Lactobacillus Salivarius REN Inhibits Rat Oral Cancer Induced by 4-Nitroquiline 1-Oxide

Ming Zhang1, Fang Wang1, Lu Jiang1, Ruihai Liu3, Lian Zhang2, Xingen Lei4, Jiyou Li2, Jingli Jiang1, Huiyuan Guo1, Bing Fang1, Liang Zhao5, and Fazheng Ren1

Abstract
Despite significant advances in cancer therapy, cancer-related morbidity and mortality are still rising. Alternative strategies such as cancer prevention thus become essential. Probiotics represent an emerging option for cancer prevention, but studies are limited to colon cancers. The efficiency of probiotics in the prevention of other cancers and the correllative mechanism remains to be explored. A novel probiotics Lactobacillus salivarius REN (L. salivarius REN) was isolated from centenarians at Bama of China, which showed highly potent antigenotoxicity in an initial assay. 4-nitroquiline 1-oxide (4NQO)-induced oral cancer model was introduced to study the anticancer activity of L. salivarius REN in vivo. The results indicated that oral administration of probiotic L. salivarius REN or its secretions could effectively suppress 4NQO-induced oral carcinogenesis in the initial and postinitial stage, and the inhibition was in a dose-dependent manner. A significant decrease of neoplasm incidence (65%–0%) was detected in rats fed with the high dose of L. salivarius REN [5 × 1010 CFU/kg body weight (bw)/d]. In vivo evidences indicated that the probiotics inhibited 4NQO-induced oral cancer by protecting DNA against oxidative damage and downregulating COX-2 expression. L. salivarius REN treatment significantly decreased the expression of proliferating cell nuclear antigen (PCNA) and induced apoptosis in a dose-dependent manner. Our findings suggest that probiotics may act as potential agents for oral cancer prevention. This is the first report showing the inhibitory effect of the probiotics on oral carcinogenesis. Cancer Prev Res; 6(7); 686–94. ©2013 AACR.

Introduction
Cancer is the leading cause of death worldwide. Despite great progress in the recent years, the overall survival rate of cancer is still low, especially for late-stage diseases (1). Cancer prevention remains an attractive strategy since the introduction of cancer chemopreventive by Sporn (2) and Wattenberg and colleagues (3). Among the various chemopreventing agents, COX-2–selective inhibitors were the most promising candidates due to their efficiency in cancer prevention and low toxicity (4–6). However, prolong administration of some COX-2–selective inhibitors was found to be associated with a significant increase in cardiovascular events (7, 8), which resulted in the withdrawal of Vioxx from the market, followed by Bextra (valdecoxib).

These facts indicate that cancer prevention with pharmacologic agents is associated with practical challenges: benefit does not always outweigh risk, especially when long-term administration is required for subjects with unknown risk factors. New candidates are heavily investigated to improve the benefit-risk ratio of cancer prevention agents. The microbial genome project and related studies have revealed that the gut microbiome may play a far more important role in human health (9–12). Probiotics are a group of health beneficial strains, which have been used for centuries in human history all over the world. Recent studies suggested that probiotics may represent an emerging option for cancer prevention and treatment. Different from chemopreventive agents, probiotics have a well-established history in safety and may act via multiple mechanisms and pathways. Several groups have reported the potential of using probiotics in cancer prevention, but studies are mainly focused on colon tumors in vitro and in vivo (13–15). Some general mechanisms have been proposed, such as antimicrobial effects against carcinogen-producing microorganisms, antigenotoxic activities against internal and external carcinogens, and activation of gut mucosal immune system (16–18). These results indicate that probiotics are effective agent for colon cancer inhibition. However, the possibility of preventing other cancers and the correllative mechanisms remain to be explored. Lactobacillus salivarius (L. salivarius) REN was a novel strain isolated from fecal samples of healthy centenarians.
living in Bama (China), one of the 5 longevity villages in the world. In a previous study, it was found that *L. salivarius* REN could decrease the 4-nitroquinoline 1-oxide (4NQO)-induced genotoxicity *in vitro* (19). To further verify these potential anticarcinogenesis effect *in vivo*, inhibitory activity of *L. salivarius* REN on 4NQO-induced oral carcinogenesis was investigated in male F344 rats. In addition, the effect of *L. salivarius* REN on DNA oxidative damage, expression of COX-2, and cell proliferation and apoptosis activity in tongue tissue were assessed.

Materials and Methods

**Microbial strains and culture conditions**

*L. salivarius* REN was isolated from fecal samples collected from centenarians who lived at Bama, Guanxi Province, China. This is one of the 5 longevity regions in the world. *L. salivarius* REN was identified on the basis of the sequences of 16S ribosomal RNA gene and the sugar fermentation pattern, using the API50 CH kit (BioMérieux) and a computer-aided identification program (version 4.0, Bio-Mérieux). Three commercial strains, *Lactobacillus rhamnosus* GG [LGG; American Type Culture Collection (ATCC) 53103], *Bifidobacterium animalis* subsp. *Lactis* Bb12 (Bb12; ATCC 27536), and *Lactobacillus casei* Shirota (LCS; ATCC 53103) were provided by Chr. Hansen Company and Yakult Central Institute, respectively. All strains were cultured in Man–Rogosa–Sharpe (MRS) liquid medium at 37°C until late log phase.

**Preparation of microbial cells and its secretions**

The strain was cultured for 12 hours in MRS liquid medium, harvested by centrifugation (4,000 g, 10 minutes), washed twice, and adjusted to appropriate concentrations in sterile saline (0.9% w/v) for 4NQO metabolism test and oral administration.

The strain collected was then resuspended in deionized water to make a final concentration. This mixture was incubated for 1 hour at room temperature, and the water soluble secretion was harvested by centrifugation (4,000 g, 10 minutes, at room temperature). The concentration of the secretion was defined as the concentration of the strain in deionized water for oral administration.

**HPLC analysis of 4NQO metabolism *in vitro***

The microbial cells were adjusted to appropriate concentrations (1 × 10⁹ CFU/mL) in sterile saline (0.9% w/v), and 4NQO was added at a final concentration of 20 ppm. Coincubation was protracted under anaerobic conditions for different period of time and with agitation (200 rpm, 37°C). The supernatant was recovered by centrifugation, filtered using a 0.45 μm membrane, and analyzed by high-performance liquid chromatography (HPLC) as described in Wang and colleagues (19).

**Animals, diets, and experimental procedure**

Male F344 rats, 4 weeks old (76 ± 17 g, Vital River Lab Animal Technology Co. Ltd.) were housed 5 in each plastic cage, in an air-conditioned room with 12-hour light/dark cycle. The temperature and relative humidity were kept at 25 ± 2°C and 50 ± 10%. After 2 weeks quarantine, the rats were randomized into 12 groups. All rats were fed with basal diet (Rodent Chow Product, KeAoXie Li feeds Co. Ltd.) and allowed free access to deionized water, with or without 4NQO (Sigma-Aldrich). A total of 170 rats were divided into 12 groups as shown in Fig. 1. From week 2, rats in groups 1 to 9 were given 20 ppm drinking water (4NQO at 20 ppm).
Cancer Prevention Research

ppm 4NQO in drinking water for 8 weeks. Groups 2 to 7 were administered with *L. salivarius* REN at $5 \times 10^6$, $5 \times 10^8$, or $5 \times 10^{10}$ colony-forming units (CFU)/kg body weight (bw) once per day, started either from week 1 or week 9, until the end of experiment. Group 8 and 9 were administrated with secretion of *L. salivarius* REN ($2 \times 10^8$ CFU/mL drinking water) w/o 4NQO (20 ppm). All of the animals were sacrificed under ether anesthesia 32 weeks after the commencement of the experiment. Tongues were cut approximately into 2 halves; one portion was used for 8-OHdG assays and the other for histopathology and immunohistochemistry assays. For histologic examination, tissue and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks, and the histologic sections were stained with hematoxylin and eosin. Epithelial lesions (hyperplasia, dysplasia, and neoplasia) in the oral cavity were diagnosed according to the criteria described by Banoczy and Csiba and Kramer and colleagues (20, 21). The diagnosis was randomized and single blinded.

8-OHdG levels of tongue epithelium tissue

8-OHdG concentration was determined using a Rat 8-OHdG ELISA kit (Cosmo Bio), according to the manufacturer’s instruction. ELISA was carried out in triplicate and in a blinded fashion.

Immunohistochemistry

The paraffin sections were deparaffinized, rehydrated, and subsequently incubated with a polyclonal primary antibody (COX-2, Cell Signaling Technology; PCNA, NOVUS) at room temperature for 1 hour. The secondary antibody was incubated for 15 minutes at room temperature, followed by incubation with strepavidin-POD (Dako) for 15 minutes. Antibody binding was visualized using AEC solution (Dako). The tissues were then counterstained by hematoxylin solution. Slides were subsequently reviewed in a blinded fashion. The overall COX-2 staining intensity was scored as follows (22, 23): −, no staining; ±, weakly positive (weaker than the immunopositivity of macrophages) over less than 10% of the area; +, weakly positive over more than 10% of the area; and ++, strongly positive (equal or more than the immunopositivity of macrophages) over more than 10% of the area. For proliferating cell nuclear antigen (PCNA) immunoreactivity, the index was determined by counting the percentage of positive cells in the total cell counts in defined fields.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted with the commercially DeadEnd Colorimetric TUNEL System (Promega) according to the manufacturer’s instructions. The apoptosis index was determined by counting the percentage of TUNEL-positive cells in the total cell counts in defined fields.

Statistical analysis

Statistical analysis on the incidence of lesions and immunoreactivity COX-2 were conducted using Fisher exact probability test. The data of 8-OHdG levels and positive cells ratio in immunohistochemistry were analyzed using the Student $t$ test. The results were considered statistically significant if the $P$ value was less than 0.001, 0.01, or 0.05.

Results

*L. salivarius* REN-induced 4NQO decomposition in vitro

The metabolism profiles of 4NQO by *L. salivarius* REN were analyzed by HPLC, and the results were shown in Fig. 2A. In the cases treated with other probiotics (LGG, Bb12, or LCS), only a single new peak (peak 2; Fig. 2A, b) was observed on HPLC in coordination with the disappearance of the 4NQO peak (peak 4), regardless of time and strain concentration. This newly formed peak was fairly stable, and no obvious decrease was observed during the experimental courses for all the other strains tested. This peak was later confirmed to be 4-hydroxy aminoquinoline-1-oxide (HAQO; found M$^+1 = 177.0$, peak 1; Fig. 2B). However, when 4NQO (peak 3) was coinubcated with *L. salivarius* REN, 2 new compounds (peaks 1 and 4) appeared (Fig. 2A, c–h), and the relative height of each peak is changing with time. If incubation time was long enough (12 hours), all the other peaks would disappear, leaving peak 1 as the only product (Fig. 2A, h). Liquid chromatography-mass spectrometry (LC-MS) analysis of 2 new compounds was 4-aminoquinoline (4AQ; expected M$^+1 146.08$) and 4-aminoquinoline-1-oxide (4AQO; expected M$^+1 161.07$). 4HAQO was the proximate carcinogen, whereas 4AQO and 4AQ were either less or nontoxic compounds compared with 4NQO.

General observations of animal

The body weights of all 4NQO treatment groups, with or without probiotics, were significantly lower than the control group (except group 8; Supplementary Table S1). In comparison of group 10 with 12, the oral administration of *L. salivarius* REN or its secretions showed no significant sign of any indexes tested, which indicates that this species is safe for the model animals.

Inhibition effect of *L. salivarius* REN on the incidence of tongue tumors and preneoplastic lesions

Neoplasms lesions were found in the tongues of all affected rats but no metastasis was observed (Table 1). Thirteen out of 20 rats in the 4NQO-treated group (group 1) were histologically diagnosed with either papilloma or squamous cell carcinoma, accounting for 65% in total, whereas a significant decrease was observed in all the other groups treated with 4NQO and *L. salivarius* REN (groups 2–7). The effects were in a dose-dependant manner, and the best effect was observed in group 4, when the highest dosage of *L. salivarius* REN (10$^{10}$ CFU/kg bw) was given to the rats for the whole experimental period. No incidence of neoplasms was observed in this group, offering 100% protection to the rats exposed to 4NQO. Reducing the dosages resulted in...
observed in all the treated groups. However, the incidence of hyperplasia, a relatively higher percentage of hyperplasia was significantly lower than that of group 1 (100%, P < 0.05). Different from the results of neoplasm and dysplasias that are considered to be preneoplastic lesions for oral cancer were observed in groups 1 to 9 but none in groups 10 to 12 (Table 1). Tongue squamous cell hyperplasia were classified into 2 categories of hyperplasia (simple and papillary) and 3 types of dysplasia (mild, moderate, and severe) according to the degree of cellular atypism. The incidences of total dysplasia in groups 2 to 9 were significantly lower than that of group 1 (100%, P < 0.05). Different from the results of neoplasm and dysplasia, a relatively higher percentage of hyperplasia was observed in all the treated groups. However, the incidence of hyperplasia in groups 4 and 8 was lower than group 1 (100%, P < 0.05). These findings suggested that *L. salivarius* REN was a powerful agent for oral neoplasms prevention.

**L. salivarius** REN protected against the DNA oxidative damage in vivo

The oxidative damage in tongue mucosa was measured by 8-OHdG assay and shown in Fig. 2C. A high level of 8-OHdG was observed in the 4NQO-treated group, indicating that DNA oxidative damage was involved in the initiation and progression of 4NQO-induced carcinogenesis. In most of the *L. salivarius* REN and its secretions treatment groups (7 out of 8), the levels of 8-OHdG were significantly decreased when compared with the control group (P < 0.05 or P < 0.01). When *L. salivarius* REN was given for the whole experimental period (group 2–4), the decrease of the 8-OHdG levels was more remarkable, whereas it had less effects when given after 4NQO exposure (group 5–7, P < 0.05). In addition, the levels of 8-OHdG in group 4 was not significantly different from the normal tissue (group 12), which suggested that long-term and high-
dosage administration of *L. salivarius* REN could effectively prevent DNA from oxidative damage in vivo.

**L. salivarius** REN downregulated the expression of COX-2 in vivo

Statistical results for COX-2 immunohistochemical staining of rat tongue mucosa are summarized in Table 2. Representative immunohistochemical expression of COX-2 in different groups were shown in Fig. 3. In normal tongue tissues, slight immunoreactivity for COX-2 was observed in tongue squamous epithelium. In group 1 (4NQO treatment alone), COX-2 expression was prominent in tongue neoplasms. Oral administration of *L. salivarius* REN (group 3 and 7) significantly decreased COX-2 immunoreactivity in the tongue neoplasms when compared with group 1 (*P* < 0.05). In addition, oral administration of *L. salivarius* REN significantly increased the incidence of preneoplastic lesions with weak (group 4, 6, 7) or negative (group 2, 4, 8) immunoreactivity of COX-2 (*P* < 0.05 or *P* < 0.01). These results indicated that the immunoreactivity of COX-2 was downregulated by administration of *L. salivarius* REN in both neoplastic and preneoplastic tissues.

**Table 1. Incidence of tongue neoplasms and preneoplastic lesions of rats during or after 4-NQO exposure**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>No. of rats (final)</th>
<th>Papilloma</th>
<th>SCC</th>
<th>Total</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Total</th>
<th>Simple</th>
<th>Hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4NQO Alone</td>
<td>20</td>
<td>1 (5%)</td>
<td>12 (60%)</td>
<td>13 (65%)</td>
<td>2 (10%)</td>
<td>1 (5%)</td>
<td>17 (85%)</td>
<td>20 (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4NQO+10⁶ cfu/kg REN</td>
<td>15</td>
<td>0 (0%)</td>
<td>5 (33%)</td>
<td>5 (33%)</td>
<td>4 (27%)</td>
<td>1 (7%)</td>
<td>5 (33%)</td>
<td>10 (67%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4NQO+10⁸ cfu/kg REN</td>
<td>15</td>
<td>1 (7%)</td>
<td>0 (0%)</td>
<td>1 (7%)</td>
<td>2 (13%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>4 (27%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4NQO+10¹⁰ cfu/kg REN</td>
<td>15</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>2 (13%)</td>
<td>1 (7%)</td>
<td>0 (0%)</td>
<td>3 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4NQO+10¹² cfu/kg REN</td>
<td>15</td>
<td>0 (0%)</td>
<td>4 (27%)</td>
<td>4 (27%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>4 (27%)</td>
<td>4 (40%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4NQO+10¹⁴ cfu/kg REN</td>
<td>15</td>
<td>0 (0%)</td>
<td>3 (20%)</td>
<td>3 (20%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>3 (20%)</td>
<td>5 (33%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4NQO+10¹⁶ cfu/kg REN</td>
<td>15</td>
<td>0 (0%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>4 (27%)</td>
<td>9 (60%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4NQO+10¹⁸ cfu/kg REN</td>
<td>20</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4NQO+10²⁰ cfu/kg REN</td>
<td>20</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4NQO+10²² cfu/kg REN</td>
<td>20</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Secretions</td>
<td>10</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>No treatment</td>
<td>10</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aSignificantly different from group 1 by Fisher exact probability test (*P* < 0.01).

**Table 2. COX-2 immunohistochemistry staining of tongue lesions**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>No. of lesions examined</th>
<th>No. of lesions with COX-2 antibody staining (%)</th>
<th>Neoplasms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4NQO Alone</td>
<td>21</td>
<td>6 (29)</td>
<td>4 (19) (16)</td>
</tr>
<tr>
<td>2</td>
<td>4NQO+10⁶ cfu/kg REN</td>
<td>24</td>
<td>6 (25)</td>
<td>9 (38)</td>
</tr>
<tr>
<td>3</td>
<td>4NQO+10⁸ cfu/kg REN</td>
<td>30</td>
<td>2 (7)</td>
<td>10 (33)</td>
</tr>
<tr>
<td>4</td>
<td>4NQO+10¹⁰ cfu/kg REN</td>
<td>18</td>
<td>4 (22)</td>
<td>12 (67)</td>
</tr>
<tr>
<td>5</td>
<td>4NQO+10¹² cfu/kg REN</td>
<td>21</td>
<td>4 (19)</td>
<td>7 (33)</td>
</tr>
<tr>
<td>6</td>
<td>4NQO+10¹⁴ cfu/kg REN</td>
<td>22</td>
<td>4 (18)</td>
<td>16 (73)</td>
</tr>
<tr>
<td>7</td>
<td>4NQO+10¹⁶ cfu/kg REN</td>
<td>19</td>
<td>3 (16)</td>
<td>14 (74)</td>
</tr>
<tr>
<td>8</td>
<td>4NQO+Secretions</td>
<td>22</td>
<td>8 (36)</td>
<td>10 (45)</td>
</tr>
<tr>
<td>9</td>
<td>4NQO+Secretions</td>
<td>23</td>
<td>4 (17)</td>
<td>8 (35)</td>
</tr>
</tbody>
</table>

NOTE: –, negative; ±, weakly positive; +, moderate positive; ++, strong positive.

*aSignificantly different from group 1 by Fisher exact probability test (*P* < 0.001).

*bSignificantly different from group 1 by Fisher exact probability test (*P* < 0.01).
**L. salivarius** REN modulated the proliferation and apoptosis activities *in vivo*

Representative immunohistochemical expression of PCNA and TUNEL in different groups were shown in Fig. 3. Results for PCNA immunohistochemical staining of rat nonlesional tongue squamous epithelium were summarized in Table 3. A high level of PCNA expression (31.8%) was observed in the 4NQO-treated group. However, in most of the **L. salivarius** REN and its secretions treatment groups (7 out of 8), the levels of PCNA expression were significantly decreased when compared with the control group (*P* < 0.05 or *P* < 0.01). The results indicated that the proliferative activity of tongue squamous epithelium cells was significantly inhibited by **L. salivarius** REN.

Results for TUNEL staining of rat lesional tongue squamous epithelium were also summarized in Table 3. In group 1 (4NQO treatment alone), apoptosis index had no significant difference when compared with the control group (*P* > 0.05). In most of the **L. salivarius** REN and its secretions treatment groups (7 out of 8), the levels of apoptosis index were significantly decreased when compared with the control group (*P* < 0.05 or *P* < 0.01). However, no significant induction of apoptosis was observed in **L. salivarius** REN treatment alone, which implied that **L. salivarius** REN had no negative effect on normal cells.

**Discussion**

The results in the current study indicated that oral administration of probiotic **L. salivarius** REN or its secretions could effectively suppress 4NQO-induced oral carcinogenesis and the inhibition was in a dose-dependent manner. Interestingly, no SCC or papilloma was detected in rats fed with high dose of **L. salivarius** REN (group 4). The strain could decompose 4NQO *in vitro*, resulted in less toxic compounds. The strain protected DNA against oxidative damage induced by 4NQO *in vivo*, significantly downregulated COX-2/PCNA expression, and induced apoptosis in a dose-dependent manner. These results clearly suggested that **L. salivarius** REN could act as a very good candidate for cancer prevention agent.

Oral carcinogenesis is a multistage, slow progress. The initiation, proliferation, and development of tumors involved many mechanism and pathways. Mutagenicity by DNA oxidative damage, overexpression of COX-2 and prostaglandin, resistance to apoptosis, continuous proliferation may all contribute to oral carcinogenesis. The intervention of these multistage processes by modulating different intracellular signaling pathways and mechanisms provides a molecular basis for chemoprevention with probiotics.

4NQO-induced oral cancer model is the best amongst the available experimental systems for sequential studies of oral carcinogenesis (24). The morphology and the molecular events showed that 4NQO-induced tumors resemble human squamous cell carcinoma of the head and neck induced by tobacco or other oral carcinogens (25). The carcinogenic action of 4NQO is thought to be initiated by enzyme reduction *in vivo* to give 4HAQO. The latter is a proximate carcinogen, which forms adduct with DNA to cause oxidative damage and mutation, which is similar with the carcinogenic progress caused by tobacco and smoking (25). Our assay revealed that **L. salivarius** REN and its secretion could effectively reduce the toxicity of 4NQO via sequential decomposition of 4HAQO to give less or no toxic product(s) 4AQO and 4AQ (Fig. 2A). This set a very solid base for further investigation to evaluate the potential of **L. salivarius** REN as cancer prevention agents. Therefore, we chose 4NQO model to further investigate these effects *in vivo*. 
Oxidative DNA damage is a preliminary step during rat tongue carcinogenesis induced by 4NQO (26). Kohda and colleagues concluded that the 8-OHdG, which was regarded as a major marker of DNA oxidative damage (27,28), can be attributed to 8-OHdQ-DNA adducts in 4NQO animal model. High level of 8-OHdG reflects the increased oxidative damage of DNA (28). Several reports have suggested that probiotics could be efficacious in reduction of DNA damage caused by carcinogenic chemical agents in vitro and in vivo (29, 30). However, this mechanism had not been proven effective in cancer prevention. In the current study, L. salivarius REN or its secretions could dose dependently decrease the levels of 8-OHdG. The reduction of 8-OHdG levels was extremely obvious in group 4, which was fed with high dose of L. salivarius REN (10^10 cfu/kg bw) together with 4NQO exposure. These results are in concordant with the 8-OHdG metabolism profile observed in vitro (Fig. 2A).

However, it must be pointed out that a significant neoplasms inhibitory effect was also observed significantly in groups treated with L. salivarius REN after 4NQO administration, where 4NQO metabolism by L. salivarius REN was not feasible. Hence, there must be other prevention mechanisms involved, in addition to 4NQO decomposition.

Increased COX-2 expression has been associated with inflammation and the development of cancers (31–34). Some COX-2–specific inhibitors can prevent these conditions, at least in part, via inhibition of the expression of COX-2 (22, 23, 35). These findings suggested that inhibition of COX-2 may be another mechanism to suppress the development of 4NQO-induced oral carcinogenesis. Several species of probiotics were also proven to reduce the inflammation response by downregulating the COX-2 expression in vitro (36, 37). However, this mechanism had not been proven effective in cancer prevention. In the current study, oral administration of REN or its secretions significantly reduced the overexpression of COX-2 in 4NQO-treated rats (Table 3).

This result is very encouraging and yet conflicting. As mentioned previously, prolong usage of COX-2 inhibitors have been reported to be associated with cardiovascular side effects (7, 8, 38). These side effects have been attributed to the prevented production of the prostacyclins in the endothelial and smooth muscle cells as a consequence of COX-2 inhibition (39). This implies that certain amount of COX-2 is expressed in these tissues and may play important physiologic functions (40). How could REN function via the same mechanism as COX-2 inhibitors but without the side effects, which has been proven by the strains’ origin? A possible explanation could be that probiotics would downregulate the overexpressed COX-2 rather than inhibit it. As part of the gut microbial composition, probiotics may benefit from a prolong supply of moderate level of COX-2–inhibiting activity, but this fine-tuning mechanism remains to be explored.

Cell proliferation and apoptosis play important roles in multistage carcinogenesis. It has been found that some probiotics could also retard the growth of tumor volume and enhance the apoptosis of tumor cells, but studies were mainly focused on colon tumors (41–43). Our results (Table 3) showed a significant antiproliferation and proapoptotic effect that correlates with suppression of 4NQO-induced oral cancer incidence by dietary L. salivarius REN are in line with these observations. No significant induction of apoptosis was observed in L. salivarius REN treatment alone, which implied that L. salivarius REN had no negative effect on normal cells.

### Table 3. PCNA, TUNEL-positive cell ratio (%) in the tongue squamous epithelium

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Nonlesional area</th>
<th>Lesional area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4NQO</td>
<td>31.84 ± 5.08^d</td>
<td>5.82 ± 3.28</td>
</tr>
<tr>
<td>2</td>
<td>4NQO+5 × 10^6 cfu/kg REN</td>
<td>26.25 ± 6.38</td>
<td>9.60 ± 3.76</td>
</tr>
<tr>
<td>3</td>
<td>4NQO+5 × 10^6 cfu/kg REN</td>
<td>19.53 ± 9.35^b</td>
<td>14.42 ± 3.03^h,c</td>
</tr>
<tr>
<td>4</td>
<td>4NQO+5 × 10^10 cfu/kg REN</td>
<td>18.91 ± 6.89^b</td>
<td>12.22 ± 2.58^a,d</td>
</tr>
<tr>
<td>5</td>
<td>4NQO+5 × 10^6 cfu/kg REN</td>
<td>31.96 ± 2.68^d</td>
<td>9.75 ± 1.33^a,c</td>
</tr>
<tr>
<td>6</td>
<td>4NQO+5 × 10^8 cfu/kg REN</td>
<td>18.69 ± 4.72^b</td>
<td>14.76 ± 1.68^a,d</td>
</tr>
<tr>
<td>7</td>
<td>4NQO+5 × 10^10 cfu/kg REN</td>
<td>22.53 ± 3.54^b</td>
<td>14.64 ± 2.84^a,d</td>
</tr>
<tr>
<td>8</td>
<td>4NQO+Secretions</td>
<td>23.65 ± 2.23^c</td>
<td>16.56 ± 3.26^a,d</td>
</tr>
<tr>
<td>9</td>
<td>4NQO+Secretions</td>
<td>19.62 ± 4.36^b</td>
<td>13.43 ± 2.95^a,d</td>
</tr>
<tr>
<td>10</td>
<td>5 × 10^8 cfu/kg REN</td>
<td>19.92 ± 1.74^b</td>
<td>7.05 ± 3.70</td>
</tr>
<tr>
<td>11</td>
<td>Secretions</td>
<td>21.14 ± 2.57^b</td>
<td>7.71 ± 4.02</td>
</tr>
<tr>
<td>12</td>
<td>Control</td>
<td>19.83 ± 2.39^b</td>
<td>5.48 ± 3.22</td>
</tr>
</tbody>
</table>

^aSignificantly different from group 1 by Student t test (P < 0.05).
^bSignificantly different from group 1 by Student t test (P < 0.01).
^cSignificantly different from group 12 by Student t test (P < 0.05).
^dSignificantly different from group 12 by Student t test (P < 0.01).
In conclusion, our results indicate that *L. salivarius* REN and its secretions have significant inhibitory effects on oral carcinogenesis induced by 4NQO, and such effects may be related to the metabolism of 4NQO, prevention of DNA from oxidative damage, suppression of cell proliferation, induction of cell apoptosis, and/or downregulation of COX-2 expression. This is the first report showing the inhibitory effect of the probiotics on oral carcinogenesis, to the authors’ knowledge. Take into account the safety profile of probiotics in general, *L. salivarius* REN could act as a very good candidate for preventive agent against oral cancer, although clinical investigations are needed.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** M. Zhang, F. Wang, L. Jiang, R.H. Liu, H. Guo, F. Ren

**Development of methodology:** M. Zhang, F. Wang, J. Li, F. Ren

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Zhang, J. Jiang, L. Zhao

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Zhang, F. Wang, L. Jiang, R.H. Liu, X. Lei, J. Li

**Writing, review, and/or revision of the manuscript:** M. Zhang, L. Jiang, R.H. Liu, X. Lei, J. Li, F. Ren

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Zhang, R.H. Liu, L. Zhang, I. Jiang, B. Fang, L. Zhao, F. Ren

**Study supervision:** L. Jiang, R.H. Liu, L. Zhang, F. Ren

**Grant Support**

This work was supported by the Ministry of Science and Technology of China (2011AA100903, 2012BAD28B08), Beijing Excellent PhD Thesis Program (YB20101092), and Beijing Science and Technology Project (D10110504601001).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 24, 2012; revised March 6, 2013; accepted April 22, 2013; published OnlineFirst May 8, 2013.

**References**


www.aacjrournals.org

Cancer Prev Res; 6(7) July 2013 693

Published OnlineFirst May 8, 2013; DOI: 10.1158/1940-6207.CAPR-12-0427

Downloaded from cancerpreventionresearch.aacjrournals.org on June 18, 2017. © 2013 American Association for Cancer Research.


Cancer Prevention Research

*Lactobacillus Salivarius* REN Inhibits Rat Oral Cancer Induced by 4-Nitroquiline 1-Oxide

Ming Zhang, Fang Wang, Lu Jiang, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-12-0427

Supplementary Material
Access the most recent supplemental material at: http://cancerpreventionresearch.aacrjournals.org/content/suppl/2013/05/08/1940-6207.CAPR-12-0427.DC1

Cited articles
This article cites 41 articles, 7 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/6/7/686.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.