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Potential of goat probiotic to bind mutagens

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ABSTRACT

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 $1–2\cdot10^6$, $1–2\cdot10^8$, and $1–2\cdot10^{11}$ 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 ($1\cdot10^6$, $1\cdot10^8$, $1\cdot10^{11}$ 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 $2\cdot10^8$–$5\cdot10^8$ 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].

% Antimutagenic activity:

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

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

removal of mixed culture and monoculture on B[α]P, 100 μL of bacterial suspension ($1 \cdot 10^{11}$ UFC mL^{-1}) was taken and added to 250 μL of pro-mutagen solution (final concentration 2.5 mg mL^{-1}) and 650 μL sodium phosphate buffer pH 7.2, incubated at 37 °C for 2 h with gentle agitation (110 rpm – SI 600 – Lab Companion) and then centrifuged at 5000 rpm for 15 min. The supernatant containing residual benzopyrene (not removed by probiotic bacterial cells) were stored at −20 °C until processed by HPLC. A stock solution of B[α]P without metabolic activation (10 mg mL^{-1}) in DMSO was used as the standard in this chromatography. The results were expressed as percentage of residual B[α]P.

For each strain, a bacterial control (bacteria suspended in PBS) and a mutagen control (working solution of mutagen without bacteria) were also incubated.

The extractions of the samples were made with hexane (3 extractions) sonicating for 30 min at each stage. They were centrifuged for 10 min at 3000 rpm, filtered (45 μm) and concentrated on rotary evaporator. Subsequently, they were resuspended in 1 mL of acetonitrile (solvent exchange). For this reason, the standard solutions were dissolved in acetonitrile.

Benzopyrene was quantified by a reversed phase column HPLC. For analysis the samples were injected into the HPLC system (Agilent) equipped with a quaternary pump (Agilent mod. 1311A); autosampler (Agilent mod. G1313A), as well as an FLD fluorescence detector (Agilent mod. 1100). A Waters PAH C18 (4.6 × 250 mm) was used for the stationary phase with a flow of 1.0 mL min^{-1}. The mobile phase was an isocratic mobile phase of acetonitrile/water (90/10).

2.9. Stability of the bacterial–mutagen complex

After incubation, each bacterial cell-mutagen suspension was centrifuged and the supernatant refrigerated until the concentration of mutagen was determined. In order to determine the stability of the bacterial–mutagen complex the mixture was washed twice with 875 μL PBS, suspended in 25 μL DMSO, vortexed for 5 min, centrifuged and the supernatant collected for quantification of released BP.

2.10. Statistical analysis

All experiments and analyses were performed in duplicate. Data analysis was carried out by ANOVA and Tukey's mean comparison tests using the MINITAB statistical package v. 14.1 to identify significant differences between bacterial strains. P values ≤ 0.05 were considered to be significant.

3. Results

3.1. Antimutagenic activity of goat probiotic bacteria against SA

The antimutagenic activity of *L. reuteri* DDL 19; *L. alimentarius* DDL 48, *B. bifidum* DDBA, *E. faecium* DDE 39 and MGP ($1 \cdot 10^6$, $1 \cdot 10^8$, $1 \cdot 10^{11}$ CFU mL^{-1}) against sodium azide (1.5 μg $plate^{-1}$) is showed in Table 1.

The amount of SA bound was strain and cells concentration specific. The percent bound ranging from 13% to 78%. The percentage of desmutagenicity rose significantly due to the increase in number of cells for *L. reuteri* DDL 19, *L. alimentarius* DDL 48, *E. faecium* DDE 39, and MGP. However, *B. bifidum* DDBA, reached the maximum level of desmutagenicity, at $1 \cdot 10^8$ CFU mL^{-1}. The highest antimutagenic activity was observed by MGP.

The percentage of antimutagenicity in cell concentrations of $1 \cdot 10^6$, $1 \cdot 10^8$ and $2 \cdot 10^{11}$ CFU mL^{-1} compared with the control was 19, 35 and 72% for *L. reuteri* DDL 19; 13, 28 and 63% for *L. alimentarius* DDL 48; 38, 48 and 46% for *B. bifidum* DDBA; 27, 31 and 67% for *E. faecium* DDE 39; as well as 36, 52 and 78% by MGP, respectively.

Table 1
Antimutagenic activity of goat's probiotic bacteria against sodium azide.

Strains	Revertants in presence of SA (colonies/plate)		
	Probiotic concentration (CFU mL^{-1})		
	$1 \cdot 10^6$	$1 \cdot 10^8$	$1 \cdot 10^{11}$
L. reuteri DDL 19	$427 \pm 1^{a,1}$	$361 \pm 1^{b,1}$	$215 \pm 8^{c,1}$
L. alimentarius DDL 48	$454 \pm 6^{a,2}$	$396 \pm 8^{b,2}$	$251 \pm 9^{c,2}$
B. bifidum DDBA	$350 \pm 11^{a,3}$	$313 \pm 4^{b,3}$	$321 \pm 2^{c,3}$
E. faecium DDE 39	$396 \pm 6^{a,4}$	$380 \pm 7^{b,4}$	$242 \pm 6^{c,2}$
MGP	$363 \pm 2^{a,3}$	$295 \pm 7^{b,5}$	$193 \pm 6^{c,4}$

Revertants of *S. typhimurium* TA 100 versus different concentrations of probiotic cells in the presence of sodium azide as mutagen. Revertants values are expressed as means ± SD. Different letters in rows ($^{a, b, c}$) indicate significant differences between the same strain at different cell concentrations ($P < 0.05$). Different numbers in columns ($^{1, 2, 3}$) indicate significant differences between the different strains at the same cell concentrations ($P < 0.05$). Concentration of sodium azide (SA) used: 15 μg mL^{-1}. Spontaneous revertants of *S. typhimurium* TA 100: 103 ± 6 (colonies/plate) are considered as negative control; numbers of revertants of *S. typhimurium* TA 100 in presence of SA without probiotic cells: 503 ± 9 (colonies/plate) are considered as positive control. MGP means mixture of goat probiotic.

3.2. Antimutagenic activity of goat probiotic bacteria against B[α]P

The results for antimutagenic property of different strains against B[a]P are presented in Table 2. The percentage of antimutagenicity against B[α]P at $1 \cdot 10^6$, $1 \cdot 10^8$ and $2 \cdot 10^{11}$ CFU mL^{-1} was 19, 29 and 70% for *L. reuteri* DDL 19; 15, 21 and 41% for *L. alimentarius* DDL 48; 17, 32 and 72% for *B. bifidum* DDBA; 24, 36 and 64 for *E. faecium* DDE 39 and 28, 45 and 74% for MGP, respectively.

In presence of B[α]P the effects of different cell concentrations by the individual cultures was only significant in presence of the higher concentration assayed.

The increase in cell concentration produced a proportional increase in the antimutagenic property of MGP. At $1 \cdot 10^6$ and $1 \cdot 10^8$ CFU mL^{-1} MGP showed the maximum dismutagenic effects against B[α]P. However at the higher cell concentration the inhibition of MGP was similar to the inhibition produced by *L. reuteri* DDL 19 and *B. bifidum* DDBA.

Table 2
Antimutagenic activity of goat's probiotic bacteria against B[α]P.

Strains	Revertants in presence benzopyrene activated with S9 (colonies/plate)		
	Probiotic concentration (CFU mL^{-1})		
	$1 \cdot 10^6$	$1 \cdot 10^8$	$1 \cdot 10^{11}$
L. reuteri DDL 19	$297 \pm 9^{a,1}$	$280 \pm 5^{a,1}$	$205 \pm 9^{b,1}$
L. alimentarius DL 48	$302 \pm 6^{a,1}$	$294 \pm 6^{a,2}$	$259 \pm 9^{b,2}$
B. bifidum DDBA	$300 \pm 7^{a,1}$	$278 \pm 6^{a,1}$	$207 \pm 10^{b,1}$
E. faecium DDE 39	$288 \pm 4^{a,1}$	$266 \pm 5^{a,3}$	$221 \pm 9^{b,3}$
MGP	$281 \pm 8^{a,1}$	$245 \pm 6^{b,3}$	$210 \pm 3^{c,1}$

Revertants of *S. typhimurium* TA 100 versus different concentrations of probiotic cells in the presence of B[α]P as mutagen. Revertants values are expressed as means ± SD. Different letters in rows ($^{a, b, c}$) indicate significant differences between the same strain at different cell concentrations ($P < 0.05$). Different numbers in columns ($^{1, 2, 3}$) indicate significant differences between the different strains at the same cell concentrations ($P < 0.05$). Concentration of benzopyrene used: 5 μg mL^{-1}. Spontaneous revertants of *S. typhimurium* TA 100: 160 ± 8 (colonies/plate) are considered as negative control; numbers of revertants of *S. typhimurium* TA 100 in presence of benzopyrene without probiotic cells: 328 ± 9 (colonies/plate) are considered as positive control. MGP means mixture of goat probiotic.

3.3. Benzopyrene binding assay

The ability to bind B[α]P through goat probiotic was determined (Fig. 1). B[α]P is *per se* a promutagen, for this reason it needs enzymatic activation triggered by the complex enzyme S9 to convert into the mutagens that usually function inside the stomach. However, if the promutagen is removed from the media, the mutagenicity is not produced. Moreover, if the complex S9 is used to convert B[α]P, the reduction of it into the media could be partially attributed to the enzymatic bioconversions. For this reason, to evaluate B[α]P bound to probiotic we did not use the activator S9. The chromatograms produced do not show any other products that were produced as a consequence of B[α]P and different goat probiotic interactions after 2 h of incubation at 37 °C. This result suggests that the goat probiotic did not metabolize B[α]P.

The amount of compounds removed was strain specific. Nearly all the B[α]P appear to be removed after 2 h incubation by *L. reuteri* DDL 19 and *B. bifidum* DDBA. *E. faecium* DDE 39 showed the least binding.

The initial binding was 77, 97, 96, 68, and 88% for *L. alimentarius* DDL 48, *L. reuteri* DDL 19, *B. bifidum* DDBA, *E. faecium* DDE 39, and MGP, respectively.

The effects of DMSO washes on release of B[α]P previously bound to probiotic suggested that the strains showing higher initial binding to B[α]P release a minor amount of B[α]P in comparison to the other strains. The irreversible amount of B[α]P bound to *L. reuteri* DDL 19, *B. bifidum* DDBA and the MGP was notably higher than in the *L. alimentarius* DDL 48 and *E. faecium* DDE 39.

4. Discussion

In the present study, *in vitro* binding of mutagens and antimutagenic activity of goat probiotic has been reported. These are the first findings about the mechanism involved in goat probiotic. The antimutagenicity effects of goat probiotic could be explained by the marked diminution of intestine cancer, putrescine and the diminution of fecal mutagens observed *in vivo* after probiotic consumption [1]. The results suggest that at least in part the antimutagenic properties observed by the MGP are a result of their ability to bind to mutagens. These results are in concordance with the previous observation that the administration of LAB has been shown to effectively reduce DNA damage, induced by chemical carcinogens in gastric and colonic mucosa in rats [13]. Lyophilized cultures of *Bifidobacterium longum* administered in the diet to rats inhibited liver, colon and mammary tumors, induced by the food mutagens [14].

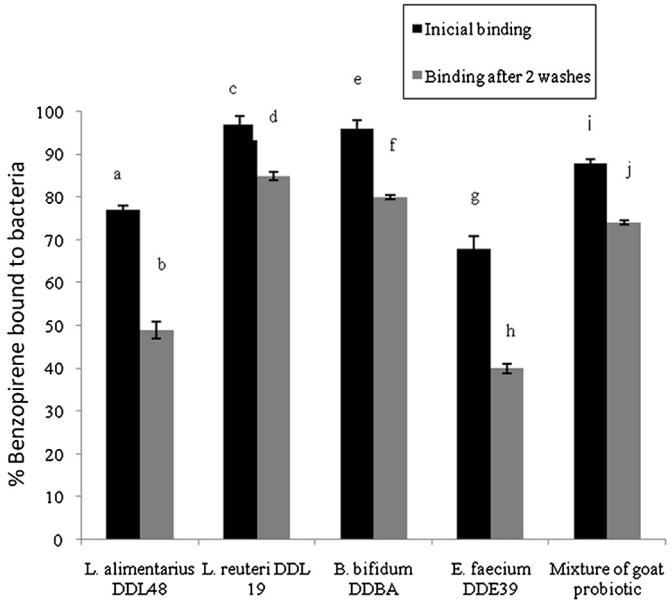

Fig. 1. Percentage of benzopyrene binding by goat probiotic and remaining binding after two DMSO solution washes. Treatments with different letters in each column are statistically different by each bacteria ($p \leq 0.05$).

It is well known that there is a strong correlation between mutagenicity and carcinogenicity [15]. Therefore, the consumption of dietary antimutagens may prevent foodborne genetic mutations and cancers [5]. Probiotic, then, could be considered as one such antimutagen [16]. LAB and their products of fermentation are claimed to be antimutagenic and anticarcinogenic [17,18]. Mechanisms of antimutagenic activity of fermented dairy products or probiotics have not been clearly understood. A strong correlation has been shown between binding and antimutagenic activity of LAB [19] and components of its cell wall play an important role in the binding and deactivation of mutagens [20,21].

The extent of the binding was dependent on the mutagen and on bacterial strain used. Maximum dismutagenic effects against SA observed in presence of MGP at the different cell concentrations used. However, in the challenge with B[α]P, the higher inhibition was observed in presence of MGP, *L. reuteri* DDL 19 and *B. bifidum* DDBA.

The antimutagenicity of culture media of *bifidobacteria* against B[α]P demonstrated that several *bifidobacteria* cultures showed more than 50% inhibitory effect on B[α]P and that *B. bifidum*, *Bifidobacterium lactis* and *B. longum* showed significantly higher antimutagenicity than *Bifidobacterium adolescentis*, *Bifidobacterium breve*, and *Bifidobacterium infantis* against B[α]P [12]. In contrast, *Lactobacillus acidophilus* seemed to bind the mutagens to greater extent than *B. longum* [22].

According to our results the effects of population density on binding capacity of B[α]P and SA were proportional. In concordance it has been previously reported that bacterial concentration influences mutagen removal. Approximately a minimum of $2–5 \cdot 10^9$ CFU mL^{-1} is required for significant AFB1 removal (13–50%), while a concentration of $2 \cdot 10^{10}$ CFU mL^{-1} is capable of reducing the mutagen level to less than 0.1 and 13% [22].

The antimutagenic activity of the strains against B[α]P studied by Ames test (Table 2) is concordant with the B[α]P removal determined by HPLC (Fig. 1) all the strains studied, with the exception of *E. faecium* DDE 39, which showed higher antimutagenic properties than ability to removal B[α]P.

Comparing the power of the probiotic strains against both mutagens, SA and B[α]P; at the same cell concentration, $1 \cdot 10^{11}$ UFC mL^{-1}, was observed, in general, greater antimutagenic power versus SA. For example *L. alimentarius* DDL 48 has a percentage of antimutagenicity of 65% against SA and 41% against B[α]P. In short, the power of removal of strains depends not only on the concentration but also on the mutagen.

Another important result is the fact that the B[α]P removal by goat probiotic is largely irreversible, even more so after washing with a universal dissolvent such as DMSO. The stability of the toxic compounds bacterial cell complex is also a key consideration when evaluating a strain's ability to reduce bioavailability of toxic compounds in foods [23].

In conclusion, the potential antimutagenic properties of MGP demonstrated after consumption by goats could be explained by the binding of mutagens. In addition, the removal of irreversible mutagens enhances the good characteristics of MGP, suggesting beneficial consequences for ruminal health.

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