Journal of Microbiology (2015) Vol. 53, No. 6, pp. 398–405
DOI 10.1007/s12275-015-5046-z
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Effects of *Lactobacillus salivarius* Ren on cancer prevention and intestinal microbiota in 1, 2-dimethylhydrazine-induced rat model

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(Received Jan 23, 2015 / Revised May 11, 2015 / Accepted May 11, 2015)

Probiotics have been suggested as a prophylactic measure in colon cancer. The aim of this study was to investigate the impact of *Lactobacillus salivarius* Ren (Ren) in modulating colonic microbiota structure and colon cancer incidence in a rat model after injection with 1,2-dimethyl hydrazine (DMH). The results indicated that oral administration of Ren could effectively suppress DMH-induced colonic carcinogenesis. A significant decrease in cancer incidence (87.5% to 25%) was detected in rats fed with a dose of 5×10^{10} CFU/kg bodyweight per day. Using denaturing gradient gel electrophoresis and Real-time PCR combined with multivariate statistical methods, we demonstrated that injection with DMH significantly altered the rat gut microbiota, while Ren counteracted these DMH-induced adverse effects and promoted reversion of the gut microbiota close to the healthy state. T-value biplots followed by band sequencing identified 21 bacterial strains as critical variables affected by DMH and Ren. Injection of DMH significantly increased the amount of *Ruminococcus* species (sp.) and *Clostridiales* bacteria, as well as decreasing the *Prevotella* sp. Administration of Ren reduced the amount of *Ruminococcus* sp., *Clostridiales* bacteria, and *Bacteroides dorei*, and increased the amount of *Prevotella*. Real-time PCR results were consistent with the results derived by t-value biplots. These findings suggested that Ren is a potential agent for colon cancer prevention. In conclusion, the results in the present study suggest a potential therapeutic approach based on the modulation of intestinal microflora by probiotics may be beneficial in the prevention of colorectal carcinogenesis.

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Keywords: colorectal cancer, microbiota, *Lactobacillus salivarius* Ren, DMH

Introduction

The human and animal gastrointestinal tract is colonized by complex and diverse microbials (Camp *et al.*, 2009), which significantly impact upon the physiology and pathology of the host (Nicholson *et al.*, 2005). A range of diseases, for instance, inflammatory bowel disease, colon cancer, and multi-system organ failure, may be associated with abnormalities or changes in the composition of intestinal microbiota (Thompson-Chagoyan *et al.*, 2007). Among these diseases, colorectal cancer (CRC) is the third most common cause of cancer mortality worldwide (Jemal *et al.*, 2011), and imbalances in the intestinal microbiota could play an important role in the initiation and development of CRC (Guarner and Malagelada, 2003). CRC animal models have indicated that microbiota is involved in the etiology of carcinogenesis (Dove *et al.*, 1997; Kado *et al.*, 2001; Uronis *et al.*, 2009). Based on these reports, to better understanding the relationship between gut microbiota and CRC, and to identify a novel tool for early diagnosis and prevention, dynamic monitoring of the changes in gut microbiota with the development of the disease is essential.

The use of probiotics is increasing in popularity for the prevention and treatment of intestinal infection and disease (Geier *et al.*, 2006). Animal models and human clinical trials indicate that probiotics may reduce intestinal inflammation and alleviate symptoms of CRC (Arthur *et al.*, 2013). However, the beneficial effects of interventional treatment remain obscure. Several mechanisms could explain the preventive action of probiotics against CRC onset. The majority of probiotic research has centered around the effects on the host, with less emphasis placed on exploring the impact of probiotics on gut microbiota. Putrefactive intestinal microbiota such as *Bacteroides* species (sp.) and *Clostridium* sp. have been implicated in the pathogenesis of CRC (Sobhani *et al.*, 2011), while some lactic acid bacteria have been shown to possess cancer-preventing attributes (Kumar *et al.*, 2010).

In our previous studies, *Lactobacillus salivarius* Ren (Ren), isolated from Bama centenarians (one of the five well-known regions in China for population longevity), was proven to counteract unfavorable 4-nitroquinoline 1-oxide-induced changes in the colonic microbiota of rats (Zhang *et al.*, 2011) and to effectively prevent oral cancer (Zhang *et al.*, 2013). We also found that Ren may significantly improve the colonic microflora structures and therefore prevent early colorectal carcinogenesis, as measured by the presence of aber-

rant crypt foci (ACF) in a 1,2-dimethyl hydrazine (DMH)-induced rat model (Zhu *et al.*, 2014). Whether such an effect operates in colorectal tumor development remains to be shown. In this study, we used this model to study the role of Ren in colorectal tumor incidence and assessed its modulating effect on colonic microbiota.

Materials and Methods

Animals and experimental design

Five-week-old male F344 rats were purchased from Vital River Lab Animal Technology Co. Ltd. and housed in an air-conditioned room controlled for temperature (21 ± 2°C), humidity (50 ± 10%), and a 12/12-h light-dark cycle for 1 week of quarantine. All rats were fed with a certified standard rat chow (Vital River Co.) and allowed unlimited access to deionized water.

After the acclimatization, a total of 24 rats were randomly divided into three groups as shown in Fig. 1. From week 2, rats in groups 2 and 3 were administered a subcutaneous injection with a DMH solution at 30 mg/kg bodyweight for 10 weeks. The DMH solution was prepared in 1 mM EDTA/0.85% (m/v) NaCl carrier (pH 6.5) and group 1 received a subcutaneous injection with EDTA/NaCl carrier of the same volume. Group 3 was administered Ren at a dose of 5×10^{10} CFU/kg bodyweight once per day from the beginning to the end of the study, and groups 1 and 2 were administered sterile normal saline. All rats were fed with the basal diet and deionized water.

Histological examination

At the end of the experiment, all rats were euthanized by cervical dislocation. After laparotomy, the colon was excised from the entire intestinal tract. For histological examination, tissue and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks, and the resulting cut histological sections were stained with hematoxylin and eosin. The diagnoses were randomized and single-blinded.

DNA extraction, PCR amplification, and PCR-denaturing gradient gel electrophoresis (DGGE) analysis

Approximately 40 mg feces were used for DNA extraction. Fresh feces were collected from each rat at 0, 16, and 32 weeks and immediately submersed in 2.0 ml RNA later. Bacterial genomic DNA was extracted from each fecal sample according to the beat-beating method (Matsuki *et al.*, 2004). Gels were stained with GelGreen (White *et al.*, 2004) and photographed with a CanoScan Lide 100 Scanner (Canon). To determine the trend of the microbial community present, Quantity One software (version 4.4; BioRad) was used to analyze the intensity of each band, calculated as a percentage of the total intensity of all the bands in each lane. Then, the data were exported to the SPSS 20.0 program. The relative intensity values for the different types of band were analyzed by hierarchical cluster analysis using the furthest neighbor method. The distances between samples were calculated using Pearson correlation (Torres *et al.*, 2006) and visualized graphically as dendrograms by the SPSS 20.0 program.

Multivariate statistical analysis

Multivariate statistical analysis was employed to assess the influence of DMH and Ren on gut microbiota via Canoco 4.5 (Biometrics). The form was imported with band type represented as species data and DMH and Ren considered as environmental variables. The linear model of redundancy analysis (RDA), with the focus scaling on interspecies distances employed as the longest gradient resulting from detrended correspondence analysis, was 1.623 (Lepš and Šmilauer, 2003; Janczyk *et al.*, 2010). Unrestricted Monte Carlo permutation tests were applied to test for significance of the microbiota response to the environmental variables (499 random permutations, $P < 0.05$). To investigate which bacteria were significantly altered with DMH and Ren treatment, t-value biplots were graphed for each variable based on RDA using CanoDraw (a module of Canoco 4.5). Species fit ranges were set according to the variability of species data explained by the first RDA axis. Species vectors (band types) in Van Dobben circles indicated the significance of their relationships with Ren or DMH (regression coefficient < −2 or > +2). Bands in the red circles indicated that the abundance of bands was positively correlated with an environmental variable, while the blue circles represent the negative correlation (Lepš and Šmilauer, 2003). The bands significantly affected by DMH and Ren treatment were marked on the DGGE gel images for further identification.

Excision and sequencing of selected bands from DGGE gels

DGGE bands related to DMH and Ren were sliced with a sterile scalpel under blue light illumination and DNA was extracted according to a procedure described previously (Sanguinetti *et al.*, 1994). The DNA was reapplied with 341F (5′-CCTACGGGAGGCAGCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) primers according to the following program: 3 min at 94°C, 30 cycles consisting of 15

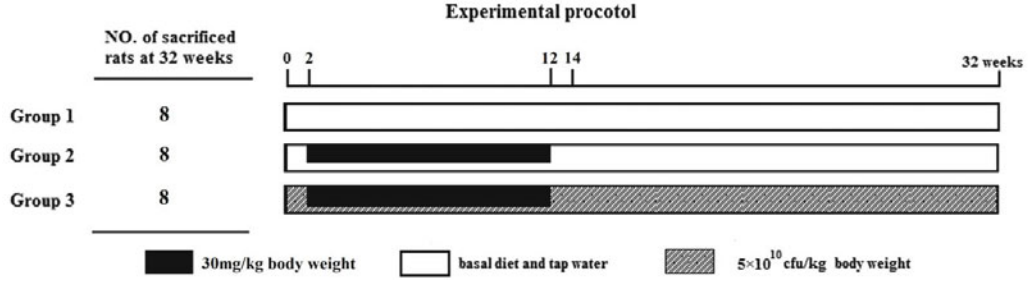

Fig. 1. Experimental protocol.

Table 1. Specific primers for RT-PCR

Target groups or organism	Amplicon size (bp)	Annealing temperature (°C)	Primer sequence (5′→3′)
All bacteria	466	58	F:TCCTACGGGAGGCAGCAGT R:GGACTACCAGGGTATCTAATCCTGTT
Bacteroides dorei	156	55	F:CACGGAGTTAGCCGATCCTT R:TGAGGAATATTGGTCAATGG
Ruminococcus sp.	94	58	F:CGAAAGCCTGATGCAGCGAC R:CTTAGTCAGGTACCGTCATT
Clostridiales bacterium	138	55	F:CACGTAGTTAGCCGTGGCTTATTC R:TGGGGAATATTGGGCAATGG
Prevotella sp.	112	55	F:CAACTCTGAACCAGCCAAGTAG R:ATTCATGCGGTACCTGCAAT

sec at 94°C, 20 sec at 55°C, and 20 sec at 72°C, and finally 5 min at 72°C. The PCR products were purified with the Universal DNA Purification Kit (Tiangen), ligated into the pMD18-T Simple Vector (TaKaRa), and cloned into *Escherichia coli* TOP10 competent cells (Tiangen). Plasmid DNA was isolated from the *E. coli* cells using the TIANprep Mini Plasmid Kit (Tiangen), and reamplified (with 341F-GC and 534R primers) as described above. The PCR product was screened by DGGE to verify the purity and the migration position of the excised band (Tannock *et al.*, 2004; Licht *et al.*, 2006). The plasmids with desirable inserts were sequenced (Invitrogen Biotech Co. Ltd) using the M13F primer. The obtained sequences were compared with known sequences in the NCBI database using the BLASTn algorithm.

Real-time (RT) quantitative PCR

The bacterial groups targeted for PCR and the specific primers are listed in Table 1. The primers were designed based on fragment sequences using Vector NTI Express software and synthesized by Invitrogen. All PCR reactions were performed in 50 µl reaction solutions including SYBR Green PCR Master Mix (TaKaRa), DNA templates (2 µl), primer (10 µM), and 509 ROX dye (0.4 µl, TaKaRa). Reactions were performed using an ABI PRISM 7000 Sequence Detection System device (Applied Biosystems), using the following PCR program: 5 min at 95°C, 45 cycles of 15 sec at 95°C, 30 sec at annealing temperature, 30 sec at 72°C, and 5 sec at 80°C for the signal collection. After PCR amplification, the dissociation curve program was run to provide values for specific product testing. Unless otherwise stated, each sample had three replicates.

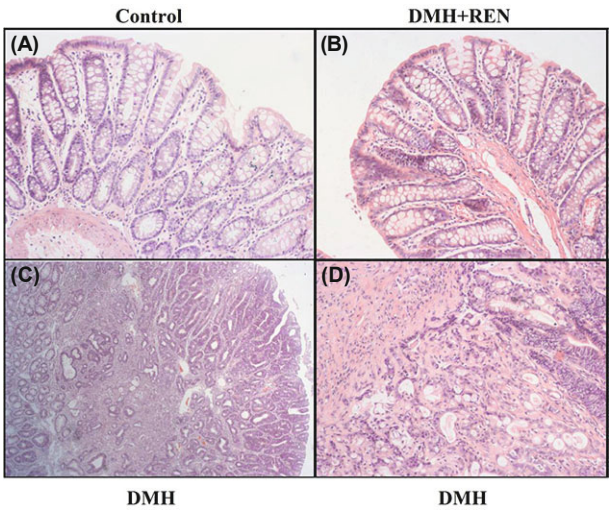

Fig. 2. Representative photomicrographs of histological (hematoxylin and eosin) cross-sections of colons from the control, Ren+DMH, and DMH groups. (A) Control, normal colorectal tissues; (B) Ren+DMH, normal colorectal tissues; (C) DMH, colorectal adenoma; (D) DMH, colorectal adenocarcinoma.

Standard curves

A 10-fold dilution series of reference bacteria genomic DNA fragments (ranging between 10^5 and 10^6 to 10^{11} and 10^{12} target genome copies) were prepared to construct the standard curves in corresponding RT-PCR groups. Quantification was performed by interpolation using a standard regression curve of C_t values generated from DNA samples of known genome copies. The C_t values were determined for the unknown samples and compared with the standard curves that were constructed by reference bacteria genome copies of the C_t.

Statistical analysis

Statistical analysis of the incidence of lesions was performed using Fisher's exact probability test. Average genome copies of bacterial groups per gram of each fecal sample (wet weight) were transformed into logarithms to achieve normally distributed data (assessed by the Shapiro-Wilk test), and the mean ± SD of each bacteria genome copy was calculated. The percentage of bacterial group and species genome copies in relation to total bacteria genome copies (relative abundance) was calculated for each individual to determine the proportion of each selected bacteria in feces, and the mean ± SD was determined to assess the relative abundance across the groups. The effects of Ren/DMH on the fecal bacterial genome copies and relative abundance were calculated using analysis of covariance in SPSS 20.0 ($P < 0.05$).

Table 2. Incidence of colorectal tumors in rats after DMH exposure

Group No.	Treatment	No. of rats	Tumor incidence (%)
1	Control	8	0[b]
2	DMH	8	87.5[a]
3	DMH+REN	8	25.0[b]

[a] Significantly different from group 1 by Fisher's exact probability test ($P < 0.05$).
[b] Significantly different from group 2 by Fisher's exact probability test ($P < 0.05$).

Results

General observations and histological examination

All rats survived the entire experiment. Intake of DMH, water, and food was similar among the three groups. Administration of Ren did not affect survival rate or lead to histological changes; indicating that the rats were tolerant of Ren administration. Figure 2 shows the typical histological classifications of tumors in the colorectum. No incidence of neoplasm was observed in group 1. Seven out of eight rats in the DMH-treated group (group 1) were histologically diagnosed with either colorectal adenoma or adenocarcinoma, while a significant decrease (87.5% to 25.0%) was observed in group 3, which was treated with DMH and Ren ($P < 0.05$) (Table 2). These findings suggested that Ren was a potential agent for colon cancer prevention.

Shifts in fecal microbiota

In this study, profiles of bacterial 16S rRNA genes of the fecal samples from 24 rats were subjected to PCR-DGGE analysis to capture the structural shifts of the fecal microbiota community. As shown in Fig. 3A, a total of 60 band types were recognized by Quantity One software and some differences were detected between the groups in the patterns of the bands. To gain insights into the variability of fecal microbiota communities, a dendrogram was obtained based on DGGE banding patterns using the relative intensity of each band type as an indicator of abundance. As shown in Fig. 3B, all subjects were divided into two clusters (A and B). All the subjects from group 1 (DMH) were grouped into cluster A. Cluster B was comprised of two sub-clusters, B1 and B2, which consisted of all the subjects from group 2 (4-NQO + REN) and group 3 (Control), respectively. These results suggest that group 2 was most similar to the control group. These results indicated that each group had a unique fecal microfloral structure with mild variation among the groups.

Effects of DMH and Ren on fecal microbiota

The relationship between the fecal microbiota and environmental variables was determined by the species-environment correlations and Monte Carlo permutation tests in the RDA. The results showed that species-environment correlations for axes 1 and 2 were 0.943 and 0.934, respectively, indicating a high relevance between microbiota and environmental variables (DMH and Ren). Followed by the Monte Carlo permutation tests ($P = 0.007$), significant effects of DMH and Ren on microbiota were confirmed. When DMH or Ren was used as an environmental variable separately (the other one being used as a covariable) in RDA, significant effects on microbiota were further verified ($P = 0.016$ and $P = 0.002$ respectively, Monte Carlo permutation tests). This indicated that both DMH and Ren were correlated with the fecal microbiota in rats.

Critical variable bands affected by DMH and Ren

A t-value biplot based on the PCR-DGGE fingerprint identified 21 bands as significant variables correlated with Ren and DMH (Fig. 4). The microbial identification of the 21 bands is summarized in Table 3.

DMH treatment increased the abundance of one *Ruminococcus* sp. strain (B23), one *Clostridiales bacterium* strain

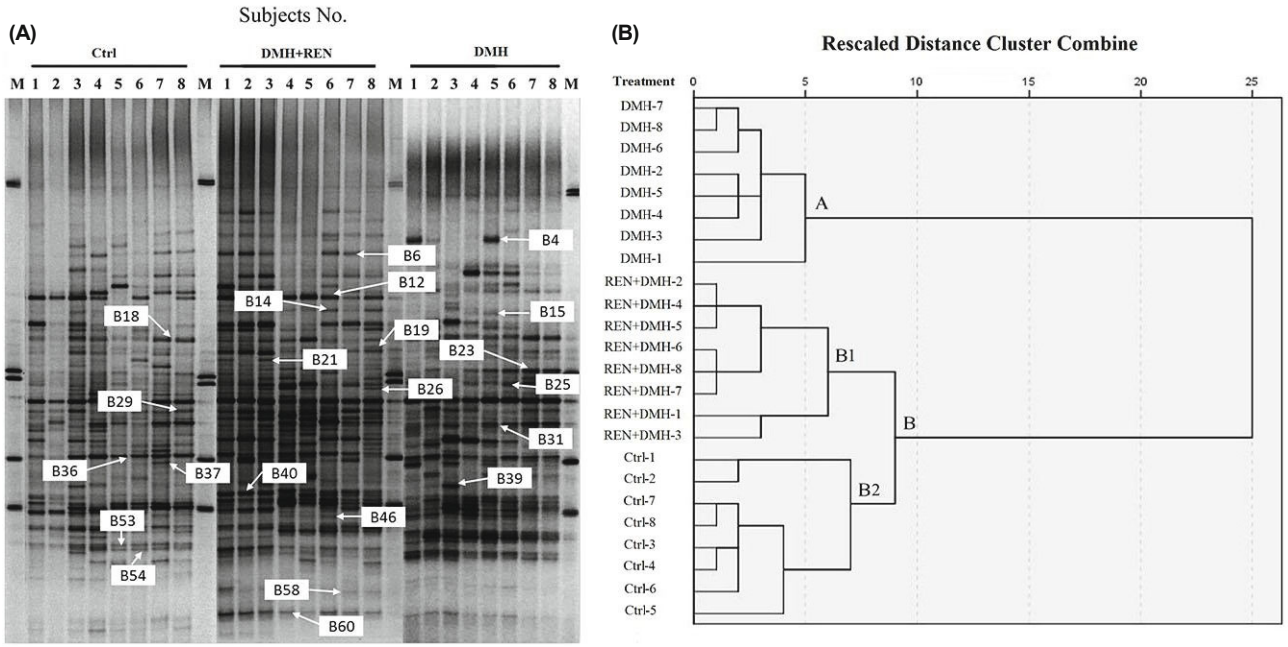

Fig. 3. Colonic microfloral communities. (A) DGGE fingerprints. Bands determined to have been significantly affected by DMH and Ren treatment were marked as "B" followed by the band type number. M, marker. (B) Dendrogram derived from DGGE analysis of colonic intestinal microbiota of rats from different treatment groups based on clustering analysis.

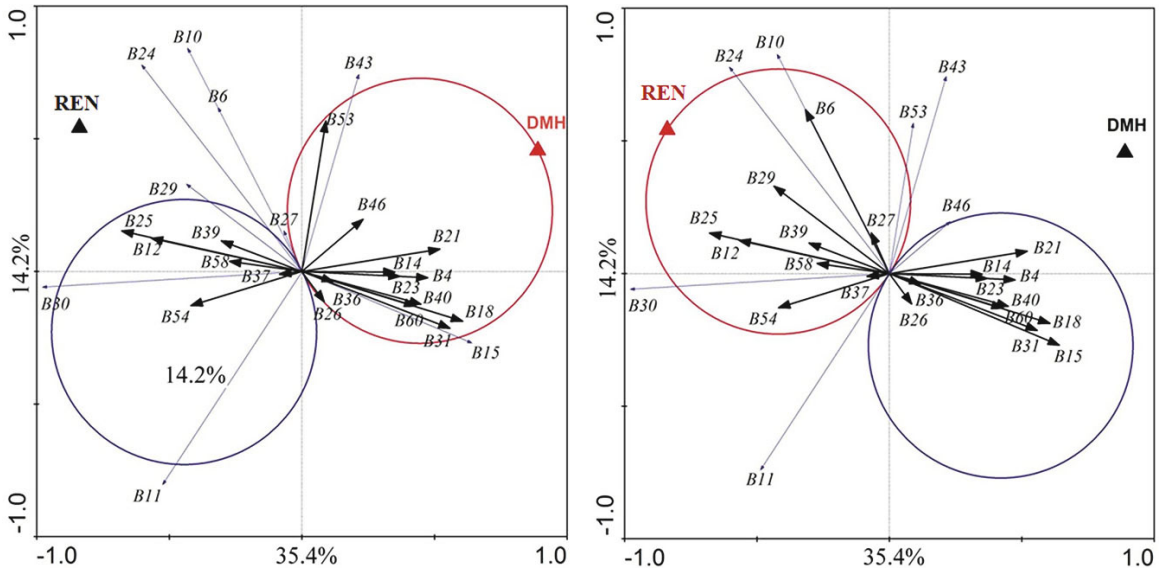

Fig. 4. T-value biplot for DMH and Ren obtained from CanoDraw. Red and blue circles indicate Van Dobben circles. The red circles are positive correlation circles while the blue ones are negative correlation circles.

(B40), and 10 uncultured bacterial strains (B4, B14, B18, B21, B26, B31, B36, B46, B53, and B60). Inversely, the abundance of one *Prevotella* sp. strain (B58) and five other uncultured strains (B12, B25, B37, B39, and B54) were reduced.

After oral administration of Ren, the abundance of one *Prevotella* sp. strain (B58) and eight uncultured bacterial strains (B6, B12, B25, B27, B29, B37, B39, and B54) were increased. Moreover, the abundance of one *Bacteroides dorei* (B15) strain, one *Ruminococcus* sp. strain (B23), one *Clostridiales bacterium* strain (B40), and eight uncultured strains (B4, B14, B18, B21, B26, B31, B36, and B60) were decreased.

Shifts of critical variable strains during tumorigenesis

ACF was detected in DMH-treated animals after 16 weeks in our previous study (Zhu *et al.*, 2014). Some strains such as *Bacteroides* sp. and *Clostridium* sp. were identified as the critical variables for discrimination after DMH injection.

Table 3. Summary of band identification and significant colonic microbiota associations with DMH and Ren

Band NO.	Closest relative	DMH	REN	Identity (%)	Accession No.
B15	*Bacteroides dorei*		-	100	HE974919.1
B23	*Ruminococcus* sp.	+	-	100	AY960569.1
B40	*Clostridiales bacterium* CIEAF 026	+	-	99	AB702939.1
B58	*Prevotella* sp.	-	+	97	DQ278861.1
B4	Uncultured bacterium clone R-9830	+	-	97	FJ881347.1
B6	Uncultured Bacteroidales bacterium clone Human431		+	100	HQ201909.1
B12	Uncultured bacterium cloneTLR1KO1.3C5_8F	-	+	99	JF912733.1
B14	Uncultured bacterium clone HRX_F23	+	-	97	EU465223.1
B18	Uncultured bacterium clone p-195-o5	+	-	100	DQ794665.1
B21	Uncultured bacterium clone DE05986F09	+	-	96	JQ694917.1
B25	Uncultured bacterium clone DE06446A11	-	+	100	JQ892741.1
B27	Uncultured bacterium clone LFDE2578FH05		+	100	JQ894297.1
B29	Uncultured bacterium clone RMAM2303		+	98	HQ321201.1
B31	Uncultured bacterium cloneTLR1KO1.3C5_8F	+	-	99	JF912733.1
B36	Uncultured Firmicutes bacterium clone TF3-8	+	-	99	GU958841.1
B37	Uncultured bacteria partial	-	+	99	AM265446.1
B39	Uncultured Firmicutes bacterium clone TCM2-39	-	+	97	GU959150.1
B46	Uncultured bacterium clone KO2_aai18c04	+		98	EU776381.1
B53	Uncultured bacterium clone T1WK15C3	+		99	HQ716145.1
B54	Uncultured bacterium clone DE06456A05	-	+	97	JQ695651.1
B60	Uncultured bacterium clone RMAM0064	+	-	98	HQ319003.1

"+" indicates an increase in the abundance of a strain in response to an environmental variable that could be detected on the t-value biplot in this study.
"-" indicates a decrease in the abundance of a strain in response to an environmental variable that could be detected on the t-value biplot in this study.

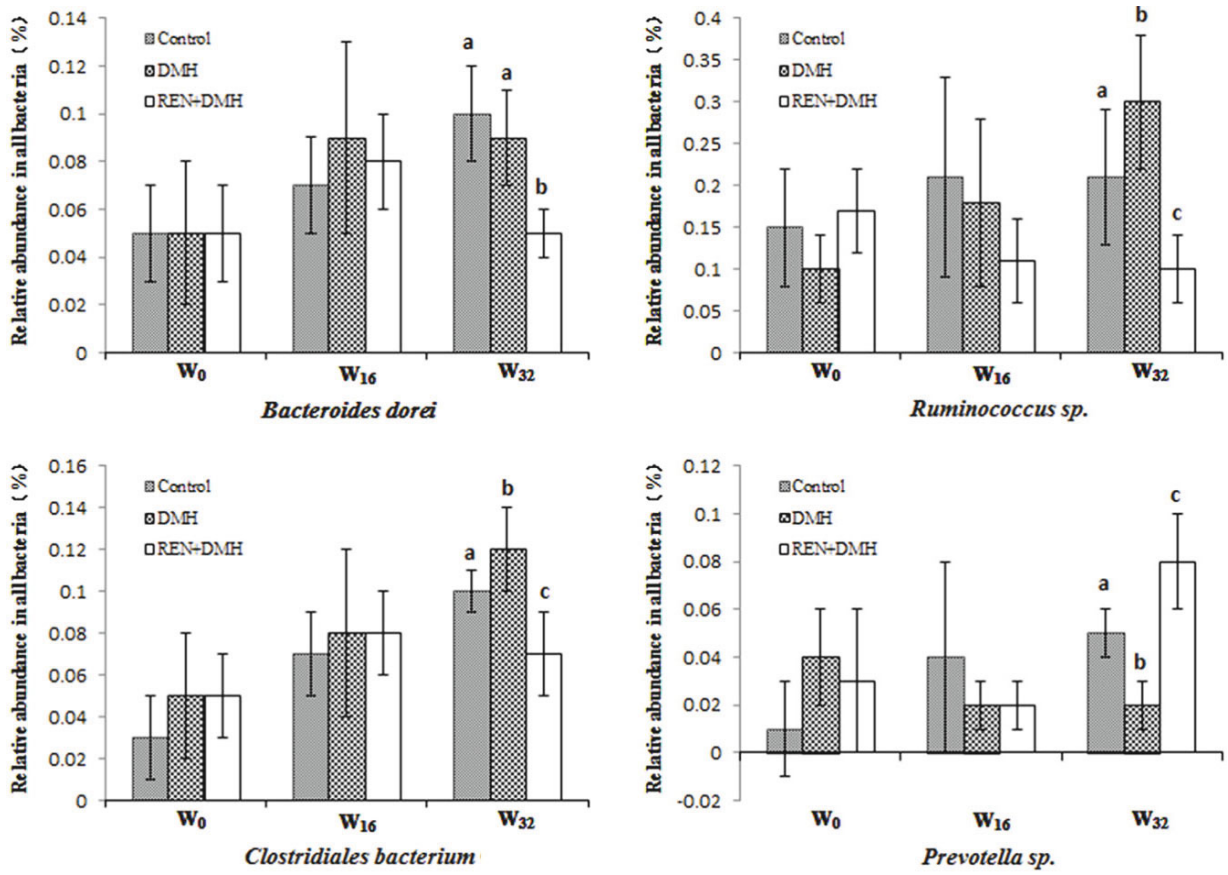

Fig. 5. Relative abundance of *Bacteroides dorei*, *Ruminococcus* sp., *Clostridiales bacterium*, and *Prevotella* sp. in all bacteria (%) at 0, 16, and 32 weeks (W_0, W_{16}, W_{32}) by RT-PCR. Values are shown as means ± SD (error bars). Values with a, b, c show statistical significance compared with the others ($P < 0.05$).

In this study, RT-PCR targeting *Bacteroides dorei*, *Ruminococcus* sp., *Clostridiales*, and *Prevotella* sp. at 0, 16, and 32 weeks (W_0, W_{16}, W_{32}) was performed, according to the *t*-value biplot.

All the standard curves of the four RT-PCR assays showed good linearity. No primer dimers with lower T_m values were observed. The relative abundance of four strains of bacteria is summarized in Fig. 5. No significant difference was observed in the relative abundance of the four strains at W_{16}. However, at W_{32}, the abundance of *Bacteroides dorei* was significantly lower in the Ren+DMH group (0.05 ± 0.01%) compared with the DMH group (0.09 ± 0.02%) and control group (0.10 ± 0.02%), with no significant difference between the DMH and control groups. The abundance of *Ruminococcus* sp. and *Clostridiales* bacteria was significantly increased after DMH treatment (0.30 ± 0.04% and 0.12 ± 0.02%, respectively) compared with the control group (0.21 ± 0.08% and 0.10 ± 0.01%, respectively), and was significantly decreased after administration of Ren (0.10 ± 0.02%). The amount of *Prevotella* sp. was significantly lower in the DMH group (0.02 ± 0.01%), while levels of the Ren+DMH group were increased (0.08 ± 0.02%). These RT-PCR results were consistent with the results derived by the *t*-value biplot.

Discussion

In our previous study, we demonstrated that Ren could modulate colonic microflora and therefore prevent early colorectal carcinogenesis in a DMH-induced rat model (Zhu *et al.*, 2014). However, it was unclear if such an effect is apparent in CRC development. In this study, we demonstrated that Ren could effectively suppress DMH-induced colon carcinogenesis. Using PCR-DGGE combined with multivariate statistical analysis, we demonstrated that Ren could counteract the unfavorable changes in the colonic microfloral communities of rats significantly altered by the injection of DMH. Based on *t*-value biplots, a total of 21 bands (bacterial strains) were identified as being significantly correlated with DMH or Ren treatment.

Probiotics are a group of health beneficial strains that have been used for centuries in human history worldwide. Recent studies suggested that probiotics may represent an emerging option for cancer prevention and treatment (Greer and O'Keefe, 2011). In our previous study, there were significant differences in the final ACF formation between the DMH-treated group and the Ren+DMH group (Zhu *et al.*, 2014). However, the suppression of ACF formation could not be fully equated with the antitumor effect. In our study, a significant decrease (87.5% to 25.0%) was observed in the group treated with DMH and Ren ($P < 0.05$). These findings sug-

gested that Ren was a potential agent for CRC prevention.

Some general mechanisms have been proposed for the antitumor effect, such as antimicrobial effects against carcinogen-producing microorganisms, anti-genotoxic activities against internal and external carcinogens, and activation of the gut mucosal immune system. However, alterations in the composition of the gut microbiota are now regarded as a driving force for the development of CRC (Arthur *et al.*, 2012). Piazzi *et al.* (2014) reported that the progression of colon cancer changed the microbiota structure that was dominated by Bacteroidetes, and induced the appearance of *Akkermansia* sp., a mucin-degrading species. Wei *et al.* (2010) also demonstrated that the gut microbiota structure of treated animals was significantly different 7 weeks after DMH injection. Accordingly, colon cancer initiates and progresses in an altered intestinal environment, including changes in gut microbiota and concentrations of short-chain fatty acids (Ohigashi *et al.*, 2013). Unlike DMH, Ren played a positive role on the gut microbiota in this study and counteracted the negative effect induced by DMH, which was directly proved by the lower tumor incidence of the Ren+ DMH group than the DMH group. Meanwhile, a dendrogram showed significant similarity between the Ren+DMH group and the control group, indicating that Ren could prevent CRC via modulation of the gut microbiota. A similar effect of Ren was found against 4-NQO, owing to the de-genotoxifying ability of Ren (Zhang *et al.*, 2011; 2013).

Specific strains of bacteria may be implicated in the risk of CRC. Putrefactive intestinal microbiota such as *Bacteroides* sp. and *Clostridium* sp. have been implicated in the pathogenesis of CRC (Sobhani *et al.*, 2011) while numerous lactic acid bacterias have been shown to possess cancer-preventing attributes (Kumar *et al.*, 2010). Wei *et al.* (2010) also proved that Ruminococcus-like and Allobaculum-like bacteria were identified as crucial variables for discrimination of DMH-treated rats from controls. In this study, *Bacteroides dorei*, *Ruminococcus* sp., *Clostridiales bacterium*, and *Prevotella* sp. were significantly changed by DMH or Ren, and may be involved in the development of CRC. Clostridial species belonging to 7α-dehydroxylating bacteria can convert primary bile acids to secondary bile acids, namely deoxycholic acid and lithocholic acid, which are well recognized co-carcinogens (Nagengast *et al.*, 1995). In this study, DMH increased the amount of one *Clostridiales* strain, and a similar result was obtained in a study that revealed bacteria of the clostridium genera increase the incidence and growth rate of colonic tumors induced in animals (Onoue *et al.*, 1997). DGGE results also demonstrated that *Ruminococcus*-related bacteria were more abundant in DMH-treated rats, which was further confirmed by RT-PCR. Studies have found similar results that indicated frequent isolation of a *Ruminococcus* sp. from high-risk populations with CRC (Moore and Moore, 1995) or rats with precancerous lesions (Wei *et al.*, 2010). Furthermore, Hamer *et al.* (2008) suggested that the *Ruminococcaceae* strain and Rumen bacteria may play an important role in the inhibition of carcinogenesis and oxidative stress because they produce butyrate. However, the effects of butyrate are still under debate. Thus, the specific mechanisms remain unclear. Changes in *Prevotella* have been reported mainly in oral and gastric cavities (Dicksved *et al.*, 2009), with few links to colon tumors identified. However, in this study, a decrease in *Prevotella* was found in the DMH group. Thus far, the association between *Prevotella* and colon cancer is unknown.

All these results suggest Ren is one of the most effective potential probiotics for preventing CRC. This study may have some practical value in the prevention of CRC. First, as the changes in gut microbiota influenced by DMH may ultimately lead to the development of CRC, modulation of the gut microbiota could possibly be one way to prevent CRC. In fact, this study demonstrated that Ren can effectively reduce the risk of developing CRC. Second, because the gut microbiota was responsible for the CRC, monitoring of the structural segregation of gut microbiota in healthy individuals could become a useful parameter for identifying groups at high risk of CRC.

Acknowledgements

This work was supported by Beijing Natural Science Foundation (6154022), Ministry of Science and Technology of China (2012BAD12B08), Beijing Municipal Commission of Education Co-constructed Program.

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