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Full Length Article

Resistant Starch Type 4 Affects Colon Morphology, Fermentation and Microflora in Rats

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Abstract

The objective of current study was to find out the best inclusion level of resistant starch Type 4 (RS4) to improve colonic fermentation, morphology and microflora of growing rats. For this purpose, thirty-six, 3 weeks old weaned female Sprague Dawley rats (66.54 ± 10.23 g body weight) were divided into four groups in such a way that each group contained 9 rats. The experimental diets had 0%, 5, 10 and 15% levels of RS4. Experimental feeds were offered to each group for 40 days. The colon tissue and colonic content were collected for analysis at the end of the trial. The results revealed that pH value and ammonia nitrogen concentration of the colonic content decreased, while short chain fatty acid concentration increased with increasing dietary RS4 level ($P < 0.05$). The villus height and the villus/crypt ratio of the colon were improved for rats fed RS4 compared with the 0% RS4 group ($P < 0.05$). The highest villus height and villus/crypt ratio were observed for rats fed 10% RS4 compared to the other groups. Rats fed 10% RS4 had higher numbers of Bifidobacteria and Lactobacillus colonies and lower numbers of *Escherichia coli* colonies ($P < 0.05$). These findings suggest that rats fed best inclusion level for RS4 is 10% to improve colonic development and health of growing rats.

Keywords: Resistant starch type 4; Colon; SCFA; Morphology; Microflora

Introduction

Starch is the major energy-producing component and the main carbohydrate of the daily diet for animals. Generally, starch is decomposed by amylase to produce glucose and absorbed in the small intestine. However, resistant starch (RS) can't be digested in the upper gastrointestinal tract, which results in an increased flow of undigested residues to the colon where they can be fermented by the microflora (Englyst *et al.*, 1992; Jia *et al.*, 2018). Its properties are similar to those of soluble fiber which is regarded as a kind of dietary fiber (Fuentes-Zaragoza *et al*., 2010).

Fermentation of RS can increase the end products of short chain fatty acids (SCFA) (Jha *et al*., 2014), which leads to a lower pH value, preventing over-growth of potentially pathogenic microorganisms and reducing several indices, including NH_3, amines, enolic, and indolic compounds which can lead to destruction of the epithelial cells (Windey *et al.*, 2012). The butyrate produced by the fermentation of RS is of great benefit to mucosal integrity (Martínez-Puig *et al*., 2007). The host can also benefit from the growth and activity of the specific microflora which are stimulated by the fermentation of RS in the large intestine.

Resistant starch type 4 (RS4) is one of the more diverse forms of RS. It is a kind of chemically modified starch with limited enzyme degradation due to a chemically altered granule structure. So far, effects of RS4 on colonic development and health of growing rats were seldom reported and the appropriate concentration of dietary RS4 is not clear. Therefore, the current experiment was designed to investigate the effects of different dietary RS4 levels (0, 5, 10 and 15%) on colonic fermentation, morphology and microflora of growing rats.

Materials and Methods

Animals, Feeds and Management

Thirty-six weaned female Sprague Dawley (SD) rats (3 weeks old) with initial body weight of (66.54 g ± 10.23) were selected and randomly divided into 4 groups in such a way that each group contained 9 replicates. Resistant starch was obtained from Hangzhou Zhiyou Science & Technology Co., Ltd. Resistant starch contained 79.8% RS4. Rats in the control group were offered an RS-free basal diet by using waxy corn starch. The RS-free basal diet was formulated according to *Laboratory animals-Nutrients for formula feeds* (GB 14924.3-2010) of National Standards of China (Table 1). The waxy corn starch was replaced by RS4 to make different dietary treatments containing 5%, 10% and 15% RS4 based

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Table 1: Ingredients and nutrition of RS-free basal diet DM basis

Ingredient	%	Nutrient level	
Waxy corn starch	47.49	DM,%	94.36
Soybean meal	40.59	CP ,%	18.00
Oil	9.32	DE[2], MJ/kg	17.99
Limestone	1.0	EE[3], %	10.00
Calcium hydrogen phosphate	0.6	Ca,%	0.68
Premix[1]	1	P,%	0.43

1. Vitamin and mineral premix contained per kilogram of DM: V_A 4000 IU, V_D 3400 IU, V_E 12 IU
V_K 1 mg, V_H 0.08 mg, Choline 200 mg, Folic acid 0.8 mg, Pantothenic acid 6 mg, VB_2 2 mg, VB_3 10 mg
VB_1 0.6 mg, VB_6 0.6 mg, Cu 15 mg, Fe 100 mg, Zn 105 mg, Mn 100 mg, I 0.3 mg, Se 0.3 mg
2. DE: digestible energy
3. EE: ether extract

on dry matter (DM) respectively. The rats were housed in individually ventilated cages (IVC), one per cage. Temperature and humidity of the cages were controlled at 25 ± 2ºC and 50 ± 5%. Rats were given three days for adaption prior to start of experimental period. No signs of toxicity or abnormal behavior were observed during the adaptation period. Experimental period of this trial was 40 days after the adaptation period. At the end of trial, rats were euthanized by CO_2 asphyxiation, and samples were collected for analysis.

Sample Collection and Analyses

Colonic fermentation: The colonic contents (approximately 0.5 g) were collected, homogenized, and diluted by using 10 mL of hyper-pure water. The pH value was tested by using a pH-meter (HJ-90B) first, then the samples were centrifuged for 20 min at 5000 rpm. After that, the supernatant was put into two 10 mL tubes. One was used to test ammonia N by spectrophotometer (UV-1700, Shimazu Corporation) using the method of Broderick and Kang (1980). The other one was used for SCFA analysis through gas chromatography (GC-2014, Shimazu Corporation) with a capillary column (Agilent HP-INNOWAX, 30 m long, 0.32 mm diameter, 0.50 μm film) following the method of Kim *et al.* (2013).

Colonic Morphology

Colons were removed from the mesentery first, and rinsed with saline after taking out of the colonic content. Then, samples of colon tissue were placed in 4% formalin solution before measurement. Morphological analyses of colon tissue were taken by using the biopsy technique as described by Wang *et al.* (2009). Samples were dehydrated through an ethanol series after rinsing with water. Then, they were cleared twice by turpentine and embedded in the paraffin. Sections of seven-μm-thick (10 sections for each sample) were stained by hematoxylin/eosin and observed through the light microscope (Olympus, Tokyo, Japan) at 50× magnification. A color video camera (Olympus, Tokyo, Japan) was used to capture the digital images. Villus heights and crypt depths were measured by the software of the Mshot Digital Imaging System (Guangzhou, People's Republic of China).

Colonic Microflora

Colonic contents (0.2 g) were collected in 5 mL sterile Eppendorf tubes. Then, samples were successively diluted (10^{-1} to 10^{-7}) by using sterile saline solution within 2 h following the method by Sangoh *et al.* (2013). A volume of 0.1 mL of each dilution was inoculated onto the surface of LBS agar, BBL agar, LB agar and EG agar plates for the cultivation of lactobacilli, bifidobacteria, colibacillusand enterococcus respectively. Lactobacilli and bifidobacteria were incubated anaerobically at 37ºC for 48 h. Colibacillus and enterococcus were incubated at 37ºC for 24 and 48 h. After incubation, the numbers of lactobacilli, bifidobacteria, colibacillus and enterococcus colony-forming units (cfu) were determined.

Statistical Analysis

SAS 9.0 was used for statistical analyses. The differences between the mean values of different groups were detected by One-way analysis of variance (ANOVA). Differences were declared significant at $P < 0.05$. When a significant effect of treatment was detected ($P < 0.05$), differences between the means of each group were tested by Duncan's multiple comparison test.

Results

Colonic Fermentation

The results of colonic fermentation are summarized in Table 2. Compared with the control treatment group, pH value and NH_3-N concentration were decreased significantly, and the butyric acid concentration was increased significantly as dietary RS4 level increased ($P < 0.05$). The rats fed 15% RS4 had the lowest pH value and NH_3-N concentration and the highest butyric acid concentration compared with the other treatments ($P < 0.05$).

Colonic Morphology

The effects of different RS4 levels on colonic morphology are shown in Table 3. Villus height and villus/crypt ratio were increased for the rats fed 10 and 15% RS4 compared with the control treatment group ($P < 0.05$). The rats fed 10% RS4 had the highest villus height and villus/crypt ratio compared to the other treatment groups.

Colonic Microflora

The numbers of four different microflora colonies are shown in Table 4. Rats fed 10 and 15% RS4 had a significantly higher number of Bifidobacteria and Lactobacillus colonies compared with the control treatment group ($P < 0.05$). A lower number of *Escherichia coli* colonies can be found for rats fed 10 and 15% RS4 than the control treatment group ($P<0.05$). Rats fed 10% RS4 had the highest number of Bifidobacteria and Lactobacillus colonies and the lowest

Table 2: Effects of different dietary RS4 levels on colonic fermentation index of growing rats

Item	0% (Control)	5%	10%	15%	P-value
pH	6.58 ± 0.10^{a}	6.51 ± 0.13^{a}	6.58 ± 0.10^{a}	5.91 ± 0.18^{b}	0.025
NH_3-N(μg/g)	294.61 ± 7.09^{a}	279.60 ± 7.65^{b}	289.93 ± 3.54^{b}	255.93 ± 4.46^{c}	0.012
Acetate acid (μmol/g)	50.46 ± 8.22	51.74 ± 7.24	52.46 ± 10.09	54.82 ± 13.19	0.140
Propionate acid (μmol/g)	16.54 ± 3.02	16.36 ± 3.60	18.18 ± 4.07	22.51 ± 6.90	0.115
Butyrate acid (μmol/g)	5.75 ± 0.33^{a}	6.74 ± 0.55^{b}	8.69 ± 0.25^{b}	9.81 ± 0.54^{c}	0.014

[a b c] Means within same row with the same superscript letter are not significantly different ($P > 0.05$)

Table 3: Effects of different dietary RS4 levels on colonic morphology of growing rats Unit: μm

	0% (Control)	5%	10%	15%	P-value
villus height	89.69 ± 2.84^{a}	88.98 ± 3.22^{a}	95.92 ± 3.65^{b}	90.97 ± 3.43^{ab}	0.036
crypt depth	20.10 ± 3.61	19.63 ± 3.46	15.63 ± 0.92	18.25 ± 2.35	0.115
V/C	4.47 ± 0.61^{a}	4.42 ± 0.42^{a}	6.14 ± 0.63^{b}	4.78 ± 1.16^{ab}	0.014

[a b] Means within same row with the same superscript letter are not significantly different ($P > 0.05$)

Table 4: Effects of different dietary RS4 levels on colonic microflora content of growing rats Unit: log10cfu/g

Item	0% (Control)	5%	10%	15%	P-value
Escherichia coli	8.86 ± 0.34^{a}	8.16 ± 0.13^{a}	7.11 ± 0.09^{b}	7.51 ± 0.76^{b}	0.043
Bifidobacteria	7.89 ± 0.04^{a}	7.69 ± 0.08^{a}	9.79 ± 0.01^{b}	9.72 ± 0.38^{b}	0.013
Lactobacillus	6.61 ± 0.29^{a}	6.81 ± 0.14^{ab}	9.10 ± 0.25^{c}	7.13 ± 0.05^{b}	0.043
Enterococci	5.96 ± 0.06	5.93 ± 0.02	5.46 ± 0.21	5.75 ± 0.05	0.137

[a b c] Means within same row with the same superscript letter are not significantly different ($P > 0.05$)

number of *E. coli* and Enterococci colonies.

Discussion

In this experiment, rats fed 15% RS4 had the lowest pH values compared with the control group (Table 2). This result may be due to fermentation of RS4, which generates SCFA that can reduce the pH value. This is consistent with the other research (Mei *et al*., 2005; Keenan *et al*., 2006; Kieffer *et al*., 2016). Studies show that a lower pH value can decrease the NH_3-N concentration in the large intestine, preventing colon carcinoma cell growth (Zaman and Sarbini, 2016). Fiber-rich food can stimulate the proliferation and differentiation of intestinal epithelial cells through SCFA (Wong *et al*., 2006; Jha and Leterme, 2012). The effect of butyric acid is the strongest, followed by acetic acid, and propionic acid is the weakest (Salminen *et al*., 1998). Butyric acid is the most important source of energy for colonic epithelium cell growth (Duncan *et al*., 2004; Tremaroli and Bäckhed, 2012; Yuan *et al*., 2017). Butyric acid also has cellular effects on stimulating development, growth, and renewal of intestinal epithelial cells by regulating gene expression of protein synthesis and maintaining the integrity of intestinal epithelial cells (Gall *et al*., 2009). Therefore, in the present study, colonic butyric acid concentrations increased in rats fed RS4, which indicates that addition of RS4 in the diet can provide positive effects on colon health.

NH_3-N is mainly produced by the fermentation of dietary indigestible protein in the large intestine. It is implicated in pathogenesis, correlating with diseases based on morphological character or physiological metabolism of large intestinal epithelial cells. NH_3-N is a potential promoting factor for colon carcinoma cell growth (Mouillé *et al*., 2003; Preter *et al*., 2006). Wutzke and Scholübbers (2013) found that adding RS in the diet could lower colonic generation of NH_3-N for humans and pigs. In the present study, rats fed RS4 in their diet had lowerNH_3-N concentration than the control group, and 15% RS4 treatment had the lowest NH_3-N concentration (Table 2). The results revealed that feeding RS4 can reduce the NH_3-N emission, which of great benefit to colon health.

The villus height, crypt depth, and villus/crypt reflect gross intestinal morphology (Liu *et al*., 2012). They are important indicators of intestinal health and integrity, reflecting the digestive and absorptive capacity of the gut (Zhang *et al*., 2013). In the present study, compared with the control treatment group, the villus height and villus/crypt ratio were significantly increased as dietary RS4 level increased. These results indicated that RS4 improved intestinal morphology. This can be explained by the trophic effect of SCFA, and especially butyric acid, as the most import energy source for the colonic epithelial cells, which can improve the growth and development of intestinal cells (Knudsen *et al*., 2003). However, the villus height and villus/crypt ratio tended to decrease for rats fed 15% RS4 than 10% RS4, which suggests that feeding too much RS4 may lead to a negative effect on colon morphology.

The RS can provide greater available substrate for the colonic microflora and can be fermented to produce SCFA, which potentially alter the composition of the microflora (Suarez-Belloch *et al*., 2013). On one hand, SCFA can lead to a lower pH value, which reduces the growth and reproduction of saprophytic bacteria that will harm the health of the large intestine. On the other hand, SCFA can promote the growth and reproduction of probiotics such as Bifidobacteria and Lactobacillus. Some previous studies found that adding resistant starch can increase the numbers of lactobacillus and bifidobacteria while decreasing the

number of enterobacteria (Silvi *et al*., 1999; Leu *et al*., 2005). In our study, the rats fed 10% RS4 had the highest number of Bifidobacteria and Lactobacillus colonies and the lowest number of *E. coli* and Enterococci colonies, which suggested that increasing dietary RS4 level can promote the growth and breeding of probiotics.

Conclusion

The RS4 has positive effects for colon health by reducing harmful end-products, improving morphology, and selectively stimulating the growth of beneficial microflora of the colon. However, feeding too much RS4 may lead to a negative effect on colon morphology. These results suggest 10% RS4 in the diet provides the most advantageous effects on colon development and health.

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