Prebiotic-like effects of chitosan on the intestinal microflora in mice

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Abstract: Food supplements can modulate the composition of human intestinal microflora due to the potential capacity of improving the host health. Chitosan (CS) possesses diverse biological and pharmacological effects and has been used as a new source of prebiotic. In this study, ERIC (Enterobacterial Repetitive Intergenic Consensus)-PCR and viable counts methods were combined to explore the prebiotic-like effects of CS on the intestinal microflora in mice. Mice were divided into 3 groups randomly, and treated with water, 1g/kg of CS, 0.25g/kg of CS, respectively for 24 days. Subsequently, they were treated with levofloxacin (65mg/kg) for 6 days. Viable counts method indicated the growth of Lactobacillus was promoted with CS treatment while at the same time Enterobacteria and Enterococcus were inhibited. ERIC-PCR fingerprint, UPGMA dendrogram, PCA and diversity analysis showed that the intestinal microflora composition was changed with antibiotics treatment, and that samples were significantly separated from those of the control and long-term CS-treated groups. Together, our results demonstrated that CS could be regarded as a potential food supplement for protecting intestinal microflora and regulate imbalance.

Keywords: Chitosan, prebiotic, ERIC-PCR, viable counts, bacterial community.

INTRODUCTION

Intestinal microflora plays a vital role in health and disease. It is essential for ensuring the proper functioning of metabolic reactions, immune regulation, epithelial development and protection against pathogens (Chen et al., 2015). Food supplements that can modulate the composition of human intestinal microflora have raised more interest because of the potential capacity of improving the host health (Li et al., 2015). For example, Bifidobacterium is selectively present in subjects who consumed oligo-fructose and inulin diets (Ramirez-Farias et al., 2009). Resveratrol could increase the growth of Lactobacilli and Bifidoacteria while at the same time decrease Enterobacteria in colitis rats (Larrosa et al., 2009). Increasingly, certain natural products have been reported for their prebiotic-like effects. They could induce microbial competition and reduce the populations of non-beneficial intestinal microbiota (Zhao et al., 2017).

At present, a range of dietary oligosaccharides, such as lacto-sucrose, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and isomalto-oligosaccharides have claimed to exert prebiotic properties (Lamsal, 2012; Peshev and Ende, 2014). Chitosan (CS), a polymer of β-(1-4)-D-glucosamine commonly found in shellfish, fungi and insects (Cárdenas et al., 2001), has possessed diverse biological and pharmacological effects, including haemostatic (Pusateri et al., 2006), antimicrobial activity (Benhabiles et al., 2012), anti-inflammatory (Xiao et al., 2014), antioxidative (Anraku et al., 2014), antitumor activity (Koide, 1998), anti-obese and antidiabetic activities (Kim et al., 2014). However, as a natural, nontoxic and biodegradable carbohydrate, no study on the prebiotic-effects of CS on the intestinal microflora has been carried out. We used an antibiotics-induced intestinal microflora imbalance mice model to evaluate the prebiotic-like effects of CS on the intestinal microflora in vivo. ERIC-PCR and viable counts obtained after bacterial growth on different selective media were combined to analyze the microbial similarity and diversity. CS could be used as a natural dietary supplement to protect intestinal microflora.

MATERIALS AND METHODS

Material and reagents
Chitosan (DD ≥ 95%) was purchased from Jinan Haidebei Marine Biological Engineering Co., Ltd. (Jinan, China). Stool DNA extract kit was purchased from ForeGenen (Chengdu, China). Polymerase Chain Reaction primers ERIC-1 (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2 (AAGTAAAGTCGCGGTAGCGG) (Chen et al., 2014) were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China). DNA markers (2000 and 100 bp) were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). PCR Mix kit was purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). Lactobacillus selection agar (LBS), Bismuth sulfite agar (BS), Eosin-methylene blue agar (EMB) and E. Coli broth (EC) mediums were purchased from Hopebio Co., Ltd. (Qingdao, China).

Animals and treatments
Male KM mice weighing 30±3g were purchased from animal experimental center of Dalian Medical University [Certificate of quality number: SCXK (Liao) 2013-0003]. They were kept under standardized conditions at 22-24°C, 20% humidity with a 12h light/dark cycle, and they had free access to standard diet and water ad libitum.

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Fig. 1: Microbial counts of *Lactobacillus* (A), *Bifidobacterium* (B), *Enterobacteria* (C) and *Enterococcus* (D). All of the data were expressed as Log CFU/g ($\bar{x} \pm s, n=10$).

\[**P < 0.01, *P < 0.05\] vs. D6 samples. \[##P < 0.01, \#P < 0.05\] vs. the normal control group (N). N: Normal mice treated with water. CS-H: Normal mice with 1 g/kg of CS. CS-L: Normal mice with 0.25 g/kg of CS. D6, D24, D30: Drug administration periods.

Fig. 2: ERIC-PCR fingerprints (A), UPGMA dendrograms (B) and PCA analysis (C).

N: normal mice treated with water. CS-H: normal mice with 1 g/kg of CS. CS-L: normal mice with 0.25 g/kg of CS. D6, D12, D18, D24, D27, D30: Drug administration periods.
Thirty mice were divided into three groups (10 mice in each group) randomly, named N, CS-H and CS-L. They were treated with water, 1g/kg of CS and 0.25g/kg of CS (Xu et al., 2013), respectively by intragastric (i.g.) administration once a day for twenty-four days. Subsequently, they were treated with 65mg/kg levofloxacin for six days (Li et al., 2014). Fecal samples were collected at 6 d, 12 d, 18 d, 24 d, 27 d, 30d of drug administration periods respectively and preserved at -80°C.

**Microbial counts**

For the isolates and bacteria counts, 0.2g of each fecal sample was transferred to sterile 4mL plastic centrifuge tube, diluted 10-fold with sterile Phosphate buffer saline (PBS), and homogenized for 120 s using a stomacher (Jingmai, Dalian, China). Serial 10-fold dilutions were then prepared using sterile PBS. Each dilution (20µL) was spread in triplicate on LBS, BS, EMB and EC agar plates, respectively. EMB and EC agar plates were incubated at 37°C for 24h, LBS and BS agar plates were incubated at 37°C for 48h under anaerobic conditions. The colonies enumerated on LBS, BS, EMB and EC agar were Lactobacillus, Bifidobacterium, Enterobacteria and Enterococcus counts, respectively. Microbial counts were expressed as Log CFU/g.

**DNA extraction**

DNA was extracted with Stool DNA kit, and analyzed by electrophoresis of 1% agarose gel containing Ethidium C: \Users\ce\Desktop\3-11-2017\Local Settings\Application\Data\youdao\DictBeta\Application\7.5.0.0\resultui\dict\bromide (EB). DNA extracts were preserved at -20°C.

**ERIC-PCR**

ERIC-PCR was performed with thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) as follows: 2µL DNA as template, 2xEasy Taq PCR SuperMix 12.5µL, primer ERIC-1 (10µM) 0.5µL, primer ERIC-2 (10µM) 0.5µL, and filled up to 25µL with sterile water. The thermal program consisted of 94°C for 5 min, followed by 35 cycles of 94°C for 50 s, 49°C for 30 s, 46°C for 30 s, and 72°C for 3 min, in which 72°C for 9 min. PCR products (10µL) were separated by electrophoresis in 3.0% agarose gels containing EB. Photography with a UV trans-illumination (Bio-Rad, USA), and compared to a molecular weight standard (DL2000).

**STATISTICAL ANALYSIS**

SPSS version 17.0 was used for analysis. P-values were calculated using Student’s t-test, P value <0.01 showed significant difference. Denaturing gradient gel electrophoresis (DGGE) gels were analyzed by Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA). Similarities were displayed as an UPGMA dendrogram. PCA was performed to group microorganisms using R 3.2.2 software for Windows. The Shannon-Wiener index of diversity (H’) was used to determine the diversity of intestinal bacterial community.

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<th>Table 1: Microflora diversity indexes analysis of different periods (x ±s, n = 10)</th>
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<td>Group</td>
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N: normal mice treated with water. CS-H: normal mice with 1g/kg of CS. CS-L: normal mice with 0.25g/kg of CS. **P<0.01 vs. the respective group of D6. H’ = - ∑ (pi) (lnpi), pi was the proportion of bands in the lane, pi = ni / ∑ni, ni was the average density of peak i.

N: normal mice treated with water. CS-H: normal mice with 1g/kg of CS. CS-L: normal mice with 0.25g/kg of CS. **P<0.01 vs. the respective group of D6. H’ = - ∑ (pi) (lnpi), pi was the proportion of bands in the lane, pi = ni / ∑ni, ni was the average density of peak i.
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resistant to majority of antibiotics. In this experiment, Enterococcus increased to (8.08 ± 0.28) Log CFU/g in antibiotics-treated group for 6 d (D30). The above results indicated that CS can indeed stimulate beneficial bacteria while at the same time inhibit pathogens, and modulate the intestinal microflora balance in the gut of the host.

ERIC-PCR analysis

The dominant intestinal microflora community of group N (D6–D30), CS-H (D6–D30) and CS-L (D6–D30) was examined by ERIC-PCR fingerprints analysis (fig. 2A). Complex bands were observed and the distribution region was extensive in groups N (D6–D24), the total number of bands were richer than those of other groups. Band numbers in groups CS-H (D6–D24) and CS-L (D6–D24) were diminished slightly, but common bands increased with increasing experimental period, the similarity of intestinal microflora community were higher than N groups. The least numbers of band were detected in antibiotics-treated groups (N27, N30, CS-H27, CS-H30, CS-L27 and CS-L30), and the intensities of them increased in CS-H27 and CS-H30 groups. It suggested that the diversity and composition of intestinal microflora were disturbed by antibiotics. Two major bands (about 300 and 500 bp) were detected in groups CS-H (D6–D24) and CS-L (D6–D24). The band about 250 bp was detected in antibiotics-treated groups (N27, N30, CS-H27, CS-H30, CS-L27 and CS-L30). The sequences obtained from the above bands will be analyzed in future.

The clustering analysis was visualized in an UPGMA dendrogram to study the community similarity among different groups. There were two main clusters (A and B) in fig. 2B, the first was almost long-term drug treated groups (N, CS-H and CS-L), and the second was antibiotics-treated groups. The minimum bacterial similarity index between cluster A and cluster B was 0.29, which suggested that the intestinal microflora community was affected obviously by antibiotics. In addition, the similarity among CS-H27, CS-H30 and CS-L27 groups was higher (0.74), but that of normal groups and CS-treated groups was 0.53, which suggested that effects of antibiotics on intestinal microflora of normal mice were obviously different to CS-treated mice.

PCA analysis (fig. 2C) of ERIC-PCR fingerprint also showed that the intestinal microflora of normal mice treated by antibiotics (N27 and N30), CS mice treated by antibiotics (CS-H27, CS-H30, CS-L27 and CS-L30) and long-term CS-treated mice (CS-H6–24 and CS-L6–24) were distributed in three different regions. But normal mice with increasing experimental period (N6–24) were completely different with other groups. It was clearly shown that a great difference existed in the intestinal microflora of normal mice which have been kept under the same conditions with increasing experimental period. The doses of CS have little effect on the intestinal microflora community of mice. Groups CS-H27, CS-H30, CS-L27 and CS-L30 distributed in the same region suggested that CS showed extremely significant differences on the intestinal microflora as compared to normal mice.

ERIC-PCR profiles showed the characteristics of general bacteria in the intestinal tract. The Shannon-Wiener indexes of \( H' \) were calculated on the basis of the number and relative intensities of bands on the gel (Wang et al., 2016) (table 1).

It was clearly shown that diversity in N27, N30, CS-L27 and CS-L30 groups decreased as compared to the respective group of D6 with statistic significance \( P<0.01 \). Compared to N (N6–24) groups, the numbers of bands were lower in CS-treated groups, especially antibiotics-treated groups produced low diversity index \( H' \), they all showed significant \( P<0.01 \) differences. But \( H' \) of groups CS-H27 and CS-H30 didn’t decrease, it appeared that intestinal microflora was protected by CS in high concentration.

DISCUSSION

Recent findings have reviewed the impacts of food supplements on the intestinal microflora composition in humans and mice, thereby affecting health outcomes. For example, obesity is associated with changes in the proportion of Bacteroidetes to Firmicutes observed both in humans and mice (Ley et al., 2005; Turnbaugh et al., 2006). In a small scale human intervention study, obese people taking low-calorie food supplements showed increased abundance of Bacteroidetes concurrent with loss of body weight. In our preliminary study (Wang et al., 2016), Maydis stigma polysaccharide promotes the growth of Lactobacillus and Bacteroides in type-2 diabetic (T2D) mice and the intestinal microflora imbalance has been improved. So the effects of food supplements on human health are positive, other, more specific effects of them are attracting people’s interests.

As reported (Hosseinnejad and Jafari, 2016), chitosan has a broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as fungi \textit{in vitro}. In the present study, an antibiotics-induced intestinal microflora imbalance mice model was built to evaluate the prebiotic-like effects of CS on the intestinal microflora \textit{in vivo}. Lactobacillus was promoted with CS in high concentration treatment while at the same time Enterobacteria and Enterococcus were inhibited. Lactobacillus, Bifidobacterium and Enterobacteria decreased significantly by antibiotics treated, but Enterococcus which resisted to majority of antibiotics still increased by antibiotics treatment. CS has the properties in accordance with prebiotics to stimulate beneficial bacteria while at the same time inhibit pathogens, and
modulate the intestinal microflora balance in the gut of the host.

ERIC-PCR fingerprint, UPGMA dendrogram, PCA and diversity analysis indicated that the composition of intestinal microflora was obviously changed with antibiotics treatment, and that samples were significantly separated from those of the control and long-term CS-treated groups.

CONCLUSION

CS could be considered as a potential candidate for developing a new food supplement which could protect the intestinal microflora and regulate imbalance.

REFERENCES


