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Oral administration of a catalase-producing *Lactococcus lactis* can prevent a chemically induced colon cancer in mice

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Reactive oxygen species, such as hydrogen peroxide (H_2O_2), are involved in various aspects of tumour development. Decreasing their levels can therefore be a promising approach for colon cancer prevention. The objective of this study was to evaluate the effect of catalase-producing *Lactococcus lactis* on the prevention of an experimental murine 1,2-dimethylhydrazine (DMH)-induced colon cancer. DMH-treated BALB/c mice received either a catalase-producing *L. lactis* strain or the isogenic non-catalase-producing strain as a control, whereas other untreated mice did not receive bacterial supplementation. Catalase activity and H_2O_2 levels in intestinal fluids and blood samples were measured, and changes in the histology of the large intestines during tumour progression were evaluated. The catalase-producing *L. lactis* strain used in this study was able to slightly increase catalase activities in DMH-treated mice (1.19 ± 0.08 U ml^{-1}) and reduce H_2O_2 levels (3.4 ± 1.1 μM) compared to (i) animals that received the non-catalase-producing strain (1.00 ± 0.09 U ml^{-1}, 9.0 ± 0.8 μM), and (ii) those that did not receive bacterial supplementation (1.06 ± 0.07 U ml^{-1}, 10.0 ± 1.1 μM). Using the histopathological grading scale of chemically induced colorectal cancer, animals that received the catalase-producing *L. lactis* had a significantly lesser extent of colonic damage and inflammation (2.0 ± 0.4) compared to animals that received the non-catalase-producing *L. lactis* (4.0 ± 0.3) or those that did not receive bacterial supplementation (4.7 ± 0.5). The catalase-producing *L. lactis* strain used in this study was able to prevent tumour appearance in an experimental DMH-induced colon cancer model.

INTRODUCTION

Reactive oxygen species (ROS) are generally small molecules (such as superoxide ions, free radicals and peroxides) that are formed as by-products of the normal metabolism of oxygen and have important roles in cell signalling. However, increased ROS levels can result in oxidative stress inducing significant damage to cell structures and macromolecular constituents, such as DNA, RNA, proteins and lipids (Berlett & Stadtman, 1997). Toxicity occurs when the concentration of ROS exceeds the capacity of cell defence systems (Farr & Kogoma, 1991). The biological sources of ROS are numerous; they can be generated in aerobiosis by flavoproteins (Condon, 1987) and by macrophages during inflammatory reactions (Roos, 1991). Large amounts of hydrogen peroxide (H_2O_2) are produced and excreted by human tumour cells (Szatrowski & Nathan, 1991), and might participate in tumour invasion and proliferation. Thus, oxidative stress plays an important role in pathologies of the gastrointestinal tract of humans, such as inflammatory bowel diseases (Kruidenier & Verspaget, 2002; Kruidenier *et al.*, 2003).

ROS are involved in various aspects of tumour metastasis, including 1,2-dimethylhydrazine (DMH)-induced colon

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Abbreviations: DMH, 1,2-dimethylhydrazine; LAB, lactic acid bacteria; ROS, reactive oxygen species.

cancers (reviewed by Nishikawa *et al.*, 2002; Monte *et al.*, 1997); therefore, decreasing their levels could be a promising approach for the anti-metastatic therapy of tumours. Several studies have shown that removal of ROS by antioxidant enzymes can inhibit the incidence of tumour metastasis in various animal models (reviewed by Nishikawa *et al.*, 2004). Catalase and superoxide dismutase have been used to prevent experimental tumour metastasis of colon cancer cells to the lungs and liver (Nonaka *et al.*, 1993; Yoshizaki *et al.*, 1994). However, these enzymes can detoxify ROS (superoxide anions and H_2O_2 for superoxide dismutase and catalase, respectively) only at local sites reached by the enzymes following their administration (Nishikawa *et al.*, 2002, 2004). This requires targeted delivery of antioxidant enzymes to prevent specific pathologies caused by ROS.

Catalases are widespread in aerobic (facultative or not) bacteria such as *Escherichia coli* and *Bacillus subtilis* (Rochat *et al.*, 2005). The two classes of catalases are distinguished according to their active-site composition: one is haem dependent and the other, also named pseudocatalase, is manganese dependent. Catalases of two lactobacilli have been successfully cloned and phenotypically expressed in heterologous bacteria lacking catalase activity (Abriouel *et al.*, 2004; Knauf *et al.*, 1992; Noonpakdee *et al.*, 2004). Strains of lactic acid bacteria (LAB) expressing high levels of catalases could be useful in both traditional food applications and new therapeutic uses. A probiotic antioxidant strain able to eliminate ROS in the digestive tract of animals and humans could have applications for the treatment of inflammatory diseases or as a post-cancer drug treatment.

LAB present in fermented foods have been consumed by humans without any obvious adverse effects for thousands of years (Fuller, 1992). Therefore, they are potent candidates for delivery vehicles of beneficial compounds such as enzymes. The most extensively studied LAB is *Lactococcus lactis*, which does not produce catalase, and over the past decades, a number of elegant and efficient genetic tools have been developed for *L. lactis*, such as the nisin-controlled expression system for both heterologous and homologous gene expression (de Ruyter *et al.*, 1996). The expression of heterologous proteins and antigens, as well as the various delivery systems developed in *L. lactis*, have recently been reviewed by Nouaille *et al.* (2003). The ability of *L. lactis* to survive in the human gastrointestinal tract allows it to deliver proteins in the gut (Klijn *et al.*, 1995). Drouault *et al.* (1999) have shown that lactococci can resist gastric acidity, but only 10–30 % survive in the duodenum. Viable cells are metabolically active in each compartment of the digestive tract, whereas most dead cells are rapidly lysed. These properties allow the use of *L. lactis* as a vector to specifically deliver proteins into the duodenum of monogastric animals. The objective of this study was to evaluate the effect of catalase-producing *L. lactis* on the prevention of an experimental DMH-induced colon cancer.

METHODS

Bacterial strains. *L. lactis htrA*-NZ9000(pVE3655) [carrying an empty expression vector containing the nisin-inducible promoter P_{nisA} (Le Loir *et al.*, 2001), hereafter called *L. lactis* NZ] and *L. lactis htrA*-NZ9000(pSEC : KatE) [containing the *B. subtilis katE* gene under the control of P_{nisA} (Rochat *et al.*, 2005), hereafter called *L. lactis* KAT] were grown in brain heart infusion broth (Laboratorios Britania), containing 10 μM haemin (Sigma) and 10 μg chloramphenicol ml^{-1}, at 30 °C without agitation. Once OD_{600} 0.5 was reached, 1 ng nisin ml^{-1} (Sigma) was added to the culture and cells were grown for an additional 3 h. These cultures were washed twice with saline solution (0.15 M NaCl), resuspended in sterile 10 % non-fat milk and administered 1 % (v/v) in the drinking water of the mice. For enumeration, appropriate dilutions of samples [prepared in (1 g l^{-1}) cold peptone water] were plated on brain heart infusion agar and incubated at 30 °C for 48 h. Under these conditions, 1.0×10^9 c.f.u. per day were orally administered to each mouse. The control group consisted of mice that received 10 % non-fat milk under the same conditions as the test groups.

Animals. BALB/c mice of 6 weeks of age, weighing 25–30 g, were obtained from the inbred closed colony maintained at CERELA. The mice were separated into six experimental groups (each group consisting of equal numbers of male and female mice): (1) DMH group, mice received injections of DMH to induce tumour growth; (2) DMH-KAT group, mice received *L. lactis* KAT after being treated with DMH; (3) DMH-NZ group, mice received *L. lactis* NZ after being treated with DMH; (4) KAT group, mice received *L. lactis* KAT; (5) NZ group, mice received *L. lactis* NZ; and (6) the non-treatment group, mice not given any specific treatment. All groups were fed *ad libitum* with a balanced rodent diet (Cooperacion;, containing 32 % protein, 5 % fat, 2 % fibre and 60 % nitrogen-free extract). Each experimental group consisted of 30–35 mice.

All animal protocols were approved by the Animal Protection Committee of CERELA and followed the latest recommendations of the Federation of European Laboratory Animal Science Associations. All experiments comply with the current laws of Argentina.

Tumour induction and feeding procedure. To induce colon tumours, mice were injected with the carcinogen DMH dihydrochloride (Sigma). Each mouse received, subcutaneously, 20 mg DMH (kg body weight)$^{-1}$ in 0.1 ml saline solution containing 1.5 g EDTA l^{-1}, pH 6.4, weekly for 10 weeks. These animals developed tumours 5 to 6 months after the first injection (DMH group). When required, bacterial suspensions in drinking water were given *ad libitum* to mice (suspension prepared freshly every day) starting at the 10th injection of DMH, considered the first day of feeding, for 6 months.

Blood and intestinal contents collection. Twice a month, animals from each group were anaesthetized with an intraperitoneal injection of [3.0 ml (kg body weight)$^{-1}$] ketamine (10 %) : xylacin (2 %) (40 : 60, v/v; Alfasan) and bled by cardiac puncture. Blood was transferred into tubes without anticoagulant, incubated at 37 °C for 1h and centrifuged (1000 *g* for 5 min), and the serum was removed and stored at −70 °C until analysed. The small intestine and colon were removed and their contents collected by adding 1 ml cold saline solution (0.15 M). The contents were homogenized then centrifuged (10 000 *g* during 15 min) and the supernatants were stored at −70 °C until analysed.

Colon histology. The large intestine was removed and washed with saline solution (0.15 M NaCl). Tissues were prepared for histological evaluation using the method described by Sainte Marie (1962). Serial paraffin sections of 4 μm were made and stained with haematoxylin–eosin for light microscopy examination. The microscope slides

Table 1. Histopathological grading scale of chemically induced colorectal cancer

Grade	Microscopic findings
0	Histological findings identical to normal mice.
1	Mild mucosal and/or submucosal inflammatory infiltrate (mixture of neutrophils) and oedema. Punctate mucosal erosions often associated with capillary proliferation. Muscularis mucosae intact.
2	Grade 1 changes involving 50 % of the specimen.
3	Prominent inflammatory infiltrate and oedema (neutrophils usually predominating) frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa. Rare inflammatory cells invading the muscularis propriae but without muscle necrosis.
4	Grade 3 changes involving 50 % of the specimen.
5	Extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells. Necrosis extends deeply into the muscularis propria.
6	Grade 5 changes involving 50 % of the specimen.
7	Tumour.

were reviewed, and the extent of colonic damage and inflammation was assessed using a modification of the histopathological grading system of Ameho *et al.* (1997) (Table 1).

Hydrogen peroxide and catalase activity. The H_2O_2 concentration of intestinal contents and blood serum were determined using an Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Invitrogen) as described by the manufacturer. Briefly, samples (50 µl appropriately diluted in the assay buffer consisting of 0.1M Tris/HCl, pH 7.5) were placed in 96-well microplates and absorbance was measured at 560 nm using a VersaMax tunable microplate reader (Molecular Devices). Afterwards, 50 µl reaction mixture [50 µl 10 mM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), 100 µl 10 U ml^{-1} horseradish peroxidase in 4.85 ml assay buffer] was added, and the microplate was incubated at 30 °C in the reader and absorbance was measured every 5 min for 45 min. Afterwards, background absorbance was subtracted and the H_2O_2 concentration of samples was calculated using a calibration curve generated with different concentrations of H_2O_2 (0 to 20 µM). Catalase activity was determined by measuring H_2O_2 degradation. Briefly, to 25 µl samples, 25 µl 40 µM H_2O_2 was added and incubated at 30 °C. The H_2O_2 concentration was determined as described above. Catalase activity (U) was expressed as µmol H_2O_2 degraded min^{-1}.

Statistical analysis. Statistical analyses were performed with the software package Minitab 14 (Minitab) using ANOVA GLM followed by a Tukey's posthoc test, and $P<0.05$ was considered significant. Unless otherwise indicated, all values ($n=15$) were the means of three independent trials ± SD (no significant differences were observed between individual replicates).

RESULTS AND DISCUSSION

Chemically induced (autochthonous) tumours in the rodent are considered as good models for obtaining results transferable to the clinical situation (Amberger, 1986); the most common carcinogen used in colon cancer induction is DMH. DMH is extensively metabolized *in vivo* and toxicity has been ascribed to metabolism-generated reactive intermediates, such as alkyldiazonium ions, carbon-centred radicals and ROS (Gamberini & Leite, 1997).

During the first 4 weeks after DMH induction, the histology of the large intestine did not change (Table 2); all groups were similar to the control group that did not receive DMH injections. Starting at week 8 post-carcinogen (DMH) administration, significant differences were observed when comparing the different experimental groups. This timeline is the same as the one previously

Table 2. Colon microscopic scores of mice

Values are means (SD).

Group	No. of weeks					
	2	4	8*	10*	14*	16*
DMH	0 (0)	0 (0)	6.7 (0.6)^{a}	4.7 (1.5)^{a}	4.0 (0.1)^{a}	6.3 (0.6)^{a}
DMH-KAT	0 (0)	0 (0)	2.7 (1.2)^{bc}	2.0 (0.1)^{b}	1.3 (0.6)^{b}	1.0 (0.1)^{b}
DMH-NZ	0 (0)	0 (0)	4.7 (2.1)^{ac}	4.0 (0.1)^{a}	2.3 (0.6)^{c}	4.0 (1.4)^{c}
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
KAT	–	0 (0)	0 (0)	–	0 (0)	0 (0)
NZ	–	0 (0)	0 (0)	–	0 (0)	0 (0)

*Means in a column without a common superscript letter (*a, b, c*) differ significantly ($P<0.05$).

obtained (de Moreno de LeBlanc & Perdigón, 2004) using the same DMH-induced tumour model as the one described in this study. At week 8 post-injection, many infiltrates in mucosa, submucosa and muscular tissues were present in animals that did not receive bacterial supplementation (DMH group) or in animals that received native non-catalase-producing *L. lactis* (DMH-NZ group). Tumours were also present in these animals starting at week 14 post-DMH. Although some infiltrates in mucosa and submucosa were present in mice supplemented with *L. lactis* KAT, most of the tissues of the large intestine were similar to those observed in the control animals (Fig. 1). No tumours were observed in animals supplemented with *L. lactis* KAT throughout the trial. Using the histopathological grading scale of chemically induced colorectal cancer (see Methods), animals receiving *L. lactis* KAT had a lesser extent of colonic damage and inflammation compared to animals that received *L. lactis* NZ. The animals that received this latter strain showed pathology of the large intestine similar to that observed in animals that did not receive bacterial supplementation (DMH group). These results show that the catalase-producing strain was able to significantly improve the morphology of the large intestine of mice that received DMH injections and also prevented tumour formation.

No differences in histology of the large intestine were observed in the animals that received either *L. lactis* NZ or *L. lactis* KAT without DMH induction compared to the non-treatment control group, showing that the strains used in this trial did not cause morphological changes in intestinal structures on their own. Also, no observable changes in the liver, spleen and kidneys were seen in the animals supplemented with either bacterial strain (data not shown).

A slight increase in catalase activity was observed in samples (small and large intestines) of animals that received *L. lactis* KAT compared to those that received *L. lactis* NZ and those that did not receive bacterial supplementation (DMH group) (Fig. 2). Even though at some time points no statistically significant differences were observed due to large intra-group variations, mean catalase activities in mice that received *L. lactis* KAT were always higher than those observed in animals that received either *L. lactis* NZ or no bacterial supplementation (DMH group), demonstrating that the catalase-producing strain was able to increase catalase activity in the gut.

No catalase activity was detected in blood serum showing that only a localized effect was obtained; the enzyme acted only where the bacterial strains transited. This result is similar to those observed by Nishikawa *et al.* (2002, 2004) where antioxidant enzymes were shown to detoxify ROS only at local sites reached by the enzymes following their administration.

H_2O_2 concentrations were slightly lower in samples (small and large intestine) from animals that were supplemented with *L. lactis* KAT compared to those that received either *L. lactis* NZ or no bacterial supplementation (DMH group). Even though at some time points no statistically significant differences were observed due to large intra-group

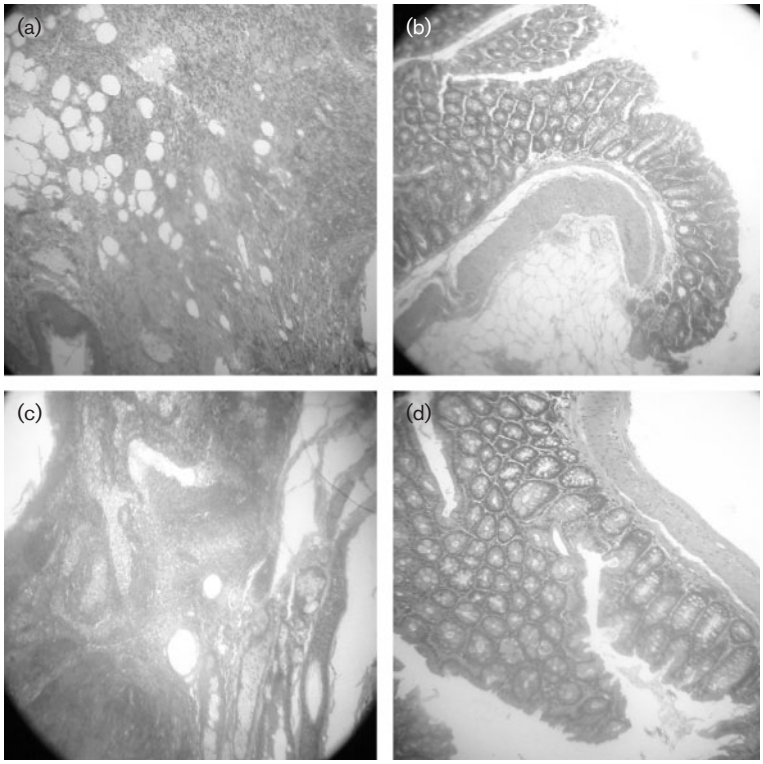

Fig. 1. Representative haematoxylin–eosin-stained paraffin-wax-embedded sections of the colon of mice from (a) the DMH group, (b) the control group, (c) the DMH-NZ group, (d) the DMH-KAT group (×100 magnification).

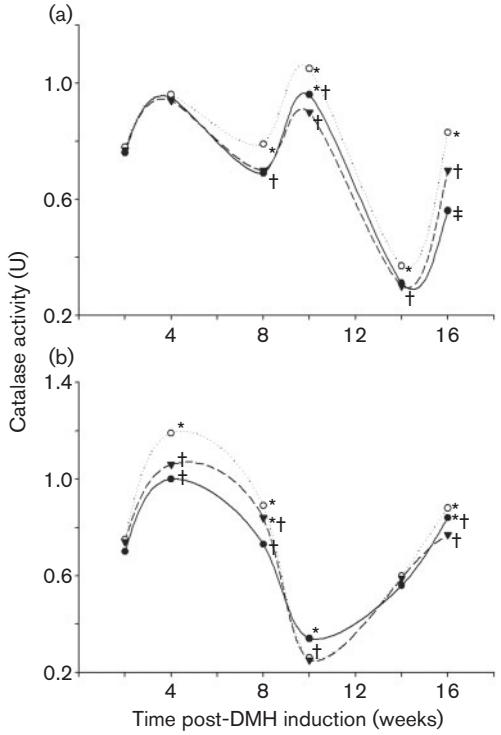

Fig. 2. Catalase activity determined in contents of the colon (a) and small intestine (b) of mice from the DMH group (●, unbroken line), the DMH-NZ group (▼, dashed line), and the DMH-KAT group (○, dotted line). Results are the means of three independent measurements of five animals (n=15). SD (lower than 10 %) was omitted to facilitate visualization. Data points without a common symbol differ significantly (P<0.05).

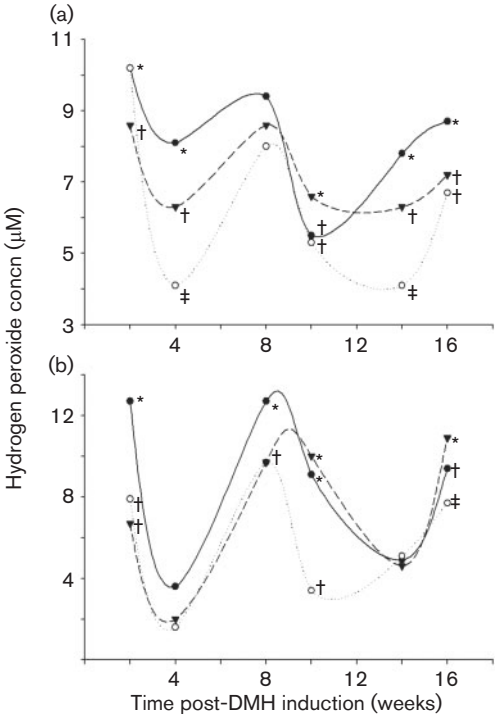

Fig. 3. Hydrogen peroxide concentration determined in contents of the colon (a) and small intestine (b) of mice from the DMH group (●, unbroken line), the DMH-NZ group (▼, dashed line), and DMH-KAT group (○, dotted line). Results are the means of three independent measurements of five animals (n=15). SD (lower than 10 %) was omitted to facilitate visualization. Data points without a common symbol differ significantly (P<0.05).

variations, the mean H_2O_2 levels of mice that received *L. lactis* KAT were always lower that those for the animals that received *L. lactis* NZ or in the DMH group (Fig. 3). These results show that the differences in catalase activity (increased catalase activity in the *L. lactis* KAT mice) were sufficient to lower H_2O_2 concentrations in the large intestine. The decrease of this ROS could explain the lower histopathologies of the large intestine and the absence of tumours in mice that received the catalase-producing strain, since it is known that ROS are involved in various aspects of tumour metastasis (reviewed by Nishikawa *et al.*, 2002; Monte *et al.*, 1997).

Conclusions

The catalase-producing *L. lactis* strain used in this study was able to increase catalase activities in mice treated with DMH. This increased antioxidant activity was sufficient to reduce levels of H_2O_2, a ROS involved in cancer promotion and progression, and prevented/regressed colon cancer promotion/progression, showing that this catalase-producing LAB could be used in novel therapeutic strategies for gastrointestinal pathologies.

Moreover, the study of other catalases produced in LAB, such as the recently described heterologous non-haem catalase produced by *Lactobacillus casei* (Rochat *et al.*, 2006; the first report of heterologous expression of a non-haem catalase in bacteria relevant to dairy industries, offering the advantage that no haem has to be added to the culture medium for enzyme activity), will open new opportunities to design novel antioxidant strains that could be able to eliminate ROS in the digestive tract of animals and humans in the treatment of intestinal inflammatory diseases or cancer.

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