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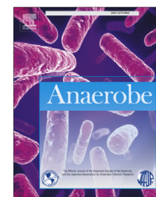

Clinical microbiology

Potential of goat probiotic to bind mutagens

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The mutagen binding ability of the goat probiotics (*Lactobacillus reuteri* DDL 19, *Lactobacillus alimentarius* DDL 48, *Enterococcus faecium* DDE 39, and *Bifidobacterium bifidum* DDBA) was evaluated. The oral administration of these probiotics reduced fecal mutagens and intestinal cancer markers in goats. Secondly, the effects of probiotics against the mutagenesis induced by sodium azide (SA), and Benzopyrene (B[α]P) by performing the modified Ames test using *Salmonella typhimurium* TA 100 was investigated. The capacity to bind benzopyrene and the stability of the bacterial–mutagen complex was analyzed by HPLC. The dismutagenic potential against both mutagens was proportional to probiotic concentration. Results showed that probiotic antimutagenic capacity against SA was ranging from 13 to 78%. The mixture of four goat probiotics (MGP) displayed higher antimutagenic activity against SA than any individual strains at the same cell concentration. This study shows that the highest diminution of mutagenicity in presence of B[α]P (74%) was observed in presence of MGP. The antimutagenic activity of nearly all the individual probiotic and the MGP were in concordance with the B[α]P binding determined by HPLC. According to our results, the B[α]P binding to probiotic was irreversible still after being washed with DMSO solution. The stability of the toxic compounds-bacterial cell binding is a key consideration when probiotic antimutagenic property is evaluated. MGP exhibits the ability to bind and detoxify potent mutagens, and this property can be useful in supplemented foods for goats since it can lead to the removal of potent mutagens and protect and enhance ruminal health and hence food safety of consumers.

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1. Introduction

Ruminant livestock breeding is one of the major industries in developing countries and in mountainous areas. Microbial ecology of the gastrointestinal tract has an important effect on goat health and development. The application of potentially beneficial microorganisms to increase host defense is a new trend to improve health. In a previous paper we found that the consumption of a mixture of goat probiotic (MGP) was able to modify microflora balance by reducing enterobacteria and increasing lactic acid bacteria (LAB) and bifidobacteria, with a significant increase in ruminant weight. Moreover, the MGP consumption was correlated with a tenfold diminution of fecal putrescine (cancer and bacterial disease marker) and a 60% reduction in mutagen fecal concentration, indicating the protective effect of the treatment [1].

Mutagens are frequently formed during stress or after viral or bacterial gastrointestinal infections and the involvement of endogenous microflora in the onset of colon cancer has been suggested in previous work [2]. On the other hand, the mammal's diet contains a wide variety of carcinogens. Benzopyrene (B[α]P) is one of the most powerful mutagens [3] and is used as an indicator of the level of environmental contamination by polycyclic aromatic hydrocarbons [4]. Sodium azide (SA), which is widely used in hospitals and laboratories as a preservative, was reported to cause partial cytochrome oxidase inhibition and learning deficiencies as detected in animal models [5].

LAB and bifidobacteria have been shown to exhibit antimutagenic activities against heterocyclic-amines, N-nitroso compounds, B[α]P and aflatoxin B [6–8]. The mechanism, by which LAB exerts antimutagenic effects, has not been proven [9]. However, it has been suggested that binding of mutagens to microbial cells could be a mechanism of antimutagenicity. With regard to the

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mentioned results, we investigated the mutagen-binding ability of goat probiotics against B[α]P and SA.

2. Materials and methods

2.1. Mutagens, chemicals and media

The mutagen sodium azide (SA) and the promutagen benzopyrene B[α]P were obtained from Sigma (Argentina). SA was prepared at a concentration of 15 μg mL^{-1} in sterile phosphate buffered saline (PBS; 100 mM pH 7.0; Sigma, Argentina). B[α]P was prepared at a concentration of 5 μg mL^{-1} in dimethyl sulfoxide (DMSO); Sigma (Argentina).

Liver-S9 homogenate (Moltox, Inc., Boone, NC, U.S.A.) was prepared from Sprague–Dawley male rats treated with Aroclor 1254. S9 mix (S9 fraction of liver homogenate with cofactors) was used for metabolic activation of B[α]P [10,11].

2.2. Bacteria strains

The probiotic strains used were isolated from healthy goat feces, (Draksler, D., Ph.D. thesis, Universidad Nacional de Tucumán, Tucumán, Argentine, 2003) and their beneficial effects against fecal mutagen were demonstrated [1]. In this study, each strain was cultured in an appropriate broth for 9 h at 37 °C. *Lactobacillus reuteri* DDL 19, *Lactobacillus alimentarius* DDL 48, and *Enterococcus faecium* DDE 39 strains were cultured in MRS at pH 5.5. *Bifidobacterium bifidum* DDBA, was cultured in the same medium plus 1% lactose at pH 7.0, but incubated at 37 °C for 24 h in an anaerobic incubator (air-jacketed DH autoflow CO_2 incubator, Nu Air, Plymouth, NH, U.S.A.) under microaerophilic conditions. Stock cultures were preserved in 10% skimmed milk at 4 °C. The mixture of goat probiotics (MGP) was constituted by *L. reuteri* DDL 19, *L. alimentarius* DDL 48, *E. faecium* DDE 39, and *B. bifidum* DDBA in a relation 1:1:1:1. *Salmonella typhimurium* strains TA 100 (hisG46, uvrB, pkm101), kindly donated by Dr. Sergio Ferrer of University of Valencia from the Spanish Type Culture Collection.

S. typhimurium TA 100 was grown in nutrient Broth II (Oxoid Australia, West Heidelberg, Australia) in the presence of 25 μg mL^{-1} of ampicillin. Tests of histidine requirement, rfa mutation, uvrB mutation and R-factor were carried out to confirm the genotypes of *S. typhimurium* TA 100. Prior to each mutagenicity test, *S. typhimurium* cells were grown at 37 °C for 10–12 h until reaching 1–2·109 UFC mL^{-1}.

2.3. Bacterial concentration used for antimutagenicity assay

The probiotic cultured solutions were centrifuged at 5000 rpm at 4 °C for 15 min, washed twice with sterile phosphate buffered saline (pH 7, 100 mM, 0.85% NaCl) and the cells were resuspended in phosphate buffer (pH 7100 mM). The cells' suspension was adjusted to 0.1, 0.4, and 0.9 at 600 nm, these absorbances correspond to 1–2·10^{6}, 1–2·10^{8}, and 1–2·10^{11} CFU mL^{-1}, respectively.

The cells were resuspended in phosphate buffer to obtain OD_{600} of 0.9, which was divided into 2 portions; one portion was used to determine the remaining mutagenic activity in the bacterial cell-mutagen suspensions using the Ames test and the other portion was used to determine the quantity of unbound/uninhibited mutagen by HPLC.

2.4. Dose response curves for mutagens

Dose response curves were prepared and the concentrations giving straight lines in dose response curves were determined [11]. TA-100 mutant of *S. typhimurium* strain was used as mutagenicity indicator organism. Dilutions ranging from 0.0015 to 2.5 μg mL^{-1} (AS) and 0.1–10 μg mL^{-1} B[α]P were used for preparing the standard curves. These standard curves were used to determine the concentration of mutagens or pro-mutagens in bacterial suspensions. Based on dose response curves, a concentration of 0.5 μg plate^{-1} was used for B[α]P and of 1.5 μg plate^{-1} for SA for antimutagenicity assays.

2.5. Antimutagenic activity assay

The antimutagenic activity of *L. reuteri* DDL 19, *L. alimentarius* DDL 48, *B. bifidum* DDBA, and *E. faecium* DDE 39 against B[α]P and SA were determined as described previously (Maron D.M., and Ames, B.N 1983), measuring the inhibition of *S. typhimurium* TA 100 mutation.

One hundred μL of the probiotic bacterial suspensions (1·10^{6}, 1·10^{8}, 1·10^{11} CFU mL^{-1}) were placed in small sterile bottles and 100 μL of each mutagen solution was added to give a final concentration of (15 μg mL^{-1}) SA and (5 μg mL^{-1}) B[α]P. A control sample was prepared for each mutagen without probiotic bacteria. Each suspension of mutagens with or without probiotic bacteria was incubated at 37 °C for 2 h in a shaker incubator, the suspension centrifuged at 5000 rpm at 4 °C using a refrigerated centrifuge Beckman J2-HS, the supernatants decanted and filtered with a 0.22 μm filter paper (Millipore, Argentina).

2.6. Ames test

An aliquot of a 100 μL 16-h culture of *S. typhimurium* TA 100 strain (approximate cell density 2·10^{8}–5·10^{8} cells mL^{-1}) was incubated with previously obtained 100 μL residual mutagen and were agitated at 150 rpm for 30 min at 37 °C in shaker and were mixed with 2 mL top agar with decanted supernatants. The top (overlay) agar for the Ames assay was prepared with 0.6% (w/v) agar and 0.5% (w/v) NaCl and was supplemented with 0.5 mM L-histidine (Sigma–Aldrich) and 0.5 mM d-biotin (Merck, Germany). The mixture was then gently mixed and finally poured onto a plate containing minimum glucose agar (glucose 2% w/v plus agar 1.5% w/v). When the top agar had solidified the plates were incubated in an inverted position at 37 °C for 48 h and HIS^{+}revertant colonies were counted.

2.7. Antimutagenic activity

Antimutagenic activity of probiotic bacteria was measured as reduction in the number of colonies on the test plates (i.e. plates prepared with each of the mutagen solutions treated with probiotic bacteria), in comparison to the control (i.e. plates prepared with mutagen and without probiotic bacteria), and was calculated as following: each assay was performed in triplicate, and antimutagenic activity was expressed as percentage of inhibition [12,7].

<u>% Antimutagenic activity:</u>

$$\text{Inhibition (\%)} = [(A - B)/(A - C)] \times 100\%$$
$$\text{Inhibition (\%)} = [(A - B)/(A - C)] \times 100\%$$

In this expression: A = Number of His^{+} revertants induced by B[α]P (positive control), B = Number of His^{+} revertants with bacteria and B[α]P, and C = Number of spontaneous His^{+}revertantes (negative control) without bacteria and B[α]P.

2.8. Benzopyrene detection by high pressure liquid chromatography (HPLC)

Removal of B[α]P by probiotic bacteria was measured for residual mutagen supernatants by HPLC. To study the power of

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