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Protective Effects of Probiotic *Lactobacillus rhamnosus* IMC501 in Mice Treated with PhIP

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The aim of the present study was to investigate the antigenotoxic properties of the probiotic *Lactobacillus rhamnosus* IMC501; DNA damage was induced by one representative food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Mice were treated orally with suspensions of lactobacilli for 10 days before administration of food mutagen. During the treatment, the abundance of lactobacilli in feces, as assessed by qPCR analysis, increased, whereas β-glucuronidase and *N*-acetyl-β-glucosaminidase activities decreased. The extent of DNA damage was measured in colon and liver cells by comet assay. In colonocytes, diet supplementation with IMC501 resulted in a significant inhibition of DNA damage induced by PhIP. The results obtained in this *in vitro* study suggest that *Lactobacillus rhamnosus* IMC501 used as a dietary supplement can provide a useful integration of antimutagen food components of the normal diet, which are generally lower than the protective level.

Keywords: Probiotics, *Lactobacillus rhamnosus*, antigenotoxicity, comet assay, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

Introduction

Consumer interest in functional foods and dietary supplements and their potential health benefits has been increasing in recent years. The scientific attention is mainly focused on products containing probiotic bacteria that can be used either in prophylaxis or therapy of gastrointestinal tract diseases [21]. A particular aspect of this issue concerns the potential role of probiotics in reducing the risk of intestinal carcinogenesis. Antigenotoxicity and antimutagenicity are therefore considered new tools for probiotics and functional foods [45], even if the evidence from human clinical trials remains quite limited [5, 16].

The human gastrointestinal tract is colonized by microorganisms (up to 1,200 species of bacteria) with peak numbers in the colon (about 10^{12} bacteria per gram) [13]. Carbohydrates and proteins are the main fermentative substrates for bacteria in the large intestine. The gut microbiota is either able to degrade undigested polysaccharides to short-chain fatty acids, and synthesize biotin, folate, and vitamin K, as well as ferment indigestible dietary residues, and cooperate in calcium, magnesium, and iron absorption [2]. Whereas products of carbohydrates fermentation are usually beneficial, protein catabolites are rather toxic [34].

Colonic microbiota can generate a wide variety of mutagens, carcinogens, and tumor promoters from dietary and endogenous precursors. Fecal bacterial enzymes such as β-glucuronidase, β-glucosidase, nitroreductase, 7-α-dehydroxylase, *etc*., mainly produced by anaerobic colon bacteria (*i.e*., *Bacteroides*, *Clostridium*) are also involved in the mutagenic activation processes [19, 41]. The expression of fecal bacterial enzymes in the colonic environment can be largely modulated by probiotics [1]. It is well known that foodborne genotoxic compounds (*e.g*., mycotoxins and plant glycosides) or genotoxins created during food processing (*e.g*., polycyclic aromatic hydrocarbons and heterocyclic amines) are capable of expressing risk in the gut [47]. It has been shown that some probiotics are capable of counteracting genotoxic and/or mutagenic effects induced by several chemical compounds [40].

At present, epidemiologic data support a role of probiotic intake in the reduction of mutagenicity/cancerogenicity in the intestinal tract. Case-control studies have shown an inverse association between yoghurt or fermented milk intake and the incidence of colon cancer, thus indicating a positive role of lactobacilli and bifidobacteria [42, 44]. Some clinical studies have demonstrated that diet supplementation with probiotic is associated with a reduction in the levels of urinary and fecal mutagens and a decrease in proliferative activity of colonic crypts in patients with an elevated activity before the treatment [40].

By *in vitro* approaches, antigenotoxic activities of probiotic bacteria such as lactobacilli, bifidobacteria, and streptococci have resulted in inhibiting or reducing the activity of genotoxic xenobiotics, such as nitrosamines, heterocyclic amines, alkylating agents, and phenolic compounds [3, 42, 45, 48, 49]. The results of our previous studies have demonstrated the ability of lactobacilli, bacilli, and yeasts to counteract *in vitro* various food mutagens, among which include 4-nitroquinoline-*N*-oxide, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, heterocyclic amines, and aflatoxin B_1 [4, 8, 51]. Carcinogenesis studies conducted in laboratory animals have shown that some probiotics (*i.e.*, lactobacilli and bifidobacteria) seem capable of counteracting the DNA damage activity exerted by potent food mutagens (2-amino-3-methylimidazo[4,5-f]quinoline, DMH, aflatoxin B_1, azoxymethane) with consequent reduction of a pre-neoplastic state and protection against tumors induced by these compounds [19, 26, 39, 54]. For these reasons, every strategy reducing mutagen presence may be effective in carcinogenesis prevention. However, the presence of compounds originating from food processing such as roasting and frying is difficult to control.

It is known that high temperatures generate various kinds of toxic substances, such as heterocyclic amines. Heterocyclic amines are formed when meat and fish are cooked at 150–300°C [15]. A number of studies have reported positive associations between higher consumption of well-done meat and the risk of colon, breast, lung, and gastric cancers [35]. PhIP, the most frequent heterocyclic amine in the human diet [10], is included in IARC Group 2B as a possible carcinogen to humans [23] and is used as a reference colon carcinogen in murine models [32]. The aim of the present study was to verify in a short-term murine model the ability of *Lactobacillus rhamnosus* IMC501, a probiotic strain already characterized for its *in vitro* antigenotoxic capabilities [53], to modulate the activity of the gut microflora and to inhibit the activity of genotoxic xenobiotics.

The experiments were carried out using a secondary exogenous food mutagen, namely PhIP. The lactobacilli protective effects were studied in liver and colon cells, targets for tumor induction by the considered compound [6, 36]. The strain efficacy was evaluated by the single-cell microgel-electrophoresis (comet) assay [50].

Materials and Methods

Chemicals

Genotoxin: 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP; CAS No. 105650-23-5) was acquired from Maxchemco Chemical Industry Ltd. (Shanghai, China).

Reagents: Dimethyl sulfoxide (DMSO), ethanol, ethylenediamine tetraacetic acid disodium (Na_2EDTA) and tetrasodium (Na_4EDTA) salts, hydrochloric acid (HCl), sodium chloride (NaCl), and sodium hydroxide (NaOH) were of analytical grade and were purchased from Carlo Erba Reagenti Srl (Milan, Italy). Ethidium bromide (EtBr), low- and normal-melting-point agarose (LMPA and NMPA, respectively), 4-methylumbelliferone (4-MU), 4-MU-β-D-glucuronide, 4-MU-β-D-glucoside, 4-MU-*N*-acetyl-β-D-glucosaminide, tris(hydroxymethyl)aminomethane (Tris), and Triton X-100 were obtained from Sigma-Aldrich Srl (Milan, Italy). The Bradford protein assay kit was purchased from Bio-Rad Laboratories (Milan, Italy).

Probiotic Microorganism

Lactobacillus rhamnosus IMC501, a probiotic strain used as ingredient of functional food and dietary supplement [52, 53], was kindly supplied by Synbiotech Srl, a spin-off of University of Camerino (Camerino, Italy).

Animals and Experimental Procedures

Male CD-1 mice, aged 4–6 weeks, weight 25–30 g, were purchased from Harlan Italy (Milan, Italy) and quarantined for 1 week before the experiments started. Mice were housed individually in plastic cages with wood chip bedding, maintained at 20°C under a 12 h light/dark cycle. Each animal was allowed free access to water and a standard pellet diet (Altromin-R, A. Rieper SpA, Vandoies, Italy). A total of 20 mice were allocated randomly to four groups (five animals per group) as follows:

Group 1: untreated (negative) control, mice given physiological saline (0.9% NaCl).

Group 2: IMC501, mice supplemented with the probiotic *Lactobacillus rhamnosus* strain.

Group 3: PhIP positive control, mice given physiological saline and treated with PhIP.

Group 4: IMC501 + PhIP, mice supplemented with probiotics and treated with PhIP.

To reduce noise in the data and ensure repeatability, a blocked design approach was used; the animal population was stratified into four homogeneous subgroups (blocks). In each repeated experimental set, a block was composed of 8 animals, with two

animals randomly assigned to each of the four experimental groups. For the experimental procedure, groups 1 and 3 received physiological saline for 10 consecutive days, whereas groups 2 and 4 received the *Lactobacillus* suspension (10^9 cells/ml, 10 ml/kg b.w.); physiological saline and probiotics were administered by gavage (at 9:00 a.m. of every day). On the 10^{th} day, 3 h after physiological saline or lactobacilli administration (12:00 a.m.), the mice of groups 3 and 4 were treated by gavage with PhIP (100 mg/kg b.w.). The genotoxin dose was derived from the literature [43], where PhIP was dissolved in corn oil at a concentration of 20 mg/ml. After a further 3 h (3:00 p.m.), the animals were killed by cervical dislocation and the colons and livers rapidly excised.

The experimental protocol was approved by the Ethical Committee for Animal Experimentation at the University of Perugia, and by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy.

Fecal Microbiota: Quantitative Analysis

Fecal samples were collected at the beginning of the experimentation (day 0), after 5 days (day 5), and on the last day, before administration of food mutagens (day 10); feces were stored at -20°C until analyses. Samples were pooled, thoroughly homogenized and analyzed in triplicate. Total community DNA was extracted from 200 mg of homogenized samples using the MoBio UltraClean Fecal DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions.

The abundance of total bacteria and of the *Lactobacillus* genus was determined by quantitative polymerase chain reaction (qPCR) analyses of 16S rRNA gene fragments. qPCR was performed on an Mx 3000P qPCR System (Stratagene, La Jolla, CA, USA) using the GoTaq qPCR Master Mix (Promega, Madison, WI, USA) following the manufacturer's instruction. PCR primers and cycling conditions used for total bacteria [14] and *Lactobacillus* group [29] were reported elsewhere. The absolute quantification of the target gene copy number was performed as previously reported [14]. Standard curves for the calibration of qPCR assays for total bacteria and *Lactobacillus* genus were constructed with serial dilutions of linearized plasmids where the full 16S rRNA genes from an *Escherichia coli* and a *Lactobacillus rhamnosus* strain, respectively, were cloned.

Fecal Enzyme Activities

The activities of three microbial enzymes (β-glucuronidase, β-glucosidase, and *N*-acetyl-β-glucosaminidase) were quantified in fecal samples of mice administered with *Lactobacillus rhamnosus* IMC501 or physiological saline. The assays were performed according to fluorimetric methods by measuring 4-MU released from 4-MU-conjugated substrates. Briefly, 50 mg of fecal sample was homogenized in 5 ml of phosphate buffer at pH 7.0, sonicated (5 cycles, 30 sec, 0°C, 1 min interval), and centrifuged (8,000 ×*g*, 5 min). Analyses were made on supernatants of cell-free extracts [7]. Fecal proteins were assayed according to the Bradford method. Results were expressed as specific activities (μmol of 4-MU/h/mg protein) and reported as the group mean ± standard error (SE).

Isolation of Colon and Liver Cells

The explanted portions of colon were emptied of feces and then washed with ice-cold homogenization buffer (75 mM NaCl, 24 mM Na_2EDTA, pH 7.5). Each colon segment was cut lengthwise and the mucosa was pulled away using a scraper, put in 1 ml of ice-cold homogenization buffer, and homogenized gently using a Potter-Elvehjem on ice to obtain a suspension of isolated cells. The explanted livers were washed with ice-cold homogenization buffer. Each liver was chopped, washed again, and resuspended in 1 ml of the homogenization buffer; an aliquot of liver was gently homogenized using a Potter-Elvehjem on ice to obtain a suspension of isolated cells [30]. Colonocyte and hepatocyte viability was checked by measuring the percentage of the cytosolic enzyme lactate dehydrogenase (LDH) activity released in the extracellular medium [55]. The activity of LDH was determined using a LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Absorbance was recorded using a Tecan Sunrise microplate reader (Tecan Italia Srl, Milan, Italy).

Single-Cell Microgel-Electrophoresis Assay

Primary DNA damage in liver and colon cells was evaluated by the single-cell microgel-electrophoresis (comet) assay performed under alkaline conditions according to the original procedure [46, 50]. The standard alkaline procedure (lysis at pH 10, unwinding and electrophoresis at pH >13) allows the detection of both single- and double-strand DNA breaks as well as apurinic/apyrimidinic sites (*i.e.* alkali labile lesions) that are expressed as frank strand breaks in the DNA under the alkaline conditions of the assay. For genotoxicity testing, suspensions of isolated cells were embedded into an agarose microgel onto a microscope slide and subsequently lysed by detergents at high salt concentration. The liberated DNA was then exposed to alkali for unwinding from the strand breakage sites and electrophoresed under alkaline conditions. Processing of cells for *ex vivo* evaluation of primary DNA damage was carried out according to the method published elsewhere [12].

After staining with EtBr, DNA results for structures resembling comets with the tail length or tail fluorescence content reflecting the frequency of DNA strand breaks and hence DNA damage [11]. The comets in each microgel were analyzed using a computer-based semi-automated image analysis system (Comet Assay III, Perceptive Instruments Ltd., Suffolk, UK). Computerized imaging, described in detail elsewhere [31], was performed on coded slides and the percent of DNA in the comet tail (*i.e.*, tail intensity; TI) was used to measure the level of DNA damage.

Each result was expressed as the group mean ± standard error (SE). Remaining PhIP-induced genotoxic activity (RGA%) was calculated according to the following formula:

$$\mathrm{RGA}(\%) = \frac{\mathrm{TI(IMC501+Genotoxin)} - \mathrm{TI(IMC501)}}{\mathrm{TI(Genotoxin)} - \mathrm{TI(NaCl)}} \times 100$$

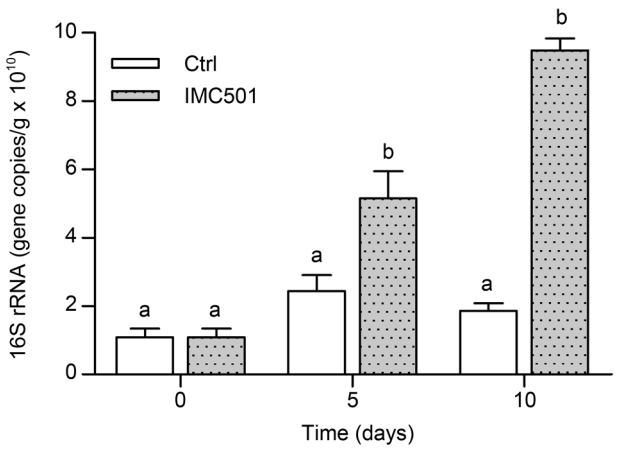

Fig. 1. Abundance of lactobacilli in mice feces during *Lactobacillus rhamnosus* IMC501 diet supplementation.
Data shown are mean ± SE (n = 3). White bars, control group; grey bars, probiotic-treated group. Values with one different superscript letter are significantly different from each other ($p < 0.05$, Student's t-test).

whereas the genotoxic inhibition rate (GIR%) was expressed as

$$GIR(\%) = \left[1 - \left(\frac{RGA(\%)}{100}\right)\right] \times 100$$

Statistical Analysis

Statistical analysis was performed with the SPSS statistical package (SPSS, Chicago, IL, USA). Comet tail intensity distributions were checked for normality by Kolmogorov-Smirnov goodness-of-fit test. Differences among groups were then evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analysis for multiple comparisons. Fecal microbiota differences (qPCR and enzyme activities) were evaluated by Student's t-test. Two-sided p values <0.05 were regarded as statistical significance.

Results

Effects on Fecal Microbiota

After IMC501 diet supplementation, the qPCR assay revealed a progressive increase of lactobacilli 16S rRNA with a slight, but significant difference with respect to the control group. The effect was already evident after 5 days administration of IMC501 (Fig. 1).

Effects on Fecal Enzyme Activities

Administration of IMC501 for 10 days was effective in modulating enzyme activities of mice fecal microflora (Fig. 2). In particular, a marked decrease was observed for both β-glucuronidase and *N*-acetyl-β-glucosaminidase specific activities ($p < 0.05$) with fold-reduction in enzyme activities, as compared with the control group, being 63% and 26%, respectively. Any significant reduction of β-glucosidase activity was however observed following IMC501 diet supplementation.

PhIP-Induced DNA Damage in Colon and Liver Cells

Percent viability observed in colon and liver cells isolated from animals administred with IMC501 and/or treated with PhIP was comparable to that observed in control (untreated) animals. Cell viability group mean values for colon cells were always around 95% (data not shown), and for hepatocytes cell viability was never found to be below 99% (data not shown).

The extent of primary (*i.e.*, basal or induced) DNA damage in colon cells, as evaluated by the alkaline comet assay, is summarized in Fig. 3. Dietary supplementation with *L. rhamnosus* IMC501 strain (group 2) did not affect primary DNA damage in mice colon cells, with tail intensity

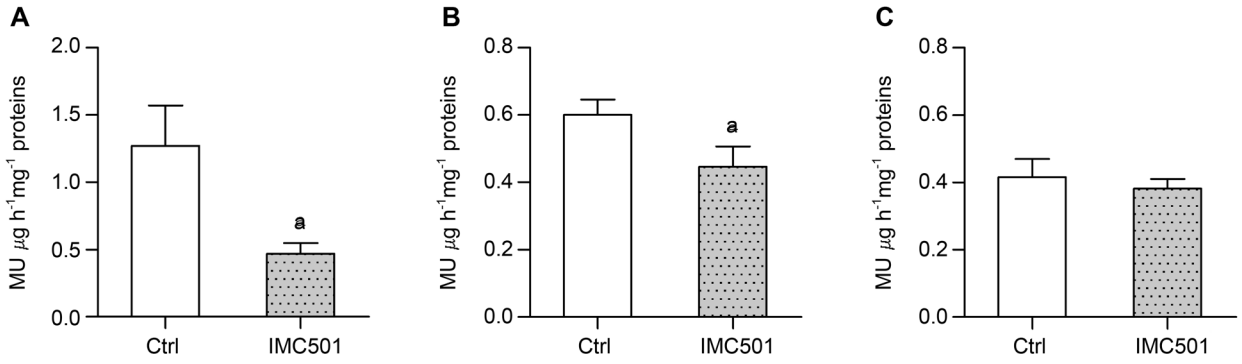

Fig. 2. Effect of *Lactobacillus rhamnosus* IMC501 diet supplementation for 10 days on fecal bacterial enzyme activities.
(**A**) β-glucuronidase; (**B**) *N*-acetyl-β-glucosaminidase; (**C**) β-glucosidase. White bars, control group; grey bars, probiotic-treated group. Values shown are the mean ± SE (n = 5). $^{a}p < 0.05$ *vs*. control, Student's t-test.

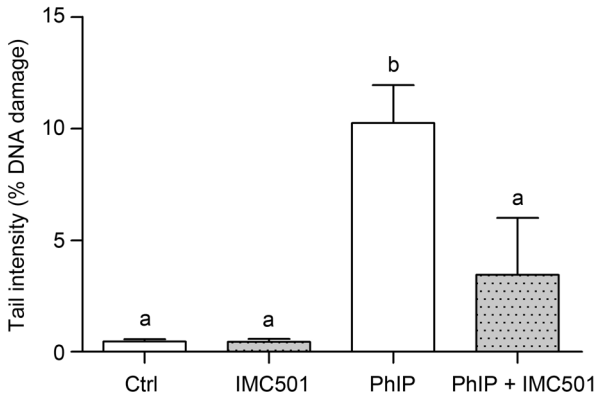

Fig. 3. Effect of 10 days of *Lactobacillus rhamnosus* IMC501 diet supplementation on PhIP-induced DNA damage in mice colon cells.

Data are summarized as the group mean value ± SE (n = 5). White bars, negative/positive (PhIP) control groups; grey bars, groups supplemented with probiotics. [a,b]Values with one different superscript letter are significantly different from each other ($p < 0.05$) by ANOVA.

values overlapping those of control animals (group 1). DNA strand breakage observed in colon cells from mice receiving PhIP (group 3) was significantly greater than in cells from negative controls (group 1). In the animals treated with IMC501+PhIP (group 4), the extent of primary DNA damage induced by the model mutagen was significantly decreased by the administration of probiotic bacteria, as compared with animals receiving PhIP only (group 3), with a GIR of 69.6%.

Dietary supplementation with IMC501 strain (group 2), as well as treatment with PhIP (group 3) did not cause any increase in the extent of primary DNA damage in mice liver cells, with tail intensity values being similar to those of control animals (group 1); consequently, any effect could be observed in mice supplemented with the *Lactobacillus rhamnosus* IMC501 strain and treated with IMC501+PhIP (group 4) (data not shown).

Discussion

The present study was aimed at investigating *in vivo* the possible protective effects exerted in mice by diet supplementation with probiotic *Lactobacillus rhamnosus* IMC501 against dietary mutagen-induced injury in colon and liver cells.

Lactobacilli abundance assessed by qPCR confirmed the capability of IMC501 to integrate on the fecal microbial ecosystem of the murine animal model used in this study. The lactobacilli increase in feces, observed already after 5 days of diet supplementation with IMC501, was associated with a reduced activity of fecal enzymes, in particular β-glucuronidase and β-*N*-acetyl-glucosaminidase that are peculiar of some components of autochthonous fecal microflora. Indeed, β-glucuronidase has the ability to hydrolyze the bond formed during glucuronidation of xenobiotics by the enzyme UDP-glucuronosyltransferase, thus reversing phase II detoxifying reactions in which noxious chemicals (*e.g.*, dietary carcinogens) undergo conjugation in the liver; as a consequence, conjugated carcinogens are not excreted and parent genotoxic xenobiotics are released in the colon lumen [22]. As a high activity of bacterial β-glucuronidase in human colon was observed in patients with colorectal cancer [24], our results highlight the IMC501-related favorable modulation of biomarkers of colorectal cancer risk indicating a harmful activity of colonic microbiota. The decrease of deconjugation activities induced by IMC501 is in line with the findings reported for humans administered with *Lactobacillus casei* and *Lactobacillus paracasei* probiotic strains [34]; more generally, species belonging to *Bifidobacterium* or *Lactobacillus* genus are reported to display a low activity of enzymes involved in carcinogens formation, as compared with other major colonic anaerobes (*e.g.*, *Bacteroides*, *Clostridium*, and *Eubacterium*) [1].

Furthermore, β-*N*-acetyl-glucosaminidase catalyzes the random hydrolysis of β-1,4 glycosidic bonds between *N*-acetyl-β-D-glucosamine and D-glucuronic acid residues in hyaluronate. The beneficial and/or detrimental effects of degradation of the gut mucus gel in the intestinal mucosa still need to be defined [17, 34]. However, as an increase in some mucolytic bacteria (*e.g.*, *Ruminococcus gnavus*, *Ruminococcus torques*) was found in subjects with diagnosis of Crohn's disease or ulcerative colitis [38], a decreased mucolytic activity could be indicative of a reduced pathogenic risk, with mucus probably protecting colonocytes from biological/chemical noxae.

Supplementation of diet with IMC501 significantly reduced the extent of DNA damage induced by PhIP in colonocytes. PhIP did not show any genotoxic effect in liver cells and, consequently, it was not possible to evaluate potential antigenotoxic properties of the tested *Lactobacillus rhamnosus* strain in this organ. The beneficial effects reported in the present paper for the challenge lactobacilli/PhIP are in agreement with previous reports indicating that oral administration of probiotics effectively reduces the extent of induced DNA damage in model animals [12, 33, 49, 54].

In vitro and *in vivo* data suggest that lactobacilli may

exert their antigenotoxic effects by both direct and indirect mechanisms. Diet supplementation with lactobacilli can modify gut microbiota metabolism, leading to the inhibition of enzyme activities involved in the re-toxification of detoxified xenobiotics [18, 20]. Direct binding of dietary carcinogens to the cell walls of probiotic bacteria has been reported and is currently considered as the most effective detoxification mechanism preventing absorption of genotoxic xenobiotics through the intestinal mucous. Several food mutagens, including PhIP, were reported to be very effectively bound to the cell walls of *Lactobacillus acidophilus* and *Bifidobacterium longum* [25, 37]. It has been also hypothesized that probiotics can prevent the absorption of genotoxic xenobiotics in the intestine [56] or reduce their bioavailability [27]. It has been also reported that probiotics adhere to colonocytes, both *in vitro* and *in vivo*, a feature that seems to play a role in limiting the uptake of xenobiotics through the intestinal barrier [28]. Moreover, probiotics can inactivate food mutagens, and/or their precursors, by several other mechanisms, such as chemical or enzymatic inactivation of mutagens (*e.g.*, scavenging) [4, 9]. However, among mechanisms by which probiotics may exert their beneficial effects, significant differences exist between different probiotic strains [33].

In this study, the protective effects of *Lactobacillus rhamnosus* IMC501 strain against PhIP-induced DNA damage were observed in colon cells, where direct positive effects of dietary probiotics could be expected, but not in liver cells, where a systemic chemopreventive activity probably involves resorbed protective agents.

In conclusion, our results confirm that the *Lactobacillus rhamnosus* IMC501 strain used in this study possesses beneficial properties. Diet supplementation with IMC501 was associated with a reduced activity of fecal enzymes associated with a harmful activity of colonic microbiota and used as biomarkers of colorectal cancer risk. Moreover, the tested probiotic bacteria were capable of reducing significantly the extent of PhIP-induced DNA damage in colon cells. The possibility of using probiotic strains as dietary supplements to counteract the activity of food mutagens in the human gut might be of interest for the production of functional foods, especially dairy products, such as yoghurts and fermented milks containing *Lactobacillus* and *Bifidobacterium* stains. With regard to the microorganism used in this *in vivo* approach, our data suggest that the incorporations of *Lactobacillus rhamnosus* IMC501 in the diet might contribute to suppress or reduce the genotoxic activity of potentially harmful compounds.

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