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Oral administration of heparin or heparosan increases the *Lactobacillus* population in gut microbiota of rats

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ABSTRACT

Heparin and heparosan have been confirmed to be effective blockers in inhibiting adhesion of pathogens *in vitro*. However, their effects on gut microbiota *in vivo* remain unknown. Here we have studied the effects of oral administration of heparin or heparosan on gut microbiota in rats by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Results showed that the predominant bacterial communities in the feces of heparin- or heparosan-treated animals were different from those of the saline-treated animals, with increased *Lactobacillus* spp. and decreased *Enterococcus* sp. Different DGGE banding patterns were also observed for the subpopulations of *Lactobacillus* and *Bacteroides* groups. In conclusion, heparin or heparosan may be used as an effective gut microbiota modulator by increasing the subpopulation of *Lactobacillus*.

1. Introduction

The human endogenous gut microbiota is essential for the health (Eckburg et al., 2005). Aberrant gut microbiota has been shown to be associated with some intestinal disorder or diseases, such as irritable bowel syndrome (IBS) and ulcerative colitis (UC). In IBS patients, both increase and decrease of variation of microbiota diversity have been reported (Codling, O'Mahony, Shanahan, Quigley, & Marchesi, 2010; Salonen, de Vos, & Palva, 2010). Noor et al. (2010) found that the presence of some *Bacteroides* spp. and *Parabacteroides* sp. in healthy volunteers distinguished them from IBS and UC patients.

Due to the complex nature of gut bacterial communities, analysis of microbiota is often conducted with molecular techniques instead of cultivation techniques. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer, de Waal, & Uitterlinden, 1993) is based on the separation of PCR-amplified fragments of genes coding for conserved 16S rRNA from a mixed sample and is able to identify the constituents which represent only 1% of the total population. The banding patterns of PCR amplicons generated by DGGE can be compared to evaluate the relative similarity of microbial communities from different treatments.

In our previous study, heparin and heparosan (the biosynthetic precursor of heparin or heparan sulfate) showed selective anti-adhesion abilities to pathogenic and probiotic strains (Chen, Ling, Duan, & Zhang, 2012). Both heparosan and heparin blocked the adhesion of *Escherichia coli*, *Pasteurella multocida*, and *Staphylococcus aureus*, but they did not block the adhesion of *Lactobacillus rhamnosus* to enterocytes and mucus *in vitro* (Chen et al., 2012). The adhesion targets of many microorganisms have been identified as heparan sulfate on mammalian cells. Exogenous heparin acting as receptor mimicry, could block the bacterial exploitation of host heparan sulfate and inhibit the adhesion and dissemination of pathogens in the host (Arciola et al., 2003; Fallgren, Andersson, & Ljungh, 2001; Fears & Woods, 2006; Frick, Schmidtchen, & Sjobring, 2003; Gu, Wang, Guo, & Zen, 2008; Henry-Stanley, Hess, Erickson, Garni, & Wells, 2003; Henry-Stanley, Hess, Erlandsen, & Wells, 2005; Hess, Henry-Stanley, Erlandsen, & Wells, 2006; Menozzi et al., 2002; Rabenstein, 2002).

The selective anti-adhesion abilities of heparin and heparosan indicated that they might affect the components of gut microbiota differently and thus modify the gut microbiota *in vivo*. There were some trials of intravenous administration of heparin to treat UC (Head & Jurenka, 2003). We think the effectiveness of heparin may be associated with the modification of gut microbiota. However, none of previous studies determined the changes of gut microbiota

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Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Reference
P2	5′-ATTACCGCGGCTGCTGG-3′	Muyzer, de Waal, and Uitterlinden, 1993
P3	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′	
LacI	5′-AGCAGTAGGGAATCTTCCA-3′	Walter, Hertel, Tannock, Lis, Munro, and Hammes, 2001
Lac2-GC	5′-CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGGATTYCACCGCTACACATG-3′	
Bfr-F	5′-CTGAACCAGCCAAGTAGCG-3′	Liu, Song, McTeague, Vu, Wexler, and Finegold, 2003
Bfr-GC-R	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCGCAAACTTTCACAACTGACTTA-3′	
Clept-F	5′-GCACAAGCAGTGGAGT-3′	Shen et al., 2006
Clept-GC-R3	5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCTTCCTCCGTTTTGTCAA-3′	

under the treatment of heparin. While intravenous administration of heparin against UC had a high risk of bleeding (Head & Jurenka, 2003), it remains unknown whether oral-administered heparin is effective on the gut microbiota. In the present study, we investigated the impact of oral administration of heparin on the gut microbiota using PCR-DGGE analysis of bacterial communities in fecal samples from rats. We also aimed to understand the acting mechanisms of heparin by comparing the sulfated heparin with nonsulfated heparosan.

2. Materials and methods

2.1. GAGs

Heparin (unfractioned) was purchased from Hebei Changshan Biochemical Pharmaceutical Co., Ltd. (Shijiazhuang, Hebei, China). The heparosan was prepared from fermentation broth of type D P-934 *P. multocida* (*P. multocida* subsp. *multocida* ATCC® 12948™) in brain–heart infusion (BHI) broth with a modified method (DeAngelis & Padgett-McCue, 2000; Chen et al., 2012).

2.2. Animals and treatments

Sprague-Dawley rats were purchased from Laboratory Animal Center of Shandong University (Jinan, Shandong, China). Forty Sprague-Dawley rats (20 male and 20 female, 75–95 g) were housed under controlled humidity (40–60%) and temperature (20–24 °C) with a 12 h light–dark cycle according to China GB 14295-2001 (Laboratory animal-requirements of environment and housing facilities). The animals were acclimated to the laboratory for two days and then randomized into five groups, *i.e.* the natural saline group, the high dose heparin group (10 mg/kg), the low dose heparin group (5 mg/kg), the high dose heparosan group (10 mg/kg) and the low dose heparosan group (5 mg/kg). Each group was composed of four male and four female rats and all animals had free access to food and water. All animals were orally administrated with aforementioned various doses of heparin or heparosan on a daily basis for two weeks. Fresh fecal samples were collected before the treatment, and on the 14th days of the treatment. The fecal samples were stored at −80 °C before preparation of total DNA.

2.3. Total bacterial DNA preparation

To extract fecal microbial cells, 1 g of feces was suspended in 35 mL of anaerobic phosphate-buffered saline (PBS, 0.1 M, pH 7.0) following steps below. A volume of 15 mL of PBS was added and homogenized by vortex for 10 min at high speed. Then another 10 mL of PBS was added and the mixture underwent further vortex for 3–5 min. The last 10 mL of PBS was added and mixed thoroughly. The suspension was centrifuged at 200 × *g* for 5 min and the supernatant was transferred into a new tube. After repeating the previous step twice, the supernatant was centrifuged at 9000 × *g* for 5 min. The pellet was washed twice with anaerobic PBS. Finally, the pellet was re-suspended in 10 mL of PBS and aliquoted into Eppendorf tubes and stored at −80 °C. Total bacterial DNA was prepared with a slightly modified method of protease K-SDS and freezing-thawing followed by chloroform/isoamyl alcohol extraction (Zijnge et al., 2006).

2.4. PCR amplification

All primers used in this study are listed in Table 1. The PCR amplification of the V3 region of the 16S rRNA gene was carried out with primers P2 and P3 according to the protocol described by Muyzer et al. (1993) in a thermocycler PCR system (MJ Mini™, Bio-Rad, USA). The 25 μL of PCR reaction mixture contained 0.5 μM of each primer, 2 mM $MgCl_2$, 0.625 U *Taq* polymerase (Fermentas, China) and 20 ng of the total fecal DNA. After the initial amplification, a reconditioning PCR method was performed to decrease heteroduplexes formation (Thompson, Marcelino, & Polz, 2002). PCR amplifications of *Lactobacillus*, *Bacteroides*, and *Clostridium* were carried out with the protocols described by Walter et al. (2001), Liu et al. (2003), and Shen et al. (2006), respectively.

2.5. DGGE

Amplicons of the V3 region of the 16S rRNA were separated by DGGE using a Dcode System apparatus (Bio-Rad, USA) in an 8% (w/v) acrylamide gel with a gradient 26.5–52%. The gel was electrophoresed at the constant voltage of 200 V and a temperature of 60 °C for 180 min. The denaturing gradients ranging 35–55% were used for the separation of amplicons of *Lactobacillus* and *Clostridium*, whereas gradients 22.5–45% were used for *Bacteroides*. After electrophoresis, the gels were stained with ethidium bromide and visualized on a GelDoc-It Imaging System (UVP, USA).

2.6. Statistical analysis

The DGGE banding patterns from three replicates in each group were digitalized by Quantity One software (Bio-Rad, USA). Each DGGE band was defined as one operational taxonomic unit (OTU) or phylotype. The intensity and relative position of each band were determined manually with background subtraction. The intensity of each band was expressed as percentage of the integrated intensity of the entire lane. The matrix of intensity and relative position was analyzed by principle component analysis (PCA) using SPSS software.

2.7. Sequence analysis of DGGE bands

Important DGGE bands were excised from the gel and incubated in 50 μL of sterile distilled water at 4 °C overnight. PCR amplifications of the DNA fragments from the excised gel were carried out according to the same protocols described above with the

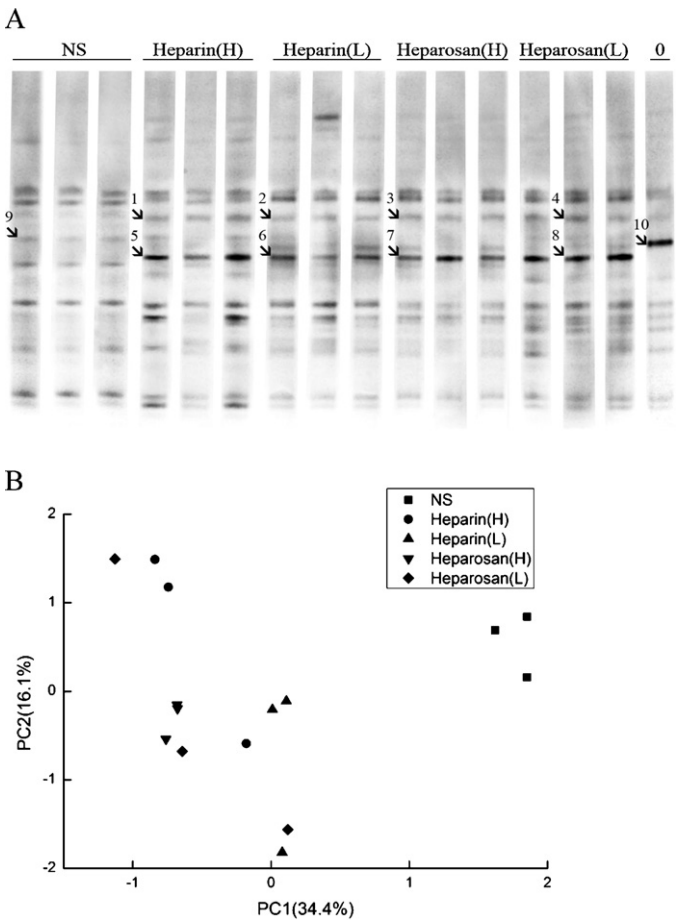

Fig. 1. Community structures assessed by DGGE analysis of the V3 region of the 16S RNA from 14th day fecal samples, (A) DGGE banding profiles of the five groups (three replicates each). NS, the natural saline group; heparin(H), the heparin-high dose group (10 mg/kg); heparin(L), the heparin-low dose group (5 mg/kg); heparosan(H), the heparosan-high dose group (10 mg/kg); heparosan(L), the heparosan-low dose group (5 mg/kg); lane 0, a sample before the treatment, included in each run as a DGGE marker. (B) PCA score plot based on the DGGE profile. Numbers in parentheses are percentages of variation explained by each principle component.

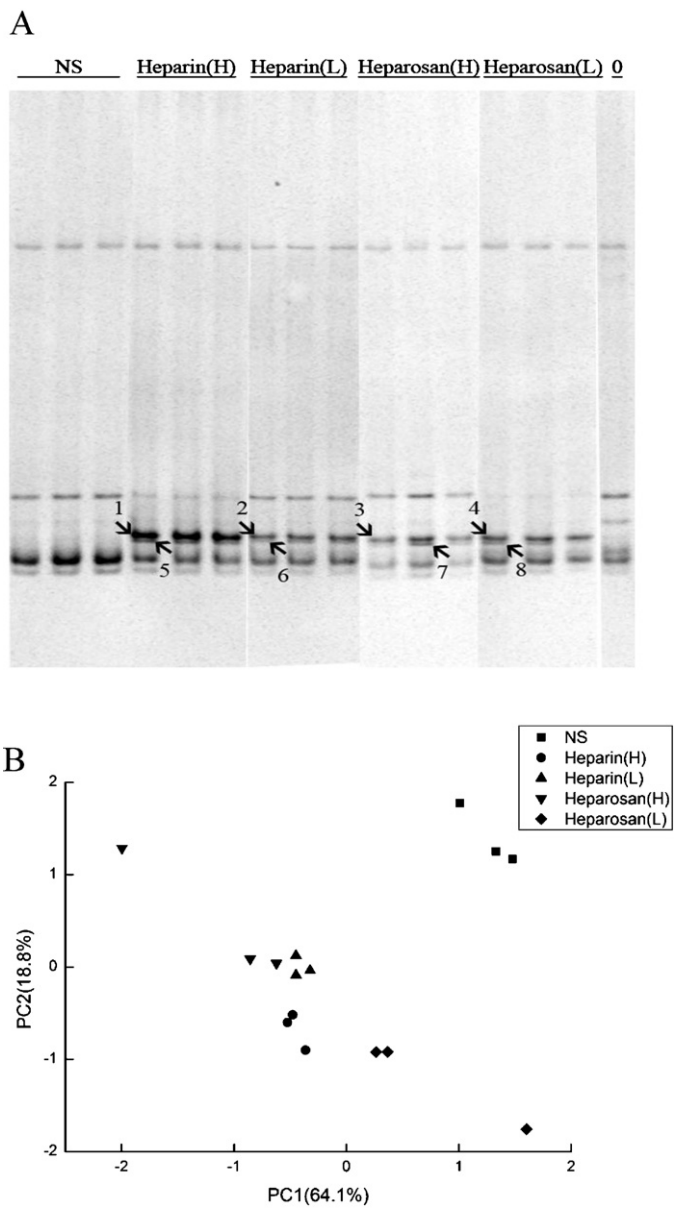

Fig. 2. Community structures assessed by DGGE analysis of *Lactobacillus* subpopulation from 14th day fecal samples, (A) DGGE banding profiles of the five groups (three replicates each). NS, the natural saline group; heparin(H), the heparin-high dose group (10 mg/kg); heparin(L), the heparin-low dose group (5 mg/kg); heparosan(H), the heparosan-high dose group (10 mg/kg); heparosan(L), the heparosan-low dose group (5 mg/kg); lane 0, a sample before the treatment, included in each run as a DGGE marker. (B) PCA score plot based on the DGGE profile. Numbers in parentheses are percentages of variation explained by each principle component.

corresponding primers and 1 μL aliquot of the gel elution. PCR products were excised from a 2.0% agarose gel and purified with a DNA Gel Extraction Kit (Tiangen, China). The DNA fragments were ligated into the pEASY-T1 vector (TransGen Biotech, China) and transformed into competent *E. coli* DH5α cells (TransGen Biotech, China). Inserted DNA was amplified using the corresponding primers and resolved by DGGE to verify the position of the original band. Then DNA fragments from three clones migrating to the same position of the original band were sequenced (Invitrogen, Shanghai, China). The sequences were submitted to GenBank and the RDP database to determine their most related bacterium. The DNA sequences are available in the GenBank database with accession numbers KC166284–KC166287.

3. Results and discussion

In order to compare the predominant bacterial communities between different treatments, the PCR amplification of the V3 region of the 16S rRNA gene was carried out with primers P2 and P3. Amplicons of the V3 region were 234 bp and the DGGE banding patterns of the V3 region of each group on the 14th day are shown in Fig. 1A. PCA score plot was used to compare the differences between banding patterns of different treatments. In the PCA score plot, "sit together" demonstrates similarity between samples. As shown in Fig. 1B, the banding pattern of the saline group was different from other groups, while heparin and heparosan groups showed similar banding patterns at both high dose and low dose. Since banding patterns were related to the bacterial communities, the above results suggested that the predominant bacterial communities in the feces of heparin- or heparosan-treated animals were different from the saline-treated animals, while bacterial communities of heparin- and heparosan-treated animals shared similarity.

To further clarify the differences, bands, which showed different percentage of density among groups, were sequenced. Although bands 1–4, 5–8 with the same migration in different lanes, higher peak density of bands can be observed in the heparin or the heparosan treated groups than the saline group (Fig. 1A). The

sequencing results showed that their most related bacteria were *Lactobacillus taiwanensis* (99%) and *Lactobacillus gallinarum* (100%), respectively. Band 9 and 10 were sequenced as well (Fig. 1A), which had higher peak density in the saline group or the sample before any treatment than in the heparin or the heparosan treated groups. The sequencing results showed that their most related bacterium was *Enterococcus faecium* (99%), which is one of the common commensal bacteria in the human intestine. These sequencing results indicated that heparin or heparosan treatment increased *Lactobacillus* spp. and decreased *E. faecium* in the gut microbiota. However, due to the limited length of amplicons of the V3 region analyzed using primers P2 and P3, further analysis was needed to confirm the initial results.

As the differences were found in the *Lactobacillus* composition, the population of *Lactobacillus* was further examined using specific primers Lacl and Lac2-GC, yielding PCR products of 385 bp, which were electrophoresed for a more detailed comparison. The DGGE banding patterns of the *Lactobacillus* population on the 14th day were shown in Fig. 2A. Limited bands in each lane indicated a relatively simple composition in the *Lactobacillus* population. Banding pattern of the saline group differed from the other groups, while banding patterns of the heparin and the heparosan groups were similar (Fig. 2B). Band 1–4 and 5–8 with the same relative position in different lanes, respectively, showed higher peak density in the heparin or the heparosan groups than the saline group (Fig. 2A). The sequencing results showed that their closest related bacteria were *L. gallinarum* (99–100%) for 1–4, *Lactobacillus antri* (100%) for 5, and *L. taiwanensis* (99%) for 6–8. The sequencing results of *Lactobacillus* specific PCR-DGGE confirmed the findings from the V3 region PCR-DGGE.

Based on the banding patterns (Figs. 1 and 2) and sequencing results (Table 2), it can be inferred that orally administered heparin or heparosan could substantially affect the gut microbiota. In the heparin or heparosan groups, subpopulations of some *Lactobacillus* spp. in the gut microbiota were increased, compared to the saline group. This is consistent with our previous results that heparin or heparosan inhibited the adhesion of some pathogenic bacteria, but did not block the adhesion of *L. rhamnosus* to enterocytes and mucus *in vitro* (Chen et al., 2012). Sugar backbones may account for this tropism, since both heparin and heparosan showed similar properties *in vitro* and *in vivo*. Heparosan, as the biosynthetic precursor of heparin or heparan sulfate, has a similar sugar backbone to heparin or HS, except its polymer is non-sulfated, and there is no epimerization of GlcUA to IdoUA (DeAngelis, Gunay, Toida, Mao, & Linhardt, 2002). Negative charges on heparin are not necessary to the selective increase of *Lactobacillus* spp., although the charges on heparin promote the biofilm formation of *L. rhamnosus in vitro* (Chen et al., 2012).

Colonization of *Lactobacillus* sp. in the intestine microbiota was beneficial to the health and could potentially inhibit the colonization of opportunistic pathogens such as *Salmonella* (Baba, Nagaishi, Fukata, & Arakawa, 1991). Effective *Lactobacillus* colonization may aid in achieving UC remission as well (Head & Jurenka, 2003). After modulation by heparin or heparosan, the gut microbiota was more resistant to infection.

Bacteroides and *Clostridium* were found to be main bacterial components of the fecal microbiota from healthy adults (Ben-Amor et al., 2005). Therefore, *Bacteroides* and *Clostridium* compositions were also analyzed in this study. The population of *Bacteroides* was further examined using PCR with primers Bfr-F and Bfr-GC-R, yielding products of 270 bp. The DGGE banding patterns and PCA analysis are shown in Fig. 3. The PCA score plot (Fig. 3B) showed that band patterns of the heparin or the heparosan groups were different from the saline group. Heparin or heparosan might be the sources of energy for *Bacteroides* spp. (Martens, Chiang, & Gordon, 2008), which could explain the different patterns of the heparin or

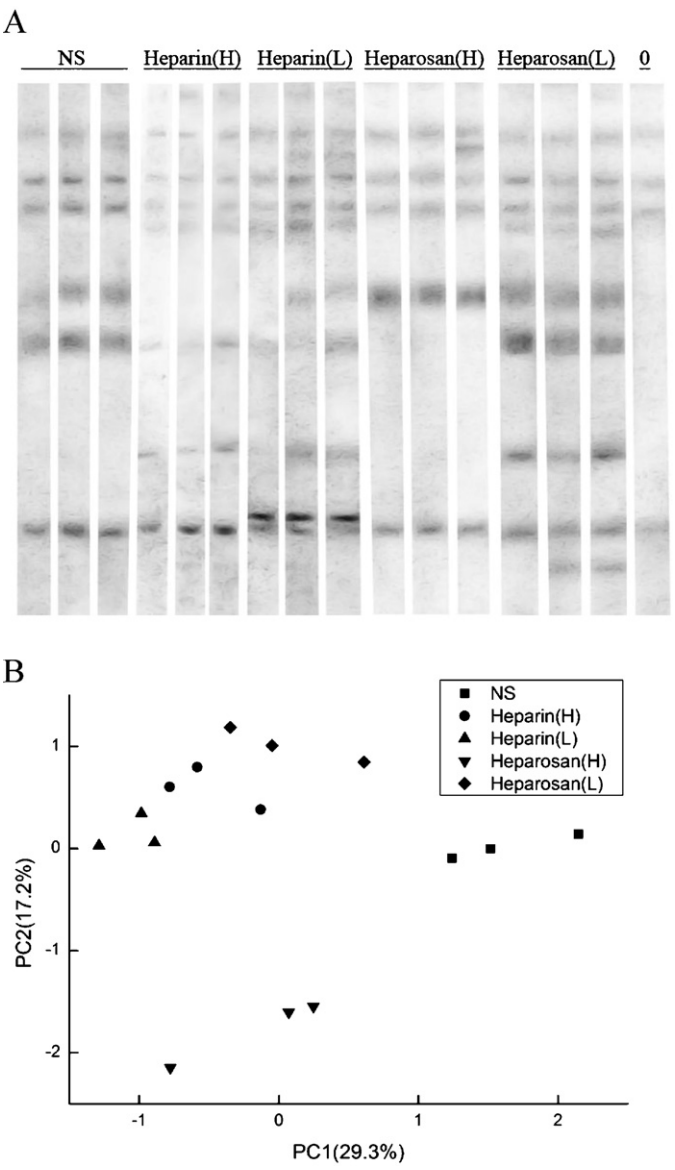

Fig. 3. Community structures assessed by DGGE analysis of *Bacteroides* subpopulation from 14th day fecal samples, (A) DGGE banding profiles of the five groups (three replicates each). NS, the natural saline group; heparin(H), the heparin-high dose group (10 mg/kg); heparin(L), the heparin-low dose group (5 mg/kg); heparosan(H), the heparosan-high dose group (10 mg/kg); heparosan(L), the heparosan-low dose group (5 mg/kg); lane 0, a sample before the treatment, included in each run as a DGGE marker. (B) PCA score plot based on the DGGE profile. Numbers in parentheses are percentages of variation explained by each principle component.

the heparosan groups from the saline group. Different patterns of *Bacteroides* species loss had been found to be associated with UC and IBS (Noor et al., 2010). Whether the change of the composition of *Bacteroides* pattern is benefit to the treatment of UC and IBS warrants further study.

Amplicons of the *Clostridium* subpopulation with primers Clept-F and Clept-GC-R3 were 279 bp. The DGGE banding patterns and PCA analysis are shown in Fig. 4. The biodiversity of the *Clostridium* subpopulation was relatively low and there were no significant difference among five groups.

In previous trials on heparin against UC *in vivo*, heparin was administered intravenously and contradictory results were reported (Head & Jurenka, 2003). In pilot studies, patients with ulcerative colitis experienced improvement when treated with heparin but a multicenter, randomized trial produced

Table 2
Sequencing results of different bands from DGGE analysis of the predominant bacterial communities and *Lactobacillus* communities.

DGGE	Band No.[a]	Genbank accession No.	Most related bacteria (identity[b])
V3-region (Fig. 1A)	1	–	*Lactobacillus taiwanensis* (99%)
	2	–	*Lactobacillus taiwanensis* (99%)
	3	–	*Lactobacillus taiwanensis* (99%)
	4	–	*Lactobacillus taiwanensis* (99%)
	5	–	*Lactobacillus gallinarum* (100%)
	6	–	*Lactobacillus gallinarum* (100%)
	7	–	*Lactobacillus gallinarum* (100%)
	8	–	*Lactobacillus gallinarum* (100%)
	9	–	*Enterococcus faecium* (99%)
	10	–	*Enterococcus faecium* (99%)
Lactobacillus specific (Fig. 2A)	1	KC166284	*Lactobacillus gallinarum* (99%)
	2	KC166285	*Lactobacillus gallinarum* (100%)
	3	–	*Lactobacillus gallinarum* (100%)
	4	–	*Lactobacillus gallinarum* (100%)
	5	KC166286	*Lactobacillus antri* (100%)
	6	KC166287	*Lactobacillus taiwanensis* (99%)
	7	–	*Lactobacillus taiwanensis* (99%)
	8	–	*Lactobacillus taiwanensis* (99%)

[a] Band No. was annotated as in the corresponding DGGE figures, respectively.
[b] Identity represents the percentage identity shared with sequences in the Genbank database. In the *Lactobacillus* specific PCR-DGGE, sequences of band Nos. 2–4 and 6–8 are identical respectively.

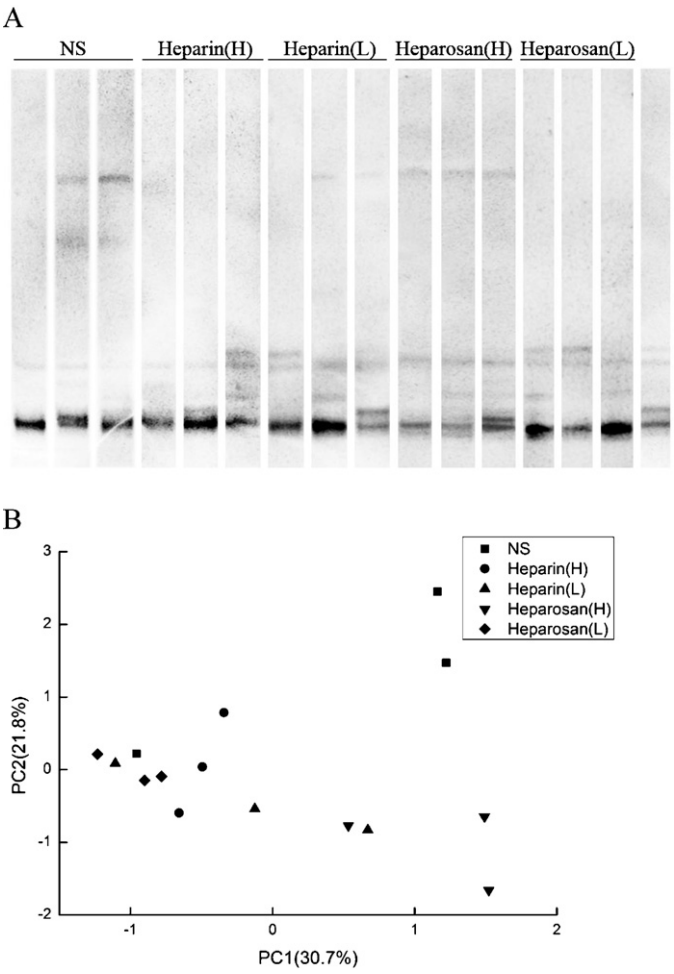

Fig. 4. Community structures assessed by DGGE analysis of *Clostridium* subpopulation from 14th day fecal samples, (A) DGGE banding profiles of the five groups (three replicates each). NS, the natural saline group; heparin(H), the heparin-high dose group (10 mg/kg); heparin(L), the heparin-low dose group (5 mg/kg); heparosan(H), the heparosan-high dose group (10 mg/kg); heparosan(L), the heparosan-low dose group (5 mg/kg); lane 0, a sample before the treatment, included in each run as a DGGE marker. (B) PCA score plot based on the DGGE profile. Numbers in parentheses are percentages of variation explained by each principle component.

poor results, together with severe rectal bleeding (Head & Jurenka, 2003). In this case, the anticoagulation effect of heparin might compromise its therapeutic effect. In our study, no rectal bleeding was observed in any group, which might due to the benefit of oral administration route. Furthermore, heparosan, without anticoagulation effects, showed similar effects on the gut microbiota, which could be expected to perform well in a trial of UC therapy. In future clinical trials on heparin or heparosan against UC, more information about the gut microbiota should be collected in order to understand their pharmacology.

In conclusion, orally administered heparin or heparosan could act as an effective gut microbiota modulator by facilitating colonization of *Lactobacillus* and this modulation was helpful against infections and inflammatory bowel diseases. The results also showed that the modulation effect of heparosan on the gut microbiota was similar to that of heparin. Considering that heparosan has no risk of bleeding and can be obtained cost-effectively by bacterial fermentation (Wang et al., 2010), heparosan or heparosan-derived polysaccharide might show more advantage over heparin as novel drug to modulate the gut microbiota.

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