Original Article

Effects of repeated low and high dosage postbiotics administration in toxicity and inflammatory responses in mice model

Efeitos da administração de baixas e altas doses repetidas de pós-bióticos na toxicidade e respostas inflamatórias em ratos modelo

DOI: 10.37111/braspenj.2023.38.2.07

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Unitermos:
Pós-bióticos. Dose repetida. Toxicidade. Suplementação nutricional. Inflamação.

Keywords:
Postbiotics. Repeated dose. Toxicity. Nutritional supplementation. Inflammation.

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Submission
07st March, 2023

Accepted for publication
12th June, 2023

ABSTRACT

Introduction: The present study evaluated the effect of repeated low-dose and high-dose postbiotics obtained from strains of *Lactobacillus gasseri* (CCT 7860), *Lactobacillus casei* (CCT 7859), *Lactobacillus paracasei* (CCT 7861), *Streptococcus thermophilus* (ATCC 19258) and *Bifidobacterium lactis* (CCT 7858) in the inflammatory effects in different tissues and damage in the liver and kidney. **Methods:** The experimental protocol consisted of fifteen probiotics administrations in the low-dose (10 mg/postbiotics/day) and high-dose (1 g/postbiotics/day) by the intragastric (i.g.) route. On the sixteen day, mice were euthanized and the blood, liver, kidney, brain and gut were removed for different analysis: myeloperoxidase (MPO) activity, nitrite levels and cytokine levels (interleukin (IL)-1β, IL-10, IL-6). **Results:** The low toxicity of postbiotics is supported by the observation that, after its administration for 15 consecutive days, there were no changes in the levels of aspartate aminotransferase (AST) and alanine transaminase (ALT), creatinine and in the histopathology of the intestine of mice, confirming the safety of its use. **Conclusion:** Our study shows that postbiotics supplementation is safe and no toxic even in high doses.

RESUMO

Introdução: O presente estudo avaliou o efeito de doses repetidas de pós-bióticos de baixa e alta dose obtidos de cepas de *Lactobacillus gasseri* (CCT 7860), *Lactobacillus casei* (CCT 7859), *Lactobacillus paracasei* (CCT 7861), *Streptococcus thermophilus* (ATCC 19258) e *Bifidobacterium lactis* (CCT 7858) em parâmetros inflamatórios em diferentes. **Método:** O protocolo experimental consistiu em administrações diárias de probióticos em baixa dose (10 mg/pós-bióticos/dia) e alta dose (1 g/pós-bióticos/dia) pela via intragástrica (i.g.) por 15 dias. No décimo sexto dia, os camundongos foram sacrificados e o soro, fígado, rim, cérebro e intestino foram removidos para diferentes análises: atividade de mieloperoxidase (MPO), níveis de nitrito e níveis de citocinas (interleucina (IL)-1β, IL-10, IL-6). **Resultados:** A baixa toxicidade dos pós-bióticos é corroborada pela observação de que, após sua administração por 15 dias consecutivos, não houve alterações nos níveis de aspartato aminotransferase (AST) e alanina aminotransferase (ALT), creatinina e na histopatologia do intestino de camundongos, confirmando a segurança de seu uso. **Conclusão:** Nosso estudo mostra que a suplementação de pós-bióticos é segura e não tóxica, mesmo em altas doses.

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INTRODUCTION

Probiotics are defined as nonpathogenic living microorganisms and, if consumed in correct amounts, confer health benefits on the host[1]. In recent years, the health-promoting effects of probiotics have already been verified through a series of studies[2–4]. However, several concerns on their use began to be discussed, such as the viability of probiotics in food products, their diverse colonizing patterns and persistence, as well as the likelihood of horizontal gene transfer of a virulence gene from a pathogenic microorganism in the intestine. Also, the processing and storage of probiotics are being discussed, as these could compromise the cell's viability if the essential requirements are not assured, which can cause the loss of the desired viability of many probiotic microorganisms[5].

These circumstances are enhancing the interest in new products using deactivated cells, as well as cell components and metabolites derived from probiotics strains. In this scenario, postbiotics began to receive great attention from researchers.

Probiotics are defined as live cells. Postbiotics, on the other hand, are characterized as cells deactivated through heat treatments, such as pasteurization or sterilization, becoming bacterial-free extracts or non-viable cells that, by offering bioactivities in addition to probiotics, provide benefits to the host, such as modulation of the immune system, secretion of metabolites, in addition to adhesion to intestinal cells, enabling the inhibition of pathogenic microorganisms[5,6].

Furthemore, postbiotics have demonstrated greater usability, because they can be safely consumed by immunodeficient and elderly people[6]. Moreover, many researchers have shown that bacterial viability is not an essential key requirement for health benefits[5,7]. However, despite being less focused on when compared to probiotics, a considerable amount of data has shown beneficial health effects assured when postbiotics are consumed. Still, there are still no reports that indicate the development of side effects when administered in large amounts.

Some authors analyzed the effects of postbiotics on cell viability and inflammatory responses using the RAW-264.7 macrophage cell line, and the results showed that *Lactobacillus gasseri, Lactobacillus paracasei* and *Lactobacillus casei*, *Streptococcus thermophilus* and *Bifidobactercium lactis* improved cell viability when compared to a lipopolysaccharide (LPS) group[8]. In addition, *S. thermophilus* and *B. lactis* decreased the myeloperoxidase activity levels. Also, the author discovered that all the probiotics analyzed showed a significant reduction in the interleukin-6 (IL-6) levels. Researchers developed a study that shows that different probiotics, including L. casei, are able of reduce the levels of interleukin-1 (IL-1), IL-6, myeloperoxidase (MPO) and levels of nitrite/nitrate[9]. In another study, the results showed that intrinsic immunomodulatory properties are available in *S. thermophilus*, expressing an antioxidant enzyme, which confers anti-inflammatory activities[10].

The present study was carried out to obtain data on the effect of repeated low-dose and high-dose postbiotics obtained from strains of *Lactobacillus gasseri* (CCT 7860), *Lactobacillus casei* (CCT 7859), *Lactobacillus paracasei* (CCT 7861), *Streptococcus thermophilus* (ATCC 19258) and *Bifidobacterium lactis* (CCT 7858) in the inflammatory effects in different tissues and damage in the liver and kidney.

METHODS

Animals

In the present study, adult male Swiss mice (25–35 g) were used. After weighing and examination of their external appearance, the animals were housed in a room under constant temperature condition 22 ± 1 °C, 12 h light/12 h dark cycle (from 07:00 a.m. to 07:00 p.m.) and with water and food ad libitum. Each experimental group consisted of 5 animals, provided by the Central Animal Facility of the Federal University of Pelotas. All manipulations were carried out during the light cycle, between 8:00 a.m. and 5:00 p.m. All procedures were approved by the Animal Care and Experimentation Committee of UNESC (Protocol 49/2021-1). Furthermore, all efforts were made to minimize the number and suffering of animals used.

Chemicals

The following postbiotics were used: NEOIMUNO Lactis Gb® (*Bifidobacterium lactis* - CCT 7858), NEOIMUNO Cas Gb® (*Lactobacillus casei* - CCT 7859), NEOIMUNO Gass Gb® (*Lactobacillus gasseri* - CCT 7860), NEOIMUNO Para Gb® (*Lactobacillus paracasei* - CCT 7861), NEOIMUNO Hilus Gb® (*Streptococcus thermophilus* - ATCC 19258). The postbiotics used were produced by Gabbia Biotechnology. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Experimental Design

The scheme of the experimental design of this study is illustrated in Figure 1. Firstly, animals were randomly separated into eleven distinct groups (5 mice/group): Control group (mice received saline solution, 10 mL/kg); *Lactobacillus gasseri* (CCT 7860) 10 mg group; *Lactobacillus gasseri* (CCT 7860) 1 g group; *Lactobacillus casei* (CCT 7859) 10 mg group; *Lactobacillus casei* (CCT 7859) 1 g group; *Lactobacillus paracasei* (CCT 7861) 10 mg group; *Lactobacillus paracasei* (CCT 7861) 1 g group; *Streptococcus thermophilus* (ATCC 19258) 10 mg group; *Streptococcus thermophilus* (ATCC 19258) 1 g group; *Bifidobacterium lactis* (CCT 7858) 10 mg group; *Bifidobacterium lactis* (CCT 7858) 1 g group.

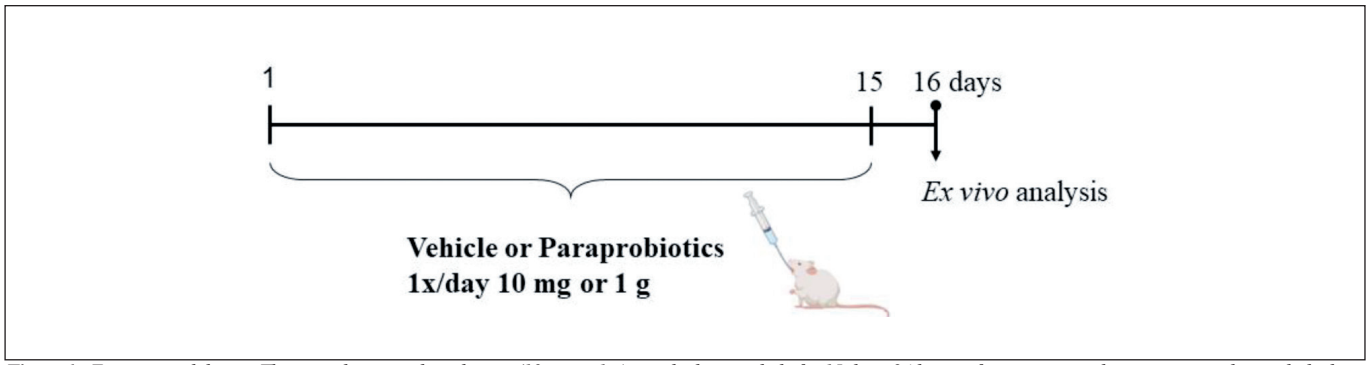

Figure 1 - *Experimental design. The animals received postbiotics (10 mg or 1 g) or vehicle once daily for 15 days. 24 hours after treatments, the mice were euthanized; the liver, kidney, brain, gut and blood were excised and used to carry out the ex vivo assays.*

The experimental protocol consisted of fifteen probiotics administrations in the low-dose and high-dose by the intragastric (i.g.) route at 8 a.m. On the sixteen day of the experimental protocol, the mice were euthanized for ex vivo analysis. So, the mice were euthanized 24 h after the postbiotics administrations.

Dissection of Structures

Immediately after the end of treatments, the mice (n = 5/group) were euthanized and the blood, liver, kidney, brain and gut of animals were surgically removed, processed, and stored in a freezer at −80°C for subsequent biochemical analysis of both samples. In these samples, the MPO activity, nitrite levels and cytokine levels (interleukin 1-beta [IL-1β], IL-10, IL-6) were evaluated.

MPO Activity Evaluation

MPO activity was evaluated as a secondary marker of neutrophil infiltration[11]. Tissues (liver, gut and kidney) were homogenized (50 mg/ml) in 0.5% hexadecyl-trimethylammonium bromide and centrifuged at 15,000×g for 40 min. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM H_2O_2. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37 °C. Data was expressed as milliunits per milligram of protein.

Oxide Nitric (ON) Level

The ON level was estimated by measuring nitrite/nitrate level, through the Griess reaction, in samples (liver, gut and kidney), by adding 100 μl of Griess reagent [0.1% (w/v) naphthylethylendiamide dihydrochloride in water and 1% (w/v) sulphanilamide in 5% (v/v) concentrated H_3PO_4, vol. [1:1] to the 100 μl sample. After 1 h of incubation at room temperature, absorbance was recorded in a spectrophotometer at 550 nm[12]. The results were expressed as nmol of nitrite levels per milligram of protein.

Cytokine Levels

The blood and gut samples were homogenized in an extraction solution containing PBS buffer PH 7.0, centrifuged 5000 rpm for 3 min, and 100 μl of supernatant as used for each assay. The level of cytokines IL-1β (DY501), IL-6 (DY506) and IL-10 (R1000) was determined by enzyme linked immunosorbent assay (ELISA) on a microplate reader using a commercial kit (R&D System), according to the manufacturer's protocol.

Assessment of Kidney and Liver Damage

To evaluate the possible toxicities of probiotic treatments, the plasma levels of AST, ALT (two markers of liver damage) and creatinine (markers of renal function) were investigated. All parameters were determined using commercial colorimetric method kits (Labtest Diagnostica).

Protein Determination

Protein content from homogenized intestine tissue was measured following the previous protocol[13]. The phosphomolybdic phosphotungstic reagent (folin phenol) was added to bind to the protein, being slowly reduced from yellow to blue with a reading obtained at 750 nm absorbance.

Histological Analyses

Another group of the animals (n=4) were euthanized with isoflurane inhalation and jejunum tissue total were immediately collected and fixed in 10% buffered formalin solution. Smaller fragments were processed routinely, embedded in paraffin, cut into sections of 3–4 μm, stained with hematoxylin-eosin (HE) and examined under an optical microscope. It is noteworthy that the histological analysis of the intestine was performed. The histological analyses were performed by one of the authors blinded to the group. Images were read in magnification 10x. Our slides were analyzed showing an ordered progression in severity according to Gibson-Corley et al.[14], where: 0 - normal; 1 - mild; 2 - moderate and 3 - severe.

Statistical Analyses

All data are expressed as means ± standard error medium (SEM), and all statistical analyses were performed using Graph Pad Prism version 9.0 Software (San Diego, CA, USA). To test a Gaussian distribution, a D'Agostino-Pearson omnibus normality test was used. Statistical analyses were performed using a one-way ANOVA followed by the Tukey's test for multiple comparison. Values of $p<0.05$ were considered statistically significant.

RESULTS

Effects of Postbiotics in the Cytokine Levels in the Blood and Brain

Regarding the blood cytokines of mice (Figures 2A, 2B and 2C), the one-way ANOVA analysis revealed effects of different postbiotics in the IL-1β ($F_{(10, 44)}= 17.60$, $p<0.0001$), IL-6 ($F_{(10, 44)}= 18.58$, $p<0.0001$) and IL-10 ($F_{(10, 44)}= 8.52$, $p<0.0001$). The results illustrated in the Figures 2A and 2B show that *Streptococcus thermophilus* (ATCC 19258) in the dose 10 mg and 1 g and *Bifidobacterium lactis* (CCT 7858) 1 g increased the IL-1β and IL-6, respectively, in the blood of animals, while the IL-10 levels (Figure 2C) was increased by *Streptococcus thermophilus* (ATCC 19258) in the dose 10 mg and 1 g treatments.

The one-way ANOVA revealed a significant effect of IL-1β ($F_{(10, 44)}= 3.93$, $p<0.0007$) and IL-10 ($F_{(10, 44)}= 3.23$, $p=0.0034$), but no effect in the IL-6 ($F_{(10, 44)}= 1.29$, $p=0.26$) in the brain of mice (Figures 2D, 2E and 2F). The results of the Figures 2D, 2E and 2F demonstrates that *Lactobacillus paracasei* (CCT 7861) 10 mg decreased the IL-1β, IL-6 and IL-10, respectively. The other postbiotics, independent of dose (10 mg or 1 g) did not alter the cytokines.

Effects of Probiotics in the MPO Activity in the Liver, Gut and Kidney

Related to the MPO activity, the one-way analysis revealed an effect of probiotics in the brain tissue (Figure 3A; $F_{(10, 44)}= 5.76$, $p<0.0001$), in the gut tissue (Figure 3B; $F_{(10, 44)}= 17.10$, $p<0.0001$) and in the liver tissue (Figure 3C; $F_{(10, 44)}= 8.34$, $p<0.0001$). In the kidney, only *Lactobacillus paracasei* (CCT 7861) 1 g increased the MPO activity and in the liver the *Streptococcus thermophilus* (ATCC 19258) 1 g and *Bifidobacterium lactis* (CCT 7858) 10 mg increased this enzyme. Interestingly, in the gut, *Lactobacillus gasseri* 10 mg (CCT 7860) and 1 g, *Lactobacillus casei* (CCT 7859) 10 mg and 1 g, *Lactobacillus paracasei* (CCT 7861) 10 mg and 1 g and *Bifidobacterium lactis* (CCT 7858) 1 g decreased the MPO activity.

Effects of Postbiotics on ON Levels in the Liver, Gut and Kidney

In relation to the effects on ON levels, the one-way analysis revealed a significant effect of probiotics in the brain tissue (Figure 4A, $F_{(10, 44)}= 2.45$, $p=0.0197$), in the gut tissue (Figure 4B; $F_{(10, 44)}= 7.64$, $p<0.0001$) and in the liver tissue (Figure 4C, $F_{(10, 44)}= 2.86$, $p=0.0078$). In the kidney (Figure 4D) and in the liver, none of the postbiotics altered the ON levels. In the gut, Lactobacillus gasseri (CCT 7860) 1 g, *Lactobacillus casei* (CCT 7859) 10 mg and 1 g and *Lactobacillus paracasei* (CCT 7861) 10 mg reduced this parameter.

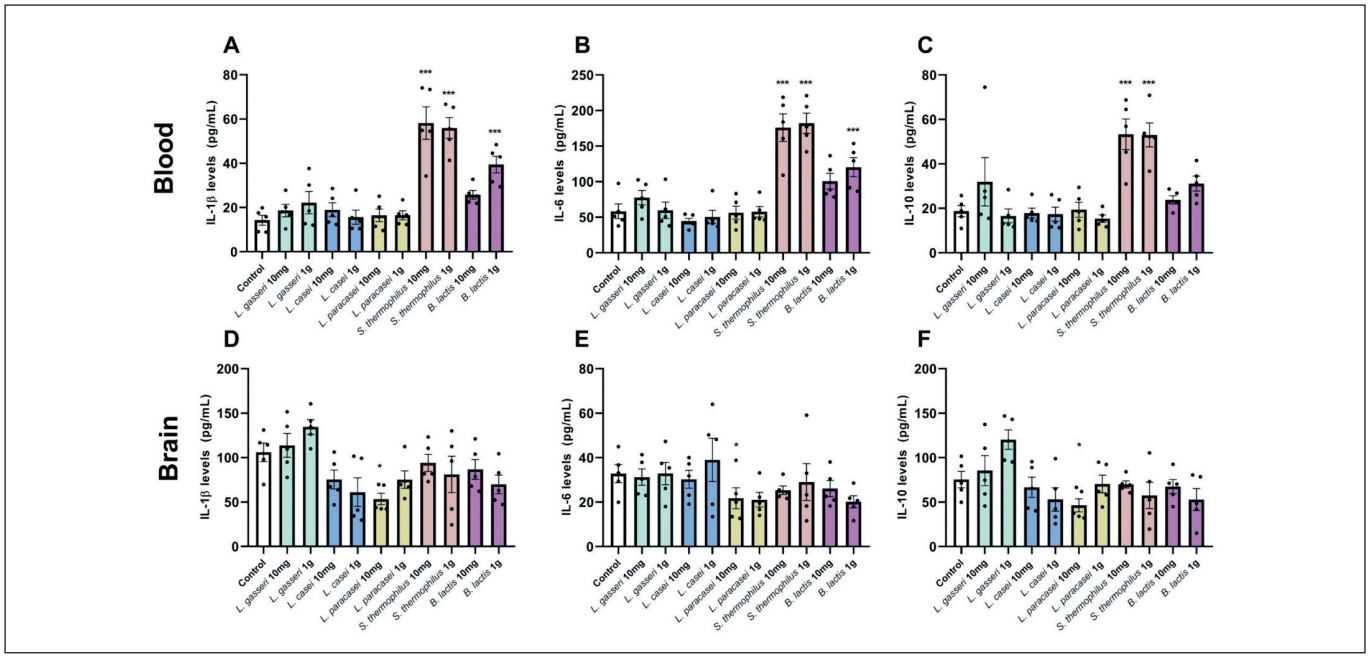

Figure 2 - *Effects of postbiotics (10 mg and 1 g; i.g) on the IL-1β levels, IL-6 levels and IL-10 levels in the blood (A, B, and C, respectively) and in the brain (D, E, and F, respectively) of mice. Each column represents the mean ± SEM. Data were analyzed by a one-way ANOVA followed by Dunnette's multiple comparison test when appropriate. (*) p < 0.05, (**) p < 0.01 and (***) p < 0.001 denote the significance levels when compared to the control group.*

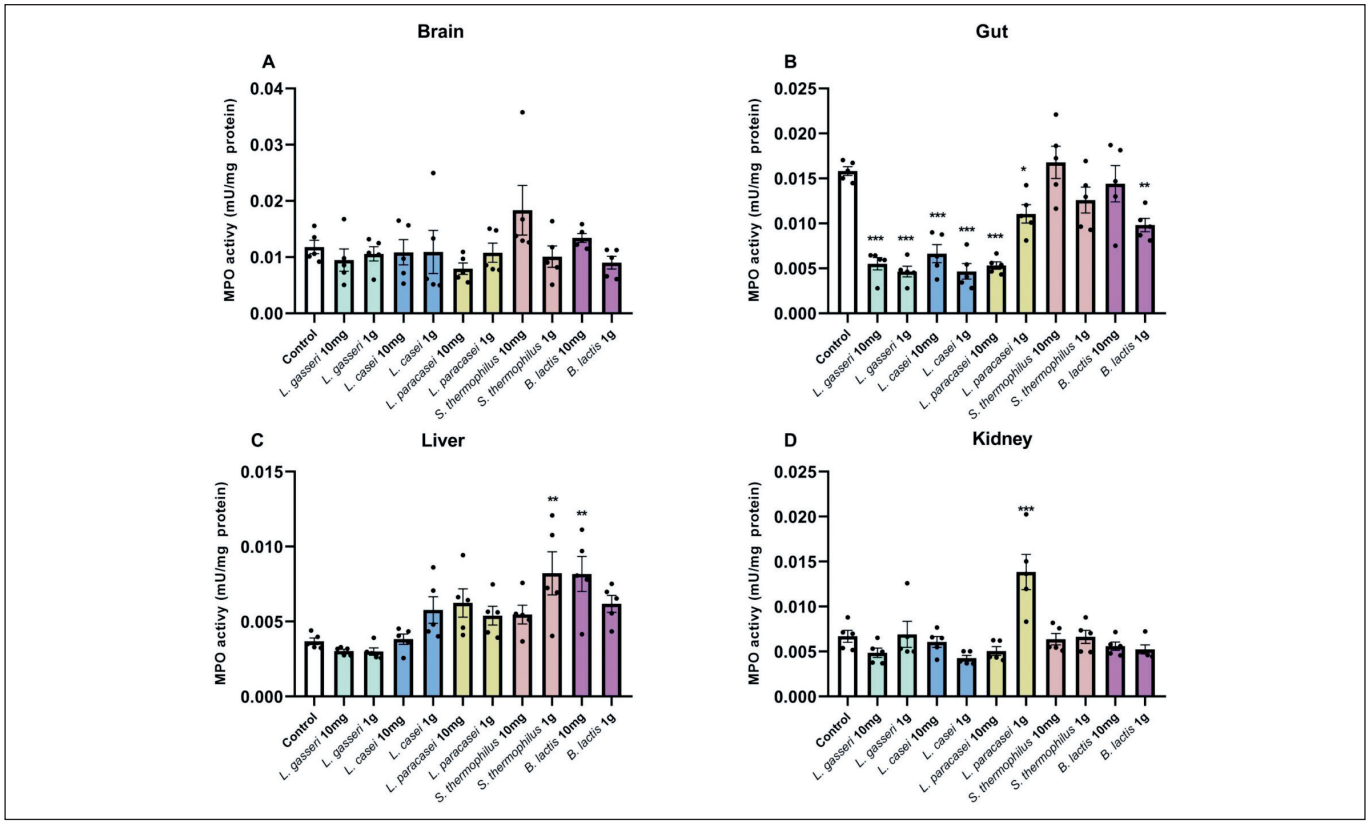

Figure 3 - *Effects of postbiotics (10 mg and 1 g; i.g) on the MPO activity in the brain (A), gut (B) liver (C) and in the kidney (D) of mice. Each column represents the mean ± SEM. Data were analyzed by a one-way ANOVA followed by Dunnette's multiple comparison test when appropriate. (**) $p < 0.01$ and (***) $p < 0.001$ denote the significance levels when compared to the control group.*

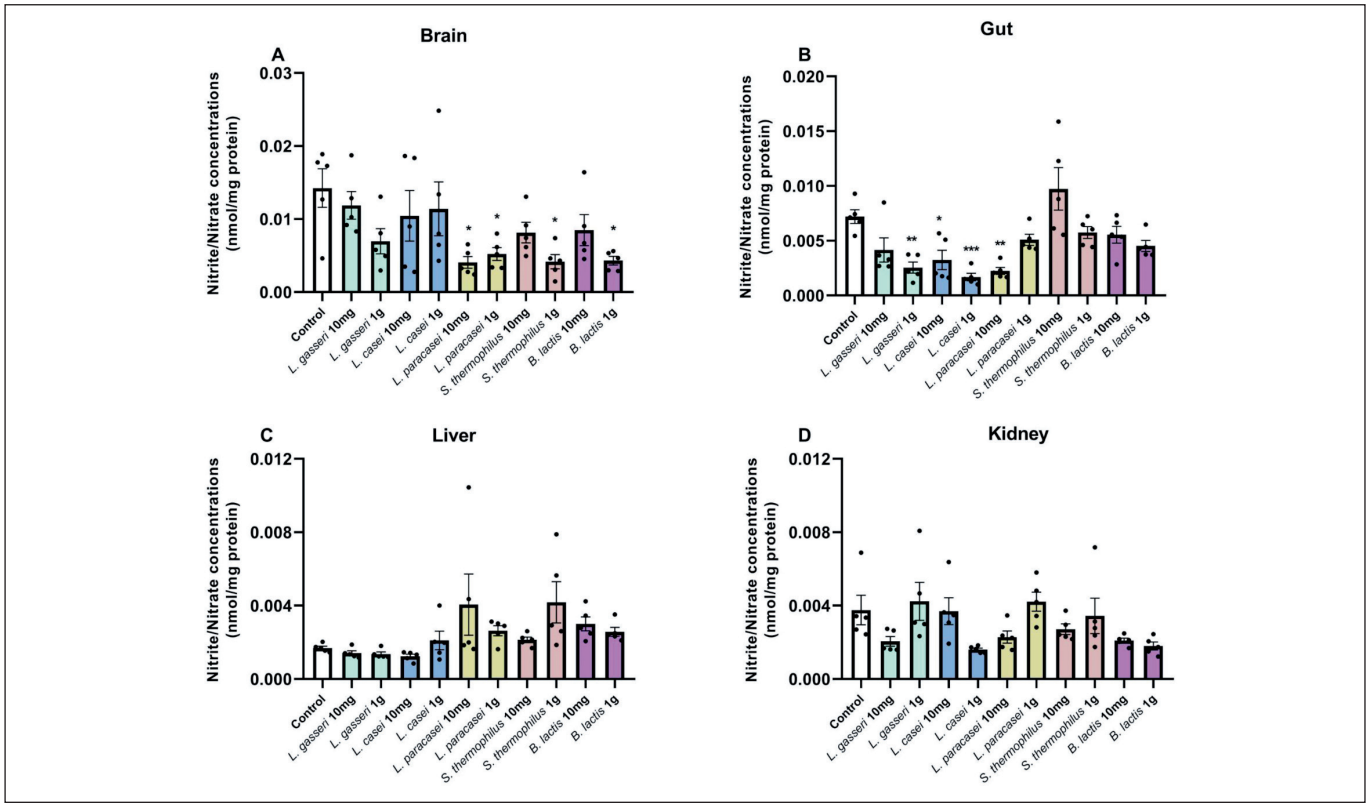

Figure 4 - *Effects of postbiotics (10 mg and 1 g; i.g) on the nitrite/nitrate levels in the brain (A), gut (B) liver (C) and in the kidney (D) of mice. Each column represents the mean ± SEM. Data were analyzed by a one-way ANOVA followed by Dunnette's multiple comparison test when appropriate. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ denote the significance levels when compared to the control group.*

Effects of Postbiotics in the Plasma Levels of AST, ALT and Creatinine

In relation to the effects in the plasma analysis (Table 1), the one-way analysis revealed no significant effect of postbiotics in the AST ($F_{(10, 44)}$= 1.47, p=0.1811) and in the ALT ($F_{(10, 44)}$= 1.57, p=0.1477) levels, but a significant effect in the creatinine ($F_{(10, 44)}$= 2.66, p=0.0122) levels. The *Bifidobacterium lactis* (CCT 7858) 10 mg, but not 1 g increased the creatinine levels.

Table 1 – Effect of paraprobiotics on AST, ALT and creatinine levels.

Treatments	AST (U/L)	ALT (U/L)	Creatinine (mg/dL)
Control	19.80 ± 0.54	21.22 ± 2.02	1.35 ± 0.09
L. gasseri 10 mg	20.88 ± 1.28	20.42 ± 0.46	1.40 ± 0.05
L. gasseri 1 g	19.88 ± 0.41	19.84 ± 0.27	1.46 ± 0.03
L. casei 10 mg	19.69 ± 0.38	20.78 ± 0.92	1.47 ± 0.03
L. casei 1 g	19.01 ± 0.59	20.34 ± 0.39	1.37 ± 0.05
L. paracasei 10 mg	22.63 ± 2.46	19.16 ± 1.03	1.44 ± 0.07
L. paracasei 1 g	25.10 ± 3.92	18.23 ± 0.70	1.80 ± 0.14
S. thermophilus 10 mg	18.49 ± 2.23	19.95 ± 1.40	1.66 ± 0.06
S. thermophilus 1 g	18.38 ± 2.08	17.20 ± 0.38	1.66 ± 0.10
B. lactis 10 mg	18.71 ± 1.04	20.26 ± 2.88	2.11 ± 0.39**
B. lactis 1 g	17.48 ± 1.03	16.15 ± 0.35	1.45 ± 0.59

Note: Values are reported as mean ± SEM for five animals per group. Data were analyzed by one-way ANOVA followed by the Dunnette's test. ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Histological Images

Figure 5 exhibits representative histological images of the gut from the animals. Preserved villi were noted in both doses and in all groups. Slides were analyzed showing an ordered progression in severity, according Gibson-Corley et al.[14]. All slides had normal grade (0) and no slides showed mild or more lesion grade (see supplementary material).

DISCUSSION

Though postbiotics have been widely used in supplementation, not enough toxicological data have been generated. Thus, the major findings of the present study were the evaluation of the safety of low-dose and high-dose of five different postbiotics. Between the five postbiotics tested, *Streptococcus thermophilus* (ATCC 19258) and *Bifidobacterium lactis* (CCT 7858) in high dosage increased the IL levels in the blood of mice, which is probably a response to repeated doses. In contrast, most postbiotics did not cause alterations in these parameters or reduce per se the IL and ON levels, such as MPO activity.

The low toxicity of postbiotics is supported by the observation that, after its administration for 15 consecutive days, there were no changes in the levels of AST and ALT (markers of liver damage), creatinine (marker of kidney damage) and in the histopathology of the intestine of mice, confirming the safety of its use. An exception is the *B. lactis* (CCT 7858) 10 mg, that increased creatinine.

The microbiota has recently been associated in the pathophysiology of many intestinal and extraintestinal diseases, including depression[15], obesity[16], diabetes[17], rheumatoid arthritis[18], Alzheimer disease[19], Parkinson disease[20] and many more. Thus, the appropriate balance of the intestinal microbiota is essential to physiological functions and the use of postbiotics could have therapeutic potential in favor of diseases. The popularity of probiotics has gained attention in the medical field, due to its reduced toxicity and improved health condition of a host.

First, our study investigated the levels of IL in the blood and brain, to verify the toxicity related to inflammatory processes in the both tissues. The inflammation is a biological response of the immune system, that is triggered by a variety of factors, including toxic compounds. The IL are inflammatory cytokines released by some cells, including monocytes, macrophages, and lymphocytes[21]. Here, only *Streptococcus thermophilus* (ATCC 19258) and *Bifidobacterium lactis* (CCT 7858) in the high-dose increased the IL6, IL1β and IL10 levels in the blood. These results suggest that repeated exposure to postbiotics can induce inflammation in the blood, and it seems that the inflammatory responses are too weak in blood to cause some alteration in the brain. Other studies also demonstrated that *Streptococcus thermophilus* (ATCC 19258) induced a significant increase of anti-inflammatory IL-6 and IL-10 cytokines, suggesting that this postbiotics is beneficial in the management and treatment of immune and inflammatory diseases[22,23].

Lactobacillus paracasei (CCT 7861) 10 mg reduced the IL1β in the brain. It corroborates a previous study, which demonstrated that *Lactobacillus paracasei* (CCT 7861) has anti-inflammatory properties[24]. Furthermore, it was previously demonstrated that several *Lactobacillus* spp. exert effect on many inflammatory disorders.

In the current study, the MPO activity was measured to evaluate the neutrophil accumulation in the liver, gut and kidney. The results were more significant in the gut, because all *Lactobacillus* spp. in both doses and *Bifidobacterium lactis* (CCT 7858) in the high-dose reduced the MPO activity, suggesting a lower infiltration of neutrophils and a possible reduction in gut inflammation. It corroborates a previous study, that showed the attenuation of gut inflammation by a probiotic mixture containing *Lactobacillus* spp. and *Bifidobacterium* spp., suggesting that this mixture can be used to protect against gastrointestinal disorders[25]. Finally, our study shows that postbiotics supplementation is safe and not toxic, even in high doses.

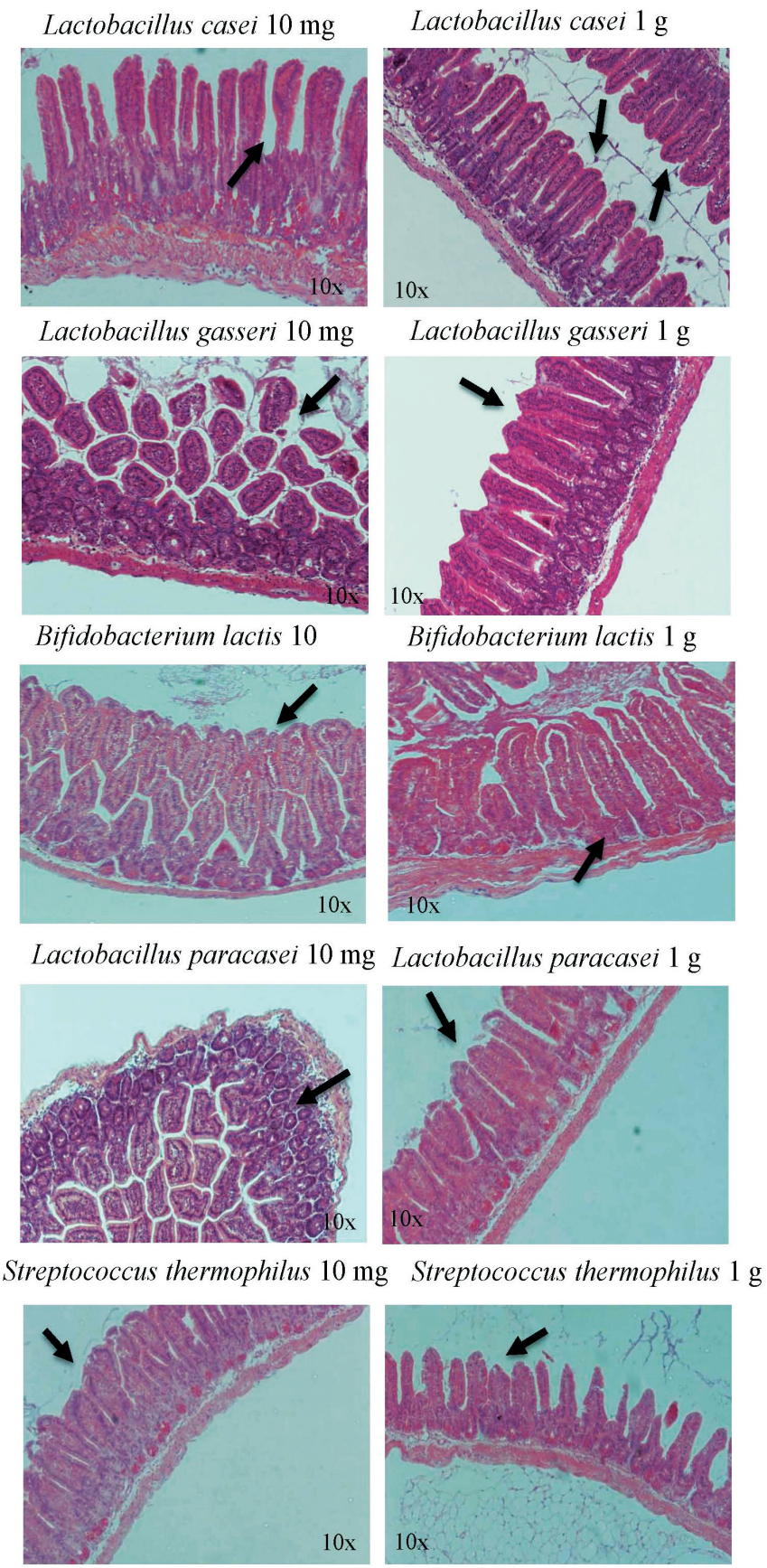

Figure 5 - *Representative histological images of the gut of mice at 10x magnitude. L. casei 10mg and 1g; L. gasseri 10mg and 1g; B. lactis 10mg and 1g; L. paracasei 10mg and 1g; S thermophilus 10mg and 1g. Arrows show intact villi. Only qualitative representations. (n=4).*

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Study location: University of Southem Santa Catarina (UNESC), Criciúma, SC, Brazil.

Conflict of interest: MPR e APV tem vínculo empregatício com a empresa Gabbia Biotechnology, que cedeu os probióticos empregados no presente estudo.