Journal of Food and Nutrition Research, 2013, Vol. 1, No. 6, 156-163
Available online at http://pubs.sciepub.com/jfnr/1/6/7

DOI:10.12691/jfnr-1-6-7

Shifts in Bacterial Community Compositions during *in vitro* Fermentation of Amylopectin and Resistant Starch by Colonic Inocula of Pigs

Xiaolin Jiang, Bo Li, Yong Su*, Weiyun Zhu

Laboratory of Gastrointestinal Microbiology, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, P. R. China
*Corresponding author: yong.su@njau.edu.cn

Received September 30, 2013; Revised November 27, 2013; Accepted December 13, 2013

Abstract Starch, which escapes the digestion of the small intestine in humans and animals, may serve as a carbon source for bacterial fermentation in the hindgut. This study aimed to compare the fermentation characteristics of amylopectin and resistant starch by the colonic microbiota of pigs, and also to reveal the shifts in bacterial community compositions during the fermentation. Two types of resistant starch (RS2 and RS4) and amylopectin were used as substances in an *in vitro* fermentation test. As compared with resistant starch, amylopectin was more fermentable by colonic microbiota, while RS4 used in this study showed very poor fermentation characteristics. Fermentation of amylopectin produced more short-chain fatty acids with a higher propionate proportion and a lower butyrate proportion. Lactate was produced in the early period of amylopectin and RS2 fermentation but consumed entirely at the end of fermentation. Pyrosequencing analysis showed that the abundance of Firmicutes decreased significantly along with the increase of Bacteroidetes during the fermentation of amylopectin and RS2. In particular, *Bacteroides* spp. (such as *Bacteroides vulgatus* and *B. uniformis*) became predominant in the bacterial community. These results suggest that *Bacteroides* spp. may play important roles in the degradation of starch in the pig hindgut.

Keywords: *resistant starch, amylopectin, Bacteroides, pig, colonic microbiota*

Cite This Article: Xiaolin Jiang, Bo Li, Yong Su, and Weiyun Zhu, "Shifts in Bacterial Community Compositions during *in vitro* Fermentation of Amylopectin and Resistant Starch by Colonic Inocula of Pigs." *Journal of Food and Nutrition Research* 1, no. 6 (2013): 156-163. doi: 10.12691/jfnr-1-6-7.

1. Introduction

It is known that starches are one of the major carbohydrates available in the human colon [1]. Starch that escapes digestion in the human and animal small intestine can enter the large intestine, where it is used as a substrate for bacterial fermentation. This fermentation results in the production of short-chain fatty acids (SCFAs), of which butyrate is considered to have beneficial effects upon hindgut health [2,3]. The reasons for these starches being resistant include physical inaccessibility (resistant starch type 1, RS1), native granular structure (resistant starch type 2, RS2) and retrogradation resulting from heating and cooling (resistant starch type 3, RS3), and chemical modification (resistant starch type 4, RS4) [4].

In recent years, the degradation of starch by hindgut microbiota has been extensively studied. A wide range of colonic bacteria, including *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium* and *Propionibacterium* can utilize amylopectin (waxy starch) and soluble starch, while only a few species of *Bifidobacterium* and *Clostridium* have the capacity to degrade amylomaize starch [5]. Although many amylolytic bacteria have been described from the human and animal gastrointestinal tract, our understanding of the microbial ecology of starch fermentation is currently limited. Based on pure culture or co-culture experiments, previous studies on starch degradation by gut microbiota were largely focused on few genera such as *Bifidobacterium* [6,7] and *Bacteroides* [8,9]. However, significantly increase of Phylotypes related to *Ruminococcus bromii* was found in the large bowel of humans fed diets containing high resistant starch [10]. By using 16S ribosomal RNA (rRNA)-based stable isotope probing (SIP), Kovatcheva-Datchary *et al*. also found populations related to *R. bromii* were the primary starch degrader, while bacteria related to *Prevotella* spp., *Bifidobacterium adolescentis* and *Eubacterium rectale* might be further involved in the trophic chain [9]. In a recent study, *R. bromii* was confirmed to possess an exceptional ability to colonize and degrade starch particles when compared with previously studied amylolytic bacteria from the human colon [11].

Cross-feeding between microbiota is regarded as a prevalent phenomenon in the gut of human and animals [12,13]. In a co-culture experiment, cross-feeding of lactate was observed between a starch-degrading species, *Bifidobacterium adolescentis*, and lactate utilizers, which suggested that a complex bacterial community may be involved in the metabolism of starch in the gut [12]. Thus, for a comprehensive understanding of this process, a high

throughput analysis is still needed to reveal the changes in the microbial community.

Although starch that enters the hindgut of human and animals is believed to be degraded by microbiota, the difference in fermentation characteristics of different starches is not well understood. The pig's intestinal tract is considered to be an appropriate model system for the human intestinal tract [14]. Therefore, by using the colonic microbiota of pigs as inocula, this study aimed to compare the fermentation characteristics of amylopectin, RS2 and RS4, and in particular, to reveal the shifts in the bacterial community during the fermentation of starch.

2. Materials and Methods

2.1. Substrates and Inocula

Three different starches purchased from National Starch Ltd. (China) were amylopectin (AMIOCATM starch), RS2 (Hi-MaizeTM starch) and RS4 (NOVELOSE 2480). Before packaging of the medium, approximately 1.00 g of each substrate was weighed and added to each fermentation bottle. Pigs donated for their colonic digesta were obtained from the experimental farm of Nanjing Agricultural University. After five pigs (50 days of age) were euthanized, the colon was immediately ligated with cotton thread and sent to the laboratory in foamed plastic containers filled with CO_2 within one hour. In the laboratory, sterile anaerobic saline (0.9% NaCl) was prepared. Equal amounts of feces from each animal were combined (by wet weight) and diluted 1:5 with prewarmed (to 39°C) sterile anaerobic saline. This diluted mixture was homogenized for 60 s to ensure proper mixing and was then filtered through a double piece of clean cheesecloth (16 threads/cm in each direction). The resultant filtrate was used as the inocula.

2.2. In Vitro Fermentation Procedure

An *in vitro* fermentation test was conducted according to the description of Awati et al. [15]. To maintain anaerobic conditions, all procedures were carried out under a constant stream of carbon dioxide. Seventy-eight ml of a semi-defined medium was added to the bottles, with 5 ml of a bicarbonate/vitamin solution and 1 ml of reducing agent [16]. Five milliliters of inocula were injected into each fermentation bottle. The bottles were incubated at 37°C for 48 h. All incubations were conducted in triplicate. For blanks, only the inoculum without any added substrate was incubated. Gas production was measured by using a pressure transducer according to the method of Theodorou et al. [17]. In all incubations, samples were harvested after 0 h, 6 h, 12 h, 24 h and 48 h and stored at -20°C for measurement of lactate and SCFA concentrations. At each time point, the pH value was also recorded. Samples in the amylopectin and RS2 groups during the fermentation in 24 h were collected for further investigation of the compositions of bacterial community by molecular analysis.

2.3. Gas Production Kinetics

The data for cumulative gas production (as ml of gas produced per g dry matter over time) were fitted to the mono-phasic model as described by Groot et al. [18] and shown in the following equation:

$$G = A / \left(1+\left(C/t\right)^{B}\right)$$

where G = total gas produced, A = estimated asymptotic gas production, B = switching characteristic of the curve, and C = time at which half of the asymptotic gas production has been reached ($T_{1/2}$). The maximum rate of gas production (R_{max}) and the time at which it occured (T_{max}), were calculated according to the following equations [19]:

$$R_{max} = (A*(C^{B})*B*(T_{max}^{(-B-1)}))/(1+(C^{B})*(T_{max}^{(-B)}))^{2}$$

$$Tmax = C*\left(\left((B-1) / (B+1)\right)^{(1/B)}\right)$$

2.4. Analysis of Lactate and Short-chain Fatty Acids

The concentration of lactate was determined by using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). SCFA concentrations in the fermentation liquids were analyzed by using a capillary column gas chromatograph (GC-14B, Shimadzu, Japan; Capillary Column: 30 m × 0.32 mm × 0.25 μm film thickness). The column, injector and detector temperature were 120, 180 and 180°C, respectively [20].

2.5. Pyrosequencing Analysis of Microbial Diversity

Total DNA was extracted from the fermentation fluids by the bead-beating method as described by Zoetendal et al. [21]. To analyze the taxonomic composition of bacterial community, the universal 16S rRNA gene primers (8F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 533R 5'-TTA CCG CGG CTG CTG GCA C-3') were chosen for the amplification and subsequent pyrosequencing of the polymerase chain reaction (PCR) products [22]. PCR was carried out in triplicate 50-μl reactions with a 10 μl five-fold reaction buffer, 25 ng of DNA, 0.4 μM of each primer, 2.5 U Pfu polymerase (TransStart-FastPfu DNA Polymerase, TransGen Biotech), and 0.25 mM dNTPs. The amplification program consisted of an initial denaturation step at 94°C for 4 min, followed by 25 cycles, where 1 cycle consisted of 94°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 30 s (extension), and a final extension of 72°C for 10 min. PCR products were visualized on agarose gels (2% in a TBE buffer) containing ethidium bromide, and purified with a DNA gel extraction kit (Axygen, China).

Prior to sequencing, the DNA concentration of each PCR product was determined using a Quant-iT PicoGreen double stranded DNA assay (Invitrogen, Germany) and was quality controlled on an Agilent 2100 bioanalyzer (Agilent, USA). Amplicon pyrosequencing was performed from the A-end using a 454/Roche A sequencing primer kit on a Roche Genome Sequencer GS-FLX Titanium platform at Majorbio Bio-Pharm Technology Co., Ltd., in Shanghai, China. All pyrosequencing reads were filtered according to barcode and primer sequences. The resulting sequences were further screened and filtered for quality. The high-quality sequences were aligned using the

'align.seqs' command and compared with the Bacterial SILVA database (SILVA version 108). The aligned sequences were further trimmed and the redundant reads were eliminated using the 'screen.seqs', 'filter.seqs', and 'unique.seqs' commands in order. The 'chimera.slayer' command was used to determine chimeric sequences. The 'dist.seqs' command was performed, and unique sequences were clustered into operational taxonomic units (OTUs) defined by 97% similarity [23]. We also calculated the coverage percentage using Good's method [24], the abundance based coverage estimator (ACE), the bias-corrected Chao richness estimator, and the Shannon and Simpson diversity indices. All the analyses were performed using the MOTHUR program.

Genera with relative abundances higher than 0.5% within the total bacteria were defined as the predominant genera and sorted for further analysis. To reveal the changes in microbial composition after 24 h of fermentation, the ratio of the relative abundance of one bacterial group in the 24-h fermentation fluid to its relative abundance in the inoculum was calculated at the genus level. OTUs in the RS2 group with the abundances higher than 0.1% in the microbial community were defined as predominant OTUs and sorted for comparing the difference during the fermentation.

2.6. Statistical Analysis

Data regarding gas production, pH value and the concentrations of SCFA and lactate were analyzed separately for each time point. The effects of the substrate (amylopectin, RS2 and RS4) on gas production, SCFA and lactate levels as well as the effect of the incubation time on the composition of bacterial community (OTU level) were tested for significance using a one-way analysis of variance (ANOVA) program in the Statistical Package for the Social Sciences (SPSS17.0). The Effects of the substrate (amylopectin and RS2) and incubation time (0 h, 6 h, 12 h and 24 h) on the composition of bacterial community were tested using a two-way ANOVA program. Significant differences were declared when $P < 0.05$.

3. Results

3.1. Cumulative Gas Production Kinetics of Different Starches

The mean values for the parameters of fermentation kinetics are shown in Table 1. Significant differences in the fermentation characteristics were observed among the three substrates. The OMCV of amylopectin was significantly higher than those of RS2 and RS4, while RS4 showed very poor fermentation characteristics. There was no significant difference in $T_{1/2}$ between amylopectin and two type of RS, while the R_{max} and T_{max} values of amylopectin were significantly higher than those of RS2.

Table 1. *In vitro* fermentation gas production kinetics of amylopectin, RS2 and RS4 by the colonic microbiota of pigs[1]

Starches	OMAV	$T_{1/2}$ (h)	Tmax (h)	RmaxG (ml/h)
Amylopectin	291.08 ± 7.20^{a}	14.20 ± 0.53^{ab}	9.48 ± 0.57^{b}	14.41 ± 0.96^{a}
RS2	162.80 ± 9.65^{b}	15.38 ± 1.14^{ab}	2.55 ± 0.66^{a}	7.00 ± 0.63^{b}
RS4	73.72 ± 5.77^{c}	17.95 ± 2.76^{a}	-	-
Blank	51.92 ± 8.70^{d}	12.04 ± 3.77^{b}	-	-

[1]The data are expressed as mean±SEM (standard error of means), n=3. OMCV, cumulative gas production (ml/g Organic Matter); T½, half time of asymptotic gas production (h); T_{max}(h), time occurrence of maximal rate of gas production; R_{max}G, maximal rate of gas production (ml/h); a different superscript letter in the same column indicates a statistically significant difference, $P < 0.05$. -, not detected.

3.2. SCFA Production of Fermentation from Different Starches

As shown in Table 2, 6 h after incubation, no significant differences in the concentrations of acetate and total SCFA were found among the three starches, while propionate and butyrate concentrations from the RS4 group were significantly lower than the other two groups. From 12 h to 36 h, fermentation of amylopectin produced significantly higher amounts of acetate, propionate, butyrate and total SCFA than RS2 and RS4 except that the difference in the concentration of butyrate between amylopectin and RS2 was not significant at 12 h. Within the two resistant starch substrates, the concentrations of SCFAs in the RS2 group were significantly higher than those in the RS4 group. A notably higher acetate to total SCFA ratio and a lower propionate to total SCFA ratio as compare to amylopectin and RS2 groups were observed in the RS4 group during fermentation. No significant differences in SCFA proportions were found between the amylopectin and RS2 groups at 6 h and 12 h after incubation, while at the time points of 24 h and 36 h, a higher butyrate to total SCFA ratio and a lower propionate to total SCFA ratio were observed in the RS2 group as compared to the amylopectin group. When compared with the SCFA proportion before fermentation, the fermentation of amylopection and RS2 produced lower acetate proportions and higher propionate and butyrate proportions in their end-products.

Table 2. The production of short-chain fatty acids (SCFA) during the *in vitro* fermentation of amylopectin, RS2 and RS4 by the colonic microbiota of pigs[1]

Incubation time	Starches	SCFA concentration (mmol/l)				SCFA proportion (%)		
		Acetate	Propionate	Butyrate	Total SCFA	AP	PP	BP
0 h		1.07 ± 0.07	0.24 ± 0.02	0.14 ± 0.02	1.45 ± 0.04	73.81 ± 2.95	16.76 ± 1.35	9.43 ± 1.66
6 h	Amylopectin	2.20 ± 1.36	2.30 ± 1.13^{a}	1.02 ± 0.43^{a}	5.52 ± 2.71	38.39 ± 6.13^{b}	41.67 ± 4.82^{a}	19.93 ± 10.03^{a}
	RS2	1.13 ± 0.34	0.90 ± 0.29^{b}	0.54 ± 0.12^{ab}	2.57 ± 0.65	43.88 ± 8.46^{b}	34.76 ± 5.21^{a}	21.36 ± 3.64^{ab}
	RS4	1.88 ± 0.42	0.12 ± 0.09^{b}	0.24 ± 0.05^{b}	2.24 ± 0.52	83.94 ± 4.93^{a}	5.14 ± 3.49^{b}	10.93 ± 2.06^{b}
12 h	Amylopectin	17.72 ± 1.57^{a}	13.44 ± 1.27^{a}	2.66 ± 0.28^{a}	33.92 ± 2.90^{a}	52.55 ± 1.56^{b}	39.63 ± 1.58^{a}	7.82 ± 0.15
	RS2	14.08 ± 2.04^{b}	9.39 ± 0.95^{b}	2.26 ± 0.28^{a}	25.73 ± 3.11^{b}	54.62 ± 1.57^{b}	36.59 ± 1.94^{a}	8.79 ± 0.75
	RS4	3.46 ± 1.21^{c}	0.41 ± 0.19^{c}	0.68 ± 0.13^{b}	4.56 ± 1.34^{c}	74.89 ± 5.92^{a}	8.75 ± 1.88^{b}	16.37 ± 7.78
24 h	Amylopectin	30.42 ± 1.92^{a}	30.77 ± 3.07^{a}	4.10 ± 0.51^{a}	65.29 ± 4.45^{a}	46.62 ± 1.62^{c}	47.09 ± 2.45^{a}	6.30 ± 0.83^{c}
	RS2	16.17 ± 2.32^{b}	8.75 ± 0.38^{b}	3.29 ± 0.11^{b}	28.21 ± 2.49^{b}	57.14 ± 3.25^{b}	31.15 ± 2.40^{b}	11.71 ± 0.86^{b}
	RS4	3.25 ± 0.34^{c}	1.05 ± 0.14^{c}	0.82 ± 0.14^{c}	5.12 ± 0.43^{c}	63.46 ± 1.44^{a}	20.62 ± 3.28^{c}	15.92 ± 2.01^{a}
36 h	Amylopectin	38.91 ± 2.31^{a}	32.84 ± 1.64^{a}	5.52 ± 0.49^{a}	77.27 ± 4.27^{a}	50.35 ± 0.52^{b}	42.52 ± 0.80^{a}	7.13 ± 0.30^{c}
	RS2	13.74 ± 2.92^{b}	9.09 ± 1.46^{b}	3.50 ± 0.35^{b}	26.33 ± 4.61^{b}	51.98 ± 1.81^{b}	34.60 ± 1.42^{b}	13.42 ± 0.62^{b}
	RS4	4.72 ± 1.05^{c}	1.39 ± 0.28^{c}	1.21 ± 0.31^{c}	7.32 ± 1.63^{c}	64.52 ± 0.94^{a}	19.05 ± 0.92^{c}	16.44 ± 0.60^{a}

[1]The data are expressed as mean±SEM (standard error of means), n=3. AP = acetic proportion; PP = propionic proportion; BP = butyric proportion. A different superscript letter of the same time point in the same column indicates statistically significant difference, $P < 0.05$

3.3. Changes in pH Value and the Concentration of Lactate during the Fermentation of Different Starches

During the fermentation, no significant difference among four groups in the pH value was found Figure 1. From 6 h to 36 h after incubation, the pH value in Am group decreased significantly as compared to other groups. A similar tendency in the changes of lactate concentration was observed when amylopectin and RS2 were used as substrates Figure 2. The concentration increased significantly in 6 h after inoculation, then decreased gradually. At 48 h of fermentation, lactate was almost entirely consumed. However, in the RS4 and blank groups, the lactate concentration maintained a low level (less than 0.5 mmol/l) during the 48-h fermentation.

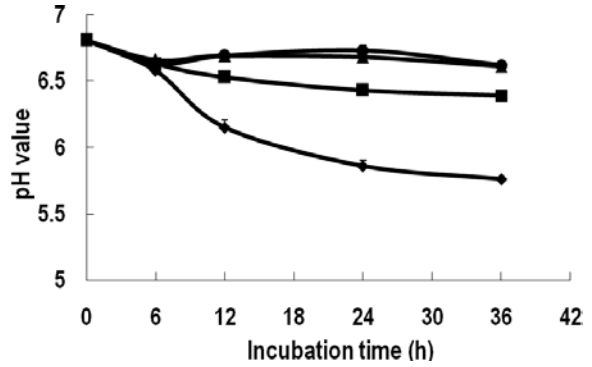

Figure 1. The pH change during the fermentation of different starches by the colonic microbiota of pigs. ,◆ amylopectin; ■, RS2; ▲, RS4; ,● blank

3.4. Changes in the Composition of Bacterial Communities during the Fermentation of Amylopectin and RS2

To reveal the changes in the composition of bacterial communities utilizing amylopectin and RS2, relative abundances of bacterial groups in fermentation fluids were determined by pyrosequencing. Across all samples, 125803 quality sequences from 137946 reads were classified as bacteria with a read length higher than 200 bp. The average length of quality sequences was 476.1 bp.

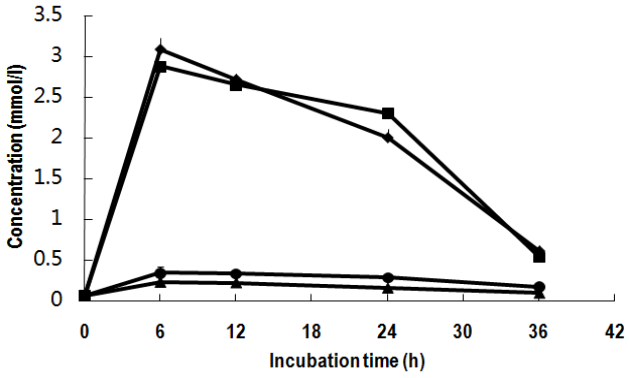

Figure 2. The lactate change during the fermentation of different starches by the colonic microbiota of pigs. ,◆ amylopectin; ■, RS2; ▲, RS4; ●, blank

Table 3. Relative abundances of phyly Firmicutes and Bacteroidetes during the fermentation of amylopetin and RS2 by colonic inocula of piglets

Substrates	Incubation time	Proportion in total bacteria (%)	
		Firmicutes	Bacteroidetes
Amylopectin	6 h	83.97	13.34
	12 h	15.01	84.54
	24 h	3.88	95.87
RS2	6 h	84.40	13.11
	12 h	48.67	49.80
	24 h	24.34	74.79
SEM		33.60	34.50
P value			
Substrate (starch type)		0.001	0.001
Incubation time		0.000	0.000
Substrate×Incubation time		0.022	0.017

Table 4. Relative abundances of some bacterial groups (genus level) during the fermentation of amylopetin and RS2 by colonic inocula of piglets

Substrates	Incubation time	Proportion in total bacteria (%)							
		Bacteroides	*Blautia*	*Lactobacillus*	*Prevotella*	*Streptococcus*	*Subdoligranulum*	UE[1]	UL[2]
Amylopectin	6 h	11.40	01.60	33.45	1.04	3.93	13.49	12.88	5.14
	12 h	59.76	2.33	2.68	23.24	0.59	3.39	1.58	1.17
	24 h	78.73	0.65	0.21	16.12	0.06	0.59	0.09	0.95
RS2	6 h	11.41	0.95	29.69	0.92	3.89	17.46	9.99	7.10
	12 h	46.95	4.89	8.66	1.96	2.49	13.96	3.49	4.63
	24 h	72.29	4.34	2.25	1.39	0.63	3.93	0.85	6.68
SEM		29.23	1.85	15.01	12.69	1.71	6.75	5.12	3.05
P value									
Substrate (starch type)		0.247	0.001	0.658	0.031	0.016	0.000	0.905	0.003
Incubation time		0.000	0.004	0.000	0.188	0.000	0.000	0.000	0.052
Substrate×Incubation time		0.623	0.005	0.463	0.24	0.049	0.010	0.016	0.321

[1]UE, unclassified Erysipelotrichaceae;
[2]UL, unclassified Lachnospiraceae

The changes in the composition of bacterial community during the fermentation of amylopectin and RS2 were calculated at the phylum level Table 3. A tendency in the decrease of the Firmicutes proportion and an increase of the Bacteroidetes proportion was observed from 6 to 24 h of fermentation for both starches. At the genus level, the abundances of *Bacteroides*, *Blautia*, *Lactobacillus*, *Streptococcus*, *Subdoligranulum* and unclassified *Erysipelotrichaceae* were significantly affected by the fermentation time. The abundances of *Blautia*, *Prevotella*, *Streptococcus*, *Subdoligranulum* and unclassified *Lachnospiraceae* were influenced by the substrates Table 4. At 6 h after incubation, *Lactobacillus* was the most abundant genus during the fermentation of amylopetin and RS2, while this group gradually disappeared from the predominant bacterial community during the middle and late period of fermentation. In contrast, *Bacteroides* gradually became the most predominant bacterial group during the fermentation. At 12 and 24 h after incubation, the abundances of *Blautia*, *Streptococcus*, *Subdoligranulum* and unclassified *Lachnospiraceae* in the RS2 groups were significantly higher than those in the amylopectin groups, while the abundance of *Prevotella* was significantly lower.

At the OTU level, the shifts of the bacterial community compositions during the fermentation of RS2 was further analyzed. Within the 59 predominant OTUs which represented 93.09% of total 16S rRNA gene sequences, 34 were significantly affected by fermentation time Table 5. Of the 13 OTUs related to the genus *Bacteroides*, 8 showed the significantly higher abundances 24 h after incubation as compared to those before incubation. OTU1158 related to *Bacteroides vulgatus* in particular became the largest abundant species, representing 37.72% of the total bacteria. A similar tendency was also found in OTU related to *Blautia producta*. In contrast, the abundances of OTUs related to *Lactobacillus amylovorus*, *L. vaginalis*, *L. johnsonii*, *Streptococcus lutetiensis* and *Enterococcus allinarum* decreased significantly during the fermentation of RS2. However, no significant changes were found in the abundances of all five *Prevotella*-related OTUs and most of OTUs were associated with the families *Lachnospiraceae* and *Ruminococcaceae*.

4. Discussion

Various factors, including the degree of gelatinization, the biological origin of the starch, the amylose/amylopectin ratio, starch-protein interaction, amylose-lipid complexes, the percentage of retrograded starch, and the presence of amylase inhibitors, have been shown to affect starch degradation [25]. In the present study, gas production kinetics indicated that amylopectin was more fermentable by the colonic microbiota of pigs than RS2. This finding is in agreement with the results of a previous study, where it was demonstrated that many strains isolated from the human colon could hydrolyze the amylopectin maize starch, while only a few of them could efficiently utilize high-amylose maize starch. It was found that the RS4 used in this study was hardly degraded, which suggests that this type of starch might only have a physical role in the gut as a prebiotic when consumed by humans or animals. Nevertheless, the etched granule of the resistant starch (including RS4) could confer physical protection on the probiotics (such as lactic acid bacteria) upon passage through the host's upper gastrointestinal tract [26]. However, an *in vivo* study in humans showed that RS4 but not RS2 induced phylum-level changes, significantly increasing Actinobacteria and Bacteroidetes while decreasing Firmicutes [27], which suggests that different modified resistant starches may have different effects on the gut ecosystem, and further study is still needed for understanding the mechanism.

The fermentation of resistant starch can produce greater amounts of fecal butyrate and enhance its proportion in the total SCFA. The present study showed that amylopectin was fermented to a greater extent as compared with RS2 and RS4, therefore it was not unexpected to find that the fermentation of amylopectin produced a higher concentration of SCFA at 12 h, 24 h and 36 h as compared to resistant starch. Based on the results of the SCFA proportion, a tendency towards fermentation of the more fermentable starch (amylopectin) to produce a higher propionate proportion and lower acetate and butyrate proportions was clearly observed, which is consistent with previous studies on ruminant animals where higher propionate:acetate ratios were observed when feeding animals a higher concentrate diet compared with a higher forage diet. Furthermore, during the middle and late period of fermentation, the butyrate proportion in the RS2 group showed an increasing tendency, which was also confirmed by the finding that the concentration of acetate fermented from RS2 began to decrease along with the increase of propionate and butyrate concentrations from 24 h to 36 h. These results indicated that cross-feeding between different bacteria

might involved in the fermentation of resistant starch. Besides acetate, lactate is also an important intermediate metabolite of bacterial fermentation in the human gut. Lactate is rarely detected as a major fermentation end-product of complex microbial communities [28], and some reports suggest that a considerable part of the lactate is converted to butyrate in both human and pig colon [29,30]. In the present study, lactate was produced from amylopectin and RS2 during the early period then was consumed entirely, which suggests that cross-feeding of lactate might contribute to the reported butyrogenic effect of resistant starch in the human colon since butyrate was regarded as one of the main products fermented from lactate by gut microbiota [12].

Table 5. Relative abundances of predominant OTUs (percentage) during the fermentation of RS2 by the colonic microbiota of pigs[1]

OTUs	0 h	6 h	12 h	24 h	SEM[2]	*P* value	Annotate[3]
OTU1158	5.81	3.43	24.74	37.27*	4.76	0.002	s: *Bacteroides vulgatus*
OTU1966	13.26	15.47	12.51	3.46*	1.60	0.001	g: *Subdoligranulum*
OTU647	10.09	15.62	4.50	0.95*	2.00	0.004	s: *Lactobacillus amylovorus*
OTU1285	0.99	0.58	5.35*	8.13*	1.06	0.001	g: *Bacteroides*
OTU648	6.10	9.18	2.68	0.74*	1.16	0.004	s: *Lactobacillus vaginalis*
OTU1109	2.28	1.03	3.78	5.96*	0.63	0.001	g: *Bacteroides*
OTU674	7.31	7.72	1.64*	0.28*	1.10	0.000	f: *Erysipelotrichaceae*
OTU1394	1.02	0.41	0.84	0.57	0.19	0.779	g: *Prevotella*
OTU1893	1.88	0.92	4.71*	4.12*	0.56	0.005	s: *Blautia producta*
OTU1157	1.40	0.42	3.23	5.49*	0.69	0.004	s: *Bacteroides uniformis*
OTU1368	1.50	0.62	1.74	3.28	0.33	0.000	g: *Bacteroides*
OTU1152	5.90	3.41	3.16	2.97	0.82	0.716	s: *Bacteroides plebeius*
OTU253	2.81	3.31	2.06	0.48*	0.38	0.002	s: *Streptococcus lutetiensis*
OTU360	1.97	2.18	1.88	1.33	0.16	0.259	p: Firmicutes
OTU490	2.20	3.00	0.94*	0.41*	0.36	0.004	s: *Lactobacillus johnsonii*
OTU1049	0.51	0.16	0.32	0.19	0.09	0.633	g: *Prevotella*
OTU1691	2.87	3.45	1.31*	0.40*	0.40	0.000	s: *Eubacterium coprostanoligenes*
OTU1189	0.35	0.18	0.34	0.11	0.08	0.704	g: *Prevotella*
OTU1970	1.47	1.28	2.23	1.90	0.19	0.290	f: *Lachnospiraceae*
OTU1275	0.26	0.12	0.33	0.15	0.07	0.784	g: *Prevotella*
OTU1883	2.57	3.99	0.46*	0.11*	0.57	0.006	f: *Lachnospiraceae*
OTU1023	0.38	0.14	1.13	2.08*	0.26	0.002	s: *Bacteroides uniformis*
OTU1271	2.44	1.41	1.19	1.30	0.34	0.694	s: *Bacteroides plebeius*
OTU522	0.99	0.83	1.72*	0.20*	0.20	0.004	g: *Catenibacterium*
OTU687	1.52	0.90*	0.44*	0.32*	0.15	0.004	g: *Parasutterella*
OTU681	0.85	1.09	0.78	0.20*	0.13	0.025	f: *Erysipelotrichaceae*
OTU1984	0.40	0.23	0.59	2.18	0.38	0.225	f: *Lachnospiraceae*
OTU352	1.34	0.80	0.84	0.49*	0.11	0.062	g: *Phascolarctobacterium*
OTU1286	0.03	0.03	0.59	1.50*	0.23	0.030	g: *Bacteroides*
OTU1070	0.87	0.54	0.50*	0.29*	0.07	0.047	s: *Parabacteroides merdae*
OTU1636	2.15	0.84	0.41	0.73	0.28	0.218	f: *Ruminococcaceae*
OTU527	0.54	0.53	0.69	0.25	0.06	0.030	s: *Clostridium innocuum*
OTU1922	0.87	0.90	0.52	0.16*	0.11	0.025	g: *Subdoligranulum*
OTU1736	1.11	1.08	0.47*	0.12*	0.15	0.004	g: *Anaerotruncus*
OTU488	0.71	0.71	0.30	0.16*	0.10	0.059	s: *Enterococcus allinarum*
OTU1159	0.06	0.03	0.30*	0.55*	0.07	0.001	g: *Bacteroides*
OTU1832	0.33	0.77	0.55	0.12	0.11	0.171	g: *Collinsella*
OTU1589	0.64	0.80	0.30*	0.09*	0.10	0.001	f: *Ruminococcaceae*
OTU496	0.41	0.67	0.17	0.02*	0.09	0.013	g: *Lactobacillus*
OTU1905	0.46	0.52	0.43	0.14*	0.06	0.046	g: *Subdoligranulum*
OTU353	0.54	0.35	0.32	0.23*	0.04	0.104	g: *Phascolarctobacterium*
OTU397	0.79	0.35*	0.20*	0.17*	0.08	0.021	f: *Alcaligenaceae*
OTU1889	0.15	0.08	0.33	0.91	0.17	0.273	f: *Lachnospiraceae*
OTU1626	0.74	0.53	0.12*	0.03*	0.09	0.001	f: *Lachnospiraceae*
OTU1249	0.00	0.00	0.31	0.59*	0.10	0.099	g: *Bacteroides*
OTU1996	0.17	0.11	0.31	0.19	0.06	0.649	f: *Ruminococcaceae*
OTU1155	0.00	0.00	0.05	0.35	0.06	0.098	g: *Bacteroides*
OTU1792	0.40	0.48	0.43	0.11	0.06	0.096	f: *Ruminococcaceae*
OTU502	0.23	0.35	0.27	0.09	0.04	0.164	s: *Streptococcus suis*
OTU1612	0.28	0.48	0.17	0.06*	0.06	0.007	g: *Mogibacterium*
OTU1216	0.00	0.00	0.05	0.26	0.05	0.115	g: *Prevotella*
OTU1664	0.50	0.52	0.11*	0.02*	0.07	0.000	f: *Ruminococcaceae*
OTU1982	0.22	0.30	0.27	0.05	0.04	0.122	o: *Clostridiales*
OTU1943	0.05	0.03	0.29*	0.44*	0.06	0.004	f: *Ruminococcaceae*
OTU659	0.18	0.39	0.04	0.02*	0.06	0.015	g: *Lactobacillus*
OTU1166	0.06	0.03	0.08	0.10	0.01	0.264	s: *Bacteroides thetaiotaomicron*
OTU1703	0.24	0.30	0.08	0.01	0.05	0.126	f: *Lachnospiraceae*
OTU1945	0.14	0.25	0.10	0.09	0.03	0.057	f: *Ruminococcaceae*
OTU1887	0.06	0.04	0.12	0.47	0.08	0.210	f: *Lachnospiraceae*

[1]Fifty-nine OTUs with abundances higher than 0.1% in the microbial community were sorted from total 2050 total OTUs and defined as the predominant OTUs. **P* < 0.05, the data were compared with the value of 0 h.

[2]SEM, standard error of means, n=3.

[3]The consensus sequence of each OTU was annotated to the closest lineage using the MOTHUR program against the SILVA 16S rRNA reference database. s: = species; g: = genus; f: = family; o = order; p = phylum.

As an important beneficial subgroup for human health, some (but not all) *Bifidobacterium* spp. were found to have the ability to degrade both amylopectin and resistant starch [5,6]. Further studies demonstrated that *Bifidobacterium* spp. played an important role in the degradation of starch in the colon of humans and animals. Feeding a resistant starch-based diet resulted in the proliferation of indigenous bifidobacteria in the hindgut of rats, pigs and humans [27,31,32]. However, in the present study, pyrosequencing of 16S rRNA genes from the total bacterial DNA of fermentation fluids failed to detect *Bifidobacterium*-related OTUs, which suggests that *Bifidobacterium* spp. might not be the key species involved in the degradation of starch in the pig gut. A possible reason might be the presence of antibiotic in the pig diet used in this study. In contrast, a high abundance of *Lactobacillus* spp. was observed at the beginning of the fermentation of amylopectin and RS2, which agrees with some previous studies on pig gut microbiota where genus *Lactobacillus* was regarded as the predominant group in the bacterial community [33]. However, this group gradually disappeared from the predominant bacterial community during the middle and late periods of the fermentation of starch. This trend is consistent with the fact that *Lactobacillus* spp. were not in the lists of amylolytic bacteria [5].

Bacteroides is the numerically dominant genus in the colonic ecosystem of humans and pigs. *Bacteroides* species are considered the primary starch-degrading colonic microbes, since high activities of neopullulanase, a-glucosidase, and amylase have been detected in cell extracts of *Bacteroides* spp. [34]. In the present study, dramatic changes in the abundance of Bacteroidetes and Firmicutes were observed during the fermentation of amylopectin and resistant starch. Within Bacteroidetes, both the numbers of *Bacteroides* and their abundances in the total bacteria increased significantly, which suggests that this genus is probably the principal degrader of both amylopectin and amylase in the pig hindgut. This observation is inconsistent with the finding that *R. bromii* was a keystone species for the degradation of resistant starch in the human colon [11]. Furthermore, another previously studied amylolytic bacterium, *Eubacterium rectale* was also not detectable during the fermentation of different starches [35]. A possible explanation for this finding is that pigs have different gut microbial structures with humans on genus and species levels due to the different diet patterns, although the intestinal tract of pigs is quite similar in both physiology and anatomy to that of humans.

In the present study, *Bacteroides vulgatus*, *Bacteroides uniformis* and other *Bacteroides* spp.-related OTUs became the predominant species during the fermentation of RS2, which is in agreement with a previous finding that *Bacteroides* spp. are also regarded as one of the main amylolytic bacteria in the human gut [9]. *B. vulgatus* is the numerically predominant *Bacteroides* species in the human colonic microflora. McCarthy *et al*. found that the activities of α-glucosidase and amylase increased 20- to 40-fold when *B. vulgatus* was grown on amylose or amylopectin compared with glucose or other monosaccharides media [36]. In contrast, the abundances of *Prevotella* spp.-related OTUs were not affected during fermentation, which is consistent with the well-known fact that this genus is responsible for the breakdown of different protein and carbohydrates in the rumen or hindgut of animals. Besides *Bacteroides* spp., the abundance of *Blautia producta*-related OTUs was also increased. *B. producta* (formerly known as *Ruminococcus productus*) share a very high 16S rRNA gene sequence similarity (99.7 %) with *Clostridium coccoides*, a species that was recognized to use H_2 and CO_2 as energy sources [37]. However, so far, no information on its role in the metabolism of starch is available, further studies are still needed to understand its function.

5. Conclusion

In conclusion, as compared with resistant starch, amylopectin was more fermentable by pig colonic microbiota and produced more SCFA with a higher propionate proportion and a lower butyrate proportion. A significant decrease in the abundance of Firmicutes and an increase in the abundance of Bacteroidetes were found during the fermentation of amylopectin and RS2. *Bacteroides* spp. such as *B. vulgatus* and *B. uniformis* became especially predominant, which suggests that *Bacteroides* spp. play an important role in the degradation of starch in the pig hindgut.

Acknowledgement

This Research has received funding from the National Basic Research Program of China (2012CB124705), the Fundamental Research Funds for the Central Universities (KYZ201153) and the National Natural Science Foundation of China (30901036).

Competing Interests

The authors have no competing interests.

Abbreviations

RS	resistant starch
SCFA	short-chain fatty acids
OUT	operational taxonomic unit
ACE	abundance based coverage estimator

References

[1] Anderson, I.H., Levine, A.S. and Levitt, M.D. Incomplete absorption of the carbohydrate in all-purpose wheat flour. *The New England Journal of Medicine*, 304, 891-892, 1981.

[2] Louis, P. and Flint, H.J. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters*, 294, 1-8, 2009.

[3] Fung, K.Y.C., Cosgrove, L., Lockett, T., Head, R. and Topping, D. L. A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate. *Brithish Journal of Nutrition*, 108, 820-831, 2012.

[4] Englyst, H.N., Kingman, S.M. and Cummings, J.H. Classification and measurement of nutritionally important starch fractions. *European Journal of Clinical Nutrition*, 46, S33-S50, 1992.

[5] Wang, X., Conway, P.L., Brown, I.L. and Evans, A.J. *In vitro* utilization of amylopectin and high-amylose maize (amylomaize)

starch granules by human colonic bacteria. *Applied and Environmental Microbiology*, 65, 4848-4854, 1999.

[6] Ryan, S. M., Fitzgerald, G. F. and Van Sinderen, D. Screening for and identification of starch-, amylopectin-, and pullulan-degrading activities in Bifidobacterial strains. *Applied and Environmental Microbiology*, 72, 5289-5296, 2006.

[7] Motherway, M.O., Fitzgerald, G.F., Neirynck, S., Ryan, S., Steidler, L. and van sinderen, D. Characterization of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. *Applied and Environmental Microbiology*, 74, 6271-6279, 2008.

[8] D'Elia, J.N. and Salyers, A.A. Contribution of a neopullulanase, a pullulanase, and an α-glucosidase to growth of *Bacteroides thetaiotaomicron* on starch. *Journal of Bacteriology*, 178, 7173-7179, 1996.

[9] Kovatcheva-Datchary, P., Egert, M., Maathuis, A., Rajilic-Stojanovic, M., de Graaf, A.A., Smidt, H., de Vos, W.M. and Venema, K. Linking phylogenetic identities of bacteria to starch fermentation in an *in vitro* model of the large intestine by RNA-based stable isotope probing. *Environmental Microbiology*, 11, 914-926, 2009.

[10] Abell, G. C., Cooke, C. M., Bennett, C. N., Conlon, M.A. and McOrist, A.L. Phylotypes related to *Ruminococcus bromii* are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. *FEMS Microbiology Ecology*, 66, 505-515, 2008.

[11] Ze, X., Duncan, S.H., Louis, P. and Flint, H.J. *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. *The ISME Journal*, 6, 1535-1543, 2012.

[12] Duncan, S.H., Louis, P. and Flint, H.J. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied and Environmental Microbiology*, 70, 5810-5817, 2004.

[13] Belenguer, A., Duncan, S.H., Calder, A.G., Holtrop, G., Louis, P., Lobley, G.E. and Flint, H.J. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Applied and Environmental Microbiology*, 72, 3593-3599, 2006.

[14] Thumbleson, M.E. and Shook, L.B. Advances in Swine in Biomedical Research. Plenum, New York, 1996.

[15] Awati, A., Williams, B.A., Bosch, M.W., Li, Y.C. and Verstegen, M.W. Use of the in vitro cumulative gas production technique for pigs: An examination of alterations in fermentation products and substrate losses at various time points. *Journal of Animal Science*, 84, 1110-1118, 2006.

[16] Lowe, S.E., Theodorou, M.K. and Hespell, R.B. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. *Journal of General Microbiology*, 131, 2225-2229, 1985.

[17] Theodorou, M.K., Williams, B.A., Dhanoa, M.S. and McAllan, A.B. France J, A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology*, 48, 185-197, 1994.

[18] Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A. and Lantinga, E.A. Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feed. *Animal Feed Science and Technology*, 64, 77-89, 1996.

[19] Bauer, E., Williams, B.A., Voigt, C., Mosenthin, R. and Verstegen, M.W.A. Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrateds. *Animal Science*, 73, 313-322, 2001.

[20] Yang, C.J., Mao, S.Y., Long, L.M., and Zhu, W.Y. Effect of disodium fumarate on microbial abundance, ruminal fermentation and methane emission in goats under different forage: concentrate ratios. *Animal*, 27, 1-7, 2012.

[21] Zoetendal, E.G., Akkermans, A.D.L. and de Vos, W.M. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Applied and Environmental Microbiology*, 64, 3854-3859, 1998.

[22] Baker, G. Smith, J.J. and Cowan, D.A. Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods*, 55, 541-555, 2003.

[23] Stackebrandt, E. and Goebel, B.M. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, 44, 846-849, 1994.

[24] Good, I.L. The population frequencies of species and the estimation of population parameters. *Biometrika*, 40, 237-264, 1953.

[25] Brown, I., Warhurst, M., Arcot, J., Playne, M., ILLman, R.J, and Topping, D.L. Fecal numbers of bifidobacteria are higher in pigs fed *Bifidobacterium longum* with a high amylose cornstarch than with a low amylose cornstarch. *Journal of Nutrition*, 127, 1822-1827, 1997.

[26] Topping, D.L., Michihiro, F. and Anthony, R.B. Resistant starch as a prebiotc and symbiotic: state of the art. *Proceedings of the nutrition society*, 62, 171-176, 2003.

[27] Martinez, I., Kim, J., Duffy, P.R., Schlegel, V.L. and Walter, J. Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. *PLoS ONE*, 5, e15046, 2010.

[28] Le Blay, G.M., Michel, C.D., Blottiere, H.M. and Cherbut, C.J. Raw potato starch and short-chain fructooligosaccharides affect the composition and metabolic activity of rat intestinal microbiota differently depending on the caecocolonic segment involved. J Appl Microbiol. 94, 312-320, 2003.

[29] Bourriaud, C., Robins, R.J., Martin, L., Kozlowski, F., Tenailleau, E., Cherbut, C. and Michel, C. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *Journal of Applied Microbiology*, 99, 201-212, 2005.

[30] Su, Y., Li, B. and Zhu, W.Y. Fecal microbiota of piglets prefer utilizing DL-lactate mixture as compared to D-lactate and L-lactate *in vitro*. *Anaerobe*, 19, 27-33, 2013.

[31] Kleessen, B., Stoof, G., Proll, J., Schmiedl, D., Noack, J. and Blaut, M. Feeding resistant starch affects fecal and cecal microflora and short chain fatty acids in rats. *Journal of Animal Science*, 75, 2453-2462, 1997.

[32] Wang, X., Brown, I.L., Khaled, D., Mahoney, M.C., Evans, A.J. and Conway, P.L. Manipulation of colonic bacteria and volatile fatty acid production by dietary high amylose maize (amylomaize) starch granules. *Journal of Applied Microbiology*, 93, 390-397, 2002.

[33] Su, Y., Yao, W., Perez, O., Smidt, H. and Zhu, W.Y. Increased abundance of *Lactobacillus* spp. and *Streptococcus suis* in stomach, jejunum and ileum of piglets after weaning. *FEMS Microbiology Ecology*, 66, 546-555, 2008.

[34] Moore, W.E.C. and Holdeman, L.V. Discussion of current bacteriological investigation of the relationship between intestinal flora, diet and colon cancer. *Cancer Research*, 35, 3418, 1975.

[35] Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C. and Flint, H.J. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Applied and Environmental Microbiology, 66, 1654-1661, 2000.

[36] McCarthy, R.E., Pajeau, M., Salyers, A.A. Role of starch as a substrate for *Bacteroides vulgatus* growing in the human colon. *Applied and Environmental Microbiology*, 54, 1911-1916, 1988.

[37] Liu, C., Finegold, S.M., Song, Y. and Lawson, P.A. Reclassification of *Clostridium coccoides*, *Ruminococcus hansenii*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus luti*, *Ruminococcus productus* and *Ruminococcus schinkii* as *Blautia coccoides* gen. nov., comb. nov., *Blautia hansenii* comb. nov., *Blautia hydrogenotrophica* comb. nov., *Blautia luti* comb. nov., *Blautia producta* comb. nov., *Blautia schinkii* comb. nov. and description of *Blautia wexlerae* sp. nov., isolated from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, 58, 1896-1902, 2008.