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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 *Bifidobacteria* 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 (AAGTAAGTGACTGGGGTGAGCG) (Chen e*t 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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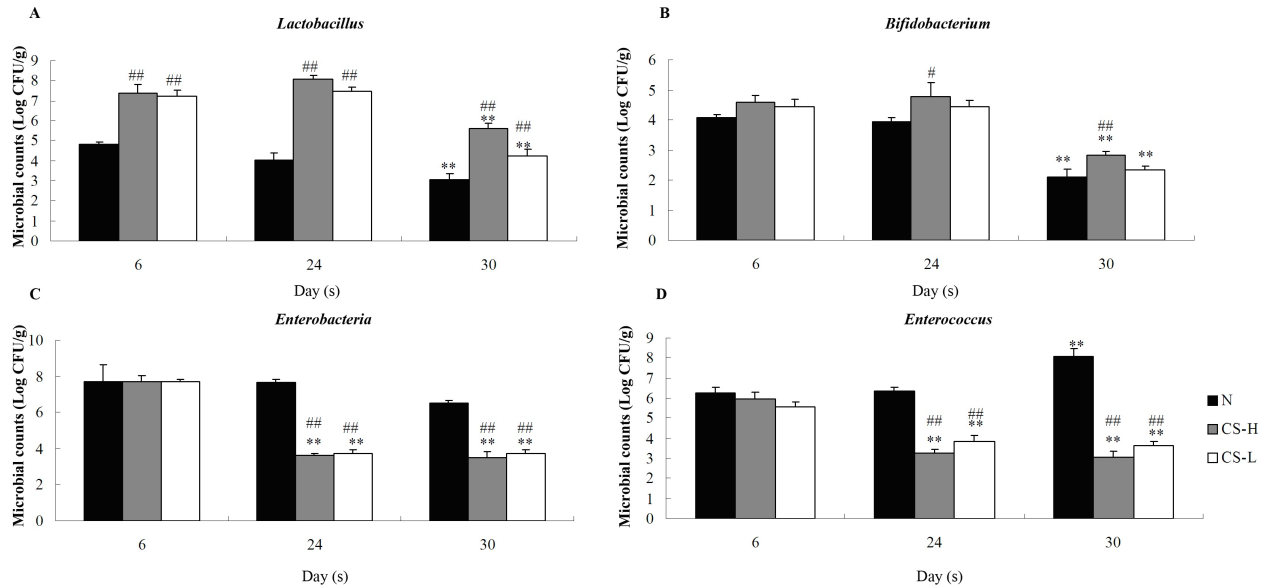

Fig. 1: Microbial counts of *Lactobacillus* (A), *Bifidobacterium* (B), *Enterobacteria* (C) and *Enterococcus* (D). All of All the data were expressed as Log CFU/g ($\bar{x} \pm$ s, n = 10).

$^{**}P$<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 0.25g/kg of CS. D6, D24, D30: Drug administration periods.

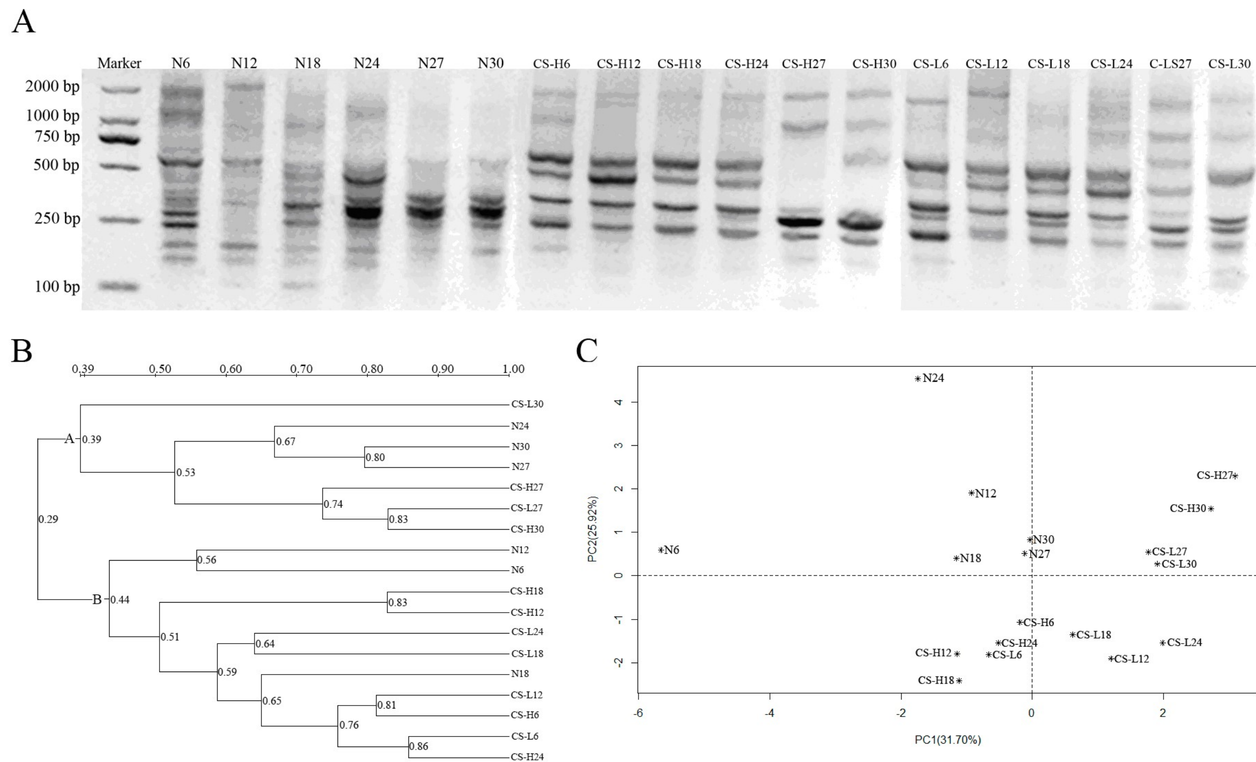

Fig. 2: ERIC-PCR fingerprints (A), UPGMA dendrograms (B) and PCA analysis (C).

N: normal mice treated with water. CS-H: normal mice with 1g/kg of CS. CS-L: normal mice with 0.25g/kg of CS. D6, D12, D18, D24, D27, D30: Drug administration periods.

Table 1: Microflora diversity indexes analysis of different periods ($\bar{x}$ ±s, *n* = 10)

Group	*H* '	Group	*H* '
N-6	2.1402±0.96	CS- H24	1.6720±0.0598
N-12	2.0276±0.82	CS- H27	1.5660±0.0284
N-18	1.8965±0.82	CS-H30	1.6803±0.0226
N-24	1.6835±1.00	CS-L6	1.6253±0.0299
N-27	1.4530±0.96**	CS-L12	1.7534±0.0267
N-30	1.3482±1.26**	CS-L18	1.5109±0.0347
CS- H6	1.2034±0.96	CS-L24	1.5400±0.0958
CS-H12	1.4459±0.82	CS-L27	0.9908±0.0340**
CS- H18	1.5811±0.50	CS-L30	1.2296±0.0150**

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. $H' = -\sum (p_i)(\ln p_i)$, p_i was the proportion of bands in the lane, $p_i = n_i / \sum n_i$, n_i was the average density of peak *i*.

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, 2×*Easy* 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.

RESULTS

Microbial counts

Four different selective media (LBS, BS, EMB and EC) were used to enumerate and detail as much as possible the cultivable fraction of the predominant intestinal bacterial groups presumably present in fecal samples: *Lactobacillus*, *Bifidobacterium*, *Enterobacteria* and *Enterococcus*. The results of bacterial viable counts are shown in fig 1. The level of *Lactobacillus* was enhanced to (8.09±0.18) Log CFU/g in CS-H-treated group at D24. *Lactobacillus* and *Bifidobacterium* decreased significantly (*P*<0.01) in antibiotics-treated groups at D30, whereas the levels of them were markedly higher in CS-treated mice than those of normal groups. In addition, two common intestinal bacteria, *Enterobacteria* and *Enterococcus* were also assessed. The levels of them decreased remarkably in CS-treated groups at D24. As reported (Beshiru et al., 2017), *Enterococcus* species have been regarded as significant pathogens liable for infections, they were

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 micro flora 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 *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 *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.

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