Research Article

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**Effect of xylitol on gut microbiota in an in vitro colonic simulation**

Ksilitolün Bagırsakta Mikrobiyota Üzerine Etkisi in vitro kolonik simülasyon

https://doi.org/10.1515/tjb-2018-0328
Received August 6, 2018; accepted December 2, 2018; previously published online September 13, 2019

**Abstract**

**Objective:** Xylitol has been commonly used as a sweetener and dental caries protective agent. However, how xylitol influences the composition and metabolism of gut microbiota is not known yet. This study aimed to dissect the changes of microbiota and their metabolites under xylitol supplementation in an in vitro colonic simulation.

**Materials and methods:** A single-phase continuous fermentation model was used to culture human fecal flora and the 16s rDNA and short chain fatty acid were analyzed.

**Results and discussion:** It was found that gut microbiota composition differentiated after xylitol supplementation only for the beginning 3 days. Xylitol significantly enhanced the relative amount of butyrate synthesizing bacteria such as Clostridium and Phascolarctobacterium. Meanwhile, xylitol increased the production of propionic acid and butyrate. An increase of Escherichia population sizes after xylitol supplementation was beyond expectation. By Spearman analysis, a positive relationship between Escherichia and Bifidobacterium was found.

**Conclusion:** xylitol can rapidly enhance the total amount of short chain fatty acids, but its influence will disappear after 3 days of fermentation. Results of this investigation can be a guideline for the further investigations on xylitol in relation to gut microbiota and the daily intake determinations.

**Keywords:** Xylitol; Colonic simulation; Gut microbiota; Bifidobacterium; SCFAs.

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**Gereç ve Yöntem:** İnsan fekal florasını kültürleme için tek fazlı sürekli bir fermantasyon modeli kullanıldı ve 16s rDNA'si ve kısa zincirli yağ asidi analiz edildi. 


**Sonuç:** Ksilitol, toplam kısa zincirli yağ asidi miktarını hızla bir şekilde artırmaktadır, ancak etkisi üç günlük fermentasyondan sonra kaybolmaktadır. Bu aradaki bulgular, bağlantılı mikrobiyotanın ve günlük alım tayinlerine ilgili olarak ksilitol ile ilgili daha ileri araştırmalar için bir rehber olabilir.
Anahtar Kelimeler: Xylitol; Kolonik simülasyon; Bağırsak mikrobiyotresi; Bifidobacterium; SCFAlar.

Introduction

Human gut microbiota contains obligate anaerobes and facultative anaerobes, which are beneficial to hosts. The benefits include regulation of immune reactions, helping hosts in disease prevention, modulating gut health and balancing intestinal ecology [1–3]. Gibson et al. recognized prebiotics as an indigestible food ingredient, and it stimulated the activity and propagation of healthy microbiota to improve the health of the host [4].

With the economic development and the improvements in living standards, consumer interests on the nutritional health and food safety have become a popular issue. Sugar alcohols, oligosaccharides, and dietary fibers are regarded as the three functional ingredients. Their main varieties are sorbitol, mannitol, xylitol, and maltitol, which are widely utilized in pharmaceutical, chemical, and food industry. Due to the metabolic functions different from that of sucrose in humans, they are also called functional sugar alcohols [5]. Sugar alcohols are not saccharides. However, these sugar alcohols bear similarity with saccharides. In America and Europe, sugar alcohols are referred to as sugar replacers or sugar replacements. Sugar-free food means food without sucrose or starch-based sugars. Compared to the regular sugars, sugar alcohols have low calories, low insulin stimulation. Also, they are suitable for diabetic patient consumption, and they are implied in the prevention of obesity and dental caries. Therefore, the sugar alcohols are regarded as healthy sugar-substitutes. The enzymes in the human gastrointestinal (GI) tract cannot digest and absorb sugar alcohols. Only the minority of sugar alcohols can enter into large intestines or are metabolized by gut microbiota. For instance, isomalt, xylitol, and lactitol have been shown to stimulate the Bifidobacteria growth [6].

Prebiotic xylitol is a white or colorless solid, a pentahydroxy sugar alcohol (C₅H₁₂O₅), readily soluble in water, slightly soluble in ethanol and methanol. Since xylitol can reach the large intestines practically undigested, its effect on the gut microbiota is of research interest. As indigestible prebiotic carbohydrate, several research studies have dealt with the xylitol and gut microbiota relationship [7].

There are two aspects of investigating the regulation of a prebiotic alcohol on gut micro-ecology. The subjects of in vivo study are usually animal or human. The subjects are supplied with the diets containing fructooligosaccharide (FOS) or other prebiotics for a designated time of duration. After this period, some parameters between control and experimental groups are evaluated regarding the regulations imposed by prebiotics on human gut microbiota [8]. However, some errors may occur due to individual differences such as diet habits and physical conditions. There are some drawbacks in in vivo simulation as well; the duration of experiments is long and a large number of samples are necessary. In vitro simulation is a methodology based on the model of GI tract favoring anaerobic fecal fermentation [9]. The source of gut microbiota obtained from human fecal excreta. Then prebiotics are added into anaerobic fermentation system to analyze the composition and amount of microbiota and SCFAs content to evaluate effects of prebiotics.

Our study investigated the effect of xylitol on human gut microbiota and metabolism by an in vitro model system. The results of this investigation can be a guideline for the studies dealing with prebiotic functions and the determinations of daily intake for people especially woman and elderly.

Materials and methods

Fecal sample collect and pre-treatment

Fecal samples were donated by six healthy adult women, ages ranging between 20 and 26, who did not receive antibiotic treatment for at least 6 months and voluntarily signed the informed consent form. Fecal samples were collected into a container and filled with a 80% N₂ and 20% CO₂ gas mixture. Under anaerobic conditions, 10 g of fresh fecal sample was collected and dissolved in 90 mL of 0.1 mol/L phosphate buffered saline solution (PBS, pH 4.7). The samples were then stirred thoroughly and filtered through three layers of gauze to discard large particulate residues. The filtrates (bacterial suspension) were collected and used as inoculum. All the media and buffer solutions were flushed with the gas mixture (80% N₂ and 20% CO₂) for 10 min before application.

In vitro single-stage colonic fermentation

In vitro single-stage, colonic reactors were constructed in Changdao Moni simulation (CDMN) in Zhejiang Gongshang University. Nutritive medium included (g/L): Peptone 3.0; Cornstarch 8.0; Yeast extract 4.5; Tryptone 3.0; Mucin 0.5; L-cysteine hydrochloride 0.8; Bile No.3 0.4; Heme 0.05; Sodium chloride 4.5; Tween 80 1.0; Potassium chloride 2.5; Potassium dihydrogen phosphate 0.4; Magnesium...
chloride hexahydrate 4.5; Calcium chloride hexahydrate 0.2; Magnesium sulfate heptahydrate 3.0; Ferrous sulfate heptahydrate 0.1; Calcium chloride dihydrate 0.1; Manganese chloride tetrahydrate 0.32; Cobalt sulfate heptahydrate 0.18; Copper sulfate pentahydrate 0.01; Zinc sulfate heptahydrate 0.18; Nickel chloride hexahydrate 0.092 [10]. The 10% fecal microbiota (v/v) (ca. 1% of fecal samples) were inoculated into first-stage (Figure 1) reactors with a total fermentation volume of about 600 mL. During the entire fermentation process, the mixing speed was set at 120 r/min to maintain broth uniformity. All reactors were kept at 37°C. The pH was maintained at 6.8 by adding at 0.5 mol/L either NaOH or HCl. The anaerobic condition was generated by flushing the headspace of all reactors and medium vessels with N₂ for 30 min three times daily. In the consecutive fed-batch fermentations the nutrient medium was replaced at a rate of 37.5 mL/h for a 24 h period.

After 7 days of continuous culturing, the fermentation broth was equally divided into two culture fermenters, which were experimental fermenters (Xyl) and control fermenters (Con). After addition of 5 g xylitol to nutrient medium tanks (3 L), in the two reactors continuous cultures were sustained for 8 days. The pH values of nutrient media were monitored. The waste liquid collected from each reactor was stored at −4°C before analysis.

**Short chain fatty acid analysis**

Gas chromatography–mass spectrometry (GC-MS) analysis was performed to determine the SCFAs, according to Tan et al. with modifications [11]. Ten milliliters batch cultures were harvested and centrifuged (TGL-16, Cence, Co., Ltd., China) at 8000 r/min for 2 min. The supernatant was collected and filtered through 0.45 um pore size membranes. 0.1 mL sulfuric acid solution (50%) and 1.0 mL ethyl-ether were added into the 0.5 mL treatment suspension. The mixture was shaken for 30 times, centrifuged at 10,000 r/min for 5 min, and stored at 4°C for 30 min. All GC-MS analyses were performed at room temperature, and each sample was placed on an FFAP elastic quartz capillary column (30 m × 0.25 mm × 0.25 μm). All parameters were set as following: Oven temperature programmed from 100°C (1 min) to 150°C (5 min) at 5°C per min; Source temperature at 200°C; interface temperature at 250°C; Carrier gas: high purity nitrogen (≥99.999%); carrier gas

![Figure 1: Changes of index and principal components analysis scores.](image-url)

(A) Changes of index Chao1 (Alpha diversity) under Xylitol and Control for 8 days. (B) Principal component analysis scores plot based on the relative abundance of OTUs (97% similarity level). (C and D) Effect of Xylitol on bacterial phylum and genera alternation. Con and Xyl stand for the control and Xylitol groups, respectively.
flow rate: 2 mL/min; inlet temperature: 270°C; injection method: no split injection; injection volume: 2.0 μL; detector temperature (FID): 280°C; mass spectrometer ion source type: electrospray ion Source (ESI); Ion Source Temperature: 350°C.

DNA isolation, PCR, and 16s rRNA gene analysis

DNA from different samples was extracted using a MicroElute Genomic DNA Kit (D3096-01, Omega, Inc., USA) according to the manufacturer’s instructions. The total DNA was eluted in 50 μL of elution buffer by a modified version of the procedure described by the manufacturer (QIAGEN), and the samples were stored at −80°C until PCR amplification (LC-Bio Technology Co., Ltd., Hang Zhou, Zhejiang Province, China). Bacteria 16s rRNA gene sequences (V3-V4 regions) were amplified from the whole genome of samples via the primer pair (319F 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′) according to a previously described method with a minor modification [12]. All reactions were conducted in 25 μL (total volume) mixtures including approximately 25 ng of a genomic DNA extract, 12.5 μL PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. PCR reactions were performed in a Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany) set to the following conditions: initial denaturation at 98°C for 30 s; 35 cycles of denaturation at 98°C for 10 s, annealing at 54/52°C for 30 s, and extension at 72°C for 45 s; and the final extension at 72°C for 10 min.

The PCR products were normalized by using an AxyPrep™ Mag PCR Normalizer (Axygen Biosciences, Union City, CA, USA), which allowed skipping the quantification step regardless of the PCR volume submitted for sequencing. The amplicon pools were prepared for sequencing with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA), and the size and quantity of the amplicon library were assessed on the LabChip GX (Perkin Elmer, Waltham, MA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The PhiX Control library (V3) (Illumina) was combined with the amplicon library (expected at 30%). The library was clustered to a density of approximately 570 K/mm². The libraries were sequenced on the 300PE MiSeq runs, and one library was sequenced with both protocols using the standard Illumina sequencing primers, eliminating the need for a third (or fourth) index readings.

16s rRNA gene sequences were processed and modified as in previously described methods [13]. The reads were controlled and confirmed by QIIME (quantitative insights into microbial ecology, http://qiime.org/tutorials/processing_illumina_data.html), Vsearch (https://github.com/torognes/vsearch, v2.3.4), FASTQC, and FLASH (Fast Length Adjustment of Short reads, v1.2.8, http://ccb.jhu.edu/software/FLASH) quality filters. A Cd-hit method was introduced to select operational taxonomic units (OTUs) by the generation of an OTU table [14]. When the similarity of OTUs was over 97%, sequences were assigned to one unit. RDP (Ribosomal Database Project) classifiers were applied to distribute the 16s rRNA gene into distinct taxonomic categories by aligning representative sequences to taxonomically annotated sequences [15]. To calculate the alpha diversity, we rarefied the OTU table and calculated two metrics: The Chaol metric, which estimates the richness, and the Shannon index. The heatmap of an important family of the gut microbiome was created by using Mev. 4-9-0. PICRUSt website to predict gene function on OTU tables (http://huttenhower.sph.harvard.edu/galaxy/). The principal component analysis and canonical correlation analysis of 16s rDNA sequencing results were performed by using Matlab software (v8.1.0.430). Principal component analysis (PCA) was carried out regarding all taxa relative abundances.

Statistics

The data were analyzed via two-way ANOVA and Duncan’s test for multiple comparisons with SPSS ver. 17.0. A value of p < 0.05 was considered statistically significant.

Results

Effect of xylitol on bacterial community

A total of 16 samples from Xyl and Con groups were chosen for microorganism profile analyses. After quality-filtering, the 16s RNA sequencing results produced 4,146,899 reads, giving a mean sample depth of 129,590.6 reads with a standard deviation of 43,704.2 reads. A total of 789 operational taxonomic units (OTUs) were obtained, and the relative abundance of OTUs showed relative abundances of the genera in the samples. Chaol is a commonly used alpha diversity index. The chaol reflects the estimated number of OTU species in the sample (Figure 1A). Alpha-diversities of the microorganisms in Xyl group was significantly lower
than that of the Con in the first 3 days, and the Chao1 index was only $201.92 \pm 17.79$, $281.74 \pm 21.62$ and $277.93 \pm 32.25$, respectively. Chao1 Index tends to be consistent with the control group. Regarding this result, xylitol reduced the alpha diversity of microbes in the colon.

According to 16s rDNA sequence of the control group and mix group by principal components analysis (Figure 1B), the primary components accounted for 90.43% while the secondary components accounted for 2.85%. These results can comprehensively reflect the distribution of the sample in the control group. After the addition of prebiotics, the distance between the control group and xylitol group increased, indicating that those two groups differed from each other. However, after 3 days, the difference tended to disappear.

The composition of gut microbiota changed quickly after the addition of prebiotics (Figure 1C, D), which can be seen from the high relative content of Firmicutes and the low relative content of Bacteroides. Furthermore, the prebiotics stimulated the relative amount of Escherichia spp. and Clostridium spp. at the beginning. The proportion of Escherichia spp. and Clostridium spp. were significantly high at the first day in the xylitol group, by 62.37% and 12.94%, respectively. Besides, xylitol also enhanced the relative abundances of Lactobacillus spp., which was observed on the second day, to the extent of 7.2%. In the bar chart of both phyla and genera, it can be seen that the modulation of the influence of prebiotics was reversible and only lasted in a short time. After 3 days the composition of gut microbiota reversed to the initial condition.

**Effect of xylitol on short-chain fatty acid (SCFAs)**

In SCFA bar chart (Figure 2), production of SCFAs in the first 2 days was low, being only 0.66 mg/L and 0.55 mg/L, followed by a dramatic increase. Xylitol had a lagging impact on short-chain fatty acids production. In the following 5 days, the concentration of SCFAs in the mix group was significantly higher than that in the control group. On the 4th day, the content of SCFAs reached the peak (4.93 mg/L); by comparison, the control group was only 2.67 mg/L. In day 8, there were no significantly differences between xylitol groups (1.00 mg/L) and control groups (0.99 mg/L). Xylitol also promoted the production of propionic acid and butyric acid.

**The relationship between microbiota, SCFA, and xylitol**

The correlation between gut microbiota can be seen after performing Spearman correlation analysis and plotting with Cytoscape. Red means a positive correlation and green means a negative correlation (Figure 3A). The solid line represented a significant correlation and the dashed...
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Figure 3: (A) The correlation between gut microbiota. (B) Canonical correspondence analysis (Label "1–24" stand for Bacteroides, Escherichia, Desulfovibrio, Parabacteroides, Pseudomonas, Clostridium, Phascolarctobacterium, Sutterella, Oscillospira, Acinetobacter, Clostridium, Collinsella, Roseburia, Lactobacillus, Blautia, Cloacibacillus, Commononas, Bilophila, Stenotrophomonas, Defluviitalea, Dorea, Bifidobacterium, Ruminococcus and Clostridium, respectively). (C) Heatmap and hierarchical clustering (Euclidean distance) on the taxonomic genus level.

line represented non-significant. Form Figure 3A, it can be concluded that Bilophila spp. and Dorea spp. are significantly and positively correlated. Bacteroides spp. were significantly negatively correlated with Escherichia spp., Bifidobacterium spp., Pseudomonas spp., and Clostridium spp. Beyond our expectation, we observed that Escherichia had a positive relationship with Bifidobacterium.

Regarding the CCA (canonical correspondence analysis) (Figure 3B), we found bacteria under xylitol supplementation had an acute angle with different SCFA, indicating a positive relationship. According to the heatmap (Figure 3C), xylitol supplemented samples on the 1st, 2nd, and 3rd day were clustered together, indicating that xylitol can only affect microbiota in the first 3 days.

**Function prediction of gut microbiota via PICRUSt**

In this study, 14 primary metabolic pathways, 24 secondary metabolic pathways, and 263 tertiary metabolic pathways of KEGG (Kyoto Encyclopedia of Genes and Genomes) were obtained through PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) prediction. We found that the majority of secondary metabolisms were not affected by xylitol. Among the tertiary metabolic pathways (Figure 4) xylitol promoted the glycolysis (Ko00010), DNA replication proteins (Ko03032), amino sugar and nucleotide sugar metabolisms (Ko00520) of gut bacteria, as well as DNA repair and recombination (Ko03400) pathways. On the other hand, xylitol interfered propanoate metabolism (Ko00640) and inhibited...
ion channel pathways (Ko04040) along with arginine and proline metabolism (Ko00330). Beyond our expectation, the expression of porphyrin and chlorophyll metabolism (Ko00860) pathways were not operating significantly in the xylitol group.

**Discussion**

Takashi et al. found that supplementation of low and medium doses of xylitol (40 or 194 mg/kg body weight/day, respectively) significantly altered the intestinal microflora composition in rats, but no significant changes in lipid metabolism were observed. Intestinal microflora can inhibit the accumulation of fat via short chain fatty acids production in the presence of dietary fibers [7]. In our study, the xylitol group increased the production of short chain fatty acids, especially propionate and butyrate. Mäkeläinen et al. also reported that the xylitol group significantly increased the production of all SCFAs in the colon model especially the concentration of butyrate. In their study, branched-chain fatty acid (BCFA) levels decreased significantly as a result of polydextrose and xylitol supplementation.

Meanwhile, biogenic amines were stable with supplementation of glucose and xylitol [16]. Motoi et al. also investigated the effects of xylitol on the intestinal microbiota and the secretion of isoflavones in the urine of mice.

Xylitol and sorbose can significantly promote the production of butyrate in vitro fecal fermentation. In our study, it was found that xylitol reduced most of the harmful bacteria, but also increased the relative butyrate synthesizing bacteria. Others also reported that the abundance of microorganisms associated with the metabolism such as *Anaerostipes hadrus* or *A. caccae* [17]. There are 12 typical bacteria can produce butyric acids in the human colon. Only *Anaerostipes A hadrus* and *A. caccae* are associated with sorbose and xylitol, but *A. hadrus* DSM 3319 does not utilize xylitol in pure culture in vitro [18]. Recent studies have shown that prebiotics promote the growth of beneficial microorganisms such as *Bifidobacteria* and lactic acid bacteria, and inhibited the harmful microorganisms such as *Clostridium* and *Shigella* [19–20]. However, in our study, it was also found that xylitol enhanced the relative amount of *Escherichia*, which may be due to the stimulation by metabolites of other bacteria. Miremadi et al. reported that *Bifidobacteria* could improve the host immune functions by the suppression of the toxin synthesis activity of harmful bacteria [21]. However, to our surprise, a positive relationship between *Escherichia* and *Bifidobacterium* was observed. Xylitol also stimulated the production of useful metabolites such as butyrate, although the prebiotic effect is obvious, the intrinsic mechanism of metabolic regulation is not yet fully understood, and how to supplement the prebiotics to achieve the optimal health effects is also uncertain, requiring a further study. After the analysis of
predicted pathways, it was found that xylitol significantly enhanced the activity of energy and DNA related pathways and inhibited the degradation of amino acid, indicating xylitol obviously brought about the adaptation of gut bacterial community. The results of this investigation can be a guideline for the clarification of prebiotic functions, as well as the daily intake determinations for people especially for woman and elderly.

Acknowledgements: This study was funded by the National Natural Science Foundation of China (31501452 and 81302781), Zhejiang Provincial Natural Science Foundation of China (LQ13C090006), the Scientific Research Foundation of Education Department of Zhejiang Province (Y201432277), Foundation of Food Science and Engineering, the most important Discipline of Zhejiang Gongshang University (2017SICR106) and Project of Xylitol research from Huakang Pharm. Co. Ltd. Xuan Zhu contributed to the design and organization. Yuanyuan Xu, Shasha Xiang, and Lihua Shi conducted the whole experiment. Kun Ye, Yi Chen, and Xuan Bao contributed to the statistics and prediction. YinGe and Mengyi Lin contributed to SCFA analysis experiment.

Conflict of interest: Authors have no conflict of interest.

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