Study Protocol and Statistical Analysis Plan

（Effect of resistant starch on symptom improvement and gut microbiota in patients with functional constipation）

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Main researcher: Huaping Xie

Research unit: Tongji Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology

1、Research background

The human gut microbiota is one of the most densely popupated microbial communities on earth and contains highly diverse microbial communities. They provide metabolic, immune, and protective functions and play a vital role in human health[1-3].Gastrointestinal microbiota is influenced by a variety of factors, including genetics, host physiology (host age, disease, stress, etc.) and environmental factors, such as living conditions and drug use. At the same time, diet is considered to be a key environmental factor mediating the composition and metabolic function of gastrointestinal microbiota[4]. In view of the fact that it is difficult to be digested and absorbed by the human body, probiotics can enter the intestinal tract through the digestive tract, so as to improve intestinal microecology and promote lipid, protein and mineral metabolism.

Food rich in anti-digestible starch has many functions, such as controlling body weight, reducing blood lipids and blood sugar, regulating intestinal flora and so on, which has attracted the interest of many scholars and become a new field of international food research in recent years. Resistant starch (RS) is the anti-digestion part of compound polysaccharide starch. According to the conditions of enzymatic hydrolysis resistance and starch source, the digestible resistant starch was mainly divided into four types, namely RS1, RS2, RS3 and RS4. From the cause of resistance, RS1 and RS2 have natural resistance to amylase, and the resistance can disappear after gelatinization, while the resistance of RS3 and RS4 is formed by the transformation of starch in the process of food processing or food production. RS1 can coexist with RS2 or RS3 in the same kind of food, and the existence of RS4 can increase the food intake of RS3. Only a small part of the indigestible starch can be digested and absorbed in the small intestine, providing a very low utilization rate of glucose. When most of the rest of the undigested starch enters the colon, it is fermented by the intestinal microflora, mainly producing short-chain fatty acids (SCFAs): acetate, propionate and butyrate[5]. Although both acetate and propionate have health effects, butyrate is particularly thought to improve health and is the fatty acid with the largest increase in resistant starch intake. Butyrate plays an important role in human intestinal health, including reducing inflammation, reducing the risk of colon cancer and improving intestinal barrier function[6]. *In vivo* and *in vitro*, resistant starch diet and butyrate significantly increased the proportion of ChAT immunoreactive intermuscular neurons, intestinal neurons expressed monocarboxylic acid transporter 2 (MCT2), small interference with RNA silenced MCT2, and prevented butyrate-induced increase in the proportion of ChAT immunoreactive neurons. Butyrate and trichostatin A increased the acetylation of histone H3 in intestinal neurons. Src signal pathway inhibitors blocked the effect of butyrate. Resistant starch diet increased colonic transport, and butyrate increased cholinergic-mediated contraction of colonic circular muscles *in vitro*[7]. Although resistant starch has been shown to be one of the best fibers to raise butyrate levels in the population, it is clear that not everyone can get the same benefits, and some people do not respond to resistant starch

supplements[8]. This suggests that differences in individual microflora play an important role in determining the outcome of resistant starch consumption, and we need to explore more deeply the mechanism of resistant starch digestion and how it leads to the production of butyrate. In addition, more work needs to be done to understand the effects beyond butyrate levels that may affect health through resistant starch consumption.

Due to the complex structure of resistant starch, some bacteria are needed to initiate the degradation of this semi-crystalline material. *Ruminococcus bromii* and *Bifidobacterium adolescence* are the two known human intestinal microorganisms with the ability to degrade resistant starch[9]. *Ruminococcus bromii* has attracted attention because of its role as a key species in resistant starch metabolism, feeding and / or enabling other members of the intestinal microbiome to obtain the substrate[9]. The amylolytic enzyme of *Ruminococcus bromii* has a unique tissue structure and forms a multi-enzyme complex. Through the adhesion protein and dockerin module, it is attached to the cell surface through the scaffold protein in the cellulose body, so it is called amylasome [10]. This system has been found in a variety of human *Ruminococcus*, and the key enzyme structure of its amylase is highly conserved among strains[11]. Despite its incredible ability to degrade resistant starch, *Ruminococcus bromii* itself does not seem to win or dominate other species in the competition, but plays a beneficial role by cross-feeding other species by releasing sugars and acetates of different lengths[12, 13]. Resistant starch supplementation increased fecal butyrate concentrations in healthy young adults from 8 to 12 mmol/kg wet feces, but responses varied widely between individuals[8]. A follow-up study by the same team found that people with increased *Ruminococcus bromii* abundance in the microbiome were more likely to have a higher butyrate response to potato starch[14], the results showed that the correct combination of primary degrading bacteria and resistant starch was needed to increase the yield of butyrate.

Constipation is one of the most common gastrointestinal dysfunction in clinical practice. About 11-20% of adults worldwide suffer from constipation[15].In clinic, it is called constipation when the frequency of defecation is reduced, or the defecation is laborious, unsmooth, difficult and the stool is dry[16]. Intractable constipation is tricky to treat and over-reliance on laxatives often leads to water-electrolyte imbalance, gastrointestinal dysfunction, colonic darkening and anal sphincter relaxation[17-19], It even leads to colorectal cancer, diabetes, anorexia nervosa and other complications in some cases[20]. Therefore, it is very important to find a safe and effective laxative medicine or dietary therapy to improve and relieve the symptoms of constipation. Resistant starch plays a health-promoting role mainly due to its short-chain fat and gas produced by microbial fermentation in the colon, and its role in the prevention of colorectal cancer and some diet-related chronic diseases is stronger than that of dietary fibre, and it can effectively overcome the drawbacks of food fortification with dietary fibre, such as bad odour, rough texture and poor quality.From the analysis of research data, as a natural, safe, "medicinal and food" food resources, like dietary fibre, has a very important role in human health. It has important industrial

application value and broad market development prospects, and opens up a new field of functional food research, greatly compensating for the drawbacks of traditional dietary fibre.

In our previous analysis, stool microorganisms of 20 constipation patients and 20 healthy people were found to be very different in structure. The difference analysis indicated that *Ruminococcus* was abundant in healthy people. A classification model of AUROC 0.967 was established using Lasso algorithm, and important features of the classification model were as follows: *Ruminococcus* is the feature with the highest weight. SPINGO notes that the genus *Ruminococcus* contains species, in which *Ruminococcus bromii* has the highest relative abundance. A search of data from the GMrepo database revealed that *Ruminococcus bromii* were found in 28,796 trials, belonging to 93 phenotypes. A total of 40,795 valid runs belonged to these phenotypes. The relative abundance of *Ruminococcus bromii* in healthy people is significantly higher than that in constipated people. Therefore, the purpose of this clinical trial is to supplement resistant starch for constipation patients with low abundance *Ruminococcus bromii*, and (1) Observe whether the symptoms of constipation patients are improved; (2) To analyze the changes of intestinal microbes in patients with constipation; (3) Verify whether the relative abundance of *Ruminococcus bromii* increases and analyze the correlation between the relative abundance of R. bromii in intestine and the improvement of constipation symptoms in patients with constipation..

2.The purpose of the study

In this study, a group of patients with functional constipation were recruited to take resistant starch: (1) to observe whether the symptoms of patients with constipation improved; (2) to analyze the changes of intestinal microorganisms in patients with constipation; (3) to verify whether the relative abundance of *Ruminococcus bromii* increased and analyze the correlation between the relative abundance of R. bromii in intestine and the improvement of constipation symptoms in patients with constipation.

3.Research and design

This study was a non-randomized controlled study without placebo. A total of 30 patients with functional constipation were recruited.

4.Subject recruitment

Inclusion criteria

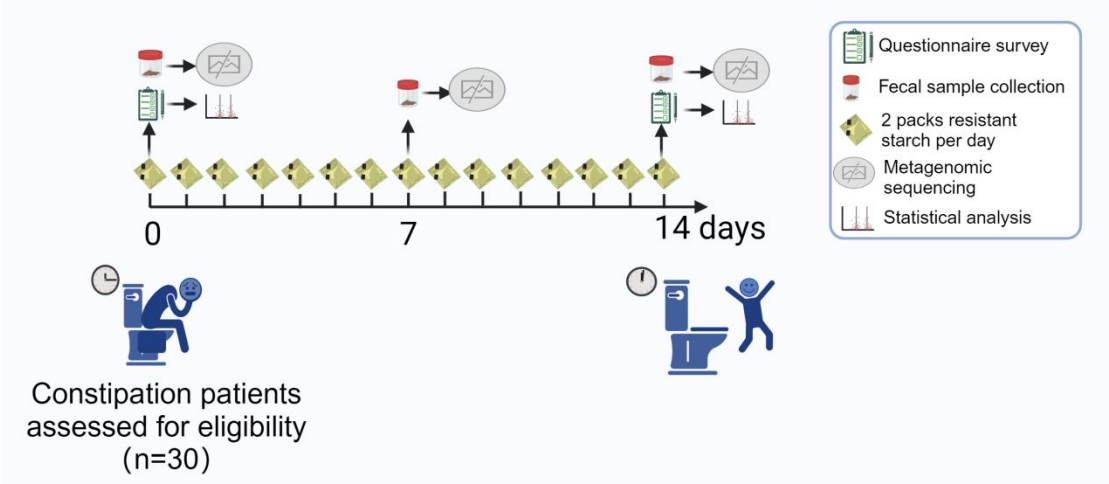
Within two years, colorectal tumors were excluded by colonoscopy, and the clinical manifestations were constipation, which met the diagnostic criteria of Rome IV constipation. Health status was assessed by having the study subjects fill out a questionnaire to assess bowel health related questionnaires and those who did not fulfil the requirements were excluded.

Exclusion criteria

(a) Patients with other gastrointestinal diseases; (b) Patients with previous abdominal surgery, cardiovascular disease, or serious medical conditions; (c) Participants have used medications (e.g., probiotics, prebiotics, antibiotics, laxatives, prokinetic medications) in the past month that could affect the results of the study.; (d) Pregnant women; (e) Patients participating in other clinical studies.

5、Research process

For recruited patients with functional constipation, stool samples were first collected and 2 sachets of resistant starch were administered per day, each pack being 10 g. This could be divided into 2 doses or 2 packs could be taken at a time. The resistant starch was brewed in 200 ml of warm water. The administration was continued for 14 days, in which fecal samples were collected from the volunteers on day 0, day 7 and day 14. On day 0 and day 14 patients filled out the questionnaire which is shown in the attached table.



A questionnaire was used to find out whether the symptoms of the constipated patients improved, while stool samples were collected and analysed by macrogenomic sequencing to investigate what changes occurred in the gut microbes of the patients before and after the resistant starch intervention, with a special focus on whether there was an increase in the relative abundance of *Ruminococcus bromii.* The study was conducted without recruitment advertisements and the recruitment of subjects was completely voluntary, with informed consent signed by the subjects after explaining the possible risks involved in the trial and obtaining their consent to join the study. Volunteers who agreed to join the trial were given a questionnaire to obtain their gut health and other health conditions, and were screened according to the above criteria for selection and exclusion of study participants. The samples collected are named with a number, which does not reveal the subject's personal information, and the subject's identity is kept confidential throughout the study, with only the number and disease phenotype visible. Subjects can withdraw from the study at any stage.

In this study, the composition and structure of the intestinal flora of the subjects will be analyzed, and the subjects can keep abreast of the progress of the test and analysis and obtain their own relevant data.The resistant starch used in this study (HiMaize260) is produced by Ingredion. HI-MAIZE®260 resistant starch is a dietary fiber derived from Ingredion's proprietary high-amylose corn starch that enhances the nutritional content of everyday foods such as white bread, muffins, cookies, cakes and pasta.

The HI-MAIZE®260 standard is GB31637-2016, which conforms to the national standards for food safety. The HI-MAIZE®260 resistant starch contains approximately 53% resistant starch (dietary fiber) and 40% digestible starch and can be easily added to standard formulations by partially replacing plain flour. In addition, because HI-MAIZE®260 resistant starch contains fewer calories than flour, it enhances the nutritional content of food.

6、Possible risks and preventive measures

During the study, if the patient had poor defecation, Kaisailu could be used to record the date and time of use.

7、 Statistical Analysis Plan

The results of the questionnaire filled out by the patients were collected and statistically analyzed, and the scores of constipation symptoms and PAC-QOL (patient assessment of constipation quality of life questionnaire) were tested by paired t-test. The higher the score, the more serious the constipation symptoms, and the lower the score means the relief of constipation symptoms. The minimum score of the scale is 0, the maximum score is 30, and a score of more than 15 can be regarded as constipation. The PAC-QOL is a specific scale for assessing the quality of life of patients with chronic constipation. The PAC-QOL consists of 28 items divided into four dimensions: worries and concerns (11 items), physical discomfort (4 items), psychological discomfort (8 items), and satisfaction (5 items). Each item was scored on a 5-point scale, and the more severe the illness, the higher the score.

The experimental data are expressed in the form of mean ±standard error (mean ±SD). The difference of intestinal flora was analyzed by Wilcoxon paired test, and it was considered that the data had significant difference. Statistical analysis uses GraphPadprism and R (version 4.1.2, https://www.r-project.org/).

Macrogenomic data were analysed using the bioBakery 3 process to obtain microbial species abundance, gene abundance and metabolic function abundance results.

(1) Data quality control: firstly, the quality of sequencing raw data was checked by FastQC software, then Trimmomatic software) was used to excise junctions, low-quality bases and filter low-quality sequences, and then host-sequence comparisons were performed by Bowtie2 to remove host sequence contamination in the sequencing data, and then finally, sequence quality test to confirm that the sequence quality had met the analysis standard.

2) Species annotation: Species annotation was performed using MetaPhlAn software, and the sequences after quality control were compared to the marker gene database using Bowtie2 to obtain the microbial community composition.

3) Gene annotation: select all the microbial genomes that have been detected by MetaPhlAn 3.0, construct the ChocoPhlAn pan-genome database, compare the sequences after quality control to the ChocoPhlAn pan-genome database by Bowtie2 to obtain the gene annotation information of all the known species, and then compare the sequences that have not been compared to the ChocoPhlAn pan-genome database by Diamond. ChocoPhlAn pan-genome by Diamond, and then translated and aligned the sequences not aligned to ChocoPhlAn pan-genome to the UniRef90 protein database, to obtain the gene annotation information of unknown species.

4) Functional annotation: according to the gene annotation results, the ko gene family was corresponded to the KEGG database to obtain the metabolic function annotation information.

5) Visual analysis: according to the species abundance table, gene abundance table and metabolic pathway abundance table of each sample, analyse the α-diversity and β-diversity of each period, and carry out the difference between groups Anosim, Wilcoxon analysis.

8、Reference

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Attachment

Attachment 1. Cleveland Constipation Scoring System（CCS)

No.		frequency	score	score
1	Defecate frequency	1-2times/1-2day	0	
		2times/week	1	
		1time/week	2	
		< 1time/week	3	
		< 1time/month	4	
2	Defecation time: squatting time for each defecation (minutes)	<5	0	
		5-10	1	
		10-20	2	
		20-30	3	
		>30	4	
3	Difficulty: Painful defecation	never	0	
		very few	1	
		sometimes	2	
		often	3	
		always	4	
4	Assisted defecation: type of assistance	no	0	
		Stimulant laxative	1	
		Finger defecation or enema	2	
5	Empty: Incomplete Empty Sense	never	0	
		very few	1	
		sometimes	2	
		often	3	
		always	4	
6	Defecation failure: the number of times defecation fails every 24 hours	never	0	
		1-3	1	
		3-6	2	
		6-9	3	
		>9	4	
7	Pain: abdominal pain	never	0	
		very few	1	
		sometimes	2	

		often	3	
		always	4	
8	History: Course of constipation (years)	<1	0	
		1-5	1	
		5-10	2	
		10-20	3	
		>20	4	

Attachment 2 .Quality of Life Scale for Constipated Patients（PAC-QOL）

The following questions are related to symptoms of constipation. In the last 2 weeks, the severity or intensity of the following symptoms	not at all	a little	usual	more serious	extremely serious
	1	2	3	4	5
1.bloat					
2.feel heavy					
The following questions about constipation domain daily life . How much time in the last fortnight	never	very few	sometimes	often	always
	1	2	3	4	5
3.discomfort					
4.Difficulty in passing stools despite having the urge to do so					
5.Feeling uncomfortable with others					
6.eating less and less because of constipation					
The following questions about constipation and daily life . In the past 2 weeks, the severity and intensity of the following problems	not at all	a little	usual	more serious	extremely serious
	1	2	3	4	5
7.Must be concerned about what to eat					
8.loss of appetite					
9.Concerns about not being able to pick food at will (e.g., at a friend's house)					
10.Feeling uncomfortable about spending too much time in the bathroom when you're out and about					
11.Feeling uncomfortable about going to the bathroom too often when you are out and about.					
12.Always worried about changing habits (e.g. travelling, going out, etc.)					
The following questions relate to feelings of constipation. In the past 2 weeks, the frequency of time the following symptoms have occurred	never	very few	sometimes	often	always
	1	2	3	4	5
13.Feeling irritable					

14.thrill					
15.It's always a problem					
16.Feel nervous					
17.Feel a lack of self-confidence					
18.Feel that life is out of control					
The following questions are related to the feeling of constipation. In the past 2 weeks, the severity and intensity of the following problems.	never	very few	sometimes	often	always
	1	2	3	4	5
19.Worrying about not knowing when to have a bowel movement					
20.Worried about not having enough bowel movements					
21.Disruption of life due to lack of bowel movement					
The following questions about constipation and daily living . In the past 2 weeks, how often have the following symptoms appeared	never	very few	sometimes	often	always
	1	2	3	4	5
22.Worried it's getting worse.					
23.Feeling physically unable to work					
24.Stools are less frequent than expected					
The following questions about satisfaction . In the past 2 weeks, the severity and intensity of the following problems	very satisfied	more satisfied	Generally satisfactory	a little upset	very unsatisfactory
	1	2	3	4	5
25.Are you satisfied with the number of stools?					
26.Are you satisfied with the rule of defecation?					
27.Are you satisfied with the time when the food passes through the intestines?					
28.Are you satisfied with the previous treatment?					