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In Vitro Inhibition of 4-Nitroquinoline-1-Oxide Genotoxicity by Probiotic *Lactobacillus rhamnosus* IMC501

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Inhibition of 4-nitroquinoline-1-oxide (4-NQO) genotoxicity by a probiotic strain of *Lactobacillus rhamnosus* (IMC501) was assessed by the prokaryotic short-term bioassay SOS-Chromotest, using *Escherichia coli* PQ37 as the target organism. Results showed the ability of strain IMC501 to rapidly and markedly counteract, *in vitro*, the DNA damage originated by the considered genotoxin. The inhibition was associated with a spectroscopic hypsochromic shift of the original 4-NQO profile and progressive absorbance increase of a new peak. IR-Raman and GC-MS analyses confirmed the disappearance of 4-NQO after contact with the microorganism, showing also the absence of any genotoxic molecule potentially available for metabolic activation (*i.e.*, 4-hydroxyaminoquinoline-1-oxide and 4-nitrosoquinoline-1-oxide). Furthermore, we have shown the presence of the phenyl-quinoline and its isomers as major non-genotoxic conversion products, which led to the hypothesis of a possible pattern of molecular transformation. These findings increase knowledge on lactobacilli physiology and contribute to the further consideration of antigenotoxicity as a nonconventional functional property of particular probiotic strains.

Keywords: Probiotics, *Lactobacillus rhamnosus*, antigenotoxicity, 4-nitroquinoline-1-oxide

Introduction

The 4-nitroquinoline-1-oxide (4-NQO) is a heterocyclic aromatic compound with high toxicity and carcinogenic properties. In relation to genotoxicity and chemical stability, this nitroarene has been frequently used as a model compound for *in vitro* toxicogenetic studies and experimentally induced oral cancerogenesis in animals [21, 24, 38]. Currently, the genotoxicity of nitroarenes has also polarized attention regarding their potential presence on the environment [22]. The most common damages caused to DNA by 4-NQO are transversion from guanine to pyrimidine [16] and dimerization of contiguous thymines [14]. The mutation mechanisms include reduction of the NO_2 group and consequent conversion of 4-NQO into 4-hydroxyaminoquinoline-1-oxide (4-HAQO) commonly operated in eukaryotic systems by hepatic nitroreductases [2]. 4-HAQO is then acetylated and forms adducts at N^2 and C8 of guanine and N^6 of adenine [26]. An alternative activation relates to the formation of 4-nitrosoquinoline-N-oxide (4-NOQO) [15].

Various microorganisms, namely bacteria and yeasts, are able to inhibit potent mutagens, in particular 4-NQO, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), heterocyclic aromatic amines (*i.e.*, IQ, MeIQ, Trp-1, and Trp-2), and aflatoxins (AFB_1) [1, 9, 18, 28, 36, 39]. Lactic acid bacteria from dairy products have been mainly investigated as model systems for their ability to prevent mutations by MNNG [17, 25] and 1,2-dimethylhydrazine (DMH) [25, 35] in animals. Microbial antigenotoxicity may therefore be attractive in protecting colonocytes from risks (*i.e.*, food and endogenous mutagens) in the intestinal tract.

In this context, our previous studies reported the ability of probiotic spore-forming bacilli [4, 7], of some lactobacilli strains isolated from dairy products [5, 8], and some food-borne yeasts [32] to counteract the biological activity of 4-NQO when microorganisms were co-incubated with this genotoxin. We have also shown, using two short-term methods, namely SOS-Chromotest and Comet assay, that the loss of genotoxicity in cell-free supernatants was not

related to 4-NQO adsorption to the cellular matrix but to cell viability, as cell thermal inactivation led to disappearance of the antigenotoxic activity [6, 8, 10, 32]. Genotoxicity inhibition was always associated to spectroscopic changes of 4-NQO, with strain-dependent behavior. There is, however, little information about the chemical-biological interactions involved.

The aim of this study was to increase knowledge concerning the inhibition of 4-NQO genotoxicity by lactobacilli and to investigate the mechanisms involved. The main expected outcome was to identify the eventual products of 4-NQO bioconversion through GC-MS spectrometry and IR-Raman analysis. To this end, a probiotic strain of *Lactobacillus rhamnosus* (IMC501), previously characterized for its protective effects against genotoxic damage induced in mice by the food mutagen 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) [13], was used.

Materials and Methods

Probiotic Microorganism and Culture Media

Lactobacillus rhamnosus IMC501, a probiotic strain used as ingredient of functional food and dietary supplements [33], was kindly supplied by Synbiotec srl (Camerino, MC, Italy). The strain is also present in Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany) as DSM 16104. IMC501 was grown on MRS broth medium (Oxoid Ltd, Basingstoke, UK) at 37°C and viability after genotoxin co-incubation was measured by plate count in MRS agar medium (Oxoid).

Chemicals

The genotoxin 4-nitroquinoline-1-oxide (4-NQO; CAS No. 56-57-5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution (1 mg/ml) in dimethyl sulfoxide was prepared and stored at −20°C. The working solution was obtained in saline just before testing. The reagents *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-phosphate (PNPP), and dichloromethane were provided by Sigma-Aldrich. All chemicals were of the highest purity grade available.

Genotoxin-Cell Co-Incubation

IMC501 was grown overnight and harvested by centrifugation (6,000 ×*g*, 15 min). The pellet was washed with saline (0.85% NaCl) and finally resuspended in saline (10^9 cells/ml). Lactobacilli and 4-NQO (0.1 mM) mixtures were co-incubated for 150 min at 37°C under agitation (200 rpm) in the dark. The genotoxin dose was chosen to induce DNA damage but avoid cytotoxicity, as previously reported [4]. Co-incubation experiments were carried out also with heat-killed cells (100°C; 15 min). The supernatant was recovered by centrifugation and 0.45 μm membrane filtration (Sartorius AG, Göttingen, Germany) and tested for residual genotoxicity and chemical analyses.

Genotoxicity Evaluation

SOS-Chromotest, a primary DNA damage assay on prokaryotic cells, was performed using *Escherichia coli* PQ37 supplied by the Institute Pasteur (Paris, France). In the presence of DNA damage, PQ37 activates the SOS-response that, due to a *sfi*A::*lac*Z fusion, induces β-galactosidase activity [27]. The SOS induction factor was defined as the β-galactosidase to alkaline phosphatase (constitutive) ratio of sample under analysis, divided by the same ratio of negative control (without genotoxin). The enzyme activities were detected colorimetrically using ONPG and PNPP substrates. Experimental details are given in a previous work [4].

Ultraviolet-Visible Spectroscopy

Analyses were performed with a UV-1204 spectrophotometer (Shimadzu, Kyoto, Japan) over the wavelength range of 300–440 nm.

IR-Raman Spectroscopy

A Coherent Innova90 C-series Ion laser, using monochromatic green laser light (λ = 514 nm) incident on the sample, was used. The sample emission was detected with an IR spectrometer (Bruker IFS28; Bruker Optik GmBH, Ettingen, Germany). Before laser analysis, the samples (culture supernatant and analytical white) were lyophilized and mixed with KBr powder.

Gas Chromatography-Mass Spectrometry (GC-MS) Analyses

Solid phase micro-extraction (SPME) was performed by a polymeric-double fiber containing dimethyl-siloxane and divinyl-benzene (PDMS-DVB, 65 mm thickness) deposited over a silica structure conditioned at 250°C. The fiber was immerged in the sample (30 min, 20°C) and then thermally desorbed at 250°C for 2 min inside the GC inlet.

Liquid/liquid extraction was performed on supernatants of 4-NQO-treated cell suspensions (5 ml), three times with dichloromethane (5 ml each time). The fractions were then collected, dried by mild stream of ultra-pure nitrogen, and then dissolved in dichloromethane (100 μl).

Analyses were performed using a gas-chromatographer coupled with a mass spectrometer. The GC was a Varian-Chromopack CP3800 (Varian, Palo Alto, CA, USA) with capillary column Factor Four VF-5ms, 30 × 0,25 mm ID, with stationary phase film thickness of 0.25 μm and with 5 m of deactivated fused silica without stationary phase pre-column EZ-guard (Chromopack, Middleburg, Netherlands). The MS was an Ion-trap detector (ITD-MS) Saturn 2000 MS-MS (Varian). The analysis of desorbed SPME samples was performed in splitless mode (injector temperature 250°C). The He carrier gas flow rate in the column was set at 0.8 ml/min. The thermal program was 50°C for the first 3 min, an increase of 5°C/min up to 145°C, an increase of 15°C/min up to 250°C, and finally 6 min at 250°C. The spectrometer was set in full-scan mode with ionization source electron impact (EI +,

10 μA, one scan per second) and acquisition range between 30 and 350 *m/z*. Liquid samples were analyzed by direct injection of 1 μl of sample into the injector heated at 260°C, operating in splitless mode. The constant flow of carrier gas in the column was 1 ml/min, and the temperature program foresaw the increase of 7°C/min from 100°C to 290°C and a stay at 290°C for 20 min. The acquisition was in full-scan mode (EI +, 10 μA), with a scan per second, in the range from 90 to 650 *m/z*.

Statistical Analysis

Cell viability and SOS IF values found before and after IMC501-genotoxin co-incubation were evaluated by the unpaired Student's *t*-test.

Results

Genotoxicity Inhibition

Fig. 1A shows that 4-NQO co-incubation with IMC501 viable cells markedly contrasts the biological activity of the genotoxin. In fact, in the tester strain PQ37 (SOS-Chromotest), the induced expression of β-galactosidase (genotoxicity) was repressed and the constitutive expression of alkaline phosphatase restored after only 15–20 min of co-incubation. In particular, the SOS induction factor (IF) went quickly down to typical values of the negative controls (without genotoxin) in the case of viable cells, whereas IF was not significantly reduced in experiments carried out using previously heat-killed cells (Fig. 1B).

Another important consideration is that the dose of genotoxin used did not significantly affect the viability of IMC501. In fact, viable cell counts passed from 5.61 ± 2.64 × 10^9 at the beginning of the incubation to 2.91 ± 1.34 × 10^9 after 150 min (t = 0.904; df = 4; p = 0.417).

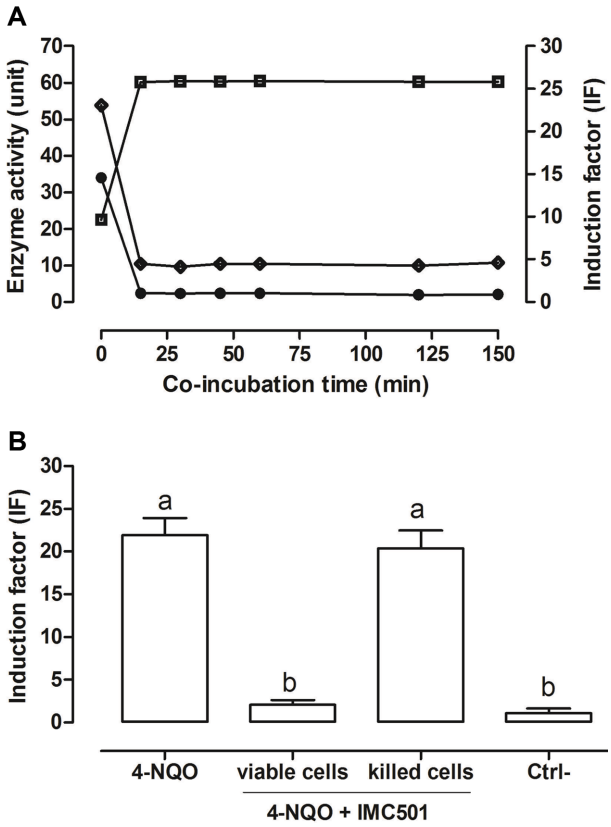

Fig. 1. Genotoxicity inhibition.
(**A**) Time course of 4-NQO genotoxicity during co-incubation with IMC501 viable cells. SOS-Chromotest parameters determined with PQ37: β-galactosidase activity (◆), alkaline phosphatase activity (■), SOS induction factor (●); mean values derived from three independent concordant experiments. (**B**) SOS induction factor for positive control (0.1 mM 4-NQO), for 4-NQO after 150 min co-incubation with IMC501 viable or heat-killed cell suspension (10^9 cells/ml), and for negative control (without genotoxin); values shown are the means (± SD) from three independent experiments. The bars marked with a and b differ from each other ($p < 0.01$ by *t*-test).

Genotoxin Modification

Spectrophotometry of cell-free filtrates showed that the antigenotoxic activity of IMC501 was associated to the modification of the absorption profile. In fact, following exposure to the bacteria, the original 4-NQO peak of maximum absorption switched from 364 to 353 nm. The hypsochromic shift was associated with a progressive increase in absorbance of the new peak (Fig. 2), demonstrating a gradual formation of a new molecular interaction complex. Co-incubation with heat-killed (100°C for 15 min) lactobacilli did not change the 4-NQO spectrum.

IR-spectroscopy confirmed the molecular changes produced by IMC501 on the genotoxin (Fig. 3). In particular, the spectrum observed before exposure to lactobacilli showed signals related to 4-NQO (stretching and bending of N=O group; bonds C-NO_2, C=C, and C-N of aromatic groups), whereas after co-incubation, with the exception of the C-C aromatic bonds, the above signals were not present. The missing signals, located between 1,450 and 1,780 nm, were related to removal of the functional group -NO_2, suggesting that the chemical transformations that prevent genotoxicity did not involve the quinoline skeleton of 4-NQO. Finally, the IR spectrum did not show any new characterizing signal related to a new functional group.

The SPME GC-MS analysis performed at time zero on the cell-free supernatant of genotoxin-lactobacilli suspension showed a profile with two chromatographic peaks at 25 and 28 min of retention time, respectively (Fig. 4A). The mass spectra of peak 1 corresponded to 4-nitroquinoline (4-

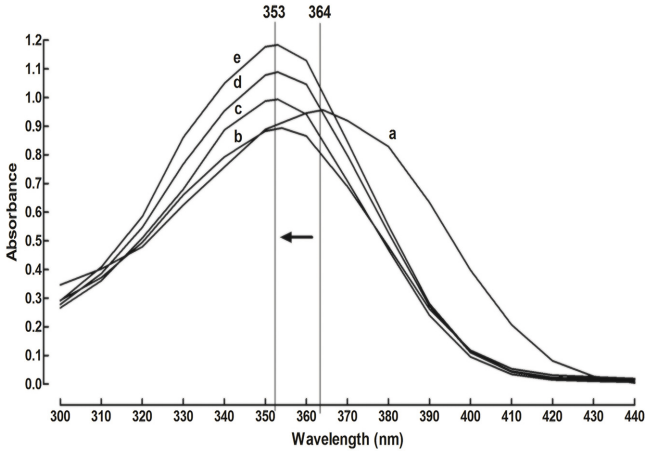

Fig. 2. UV-visible spectra of 4-NQO before (a) and after 30 (b), 60 (c), 120 (d), and 150 (e) min of co-incubation with IMC501 viable cells.

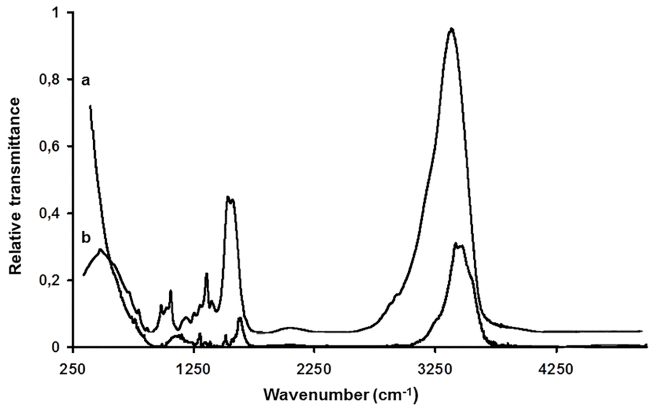

Fig. 3. IR-Raman spectra of aqueous solution of 4-NQO before (a) and after (b) 150 min of co-incubation with IMC501.

NQ), having diagnostic ions 174 and 128 *m/z*, whereas peak 2 is 4-NQO, with 160 and 190 *m/z* molecular ions. 4-NQ was an artifact originated from thermal shock during injection, and its presence is due to loss of oxygen from the molecule. The analysis performed on IMC501 co-incubation supernatant highlighted the disappearance of both peaks (Fig. 4B).

The GC-MS profile obtained after extraction with dichloromethane partition revealed a cluster of seven chromatographic peaks with different intensities and the related mass spectra (Fig. 5). A semi-quantitative analysis of the cluster was compatible with the initial concentration of 4-NQO.

Discussion

In this study, 4-NQO was chosen as a model genotoxin owing to its high carcinogenicity and molecular stability, as well as the effectiveness on toxicogenetic studies, through production of charge transfer DNA adducts [23] and its direct role in SOS-Chromotest [12].

We confirmed that *L. rhamnosus* IMC501 was effective in the transformation of this genotoxin into inactive compounds. In particular, our data clearly show that the loss of genotoxicity observed by the SOS-Chromotest bioassay was achieved not by a passive and permanent adsorption in the bacterial matrix, different from that reported for other mutagens (*i.e.*, protein pyrolysates [39],

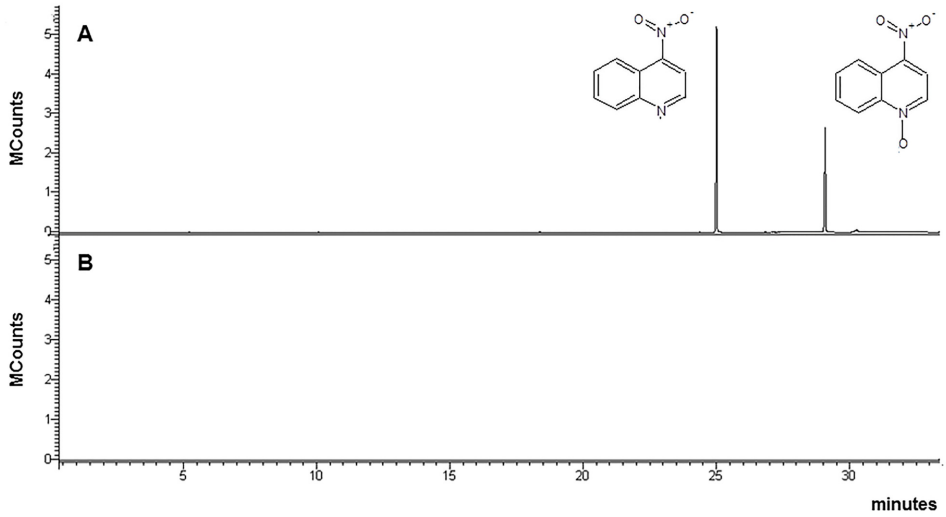

Fig. 4. GC-MS profile before (**A**) and after (**B**) co-incubation of 4NQO (water solution) with IMC501.
4-NQ is a thermal artifact produced during the sample injection on the GC-MS port.

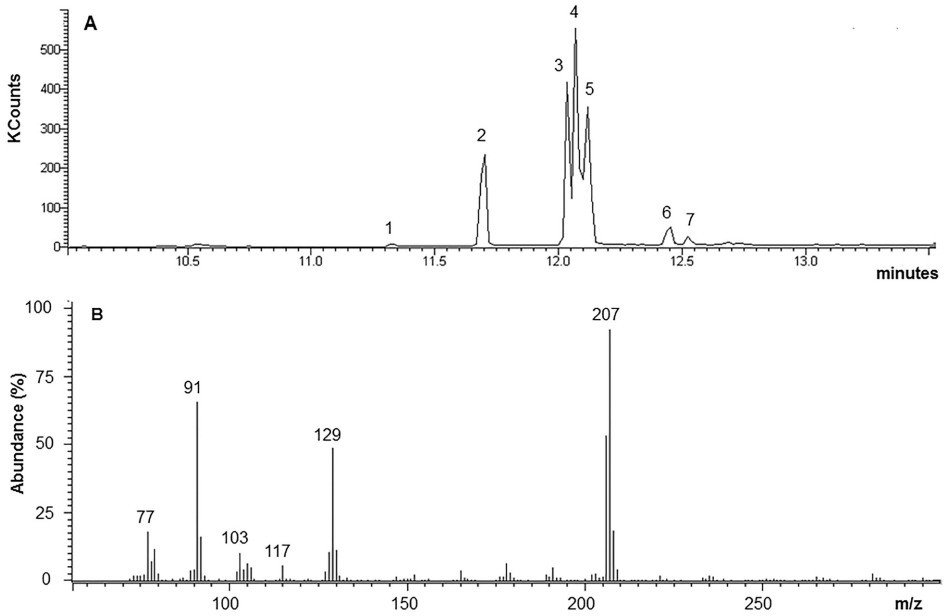

Fig. 5. (**A**) GC-MS profile after liquid/liquid partition with dichloromethane and (**B**) related mass spectra.

heterocyclic amines [29], and aflatoxin B_1 [18, 20]). The result obtained with IMC501 is consistent with findings of Wang *et al*. [37] concerning the antigenotoxic effect of *Lactobacillus salivarius* FD889 against 4-NQO. The observed absence of residual genotoxin in the aqueous phase, after washing of the exposed cells, would explain the lack of genotoxicity after 4-NQO incubation with IMC501 and supports the microbial conversion of the genotoxin.

The specific conversion product we identified, namely phenyl-quinoline (PhQ), is in fact a nonpolar compound, without functional groups on the aromatic rings, as evidenced by IR-Raman spectroscopy, and having no genotoxic properties, consistent with bioassay results obtained in this study. To the best of our knowledge, this is an original finding since the only published evidence on bacterial conversion of 4-NQO to inactive products refers to the formation of 4-aminoquinoline [4, 34]. Moreover, genotoxicity inhibition was obtained in the presence of living cells, whose viability was maintained above 80% during the experiments, but not with heat-inactivated ones, according to that reported by Walia *et al*. [36] for potential probiotic lactobacilli and yeasts. On the contrary, we demonstrated the persistence of 4-NQO when incubated with IMC501 heat-killed cells. In line with our results, other authors showed that synthetic C-2 substituted quinolines (*i.e.*, phenyl, pyridinyl, methyl, and 4-nitrophenyl quinolines) reduced induction of the SOS response, suggesting that these derivatives have reduced genotoxic potential [12].

An important step in 4-NQO conversion by IMC501 may thus be hypothesized as involving either the activity of cellular reductase-like proteins able to reduce the nitro-group [11, 19, 30], or by cellular –SH compounds and/or nucleophilic moieties able to displace 4-NO_2 [3]. The ability of many microorganisms to catalyze the nonspecific reduction of nitroaromatic compounds and formation of aminoderivatives or condensation products is known [31].

A further consideration, emerging from GC-MS study, is the absence of the peak related to 4-NQO. The mass spectra of the seven peaks highlighted in the chromatographic profile showed marked similarity, presenting characteristic ions at 103, 117, and 129 *m/z*, related to the quinoline structure. Furthermore, signals present at 77 and 91 *m/z*, typical of a tropylium ion, are indicative of the presence of a phenyl group in the investigated molecule. The ion at higher mass, 207 *m/z*, was protonated molecular ion $[M+H]^{\circ+}$ of the new compound originated by the co-incubation of 4-NQO with IMC501. Therefore, the mass spectrum is compatible with the molecular structure of PhQ and its possible isomers. The finding of PhQ isomers with a different localization of the phenyl group, characterized by a different degree of polarity, can be related to the particular reactivity of quinoline after the loss of the NO_2 group, as clearly indicated by the presence of the above-mentioned seven peaks in the chromatographic profile. We propose that a possible bioconversion pathway of 4-NQO

by IMC501 might involve the loss of the electrophilic group (NO_2) deactivating the aromatic ring with the acquisition of a less electrophilic and weakly deactivating group (phenyl). The reaction would proceed in two stages, with the formation of an intermediate with aromatic positive charge and possible production of seven isomers with different polarity. Further work, however, is still necessary to detail the bioconversion dynamic in terms of the enzymes involved.

The formation of phenyl-quinoline from 4-NQO, as the main metabolite, can be explained by considering both the microaerophylic conditions of co-incubation with IMC501, compatible with reductive change, and the presence of aromatic amino acids (*i.e.*, phenylalanine and tyrosine) in *L. rhamnosus* as intermediates for the synthesis of pyrimidine, found also in this study. It should also be noted that the fast microbial conversion observed can positively be responsible for the prevention of the metabolic activation that, in eukaryotic organisms, leads to the formation of 4-hydroxyaminoquinoline-1-oxide (4-HAQO) and 4-nitrosoquinoline-1-oxide (4-NOQO) implicated in the formation of DNA adducts [21]. The above compounds were not detected also extending the co-incubation time.

In conclusion, the results of the present *in vitro* study, besides of significance as basic knowledge, provide new information on a possible chemopreventive strain-dependent effect of *L. rhamnosus* IMC501 in the gut. This evidence, although not directly transferable to humans, is in line with the expected efficacy to exert a bioprotective role. For this reason, it may be worthwhile to further investigate IMC501's ability in reducing carcinogenic risk against the potential DNA damage produced by endogenous or food-borne mutagens. The lactobacilli antigenotoxicity can be therefore reasonably considered as a new attractive nonconventional property when strains possessing this characteristic are used as probiotics or included in functional foods.

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