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Lactobacillus delbrueckii subsp *lactis* strain CIDCA 133 inhibits nitrate reductase activity of *Escherichia coli*

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Abstract

The aim of the present work was to investigate the effect of strain CIDCA 133 on the nitrate reductase activity of a non-pathogenic *Escherichia coli* strain. Suspensions containing different ratios of the strains under study were coincubated in MRS or MRS without glucose. In some experiments lactobacilli were killed by UV treatment. The nitrate reductase activity was determined by using a diazotization reaction for nitrite. Presence of live lactobacilli leads to a dose–response diminution in the specific nitrate reductase of *E. coli* even when no acidification occurred. Killing of lactobacilli by UV treatment completely abolished the anti-nitrate reductase effect. In addition, the effect was only partially observed with filtered spent culture supernatants of lactobacilli.

Lactobacillus delbrueckii subsp *lactis* strain CIDCA 133 is able to antagonize the nitrate reductase activity of *E. coli*. This effect is neither due to a diminution of the viability of *E. coli* nor is depending on the acidification of the medium by the lactobacilli. Viability is needed for maximal anti-nitrate reductase activity. Modulation of undesirable enzymatic activities of intestinal microorganisms by means of selected microorganisms constitutes a further insight on the mechanisms by which probiotics lead to beneficial effects. Administration of probiotic strains able to modulate microbial intestinal activities could lead to a protection of the host against harmful effects of some members of the intestinal microflora.

Keywords: *Lactobacillus*; Probiotics; Nitrate reductase; Intestinal microbiota; Gut homeostasis

1. Introduction

The effect of the intestinal microflora on the health of humans and animals is largely documented (for a review see Tannock, 1995) and the activity of intestinal microorganisms leads either to adverse or beneficial effects depending on the metabolic capabilities of the microorganisms and the presence of virulence factors.

Colonization of the gut after birth provides a defense against harmful microorganisms. Also, commensal microorganisms constitute essential players in the maturation of the mucosal immune system as well as in the modulation of the immune response (Blum et al., 1999; McCracken and Lorenz, 2001).

However, metabolic activity of some intestinal microorganisms inhabiting the gut leads to the production of harmful substances. These substances could be metabolically undesirable (e.g D-lactic acid for the newborn) or might lead to potentially carcinogenic substances such as *N*-nitroso compounds (Goldin, 1986).

In this context, nitrate reductase activity appears as a common theme to account for the adverse effects of some members of the intestinal microflora. It is well known that nitrites could lead to an increase in the concentration of methemoglobin in blood. In vitro and in vivo studies have demonstrated that nitrites are mutagenic and carcinogenic (Anonymous, 2001; Spencer et al., 2000). This effect could be related to the nitrosative deamination of DNA nucleotides. Interestingly, microorganisms that are nitrate reducers are nat-

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urally protected from the mutagenic effect of nitrite (Weiss, 2001).

In addition, large amounts of nitrogenous compounds that gain access to the intestine are made available for *N*-nitrosation by colonic bacteria. These events could be related to the nitrate reductase activity of intestinal microorganisms (Calmels et al., 1985, 1996). *N*-nitroso compounds are involved in carcinogenesis since many classes of such compounds participate in alkylating reactions known to induce mutations related to colorectal cancer (Bos, 1989).

To modulate the metabolic activity of the intestinal microflora, different strategies have been used, i. e. modification of the diet (Goldin and Gorbach, 1976; Teramoto et al., 1996), administration of probiotic microorganisms (Djouzi et al., 1997; Naidu et al., 1999; Romond et al., 1998) and/or administration of prebiotics (McBain and MacFarlane, 2001; Gudiel-Urbano and Goñi, 2002). Diminution of undesirable enzymatic activities by probiotics has been mainly ascribed to the modification of the concentration of harmful microorganisms. However, it is likely that probiotic microorganisms can antagonize enzymatic activities by mechanisms other than displacement of undesirable microflora.

The characteristics of *Lactobacillus delbrueckii* subsp. *lactis* strain CIDCA 133 indicate that this microorganism could be interesting to formulate probiotic products. Indeed, when grown in different culture media (Kociubinski et al., 1996, 1999), this strain is resistant to high acid and bile concentrations and it is able to inhibit growth of food contaminants.

The aim of the present work was to investigate in vitro the effect of strain CIDCA 133 on the nitrate reductase activity of a non-pathogenic *Escherichia coli* strain in liquid culture medium.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli CM1 belongs to the culture collection of the Cátedra de Microbiología of the Facultad de Ciencias Exactas (Universidad Nacional de La Plata, Argentine). *L. delbrueckii* subsp *lactis* CIDCA 133 as well as *L. delbrueckii* subsp *delbrueckii* strain LBB belongs to the culture collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina). Pure strains of the bacteria under study were individually maintained at −80 °C and each microorganism was reactivated twice in liquid medium before the experiments.

E. coli CM1 was grown statically in nutrient broth containing 3g/l meat extract, 5 g/l meat peptone and 0.1% (w/v) sodium nitrate at 37 °C for 16 h. The presence of nitrate is important for proper induction of the nitrate reductase activity (Shoeb et al., 1991).

L. delbrueckii subsp *lactis* was propagated in anaerobic conditions in MRS broth (Biokar Diagnostics, Beauvais, France) at 37 °C for 16 h. Bacteria were harvested by centrifugation at 10,000 g for 10 min. *E. coli* was suspended in PBS to a density of 2×10^7 cfu/ml. Strain CIDCA 133 was suspended in MRS medium with or without glucose (20 g/l). Suspensions containing 4×10^8 cfu/ml lactobacilli were serially diluted in order to obtain different bacterial densities.

Counts of viable microorganisms were performed by plating appropriate dilutions of the bacterial suspensions onto nutrient agar (for strain CM1) or MRS agar (for strain CIDCA 133). Incubations were done at 37 C for 24 h.

2.2. Nitrate and nitrite reductase assays

The nitrate reductase activity was determined by using a diazotization reaction for nitrite with sulphanilic acid and naphthylene diamine (McNamara et al., 1971). Formate was added as reducing substrate because it is known that formic dehydrogenase is closely linked to the nitrate reductase system (Payne, 1973).

Samples were incubated with a mixture of 5 parts of 1% (w/v) sodium nitrate and 3 parts of 1 mol/l sodium formate for two hours at 37 °C. Afterwards, samples were centrifuged 1 min at 14,000 g and 0.1 ml of the supernatants were taken and mixed with 0.1 ml of 1% (w/v) sulphanilic acid and 0.1 ml 0.02% (w/v) *N*-naphthyl-ethylene-diamonium dichloride (both in 1.5 mol/l HCl). The mixtures were allowed to stand 10 min at room temperature for color development and optical density at 540 nm (OD_{540}) was measured in a plate reader (ELISA Plate Reader SLT Rainbow Reader, Wien, Austria).

To assess nitrite reductase activity, ammonia production was tested by a colorimetric adaptation of the Nessler's method (Clescerl et al., 1999). The specific nitrate reductase activity was calculated as the ratio: OD_{540}/log cfu/ml.

2.3. Coincubation experiments

Different concentrations of strain CIDCA 133 suspended in MRS with or without glucose were mixed with an equal volume of the suspension of strain CM1 in PBS. Mixtures were incubated at 37 °C for 1 h. Afterwards, the nitrate–formate solution was added and further incubation for 2 h at 37 °C was performed. Nitrite concentration was determined as indicated above in the supernatant after centrifugation. No nitrate reductase activity was detected in strain CIDCA 133.

Another set of experiments was performed with dead lactobacilli. To this end, bacterial suspensions were irradiated with an UV germicidal lamp before the experiments. After this treatment, viable counts were below 10^2 cfu/ml. This represents more than 6-log reduction of viability.

Bacteria-free supernatants of strain CIDCA 133 were obtained after incubation of 4×10^8 cfu/ml of lactobacilli in MRS without glucose during 3 h. Afterwards, suspensions were centrifuged at 10,000 ×*g* for 10 min and filtered by 0.45 μm Millipore membrane.

2.4. Determination of organic acids

Organic acid concentration of spent cultures of CIDCA 133 incubated at 37 °C for 3 h in MRS with and without glucose was

determined by HPLC. Acid separation was performed with an AMINEX HPX-87H ion exchange column (Biorad Laboratories. Richmond, California); and organic acids were detected with a wavelength detector at 214 nm (Waters 996, Millipore). Acid identification was based on matching the retention times with standard acids. Ten milliliter samples were added to 40 ml of 0.01 N H_2SO_4, shaken for one hour, and centrifuged at 14,000 ×*g* for 10 min. The resulting supernatants were filtered through a 0.45 μm membrane filter (Millipore). Ten microliters of the resulting filtrate was injected in the chromatograph (Waters 716, Millipore). Analyses were performed at a flow rate of 0.7 ml/min at 60 °C by using 0.009 N H_2SO_4 as the mobile phase. HPLC grade reagents were used as standard acids. Solvents were degassed under vacuum.

2.5. Statistics

The effect of the different conditions on the nitrate reductase activity was assessed by ANOVA and two tailed paired Student's *t* test with equal variances. Analysis was performed with SYSTAT software (SYSTAT, Inc., Evanston, Ill.).

3. Results

When *E. coli* CM1 was incubated at 37 °C for 3 h with strain CIDCA 133, a significant reduction of the specific nitrate reductase activity of *E. coli* was observed (Fig. 1A). This effect was dependent on the lactobacilli concentration. In MRS with glucose, the drop of the enzymatic activity correlated with the decrease in pH that, in turn, depends on the concentration of lactobacilli added (Fig. 1B, filled symbols). When no glucose was present in the culture medium, pH values remained almost unchanged irrespective of the concentration of lactobacilli (Fig. 1B, open symbols). However, still an effect on the nitrate reductase activity was observed (Fig. 1A, open symbols). It is worth to note that in MRS without glucose, no effects on the viability of *E. coli* were observed. In contrast, in MRS with glucose both the viability of *E. coli* and pH decreased (Fig. 1C).

Above results prompted us to perform further experiments in MRS without glucose. As shown in Fig. 2, viability of the lactobacilli was necessary for reducing nitrate reductase activity. Indeed, whereas only 20% of the enzymatic activity was measured in the presence of 10^8 viable lactobacilli per ml, the activity remained almost unchanged in the presence of 10^8 UV-killed lactobacilli. Interestingly, no effects were detected when experiments were performed with live *L. delbrueckii* subsp *bulgaricus* (Fig. 2).

To assess the need for the presence of bacterial cells to decrease the nitrate reductase activity, filtered spent culture supernatants were used. To mimic the conditions of the assays performed in the presence of lactobacilli, these filtrates were obtained from bacterial suspensions of different concentrations incubated for 3 h at 37 °C in MRS without glucose. When assays were conducted with these spent culture supernatants, the effect on the enzymatic activity was significantly reduced ($p<0.05$; Fig. 2).

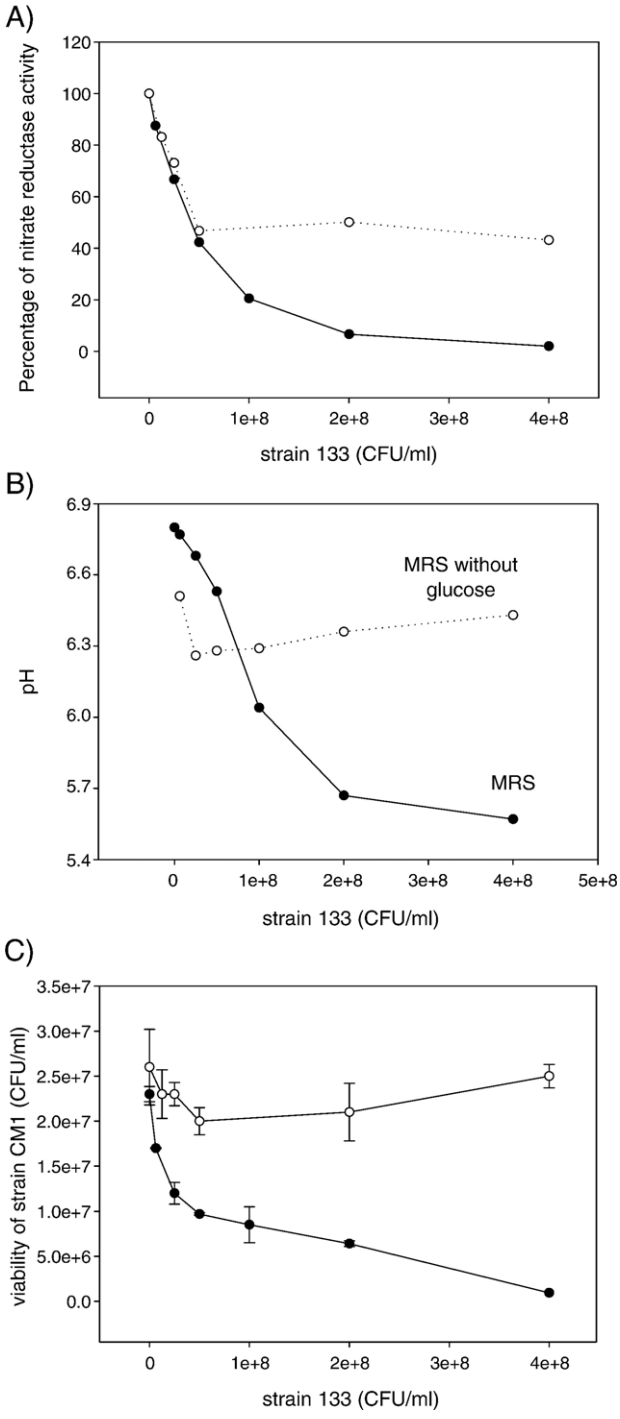

Fig. 1. Percentage of specific nitrate reductase activity (A), pH values of the suspensions (B) and viability (C) of *Escherichia coli* strain CM1 coincubated with *Lactobacillus delbrueckii* subsp *lactis* strain CIDCA 133 in MRS with (●) or without (○) glucose. Determinations were done after 1 h incubation of the bacterial suspensions at 37 °C and 2 h incubation with the nitrate–formate solution. Values of enzymatic activity were referred to the absorbance values of the reaction for nitrite detection when no lactobacilli were added (100%). Initial concentration of *Escherichia coli* was 2×10^7 cfu/ml. The concentrations of lactobacilli are indicated in the abscise. Results are means of two independent experiments. Error bars represent standard deviation.

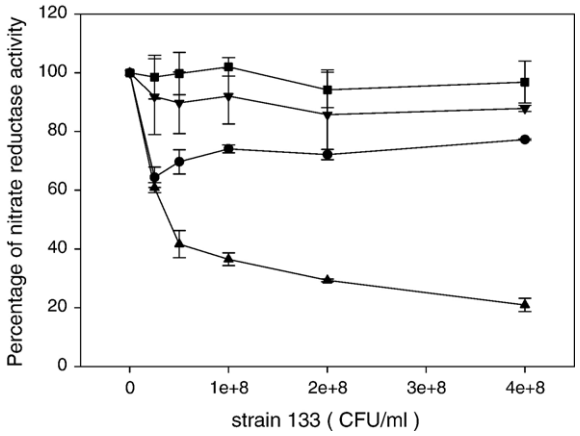

Fig. 2. Effect of different treatments on the nitrate reductase activity of *Escherichia coli* CM1. Strain CM1 was coincubated in MRS without glucose with: strain CIDCA 133 untreated (▲), CIDCA 133 killed by UV irradiation (•), cell free spent culture supernatant of CIDCA 133 (●), *Lactobacillus delbrueckii* subsp. *bulgaricus* strain LBB (▼). Results are means of two independent experiments. Error bars represent standard deviation. In the case of spent culture supernatants of strain 133, the number of bacteria the suspensions were prepared from, are indicated in the abscissa.

In *E. coli*, nitrate reduction to nitrite is coupled with nitrite reduction to ammonia through nitrite reductase activity. Therefore, to assess the possible contribution of the nitrite reductase activity in the decrease of nitrite concentration, we determined ammonia levels. As shown in Fig. 3, ammonia concentrations diminished from around 3.5 to 2 mM when 4×10^8 cfu/ml of strain CIDCA 133 was added. It is important to note, that no nitrite reductase activity was detected in strain CIDCA 133 (data not shown).

As shown in Fig. 4A, lactic acid added to MRS at the final concentration of 190 mM and a pH of 5.28±0.02 was equivalent to that found in the spent culture of strain CIDCA 133 incubated at 37 °C for 3 h in MRS with glucose. This treatment produced a significant reduction of the nitrate reductase activity in comparison to the control without lactobacilli. In addition, a significant difference is observed between organic acid action and CIDCA 133 suspension. In contrast, lactic acid at the concentration (4 mM) and pH (6.20± 0.02), obtained after incubation in the same conditions as above in MRS without glucose, does not show any reduction in the enzymatic activity (Fig. 4B).

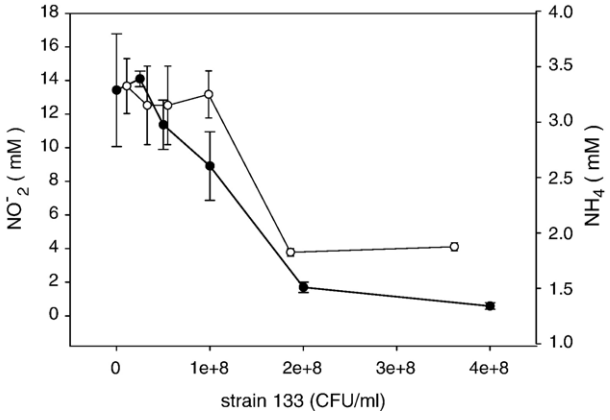

Fig. 3. Effect of *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 on nitrite (●) and ammonia (○) production by *Escherichia coli* CM1. Results are means of two independent experiments and bars represent standard deviation.

4. Discussion

Many intestinal microorganisms are able to exert beneficial effects on the host (Naidu et al., 1999). These probiotic effects could be related to the displacement or inhibition of pathogenic microorganisms, modulation of the mucosal immune response and improvement of the intestinal enzymatic activity. Increasing scientific evidence accounts for some probable mechanisms involved in such capabilities (Pérez et al., 2001; Blum et al., 1999; Liévin-Le Moal et al., 2002; Perdigón et al., 2002; Benyacoub et al., 2003).

The effect of beneficial microorganisms on the intestinal enzymatic activity has been so far ascribed either to the displacement or inhibition of microorganisms (Gudiel-Urbano

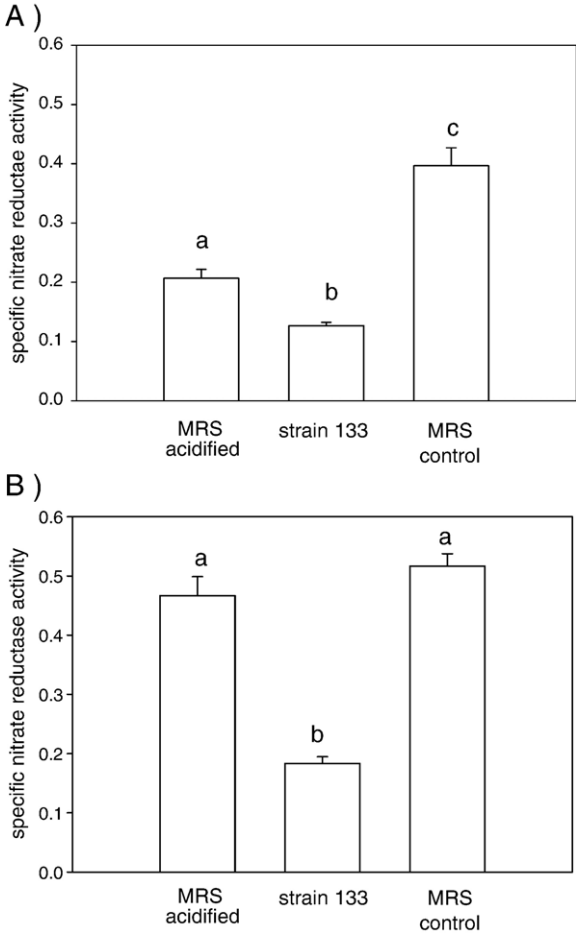

Fig. 4. Comparison of specific nitrate reductase activity of *Escherichia coli* CM1 in the presence of strain CIDCA 133 and lactic acid. A) Coincubation experiment performed in MRS with glucose. B) Coincubation experiment performed in MRS without glucose. The amount of lactic acid added was equivalent to the acid produced by the strain CIDCA 133 (4×10^8 cfu/ml) during 3 h at 37 °C in MRS with glucose (190 mM lactic acid) and MRS without glucose (4 mM lactic acid) determined by HPLC. Different letters indicate significant differences at $p \le 0.05$ (Student's test).

and Goñi, 2002; McBain and MacFarlane, 2001). In the present paper we show for the first time that a potentially probiotic *Lactobacillus* is able to antagonize the nitrate reductase activity of a non-pathogenic strain of *E. coli*. This gram negative microorganism was chosen as a paradigm of a non-pathogenic intestinal microorganism that could lead to harmful effects through their metabolic activity.

The anti-nitrate reductase effect was also observed when a medium without glucose was employed. Since in these conditions acidification was prevented (Fig. 1A), these findings demonstrate that the effect on the nitrate reductase activity can be ascribed to factors other than low pH. In addition, presence of viable lactobacilli is necessary for maximal inhibition of the anti-nitrate reductase activity (Figs. 1, 2 and 4).

In parallel with the diminution of nitrite production, we found a decrease in ammonia concentrations (Fig. 3). These findings make sense because nitrite is the substratum for nitrite reductase. Since ammonia concentration decreases together to the nitrite concentration, we can conclude that the reduction in the concentration of nitrite is not due to an increase in the nitrite reductase activity.

In *E. coli* there are three genetically and biochemically distinct nitrate reductases. Two of them are membrane-bound and they are named nitrate reductase A (NRA) and nitrate reductase Z (NRZ). The third is a periplasmic enzyme (Nap) (Richardson and Watmough, 1999).

Synthesis of NRA is activated during anaerobic growth in the presence of nitrate. On the other hand, NRZ is expressed at very low levels and its synthesis is unaffected neither by oxygen nor nitrate. Nap is effective in scavenging low concentrations of nitrate whereas NRA favors more rapid and efficient growth when nitrate is available in excess (Potter et al., 1999).

Electron transport related to nitrate reduction is coupled to the generation of a transmembrane proton motive force (Richardson and Watmough, 1999). We could hypothesize that exocellular factors produced by strain CIDCA 133 modify chemical composition of the periplasm of *E. coli* thus interfering in the nitrate reductase activity. In addition, need for the presence of lactobacilli for maximal anti-nitrate reductase activity suggests the possibility of a direct transfer of some substances from the lactobacilli to *E. coli*. Interestingly, not all the lactobacilli were able to antagonize nitrate reductase activity. Indeed, *L. delbrueckii* subsp. *bulgaricus* strain LBB did not modify the nitrate reductase activity of *E. coli* (Fig. 2).

Results shown in Figs. 1 and 4 indicate that lactic acid at high concentration (190 mM) contributes to the reduction of the enzymatic activity. However, other metabolites not yet identified could be also involved in this effect since a lower nitrate reductase activity was observed when lactobacilli suspensions (Fig. 4) were used instead of acidified culture medium.

Even though our interpretation of the effect observed is merely speculative, the ability of some lactobacilli to antagonize nitrate reductase activity of *E. coli* seems to be an important finding to better understand the interaction between bacteria in the context of intestinal homeostasis.

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