byproducts.

After the simulated digestion finished, the undigested residues in the dialysis tubing were transferred into glass petri dish and dried overnight at 55℃, cooled in the desiccators, and used as substrates to *in vitro* fermentation.

### *2.3. In vitro fermentation*

#### *2.3.1. Inoculum preparation and medium composition*

Ten healthy pigs (Duroc × Landrace × Large White, approximately 50 kg) were selected and used to collect feces, which served as the sources of inoculum. All pigs consumed a standard commercial diet and had no antibiotics. Feces of pigs were manually collected from the rectums into a $CO_2$ pre-flushed plastic container after feeding, and stored at -80℃. Before fermentation, an equal amount of frozen feces from each pig were mixed and homogenized with pre-warmed (39℃) phosphate buffer solution at pH 6.8 (1:5, w/v). The diluted mixture was filtered through the four layers of sterile gauze to serve as the inoculum. All processes were carried out in an anaerobic glovebox, supplied with $CO_2$ continuously. The fermentation medium was a buffer solution composed of salts and minerals, which prepared according to the previous study [21]. One liter of medium contained: 8.3 g $NaHCO_3$, 0.95 g $NH_4HCO_3$, 3.30 g $Na_2HPO_4$, 3.29 g $KH_2PO_4$, 0.14 g $MgSO_4 \cdot 7H_2O$, 0.34 g $Na_2S \cdot 9H_2O$, 80 mg NaOH, 1.6 mg $CaCl_2 \cdot 2H_2O$, 1.2 mg $MnCl_2 \cdot 4H_2O$, 0.12 mg $CoCl_2 \cdot 6H_2O$, 0.96 mg $FeCl_3 \cdot 6H_2O$ and 1.19 mg resazurin. The fermentation medium was sterilized using high pressure sterilizer at 121℃ for 15 min, then flushed with $CO_2$ to adjust the pH to 6.8.

*2.3.2. In vitro fecal fermentation*

According to the previously described process [21], a total of 500 mg of each ingredient or 200 mg of each residue was accurately weighed, and added into 100 mL glass bottle. Five replicate bottles were prepared for each substrate and blank (without substrate). All bottles were transferred into an anaerobic glovebox filled with $CO_2$. Then, 60 mL medium and 5 mL inoculum was added sequentially into the bottles. All bottles were capped, then transferred to a pre-warmed incubator at 39℃ for 36 h, which according to the hindgut transit time of growing pigs [22, 23].

*2.4. Gas production, SCFAs and IVDMF determination*

Air pressure transducer (SUAY30, RFS, China) and syringe were used to determine the volum of gas production in each bottle at 12, 14, 16, 18, 20, 24 and 36 h of *in vitro* fermentation, respectively. The initial atmospheric pressure was recored using air pressure transducer at the beginning of fermentation. At the determing time point, the needles of the syringe and air pressure transducer were simultaneously inserted into the bottles through the rubber cap. The pressure inside the bottle was real-time read by the air pressure transducer, and the produced gas was evacuated from bottle by syringe until the pressure in bottle equal to the initial atmospheric pressure. The volume of gas in syringe (mL) is the gas production.

At the end of fermentation, the bottles were placed in an ice-water bath for 30min to terminate the fermentation. Concentration of SCFA in fermentation broth were analyzed accroding to our previous study [21]. In brief, one milliliter of the fermentation broth was pipetted into tubes and centrifuged at 10,000g for 10 min at 4℃. A mount of 0.8 mL of the supernatant was transferred into new centrifuge tube, the residues was stored at -80 ℃ for 16S rDNA sequencing. Then, 0.2 mL of 25% (w/v) metaphosphoric acid was added to the new centrifuge tube. The tubes were shaken for 30 min, centrifuged at 12,000 g for 10 min at 4℃. The supernatant was filtered (0.45 μm filtration membrane) for SCFA analysis using a Agilent 7890 Gas Chromatograph System (Santa Clara, CA, USA).

All other fermentation broth was suction filtrated (40 μm filter paper; Whatman 1541) with a circulating water type vacuum pump (SBH-III, China), and the residue was washed 3 times with 20 mL ethanol and acetone, respectively, then oven-dried at 105℃ for 4 h, weighed for determination of IVDMF.

*2.5. 16S rDNA amplicon sequencing*

Microbial genomic DNA in the fermentation residues was extracted with Qiagen DNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The V3–V4 hypervariable regions of the 16S rDNA was amplified using an ABI GeneAmp® 9700 thermocycler (ABI, CA, USA). The PCR products were detected using a 2% (w/v) agarose gel and purified with AxyPrep DNA Gel (Axygen). The purified amplicons underwent paired-end sequencing (2 × 300 bp) using the Illumina HiSeq platform (Illumina, San Diego, USA), following the standard protocols provided by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China), as previously documented [24]. Clean sequences were clustered into operational taxonomic units (OTUs) utilizing UPARSE (version 7.0.1090, http://drive5.com/uparse/) with a 97% similarity threshold [25]. The OTUs were classified using the Ribosomal Database Project (RDP) classifier (version 11.1, http://rdp.cme.msu.edu/) with a confidence threshold of 0.7 [26].

*2.6. Statistical analysis*

TTEST procedure of SAS 8.0 (SAS Institute, Inc., Cary, NC, USA) was conducted to compare the gas production, IVDMF, and SCFA production between ingredients and corresponding residues fermentation. Difference in SCFAs and IVDMF among ingredients or residues was also analyzed using one-way ANOVA with Duncan's multiple comparison test. Alpha-diversity (Sobs, Shannon, ACE, and Chao indexes) was analyzed by student's *t*-test between ingredient and residue, and also analyzed by Wilcoxon rank-sum test among ingredients or residues. Beta-diversity (the principal coordinate analysis, PCoA) based on the Bray-Curtis distance and ANOSIM test was employed using Majorbio Cloud Platform (www.i-sanger.com). The significant difference in microbial community between ingredients and residues was tested by the Mann–Whitney *U* test, and FDR value (<0.05) was used to correct the multiple testing. $P < 0.05$ was considered as a significant difference, and $0.05 < P < 0.1$ was considered as a tendency toward significance.

## 3. Results

*3.1. Difference in gas production between fiber-ingredients and their corresponding residues*

According to the cumulative gas production during *in vitro* fermentation (Fig. 2), the results showed that the gas production of ingredients was changed after simulated gastric and small intestinal digestion. Gas production of WB was reduced ($P < 0.05$, Fig. 2A) after *in vitro* simulated digestion. The cumulative gas production of simulated residue of SH, SBP, DDGS, and AM were improved ($P < 0.05$, Fig. 2B-E). During the early stage of *in vitro* fermentation, simulated digestion improved the gas production of RH ($P < 0.05$, Fig. 2F), after 16 h of fermentation, the gas production between RH and RRH was similar (Fig. 2F). In addition, the sorting of the ingredients based on gas production was altered after *in vitro* digestion.

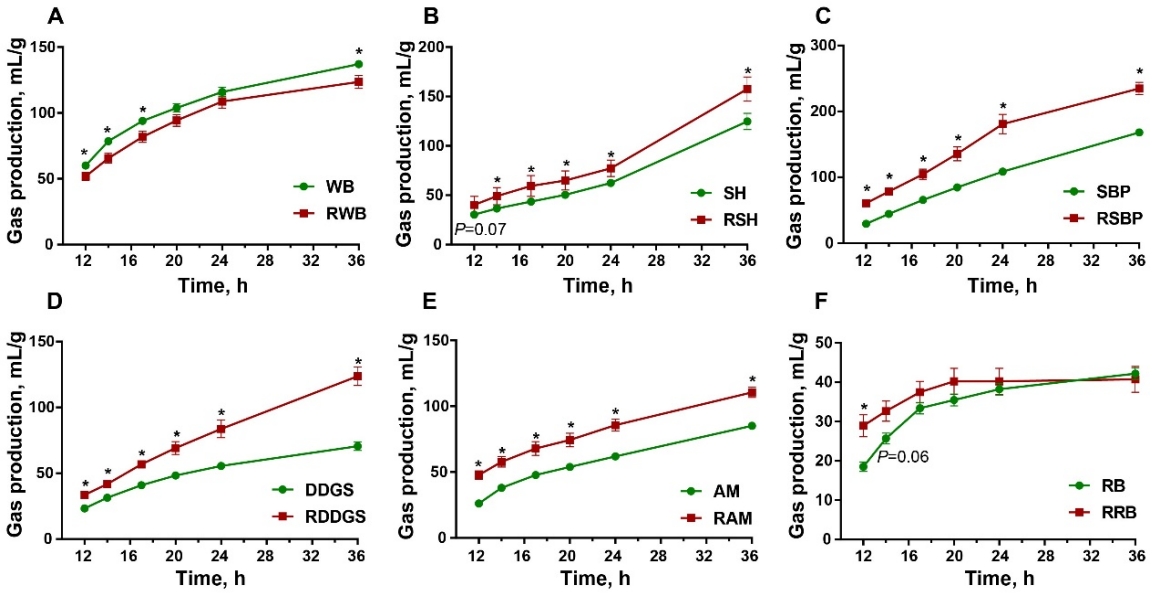


**Figure 2** Gas production of fiber ingredients and simulated digestion residue over time during *in vitro* fermentation (n = 4-5 bottles/substrate, Mean ± SEM). (A) Gas production of WB and RWB fermentation. (B) Gas production of SH and RSH fermentation. (C) Gas production of SBP and RSBP fermentation. (D) Gas production of DDGS and RDDGS fermentation. (E) Gas production of AM and RAM fermentation. (F) Gas production of RB and RRB fermentation. * indicates $P < 0.05$. WB, wheat bran; DDGS, corn distillers dried grains with soluble; SBP, suger beet pulp; SH, soybean hull; AM, alfalfa meal; RH, rice husk; RWB, residual of WB; RDDGS, residual of DDGS; RSBP, residual of SBP; RSH, residual of SH; RAM, residual of AM; RRH, residual of RH.

### *3.2. Difference in IVDMF and SCFAs between fiber-ingredients and their residues*

The IVDMF and SCFAs production were altered after the ingredients performed a prior *in vitro* simulated gastro-intestinal digestion. After *in vitro* digestion, IVDMF of WB, AM, and RH was reduced, whereas that of SH, SBP, and DDGS was increased ($P < 0.05$, Fig. 3A). After simulated *in vitro* digestion, the production of acetate, propionate, butyrate, and total SCFAs was decreased for WB and RB fermentation ($P < 0.05$, Fig. 3B-D, F), whereas valerate production was increased for RB fermentation ($P < 0.05$, Fig. 3E). After simulated *in vitro* digestion, SH fermentation reduced butyrate yield and improved valerate yield ($P < 0.05$, Fig. 3D, E), whereas SBP fermentation improved the butyrate and valerate production, reduced propionate production ($P < 0.05$, Fig. 3C-E). *In vitro* digestion of DDGS improved SCFAs except propionate production ($P < 0.05$, Fig. 3B, D-F). No significant difference in SCFAs production between AM and its digestion residue ($P > 0.05$, Fig. 3B-F).

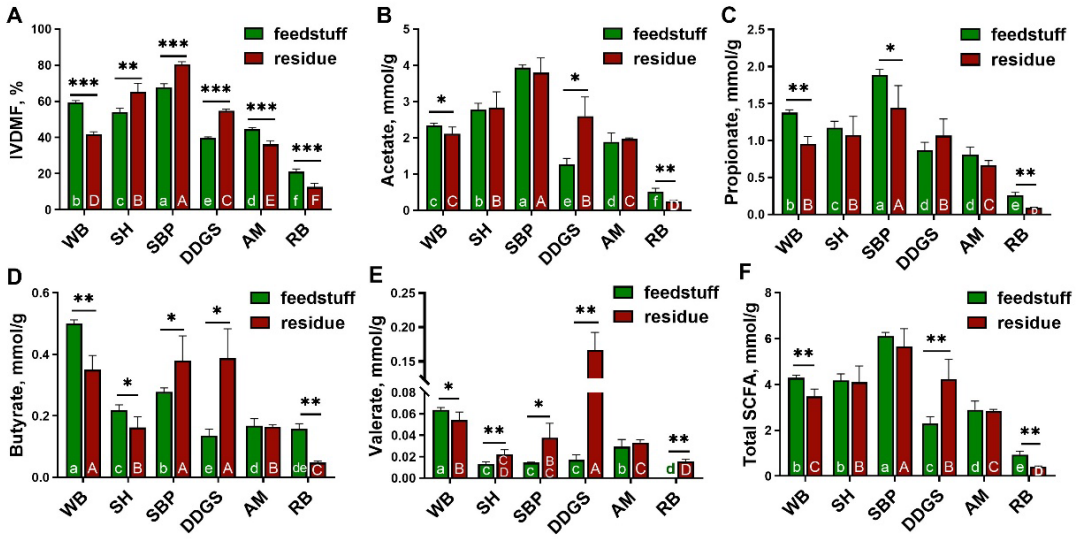


**Figure 3** Effect of gastro-intestinal simulated digestion on IVDMF and SCFA production of fiber-rich ingredients fermentation. (A) The in vitro dry matter fermentability of ingredients and residues fermentation. (B) Acetate production of ingredients and residues fermentation. (C) Propionate production of ingredients and residues fermentation. (D) Butyrate production of ingredients and residues fermentation. (E) Valerate production of ingredients and residues fermentation. (F) Total SCFAs production of ingredients and residues fermentation. * indicates $P < 0.05$, ** indicates $P < 0.01$. Feedstuffs or residues with different letters (a, b, c, d or A, B, C, D) are significant different at $P < 0.05$ among 6 feedstuff groups or 6 residue groups.

### *3.3. Differences in microbial diversity between ingredients and their simulated digestion residues*

After simulated gastro-intestinal digestion, the Sobs, Chao1, and Ace indexes of WB, SBP, and AM significantly increased ($P < 0.05$, Fig. 4A-C). The Shannon index of WB, SH, and SBP were increased, whereas that of AM and RB were decreased ($P < 0.05$, Fig. 4D). In addition, microbial alpha-divestiy (Sobs, Chao1, Ace, and Shannon indexes) has significant difference among ingredients or among their simulated digestion residues ($P < 0.05$, Fig. 4).

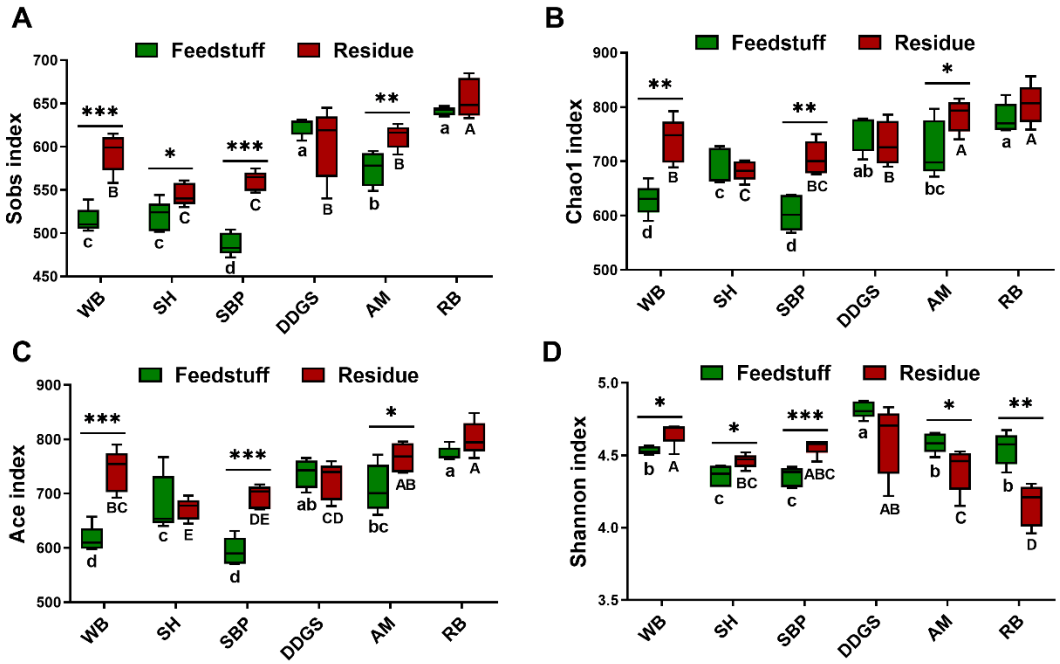


**Figure 4** Difference of in vitro fermentation of fiber-rich ingredients and simulated digestion residues on Alpha-diversity. (A) Sods index. (B) Chao1 index. (C) Ace index. (D) Shannon index. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. Boxes (a, b, c, d or A, B, C, D) of feedstuffs or residues with different letters are significant different at $P < 0.05$ among 6 groups.

PCoA also showed that community of microbiota was clear difference between ingredients and residues fermentation ($P < 0.01$, Fig. 5A-F), indicating a prior *in vitro* digestion of dietary fibers could affect their fermentation properties to form different microbial communities. The community of microbiota was also different among different ingredients or residues ($P < 0.01$, Fig. 5G, H).

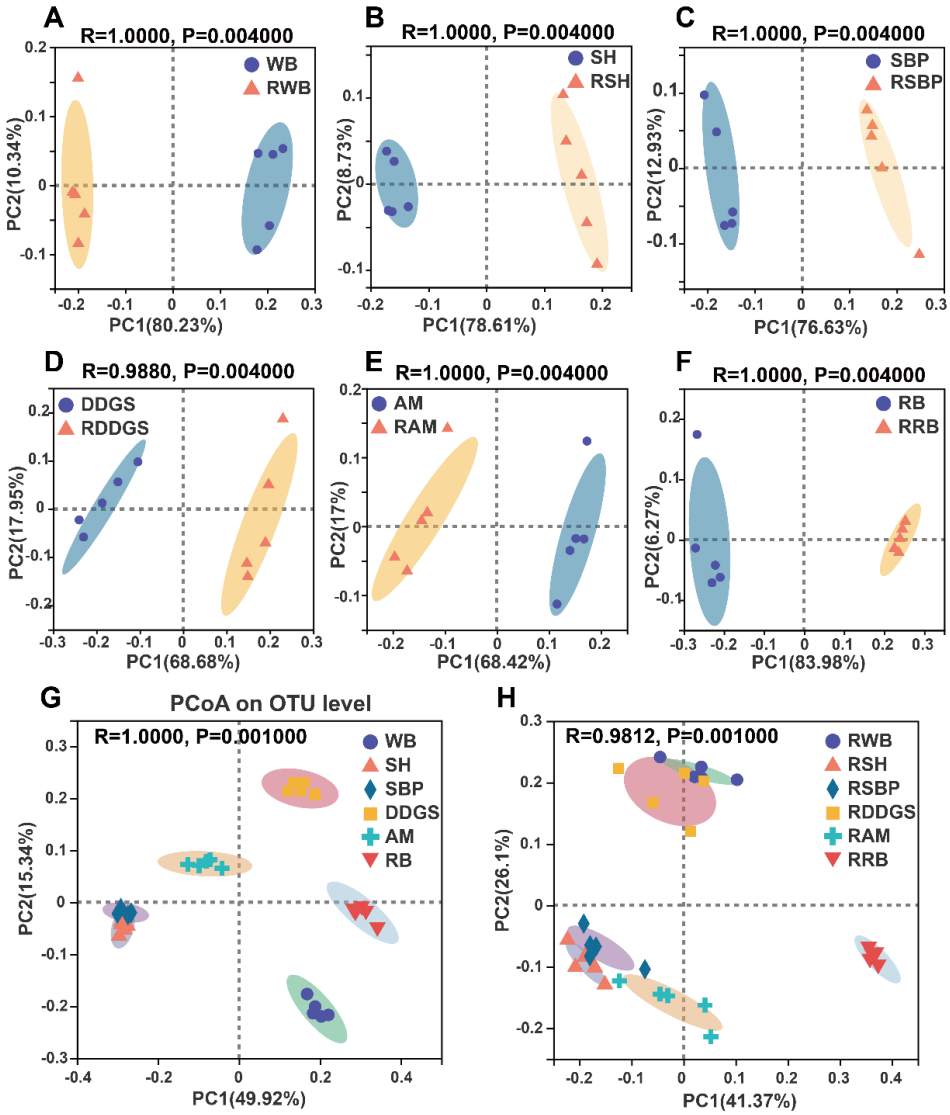


**Figure 5** Difference between fiber-rich ingredients and simulated digestion residues on Beta-diversity. (A) PCoA analysis of microbiota in WB and RWB fermentation. (B) PCoA analysis of microbiota in SH and RSH fermentation. (C) PCoA analysis of microbiota in SBP and RSBP fermentation. (D) PCoA analysis of microbiota in DDGS and RDDGS fermentation. (E) PCoA analysis of microbiota in AM and RAM fermentation. (F) PCoA analysis of microbiota in RB and RRB fermentation. (G) PCoA analysis among 6 ingredients fermentation. (H) PCoA analysis among 6 residues fermentation.

### *3.4. Mcrobial composition and Alteration of Specific Microbiota*

At the phylum levels (Fig. 6A), the domainate phyla were Firmicutes, Bacteroidetes, Spirochaetota, Proteobacteria, and Fibrobacterota, accounting for more than 96% on average in the each group. After *in vitro* digestion, SBP and RB fermentation improved (FDR < 0.05, Fig. 6D, G) the Firmicutes abundance, SBP fermentation increased Proteobacteria abundance (FDR < 0.05, Fig. 6D), whereas RB fermentation decreased Proteobacteria abundance (FDR < 0.05, Fig. 6G). RWB and RAM fermentation also reduced the abundance of Proteobacteria (FDR < 0.05, Fig. 6B, F). RSBP, RAM and RRB fermentation reduced the Spirochaetota abundance (FDR < 0.05, Fig. 6D, F, and G). The abundance of Fibrobacterota was decreased

in RSBP fermentation (FDR < 0.05, Fig. 6D) and tended to decrase in RSH fermentation (FDR < 0.1, Fig. 6C).

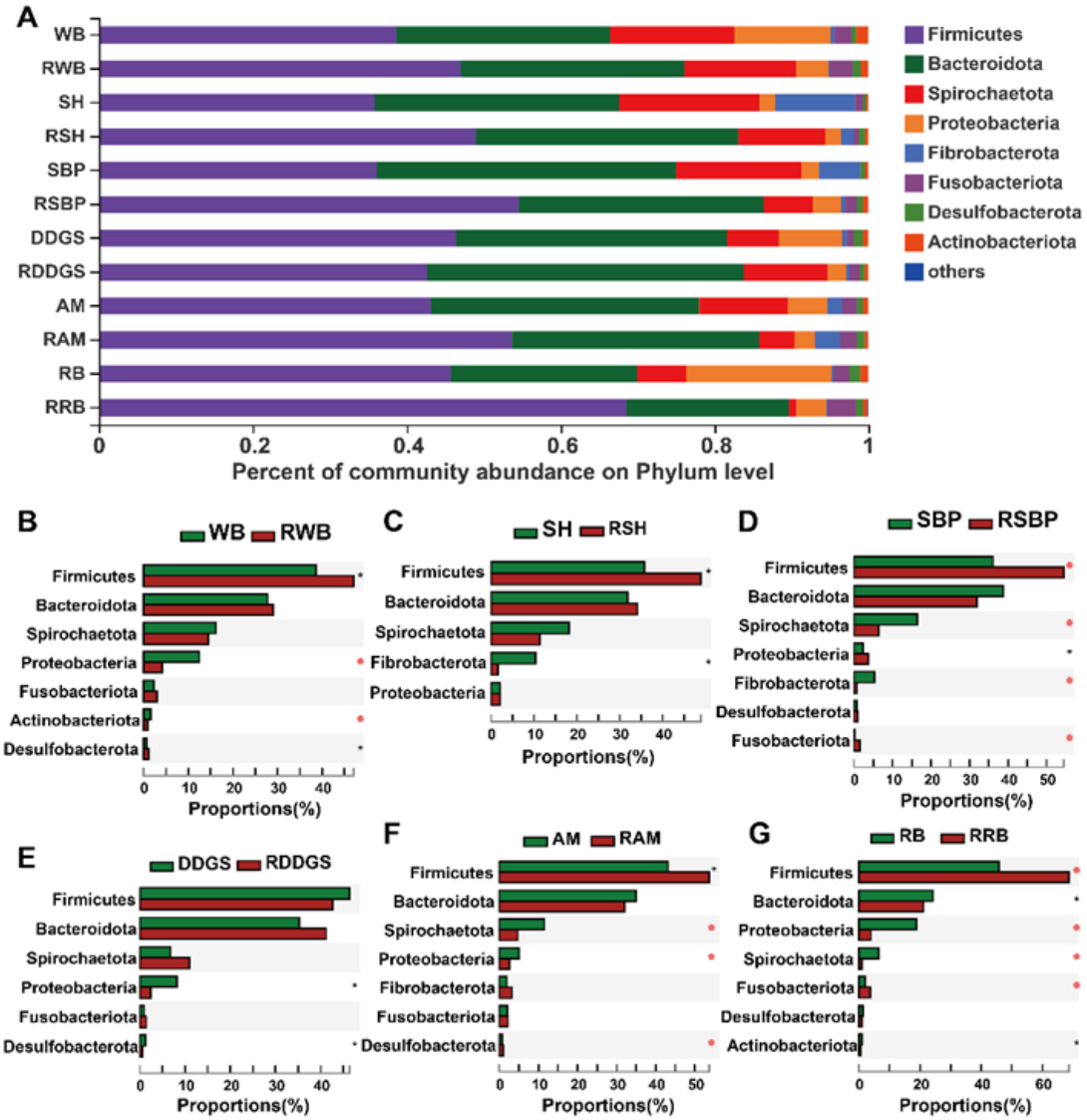


**Figure 6** Community composition analysis and diferential microbiota analysis at phylum levels. (A) The microbial composition at the phylum level. (B) Differentially abundant phylum between WB and RWB fermentation. (C) Differentially abundant phylum between SH and RSH fermentation. (D) Differentially abundant phylum between SBP and RSBP fermentation. (E) Differentially abundant phylum between DDGS and RDDGS fermentation. (F) Differentially abundant phylum between AM and RAM fermentation. (G) Differentially abundant phylum between RB and RRB fermentation. Red star indicates corrected *P* value (FDR) < 0.05, Black star indicates 0.05 < *P* value (FDR) < 0.1.

At the genus levels (Fig. 7), the top ten dominant genera were *Treponema, Prevotellaceae_NK3B31_group, Prevotella, Lysinibacillus, Ruminococcus, Lactobacillus, Escherichia-Shigella, Lachnospiraceae_AC2044_group, Lachnospiraceae_NK4A136_group, Clostridium_sensu_stricto_1*, which consists of 52% - 70% of total genera.

The top 10 genera of the fermentation broth attracted more attention, and were analyzed using Mann–Whitney *U* test with corrected *P*-value to identify the differential genera. After simulated digeston, the abundance of *Prevotellaceae_NK3B31_group* was improved in RWB fermentation (FDR < 0.05, Fig. 7B), and were reduced in RRB fermentation (FDR < 0.05, Fig. 7G). The abundance of *Succinivibrio* was decreased in RWB and RRB fermentation (FDR < 0.05, Fig. 7B, G). The relative abundance of *Treponema*

was improved in RDDGS fermentation (FDR < 0.05, Fig. 7D), but was reduced in RSBP, RAM, and RRB fermentation (FDR < 0.05, Fig. 7E-G), in which the abundance of *Lysinibacillus* was improved (FDR < 0.05, Fig. 7E-G). The abundance of *Lactobacillus* was decreased in RDDGS fermentatation (FDR < 0.05, Fig. 7D) and was increased in RRB fermentation (FDR < 0.05, Fig. 7G).

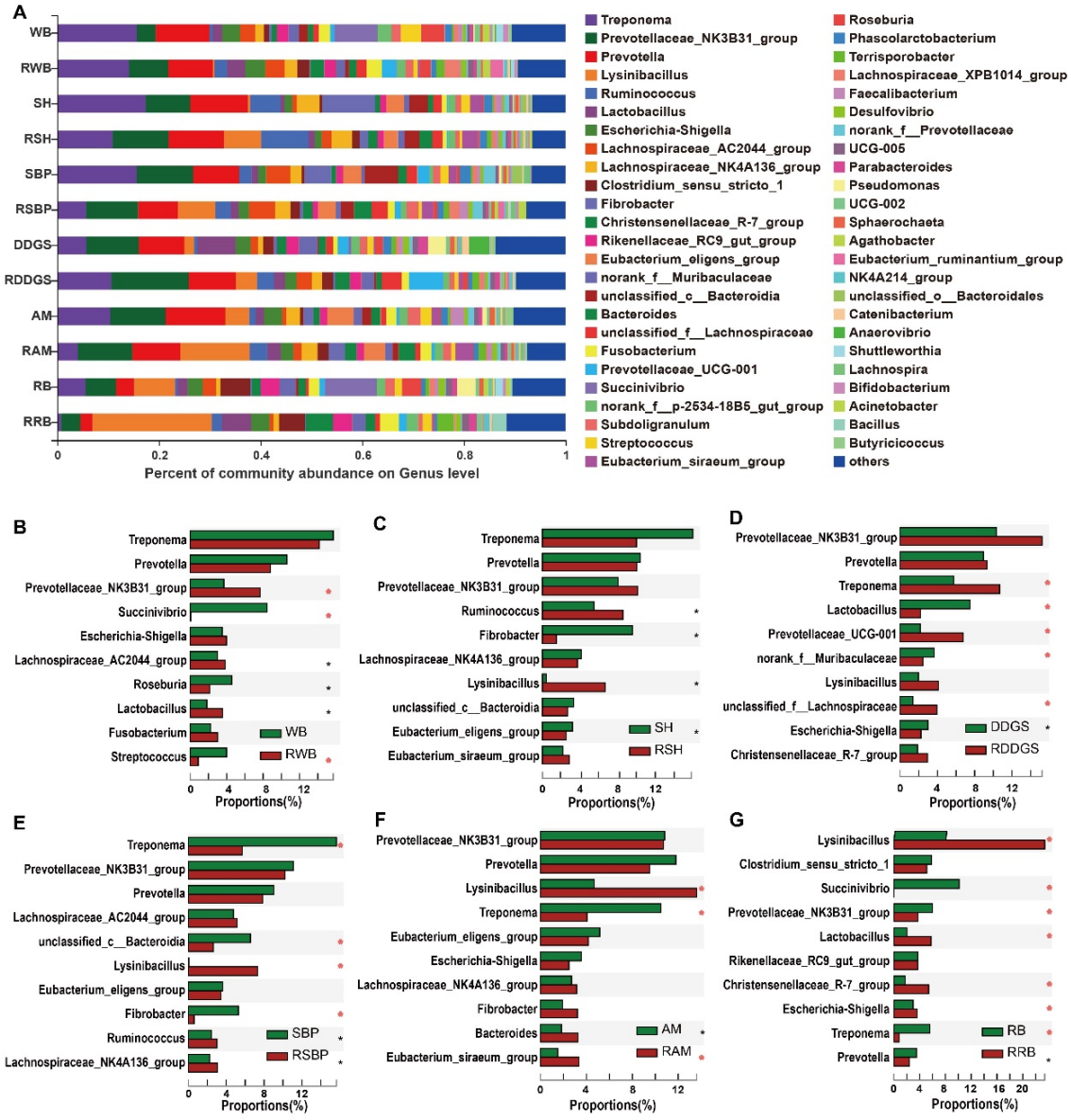


**Figure 7** Community composition analysis and differential genera analysis at genera levels (Top 10). (A) The microbial composition at the genera level. (B-G) Differentially abundant genera between WB and RWB fermentation. (C) Differentially abundant genera between SH and RSH fermentation. (D) Differentially abundant genera between SBP and RSBP fermentation. (E) Differentially abundant genera between DDGS and RDDGS fermentation. (F) Differentially abundant genera between AM and RAM fermentation. (G) Differentially abundant genera between RB and RRB fermentation. Red star indicates corrected *P* value (FDR) < 0.05, black star indicates 0.05 < FDR < 0.1

## 4. Discussion

Gas production, IVDMF and SCFA production typically associated with fermentation, which can reflect the fermentation rate and extent of DF by microorganisms [27, 28]. The indicator of IVDMF can be used to rapidly estimate apparent total tract digestibility of DM and gross energy (GE) in ingredients [29]. The relevance of gas production is usually correlated with the abdominal discomfort caused by DF fermentation in the gut, which due to excessive ingestion of fast fermented fiber [30]. For example, SBP and WB are already

used as fiber supplements in baked goods and cereals. Understanding how pre-digestion alters their fermentation kinetics could inform strategies to design "slow-release" fibers for human diets that minimize abdominal discomfort while maximizing distal SCFA delivery. The metabolites production during fermentation, such as SCFAs, are more stable at slower fermentation rates, resulting in more even distribution of substances in the intestine, an increased likelihood of meeting the energy requirements of the distal colon, and a reduced colonic inflammation [31]. Therefore, dietary fiber with a slow and steady fermentation rate may be preferred. The physicochemical properties of dietary fibers (nutrient content, chemical structure, and physical form) are critical factors determining fermentation rate/extent [28], indicating that the changes of those factors may affect the fermentation properties of DF. It has been reported that *in vitro* simulated digestion modified the cell-wall matrix and predisposing the fibers to further fermentation [32]. In the present study, the gas production kinetics and the ranking order of the fermented substrates after simulated *in vitro* digestion were changed, which may be due to the reduction of the amounts of digestible material and increment in indigestible fiber content [15]. Moreover, water-retention capacity and swelling capacity are important properties of fiber. The fiber substrates, such as SBP, would swell caused by water absorption during *in vitro* digestion, making fiber structure becomes loose and increasing nutrient accessibility for microbes, thereby affecting fermentation rate. In our results, the gas production of WB after *in vitro* digestion was reduced, whereas other ingredients after *in vitro* digestion had higher gas production during fermentation. In line with our results, Bindelle et al. [15] reported that after enzymatic hydrolysis, gas production of WB fermentation was decreased, while that of soybean meal (SBM) fermentation was increased. This result may be because WB has higher starch content, which was largely degraded and removed during *in vitro* enzymes digestion [33]. Consistent with the results of gas production, the IVDMF of SH, DDGS, and SBP was also improved after *in vitro* enzymatic hydrolysis. However, in addition to WB, IVDMF was also reduced in RB and AM fermentation after simulated gastrointestinal digestion. Study has reported the hydrolysis prior to the *in vitro* fermentation of SBP resulted in an increment in fermentation extent [32], which is consistent with our results. Moreover, SBP and SH have higher SDF content and better swilling capacity, which causes largely changed in fiber structure. While the fiber of WB, RB and AM have high degree of lignification, which difficult to ferment. After simulated *in vitro* digestion, starch and proteins are hydrolyzed and removed from raw materials, resulting in the amount of substrates that can be directly utilized by microbiota was reduced, which increases the difficulty of fermentation [32]. Whereas, higher gas production of RB and AM residues may be ascribed to the inclusion of non-dietary materials, mainly simulated digestive enzymes, during in vitro digestion. Some studies have reported that non-dietary materials interfere with the dietary fiber fermentation [34]. In previous study, SH, SBP, and OB showed higher fermentability and SCFA concentration than CB and WB in the intestine of pigs, whereas the inconsistent results were obtained in vitro [9, 10]. In the present study, after simulated *in vitro* digestion, the fermentability of SBP and SH was superior to WB, which was consistent with the results of in vivo [9]. The results indicated that the residues obtained from in vitro digestion using CCSDS might be similar to ileal digesta in fermentation properties.

SCFAs, mainly composed by acetic acid, propionic acid and butyric acid, are physiologically important metabolites of gut microbiota fermentation with multiple functions for human health [35]. For example, acetate and propionate regulate systemic metabolism and immune responses [36, 37]. Butyrate is a key energy source for colonocytes, enhances gut barrier integrity, and exhibits anti-inflammatory effects [4, 5]. The production of SCFAs is associated with microbiota compositions, which could indicate the microbial fermentation capabilities [38]. In the present study, the SCFA production in WB and RB fermentation after simulate digestion was reduced, which in line with the lower IVDMF of WB and RB residues fermentation. DDGS residues fermentation improved the SCFAs production, especially acetate and butyrate. SBP residues fermentation increased the butyrate yield, whereas decreased the propionate yield. Except for the changes of chemical and physicochemical characteristics of dietary fibers after in vitro simulated digestion, this result can be further explained by the changes in the microbiota composition during fermentation. In this study, the microbial α-diversity in simulated digestion residues as fermentation substrate was improved, which is likely due to the concentration of fiber components. Several animal studies have showed that a high fiber diet increased gut microbial diversity [39, 40]. However, microbial diversity cannot fully explain the fermentation capacity. The fermentation ability depends mostly on the composition of microbiota in the gut [41]. In this study, we found that ingredients after in vitro simulated digestion improved the Firmicutes abundance and reduced Bacteroidetes abundance during in vitro fermentation. Firmicutes are the predominant group in microbiota that degrade fibers to produce butyric acid [42], and Bacteroidetes are the main bacterial groups that degrade carbohydrates to produce propionic acid [43]. In present study, after the pre-digestion, the decrease of propionic acid production may be related to the decrease of Bacteroidetes abundance in RRB fermentation, while the increase of butyric acid production in RSBP fermentation may be related to the increase of Firmicutes abundance. However, the abundance of Firmicutes in RRB fermentation increased while butyric acid decreased, which may be due to the low fermentability of RRB, and the produced SCFA was utilized by the microbiota to regulate growth and metabolism [44]. *Succinivibrio* is fiber-degrading bacteria, and is positively correlated with the concentration of butyric acid [45], which was reduced in RB and WB residues fermentaion. The result is also consistent with the less butyric acid produced during RB and WB residues fermentation than ingredients fermentation.The higher abundance of *Fibrobacterota* in SH and SBP fermentation may be related to the high fermentable oligosaccharides content, which is consistent with previous reports [9]. *Treponema* is reported to be a cellulose-degrading bacteria, usually involved in the degradation of soluble fibers and plant polysaccharides [46], *Lysinibacillus*, a genus of Bacillus, can also secrete fiber-degrading enzymes to improve fiber utilization. In this study, we found that the abundance of *Treponema* increased in RDDGS compared to DDGS fermentation. A lower total amount of SDF (substrate weight × SDF content in substrate) was also observed in the RDDGS compared to DDGS, suggesting that SDF concentration alone did not drive the observed *Treponema* increase. It is known that the microbial population dynamics are also affected by substrate contents [47]. Therefore, this variation may be explained by the complex interplay of substrate contents and multiple components, not solely by SDF content. The abundance

of *Lysinibacillus* increased in RRB and RAM fermentation, whereas it remained unchanged in RWB, RDDGS and RSH fermentation, which might appear to be primarily influenced by the polysaccharide profile of the fiber rather than the levels of poorly fermentable fibers (ADF). Consequently, after bionic pre-digestion of different substrates, the microbial changes are not consistent. Bindelle et al. [48] reported that NSP enzyme added in pre-digestion increased the cellulolytic *Ruminococcus*- and xylanolytic *Clostridium*-like bacteria, and the shifts were different in microbial community and the fermentation patterns for different fiber sources, indicating that pre-digestion modifies both the physical architecture and chemical makeup of the substrate, accordingly shifting the fermentation dynamics and patterns of the fibrous sources. Our study confirms that following simulated gastrointestinal digestion, the fermentation properties of fibrous ingredients undergo changes, including fermentation kinetics, SCFA production, and microbial composition. Therefore, evaluating the fermentation characteristics using ingredients that have undergone simulated gastriointestinal digestion as substrates may yield results that are more closely with the actual physiological conditions, providing more meaningful guidance for the selection of dietary fiber for humans.

Studies have shown that in vitro enzymatic hydrolysis before fermentation can significantly affect the fermentation patterns of substrate [15, 48]. However, among the reported enzymatic hydrolysis methods, the in vitro digestion products were removed by filtration with filter paper, which results in the disappearance of part of the soluble fiber [49]. In this study, the in vitro digestion was conducted using CCSDS with a novel in vitro digestion procedure according to *in vivo* physiological conditions including digestion time, enzyme activities, and the clearance of byproducts in the tract of growing pigs [17], which the determinded in vitro digestibility of protein and energy was similar to that of in vivo values. In addition, the removed of enzymatic hydrolysis products was controlled by dialysis tubes (14 kDa molecular-mass cutoff) with circulated buffer and deionized water, which prevent the loss of soluble dietary fiber in in vitro digestion. Therefore, the residues used in our study may be similar to ileal digesta in terms of dietary fiber composition and content. However, whether the fermentation properties were similar between in vitro digestion residues and ileal digesta still need further studies.

## 5. Conclusion

This study demonstrates that simulated gastrointestinal digestion using CCSDS critically modified the fermentation characteristics of the fiber-rich ingredients, with gas kinetics, IVDMF, SCFA profiles, and microbial communities altered. Specifically, gas production, IVDMF, or SCFAs yield was increased in the fermentation of SH, DDGS, and SBP residues, but decreased in WB and RB residues fermentation compared to their corresponding untreated ingredients. Additionally, microbial communities shifted significantly, with Firmicutes increased in most residues. Genera like *Treponema* and *Lysinibacillus* were differently enriched, driving substrate-specific SCFA patterns. These findings highlight the importance of considering prior gastrointestinal digestion when evaluating the fermentation properties of DF, providing critical reference for the selection of fiber-rich ingredients and to promote gut health.

## Conflicts of interest

The authors declare no conflicts of interests.

## Acknowlegments

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