SHORT COMMUNICATION

# INVESTIGATION OF THE ANTIGENOTOXIC PROPERTIES OF THE PROBIOTIC *LACTOBACILLUS RHAMNOSUS* IMC 501® BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A method was set up to study the antigenotoxic property of the probiotic Lactobacillus rhamnosus IMC 501® against 4-nitroquinoline-1-oxide (4-NQO). The method is based on liquid-liquid extraction followed by gas chromatography-mass spectrometry (GC-MS) analysis. The GC-MS analysis was used to determine the quantitative change of 4-NQO before and after co-incubation with Lactobacillus rhamnosus IMC 501®. The results from GC-MS analysis showed that 4-NQO genotoxicity was inhibited and then converted to less toxic compounds. These preliminary results from GC-MS analysis indicate that the method described is able to detect physicochemical modifications of the genotoxic agent, which occur after co-incubation with bacteria cells. The method, therefore, is a novel alternative to biological assays for detecting the antigenotoxicity of lactobacilli.

- Key words: antigenotoxicity, functional foods, GC/MS analysis, Lactobacillus, 4-nitroquinoline-1-oxide, probiotics -

## INTRODUCTION

Food contaminants entering the body through the oral route are directly exposed to the action of the gut microflora. Normal healthy intestinal microflora contains many strains of lactic acid bacteria (LAB), some of which have been identified, and termed probiotic strains with ascribed health benefits (SALMINEN et al., 1998). Probiotic bacteria have generated much scientific and commercial interest as ingredients in several foods. This interest is due to a range of possible health benefits of these bacteria, which include the improvement and balance of intestinal microflora, suppressing enhanced inflammatory responses, immune system stimulation, cholesterol lowering and prevention of cancer recurrence (BURNS and ROWLAND, 2000; MARTEAU, 2001). The protective effect of LAB against food mutagens such as heterocyclic amines, N-nitroso compounds, and aflatoxins has also been reported (ORRHAGE et al., 1994; EL-NEZAMI et al., 2000). Consequently, the antigenotoxicity and antimutagenicity of LABs begin to be considered when characterizing the functional properties of probiotic bacteria. Remarkably, the inhibitory activity of LABs against some genotoxins has been shown for various strains of L. rhamnosus, L. casei, L. plantarum, L. brevis, L. acidophilus and Lactobacillus spp. isolated from dairy and pharmaceutical products or obtained from culture collections (CENCI et al., 2005; CALDINI et al., 2008). In these studies the antigenotoxicity was demonstrated by short-term biological assays (SOS-Chromotest and Comet assay) and was strain-dependent. The mechanism of antimutagenic activities of probiotics is not clearly understood. There is, however, evidence to associate some known modes of activity such as binding of mutagens to microbial cells (ORRHAGE et al., 1994), mutagen conversion or direct inhibition of tumorogenesis by these metabolites (HIRAYAMA and RAFTER, 2000) to explain the mechanism of action. The antigenotoxicity of probiotics as evaluated by short-term biological assays only reflects not more than the relative activity of probiotics, but no information about antigenotoxicity mechanisms could be obtained. In particular, it could not differentiate between the absorption or the conversion of the genotoxin.

The aim of the present work was to set-up a gas chromatography-mass spectrometry method that is able to delineate the mechanisms involved in the LAB inhibition of 4-NQO, a direct-acting agent which produces strand scission and formation of charge-transfer adducts on DNA. Specifically, the 4-NQO antigenotoxicity of a LAB probiotic strain (Lactobacillus rhamnosus IMC 501®) revealed by short-term biological assays (data not shown), was evaluated by GC-MS analysis.

## MATERIALS AND METHODS

### Bacterial strain

Lactobacillus rhamnosus IMC 501® strain was used in this study. The bacterial strain was isolated in our laboratory from faecal samples of elderly Italian subjects during an EU project named Crownalife, as previously described (SILVI et al., 2003; MUELLER et al., 2006). L. rhamnosus IMC 501® has been studied for its probiotic properties such as gastro-intestinal transit (acid and bile tolerance), in vitro adhesion to cell line, antimicrobial activity, antibiotic susceptibility, plasmid profile and in vivo colonization and persistence in the human gut. The bacterial strain was phenotypically and genotypically characterized and deposited in the culture collection Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) with number DSM 16104 and it was assigned Italian patent no. RM2004A000166 (VERDENELLI et al., 2009).

### Viability

The viable number of L. rhamnosus IMC 501® after co-incubation with 4-NQO was determined by plate count on MRS agar (Oxoid Ltd, Basingstoke, Hampshire, UK) under aerobic incubation conditions. The experiment was conducted in triplicate.

### Chemicals

The genotoxin, 4-nitroquinoline-1-oxide, was obtained from Sigma (CAS no. 56-57-5, Sigma-Aldrich, St. Louis, MO, USA). Stock solution (1 mg/mL) was prepared in dimethyl sulphoxide (DMSO) and aliquots were stored at -20°C, until the test. Working solutions were obtained with suitable dilution in saline.

### Cell preparation and genotoxin-cell co-incubation

Cultures were grown overnight in L-broth at 37°C and harvested by centrifugation (6,000 g for 15 min). The pellet was resuspended in physiological saline ($10^8$-$10^9$ CFU/mL) and 4-NQO was added at a final concentration of 0.1 mM. Co-incubation was carried out at 37°C for 15, 30 and 135 min under agitation. The supernatant was recovered by centrifugation followed by filtration through a 0.45 μm membrane filter (Sartorius AG, Göttingen, Germany).

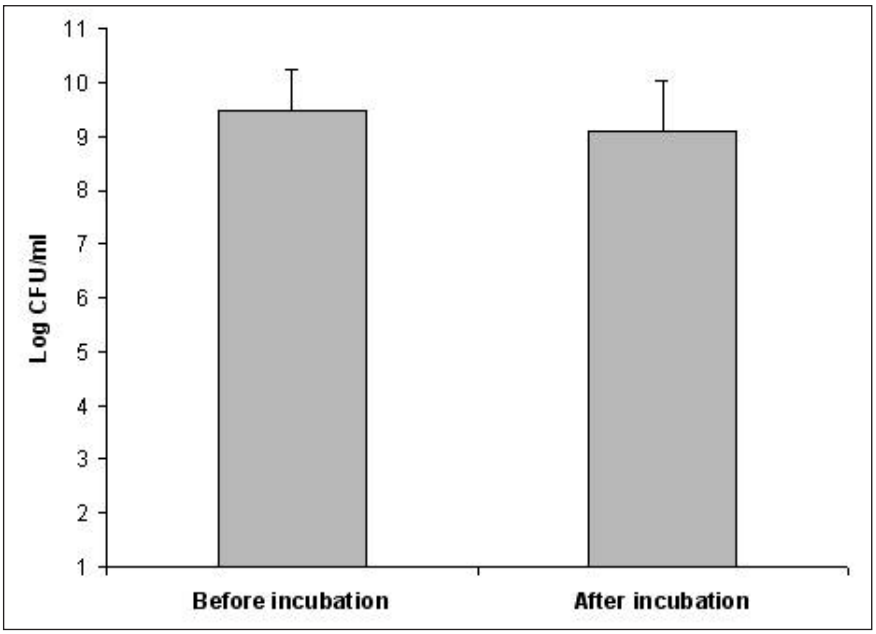


Fig. 1 - The bacterial count of Lactobacillus rhamnosus IMC 501® after co-incubation with 0.1 mM 4-NQO at 37°C for 135 min. Mean values ± standard deviation of three measurements.

Physicochemical analyses

Supernatants of 4-NQO-treated cultures were examined by both absorbance profiles (Shimadzu UV-1204 spectrophotometer, Columbia, MD, USA) and gas chromatography/mass spectrometry.

Fifty mL of 4-NQO-treated culture supernatant was extracted three times in a separatory funnel with dichloromethane by using 15, 15 and 20 mL, respectively. The solvent was evaporated under vacuum and dried samples were re-dissolved in 1 mL of dichloromethane. The GC-MS analyses were performed on a gas chromatography (Agilent Technologies 6890N, Santa Clara, CA, USA) coupled to a mass spectrometer (Agilent Technologies 5973N). A HP-5MS Agilent Techonologies (30 m - 0.25 mm I.D. - 0.1 um film) column was used with helium as carrier gas (flow rate 1 mL/min). The sample (2.0 μL) was injected in the splitless mode at 250°C (purge time 2.0 min). The GC oven was operated with the following temperature program: initial temperature 70°C held for 3 min, increased to 320°C at 30°C/min and held at 320°C for 10 min. The ionizing energy was 70 eV and all data were obtained by collecting full-scan mass spectra at a scan range of 29-350 amu. The compounds were identified with NIST 08 mass spectra library using the chemStation Software.

Statistical analysis

The mean values and the standard deviation were calculated from the data obtained in three separate experiments. A Student t-test was used to determine the significance of differences in bacterial counts before and after genotoxin-cell co-incubation.

## RESULTS

Survival of probiotic bacteria in the presence of 4-NQO

It was observed that the viability of the probiotic bacteria, L. rhamnosus IMC 501®, remained comparatively high after co-incubation with the genotoxin. In fact, cell survival remained above 90% (Fig. 1) and there were no significant differences.

Spectroscopic characteristics of 4-NQO before and after cell co-incubation

Bacteria-mutagen co-incubation was accompanied by modifications in the spectroscopic characteristics of the supernatants. Representative absorbance profiles indicate that the maximum absorbance peak of 4-NQO was shifted to a short wavelength (hypsochromic shift), compared to the normality typical of the compound (Fig. 2).

Fig. 3 illustrates mass spectral data from supernatants of the strain L. rhamnosus IMC 501® treated with 4-NQO at different incubation times. The spectra showed the gradual disappearance of 4-NQO and the appearance of 4-aminoquinoline and 4-nitroquinoline at 15 min as depicted in the standards shown in Fig. 4. The concentration of these two products also showed a gradual reduction at 30 and 135 min. Fig. 5 shows the decrease of 4-NQO peak

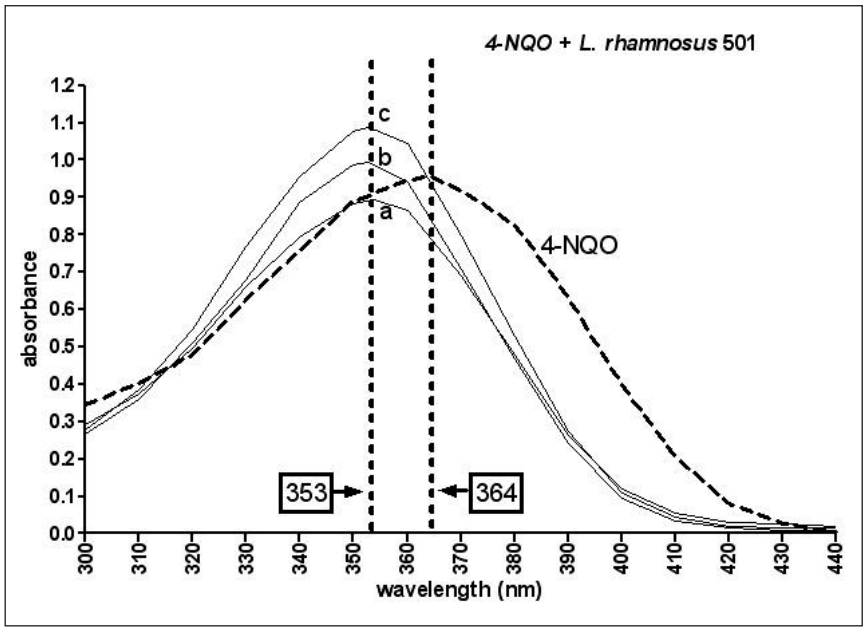


Fig. 2 - Representative absorbance profiles of 4-NQO without and with Lactobacillus rhamnosus IMC 501® co-incubation at different times. (a) 15 min, (b) 30 min, (c) 135 min. The dashed profile represents genotoxin spectrum without bacteria co-incubation.

areas at different times of genotoxin-cell co-incubation.

## CONCLUSIONS

The data from the study of the co-incubation of L. rhamnosus IMC 501® with the reference genotoxin 4-NQO shows that this probiotic bacterial strain has potential inhibitory activity against this genotoxin as revealed by GC mass spectrometry. Lactic acid bacteria have been reported to have antimutagenic/anticarcinogenic properties in vitro and in vivo. The mechanisms for such effects have been hypothesized to include the physical binding of the mutagenic compounds to the bacteria, genotoxin bioconversion or conjugation (BURNS and ROWLAND, 2000; COMMANE et al., 2005). The GC-MS protocol set-up in this study has demonstrated the inhibition of 4-NQO genotoxicity by the strain L. rhamnosus IMC 501®, revealing physicochemical modifications of the genotoxic agent that originated from bacterial preincubation. In fact, the spectra show appearance of 4-amino-quinoline as a bioconversion product, which is a widely known inactive compound. First of all, the modification seen in the absorbance profile of 4-NQO after co-incu-

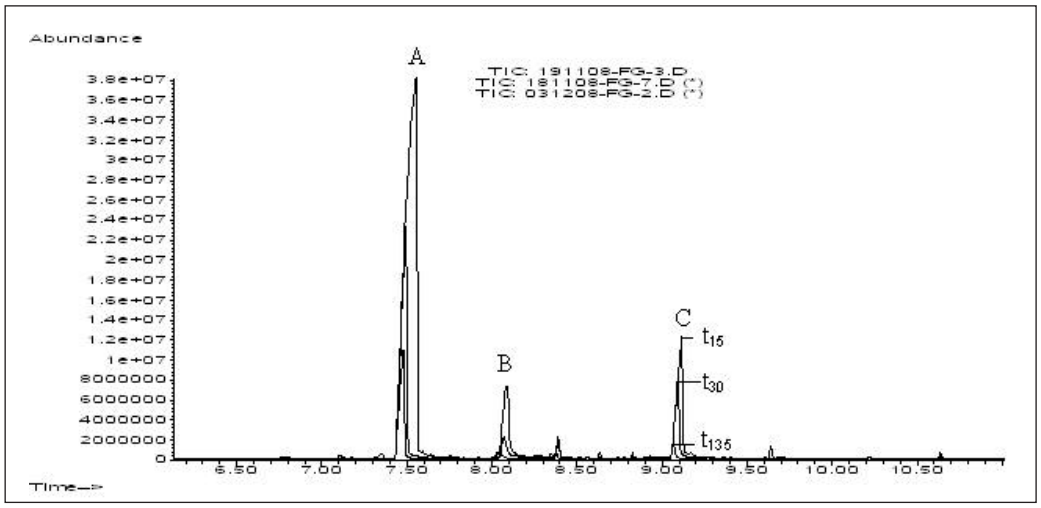


Fig. 3 - Mass spectra of supernatants from the Lactobacillus rhamnosus IMC 501® strain co-incubated with 4-NQO for different times of incubation (15, 30 and 135 min). A, 4-nitroquinoline; B, 4-aminoquinoline; C, 4-nitroquinoline-1-oxide.

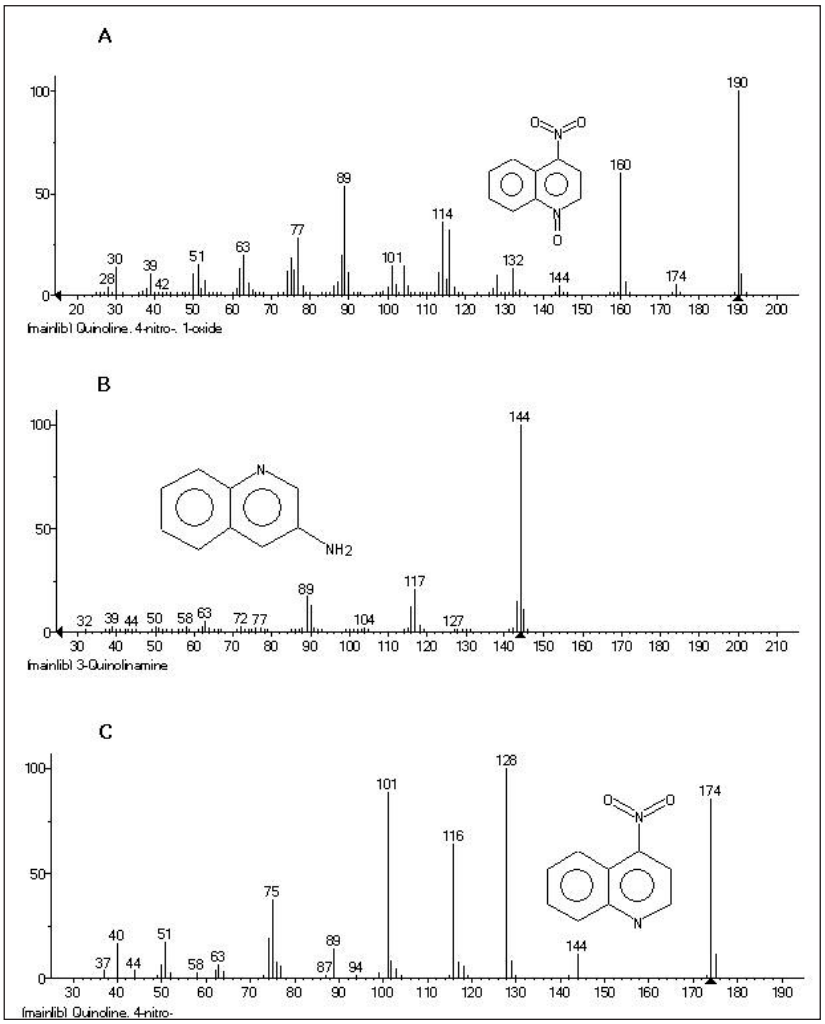


Fig. 4 - Mass spectra of 4-nitroquinoline-1-oxide (A), 4-aminoquinoline (B) and 4-nitroquinoline (C).

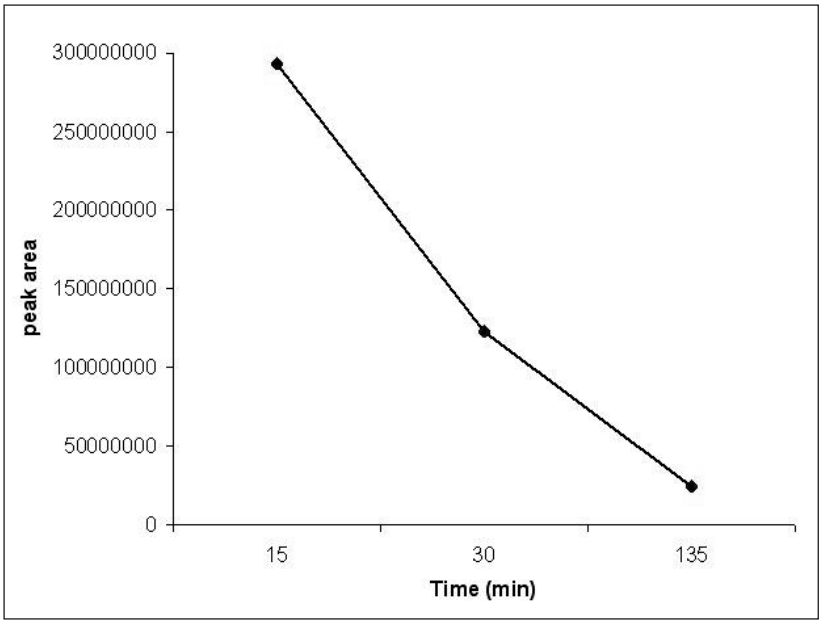


Fig. 5 - Peak area reduction of 4-nitroquinoline-1-oxide after 15, 30 and 135 min of co-incubation with Lactobacillus rhamnosus IMC 501®.

bation with L. rhamnosus IMC 501® proves the involvement of chemico-biological interactions. It is notable that the antigenotoxicity is a strain-dependent characteristic as demonstrated by the observable spectroscopic modification profile of the genotoxins during co-incubation with different bacterial strains (CORSETTI et al., 2008). The results obtained in this study are in accordance with those of CALDINI et al. (2002) who demonstrated a blue shift in the maximum absorbance wavelength of 4-NQO after co-incubation with Bacillus strains. Moreover, the GC-MS method was able to monitor the disappearance of the toxic agent and the appearance of other possible metabolic products, as also shown by other authors (CALDINI et al., 2002). In this study GC-MS spectra showed that the antigenotoxic process was accompanied by a decrease in the content of 4-NQO and a simultaneous appearance of a new product peak. It could be deduced from the GC-MS results that the mechanism of antigenotoxicity for L. rhamnosus IMC 501® was the conversion of 4-NQO to an inactive end product, and not the simple absorption or binding onto cell wall components. In this context, it is interesting to highlight that the inhibition of the tested genotoxin was related to the maintenance of cell viability after co-incubation. This fact, together with the capability of resistance to gastrointestinal stress by L. rhamnosus IMC 501® suggests the possibility of using this probiotic strain as an ingredient in functional foods where it could serve as a source of protection against genotoxic risk in the human gut. These results are of considerable interest with the increasing demand for functional foods, especially dairy products, such as yoghurts and fermented milks, containing Lactobacillus and Bifidobacterium. Results suggest that GC-MS may be considered an excellent methodology for revealing genotoxin deactivation by probiotic bacteria. The GC-

MS analysis is in fact applicable to different genotoxic compounds which may be present in foods or originate from cooking (exogenous genotoxins) and/or be produced by host metabolism (endogenous genotoxins). Finally, with regard to the microorganism used, the results of this in vitro study suggest that the incorporation of L. rhamnosus IMC 501® in the diet might be able to suppress or reduce the genotoxic activity of potentially harmful compounds. In this context it is important to emphasize the excellent tolerance to gastro-intestinal transit demonstrated by this microorganism (VERDENELLI et al., 2009). The evidence from the above-described in vitro approach, although not directly transferable to humans, is in line with the hypothesised efficacy of probiotics in providing a protective effect against genotoxins in the gut. Further trials are in progress to confirm in vivo antigenotoxicity of L. rhamnosus IMC 501® using an animal rat model.

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