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Lactobacillus salivarius REN Counteracted Unfavorable 4-Nitroquinoline-1-Oxide-Induced Changes in Colonic Microflora of Rats

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Probiotics and carcinogens both have a significant effect on the microfloral composition of the human intestine. The objective of this study was to investigate the impact of an important carcinogen, 4-Nitroquinoline-1-Oxide on colonic microflora and the efficacy of the probiotic *Lactobacillus salivarius* REN as an agent of counteracting these effects. Using denaturing gradient gel electrophoresis (DGGE) combined with redundancy analysis, we demonstrated that both 4-Nitroquinoline-1-Oxide and *L. salivarius* REN significantly altered the bacterial communities of rat colons. A total of 27 bacterial strains were identified as being affected by treatment with 4-Nitroquinoline-1-Oxide or *L. salivarius* REN using a t-value biplot combined with band sequencing. 4-Nitroquinoline-1-Oxide treatment increased the abundance of two potential pathogens (one *Helicobacter* strain and one *Desulfovibrio* strain), as well as reducing the abundance of two potentially beneficial strains (one *Ruminococcaceae* strain and one Rumen bacteria). The *Helicobacter* strain was initally detected in carcinogen-treated rat intestinal microflora, but *L. salivarius* REN treatment effectively suppressed the growth of the *Helicobacter* strain. These results suggested that *L. salivarius* REN may be a potential probiotic, efficiently acting against the initial infection with, and the growth of pathogenic bacteria.

Keywords: colonic microflora, 4-nitroquinoline-1-oxide, *Lactobacillus salivarius* REN, DGGE

The human gastrointestinal tract harbors a complex microbial community that has a significant impact on the host physiology and pathology (Nicholson *et al*., 2005). Many chronic diseases, such as inflammatory bowel disease, obesity and colon cancer can be related with disorders of the intestinal microflora (Thompson-Chagoyan *et al*., 2007). There is growing evidence that probiotics are effective in the prevention and treatment of intestinal disorders (Gareau *et al*., 2010).

Carcinogens in food and the environment are an important etiological factor in human gastrointestinal tract disease (Ramesh *et al.*, 2004; Turesky, 2004). However, studies of the impact of carcinogens on intestinal microflora are limited. Maciorowski *et al.* (1997) suggested that azoxymethane, a gastrointestinal carcinogen, could alter fecal microbial populations. Treatment with another carcinogen, 1, 2-dimethyl hydrazine (DMH) significantly altered the microfloral structure; *Ruminococcus*-like and *Allobaculum*-like bacteria were identified as key variables (Wei *et al.*, 2010). 4-Nitroquinoline-1-Oxide (4-NQO) is a typical carcinogenic aromatic nitro compound, and can be formed during incomplete combustion of organic substance; it has been widely used in cancer studies (Shiotani *et al*., 2001; Yoshida *et al*., 2005). However, previous reports have not considered the effect of 4-NQO on intestinal microflora communities.

The use of probiotics for both the prevention and treatment of gastrointestinal infections and diseases is becoming increasingly popular. The detoxification of genotoxins in the gut has been proposed as a possible mechanism of action (Wollowski *et al*., 2001). Several lactic acid bacteria have been shown to be capable of binding or inactivating carcinogens *in vitro* (Cenci *et al*., 2002; Burns and Rowland, 2004). Whether such a mechanism operates *in vivo* is still questionable.

Lactobacillus salivarius REN (REN) was recently isolated from Bama centenarians (China, one of the five well- known longevity regions). In our initial study using a SOS-Chromotest, REN was shown to inhibit 4-NQO genotoxicity by 98%. The subsequent HPLC assay suggested that REN detoxified 4-NQO by converting it to less toxic compounds after co-incubation (Wang *et al*., 2008). The objective of this study was to investigate the impact of 4-NQO on the microbial communities of rat colons and the efficiency of REN as an agent counteracting these changes. Denaturing gradient gel electrophoresis (DGGE) was employed to analyze the colonic microfloral communities. Multivariate statistical analysis was used for assessing the effects of 4-NQO and REN, and the related bacteria were characterized by band sequencing.

Materials and Methods

Lactic acid bacteria

Lactobacillus salivarius REN was isolated from the fecal samples of healthy centenarians (China).

Animals, diets, and experimental procedure

Four week old, male F344 rats (76±17 g, Vital River Lab Animal Technology Co. Ltd., China) were housed four per plastic cage, in

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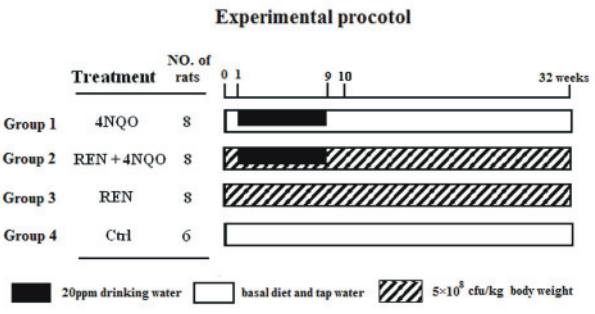

Fig. 1. Experimental protocol

an air conditioned room with a 12 h light/dark cycle. Temperature and relative humidity were kept at 25±2°C and 50±10%, respectively. After two weeks quarantine, the rats were randomized into twelve groups, corresponding to experiment week 1. All rats were fed with a basal diet (Rodent Chow Product, Ke Ao Xie Li feeds Co. Ltd., China) and allowed unlimited access to deionized water.

4-NQO (CAS 56-57-5; 98% pure) was purchased from Sigma-Aldrich (China), and was given to rats in tap water at a concentration of 20 ppm. Black bottles were used to protect 4-NQO from decomposition by light. 4-NQO solution was made on a weekly basis and stored in a dark, cold room (4-8°C).

A total of 30 rats were divided into four groups as shown in Fig. 1. From week 2, rats in groups 1, 2 were given 20 ppm 4-NQO in drinking water for 8 weeks. Groups 2, 3 were administered with REN at 5×10^8 CFU/kg body weight once per day. Group 4 was fed with the basal diet and deionized water.

Colonic contents sampling and bacterial genomic DNA extraction

At the end of the experiment, all the rats were euthanized by cervical dislocation. After laparotomy, the colon was excised from the entire intestinal tract, and its contents were immediately submersed in 2.0 ml RNAlater. Bacterial genomic DNA was extracted according to the method described by Matsuki *et al.* (2004).

PCR amplification of 16S rDNA V3 region

The V3 variable region of 16S rDNA was amplified by PCR using those primers described by Muyzer *et al*. (1993). The primers 341F-GC (5′ CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGGCCTACGGGAGGCAGCAG) and 534R (5′ ATTAC CGCGGCTGCTGG) were synthesized by Invitrogen (Beijing) Biotech Co. Ltd. The PCR reaction mixture contained 2.5 μl template DNA, 0.25 μM (final concentration) of each primer (25 ml premixed Ex Taq polymerase solution), 4 μl of dNTP mixture, 5 μl of 10× Ex Taq buffer, and 0.25 μl of Ex Taq polymerase (TaKaRa, Japan). The final volume of the reaction mixture was adjusted to 50 μl with sterile deionized water. A "touchdown" PCR was performed in a PTC-200 Thermo Cycler (Bio-Rad, USA) following the program described by Lubbs *et al*. (2009). The length of the amplicons was approximately 200 bp, as determined by electrophoresis in 2% (w/v) agarose gels and ethidium bromide staining. We added 10 μl of DGGE gel loading buffer [0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol (w/v) in H_2O] to 20 μl of the PCR product and the mixture was stored at -20°C until DGGE analysis.

DGGE analysis

DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad, USA). Amplicons were separated on 8% (w/v) polyacrylamide gels (acrylamide-bisacrylamide stock solution 37.5:1, Amresco, USA) in 0.5× TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2-EDTA) (Muyzer *et al.*, 1993), along a 35-60% linear denaturing gradient (A 100% denaturant corresponds to 7 M urea and 40% to (v/v) deionized formamide). Electrophoresis was performed in 0.5× TAE at 150 V and 60°C for 10 h. Gels were silver stained (White *et al*., 2004) and scanned using a CanoScan Lide 100 Scanner (Canon, Japan).

Gel images were analyzed using Quantity One software (version 4.2, Bio-Rad, USA) according to the user guide. The intensity of each band in the same lane was calculated was a percentage of the total intensity of all the bands in each lane. These values were exported to SPSS 16.0 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 (SPSS Inc., USA). The similarities between the analyzed samples have been presented in dendrograms (SPSS Inc.) (Da Silva Torres *et al*., 2006).

Multivariate statistical analysis

To assess the effects of 4-NQO and REN on colonic microflora, multivariate statistical analysis was performed using Canoco 4.5 (Biometrics, Netherlands). The band type table was imported as species data, and 4-NQO and REN were considered environmental variables. Because the longest gradient resulting from detrended correspondence analysis (DCA) was 1.219, the linear model of redundancy analysis (RDA), with the focus scaling on interspecies distances, was employed (Lepš and Šmilauer, 2003; Janczyk *et al*., 2010). Unrestricted Monte Carlo permutation tests were applied to test for significance of the microfloral response to the environmental variables (499 random permutations, $p<0.05$). To investigate which bacterial communities significantly responded to 4-NQO and REN treatment, t-value biplots for each variable were graphed 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 (Lepš and Šmilauer, 2003). Species vectors (band types) enclosed in Van Dobben circles indicated the significance of their relationships with 4-NQO or REN (regression coefficient <-2 or $>+2$) (Lepš and Šmilauer, 2003). Bands within the positive circles indicated an increased abundance associated with an environmental variable and the opposite effect was associated with bands in negative circles (Lepš and Šmilauer, 2003). The bands significantly affected by 4-NQO and REN treatment were marked on the DGGE gel images to identify them for later sequencing.

Excision and sequencing of selected bands from DGGE gels

Bands of interest were excised with a sterile scalpel from the DGGE gels and the DNA was extracted according to the procedure described by Sanguinetti *et al*. (1994). The DNA was subjected to PCR applification as templates with 341F (without GC-clamp, the sequence was 5′ CCTACGGGAGGCAGCAG) and 534R primers according to the following program: 3 min at 94°C, 30 cycles consisting of 15 sec at 94°C, 20 sec at 55°C, and 20 sec at 72°C, and finally 5 min at 72°C. The resulting PCR products were cleaned with the Universal DNA Purification kit (Tiangen, China) and cloned into *Escherichia coli* TOP10 competent cells (Tiangen) with the pMD18-T Simple Vector (TaKaRa). Plasmid DNA was isolated from the *E. coli* cells using

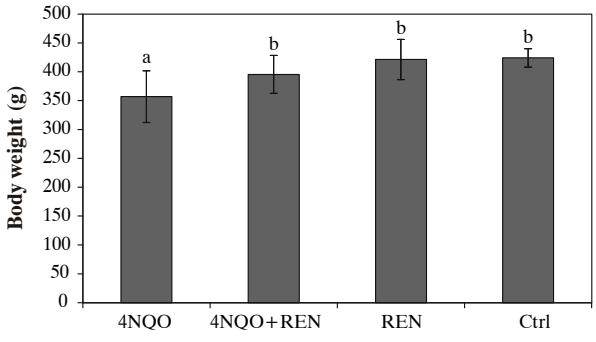

Fig. 2. Average individual body weight for each group. The data were analyzed using the Duncan test. The results were considered significantly different if the *P* value was less than 0.05.

the TIANprep Mini Plasmid kit (Tiangen), and subjected to PCR (with 341F-GC and 534R primers) as described previously. The PCR product was checked by DGGE to confirm 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 by Invitrogen (China) Biotech Co. Ltd using the M13F primer. The obtained sequences were compared with known sequences in the GenBank database using the BLASTn algorithm.

Results

General observations

All the rats in groups 1 and 2 (Fig. 1) were tolerant of 4-NQO exposure. There were no significant differences in the total intake of 4-NQO, water or basal diet per rat among the four groups (data not shown). Oral administration of REN (groups 2, 3) did not reduce survival rate or result in histological changes; suggesting that these rats were tolerant of REN administration.

The mean body weights of group of rats at the end of the experiment are provided in Fig. 2. The mean body weight of group 1 (4-NQO alone) was significantly lower than those of the other groups ($P < 0.05$).

Structural variations in colonic microflora

PCR-DGGE fingerprinting analysis of the predominant bacterial strains was used to capture the structural response of the colonic microfloral community. In this study, the colonic microflora of 30 rats was successfully represented by DGGE.

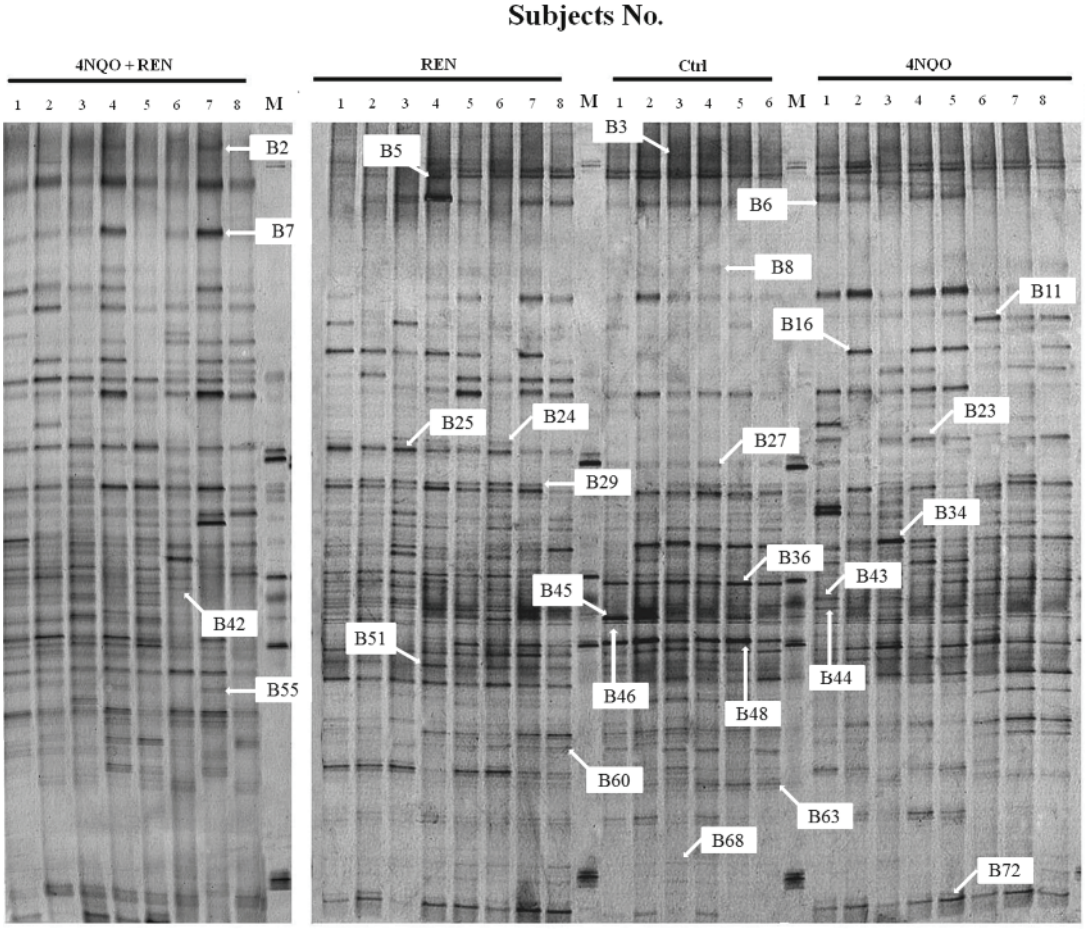

Fig. 3. DGGE profiles of colonic microfloral communities. Bands determined to have been significantly affected by 4NQO and REN treatment were marked as "B" followed by the band type number. M, marker.

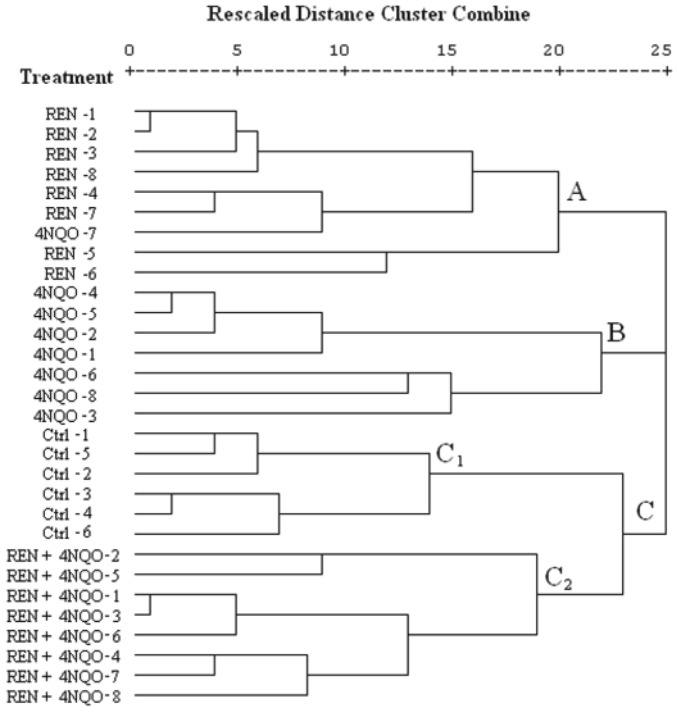

Fig. 4. Dendrogram derived from DGGE analysis of colonic intestinal microflora of rats from different treatment groups, based on clustering analysis.

As shown in Fig. 3, some inter-group variability was detected in the patterns of the bands.

To further determine the variability between colonic microfloral communities, a total of 72 band types were recognized by Quantity One software. The relative intensity of each band types was used as an indicator of abundance for the hierarchical cluster analysis. As shown in Fig. 4, the 30 subjects were grouped into three clusters (A, B, and C). Cluster A consisted of all the subjects from group 3 (REN) and one subject from group1 (4-NQO). All the subjects from group 1 (4-NQO) were grouped into cluster B. Cluster C comprised of two sub-clusters, C_1 and C_2, which consisted of subjects

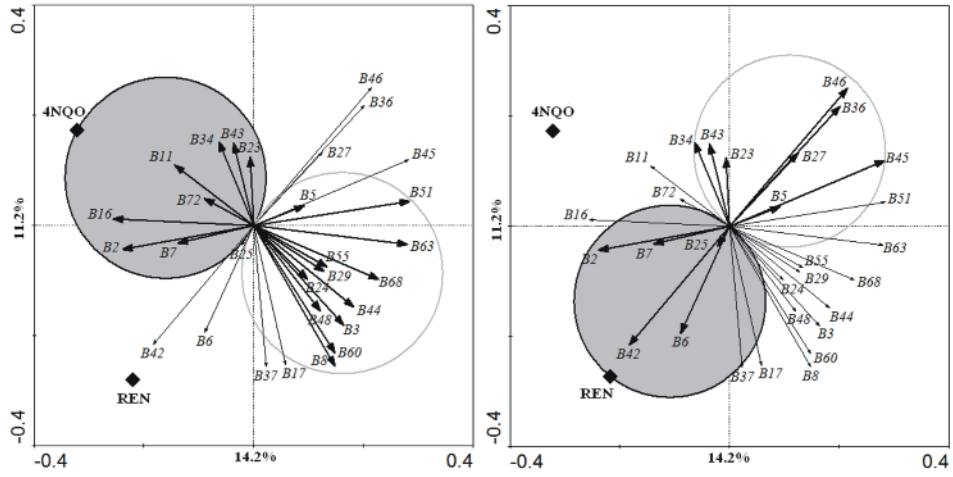

Fig. 5. T-value biplot for 4-NQO and REN obtained from CanoDraw (a module of Canoco 4.5). The first and the second axes explained 14.2% and 11.2% of total variation respectively. Gray line circles indicate Van Dobben circles. The grey circles are positive correlation circles while the transparent ones are negative correlation circles. Vectors indicated species (band types in DGGE profile). Bold vectors indicate those bands significantly altered by 4-NQO and REN treatment. Tetragonum indicates environmental variables (4-NQO and REN).

from group 2 (4-NQO + REN) and from group 4 (control), respectively. These results suggest that group 2 was most similar to the control group. According to the hierarchical cluster analysis, each treatment group had a distinctive colonic microfloral structure, though there was slight inter-individual variation within groups.

Effects of 4-NQO and REN on colonic microflora

Both species-environment correlations and Monte Carlo permutation tests were used to analyze the relationship between the structure of colonic microbial communities and the treatment procedures. In this study, when both 4-NQO and REN were treated as environmental variables, the first axis of the RDA explained 14.2% of total variation and the first and the second axes together explained 25.4%. Species-environment correlations for axes 1 and 2 were 0.944 and 0.977, respectively, indicating a high correlation between the changes in microfloral communities and the environmental variables. Significant effects of 4-NQO and REN on microflora were confirmed by Monte Carlo permutation tests (P=0.002). When 4-NQO or REN were separately considered environmental variables (with the other as a covariable) in RDA, significant effects on microflora were also detected (P=0.002, Monte Carlo permutation tests); suggesting that both 4-NQO and REN significantly influenced colonic microfloral communities in rats.

Sequencing of the bands correlated with 4-NQO or REN

A total of 27 bands were observed on the t-value biplots to significantly correlate with 4-NQO and REN treatment based on RDA (Fig. 5). The positions of these bands in the DGGE profile are represented in Fig. 2, and the microbial identification of the bands is summarized in Table 1.

4-NQO treatment increased the abundance of one *Helicobacter pullorum* strain (B23), one *Desulfovibrio* strain (B16) and one *Bacteroides chinchillae* strain (B43), and reduced the abundance of one *Ruminococcus* strain (B60), one Rumen bacterial strain (B63), four Bacteroides strains (B3, B8, B24, and B48) and two Firmicutes strains (B44 and B51). Furthermore, the increase of five (B7, B11, B34, B43, and B72) and the decrease of four (B5, B29, B55, and B68) uncultured bacterial strains were also correlated with 4-NQO treatment. Oral administration of REN increased the abundance of one *Lactobacillus salivarius* strain (B25), three Bacteroides strains (B2, B6, and B42), and reduced the abundance of one *Helicobacter pullorum* strain (B23), one *Clostridiaceae* strain (B45) and two Bacteroides strains (B27 and B46). In addition, the increase of the B5 strain and the decrease of the B7 strain were also correlated with the administration of REN.

Table 1. Summary of band identification and significant bacterial associations with 4-NQO and REN in colonic microflora

Band NO.	Closest relative	4-NQO	REN	% identity	Accession no.
16	*Desulfovibrio simplex*	+		98	AB603520.1
23	*Helicobacter pullorum* strain MIT 09-6635	+	-	100	HD319257.1
25	*Lactobacillus salivarius* strain LS(2)		+	99	HQ312979.1
60	*Ruminococcus* sp.ID1	-		100	AY960569.1
63	Rumen bacterium NK4A136	-		100	GU324401.1
45	*Clostridiaceae bacterium* WN011		-	100	AB298726.2
2	*Bacteroides chinchillae*	+	+	95	AB547637.1
3	*Bacteroides chinchillae*	-		95	AB531491.1
24	*Bacteroides rodentium*	-		100	AB547646.1
27	*Bacteroides chinchillae*		-	97	AB547637.1
42	*Bacteroides chinchillae*		+	95	AB547637.1
6	Uncultured Bacteroidetes bacterium clone CM2-172		+	96	GU959413.1
8	Uncultured Bacteroidetes bacterium clone MS054A1_C01	-		99	EF696853.1
46	Uncultured Bacteroidetes bacterium cloneTCM1-76		-	97	GU958635.1
48	Uncultured Bacteroidetes bacterium clone CM1-122	-		98	GU958451.1
44	Uncultured Firmicutes bacterium clone CM1-116	-		99	GU958436.1
51	Uncultured Firmicutes bacterium TF3-120	-		98	GU959458.1
5	Uncultured bacterium clone R-8963	-	-	96	FJ880966.1
7	Uncultured bacterium	+	+	100	AB606289.1
11	Uncultured bacterium clone H75N1_02e06	+		100	EU451751.1
29	Uncultured bacterium clone CF5956	-		98	GU605944.1
34	Uncultured bacterium clone RMAM 0327	+	-	99	HQ319257.1
36	Uncultured bacterium clone RMAM0346		-	100	HQ319276.1
43	Uncultured bacterium clone HFV01_467	+	-	99	GU103547.1
55	Uncultured bacterium clone HF7057	-		96	GU632566.1
68	Uncultured bacterium clone nby265d11c1	-		97	HM813486.1
72	Uncultured bacterium clone HFV01_243	+		98	GU103323.1

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

Discussion

Using PCR-DGGE, combined with multivariate statistical analysis, we demonstrated that the structures of colonic microfloral communities were significantly altered by treatment with 4-NQO or REN. Group 2 (4-NQO + REN) was most similar to the control group (Group 4), which suggested that the administration of REN could effectively counteract the negative effects for 4NQO exposure. Based on t-value biplots, a total of 27 bands (bacterial strains) were identified as being significantly correlated with 4-NQO or REN treatment.

In our study, 4-NQO had a significant effect on the microbial community structure of rat colons. This was consistent with a previous study that demonstrated that rat gut microfloral community structure was significantly altered by treatment with DMH, another carcinogen (Wei *et al*., 2010). In this study, 4-NQO treatment increased the abundance of two potential pathogens, one *Helicobacter* strain (B23) and one *Desulfovibrio* strain (B25). Infection with *Helicobacter* spp. is a strong risk factor, and affects the prognosis of gastrointestinal tract malignancies (Fox *et al*., 2001; Keenan *et al*., 2010; Polk and Peek, 2010). Similarly, *Desulfovibrio* species are opportunistic pathogens that may be involved in human colorectal cancer and intestinal infections (Scanlan *et al*., 2009; Ichiishi *et al*., 2010). Conversely, 4-NQO treatment reduced the abundance of two potentially beneficial bacterial strains, one *Ruminococcaceae* strain (B60) and one Rumen bacteria (B63). The *Ruminococcaceae* strain and Rumen bacteria potentially produced butyrate, which may play an important role in inhibition of carcinogenesis and oxidative stress (Hamer *et al*., 2008). Therefore, 4-NQO treatment induced unfavorable changes in the structure of colonic microfloral communities in rats, potentially increasing the risk of colorectal cancer and intestinal infections.

The mechanism by which carcinogens affect intestinal microflora is currently unclear. 4-NQO treatment has been shown to result in the production of a substantial amount of reactive oxygen species (ROS), which might cause oxidative damage to vital cellular components and result in the death of sensitive cells (Arima *et al*., 2006; Mozzeti *et al*., 2010). In our study, the abundance of two potential pathogens increased. The phenomenon might be partly explained by the fact that *Helicobacter* and *Desulfovibrio* strains exhibit strong defensive mechanisms against oxidative stress (Dolla *et al*., 2006; Handa *et al*., 2010). The structural divergence in the microfloral communities may have resulted from differences in the adaptability of different bacterial strains to ROS.

REN also played an important role in structuring colonic microfloral communities in our rat study population. Opposed to the effect of 4-NQO, no potential pathogenic strains were observed among those strain that responded positively to REN treatment. The hierarchical cluster analysis results revealed that the microbial community of group 2 (4-NQO + REN) was most similar to that of group 4 (Control), indicating a counteractive effect of REN against 4-NQO treatment. Further investigation revealed that REN significantly suppressed the growth and colonization of the pathogenic *Helicobacter* strain, which had been bolstered by 4-NQO. These effects could be partially explained by the de-genotoxifying effect of REN against 4-NQO previously reported by Wang *et al*. (2008). The high binding ability of *L. salivarius* to intestine epithelial cells may have contributed to the suppression of the *Helicobacter* strain. With this ability, *L. salivarius* may have discharged a sufficient amount of lactic acid at *Helicobacter* to inhibit its growth (Aiba *et al*., 1998). In addition, *L. salivarius* was identified by sequencing in both REN treatment groups (Groups 2 & 3). Combined, these results suggest the potential probiotic REN adhered to the intestinal mucosal and epithelial cells and exhibited sufficient survival and growth within the colon; regarded as one of the most important traits for probiotic microorganisms (Lee and Salminen, 2009).

It should be noted that, in the RDA, the first and the second axes only explained up to 25.4% of all variation, indicating that there were significant and unaccounted for effects on the microfloral communities studied in this report. Inter-individual variation may have contributed to these effects. However, a relatively low percentage of explained variance when using RDA is commonly found in the literature (Fuentes *et al.*, 2008; Janczyk *et al.*, 2010).

This study focused on the effect of 4-NQO and REN on colonic microflora, both of which were found to significantly alter intestinal microfloral communities in rats. Sixteen previously uncultured strains were observed to be significantly affected by 4-NQO and REN treatment, suggesting that 4-NQO or REN treatment can influence the growth of novel bacterial strains. Previous studies have suggested that approximately 75% of intestinal bacteria are novel phylotypes (Suau *et al.*, 1999); further research should focus on the large fraction of novel bacteria in the intestine and their affect on host health.

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