Effects of Different Probiotic Strains B. Lactis, L. Rhamnosus and L. Reuteri on Brain-Intestinal Axis Immunomodulation in an Endotoxin-Induced Inflammation

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Research Article

Keywords: probiotics, gut-brain axis, inflammation, immunomodulation

Posted Date: December 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1096782/v2

Abstract

Aim: The study evaluated the effects of supplementation with three different probiotic strains *B. lactis* (LACT GB^{TM}), *L. rhamnosus* (RHAM GB^{TM}) and *L. reuteri* (REUT GB^{TM}) on brain-intestinal immunomodulation in an animal model of LPS-induced inflammation.

Methods: 50 mice Balb/C were distributed into five groups: Control; lipopolysaccharide (LPS); LPS + *B. lactis* (LACT GB^{TM}); LPS + *L. rhamnosus* (RHAM GB^{TM}); LPS + *L. reuteri* (REUT GB^{TM}). The animals were supplemented with their respective probiotic microorganisms daily, for 30 days, at a concentration of $1x10^9$ CFU/animal/day. After 30 days of supplementation, animals received the inflammatory insult by LPS (15mg/kg). Behavioral tests, oxidative stress and inflammation were performed, as well as gut and brain histology.

Results: In the behavioral test, LPS+ B. lactis group was less anxious than the other groups. Serum interleukin IL-1β and IL-6 levels increased in all groups that received the LPS insult and there was a reduction in inflammation in the supplemented groups when compared to the LPS group in brain and gut. A reduction in myeloperoxidase activity and oxidative stress in groups supplemented with probiotics. Intestine histological analysis, damage to tissue integrity in the LPS group and preservation of integrity in the supplemented animals. In the brain, infiltrates of perivascular inflammatory cells can be seen in the LPS group.

Conclusion: The three probiotic studies showed efficient immunomodulating activity and ensured integrity of the intestinal barrier function, even after the severe insult by LPS. These results show the important role of probiotics in the gut-brain axis.

Introduction

The supplementation of probiotics has attracted the population's interest in health promotion and disease prevention. Probiotics are live microorganisms that provide health benefits to the host [1]. The main advantages of probiotic ingestion reported in systematic reviews are related to several different conditions. Those include prevention and treatment of necrotizing enterocolitis [2]; decreased incidence of diarrhea associated with antibiotic use [3] ; decreased duration of infectious and inflammatory diseases [4]; regulation of intestinal transit [5]; relief of irritable bowel syndrome symptoms [6] ; decrease in the incidence of upper respiratory tract diseases [7], reduction in allergy symptoms, serum cholesterol concentration, stmulation and modulation of the immune system and modulation of gene expression [8].

Probiotics have been widely studied and characterized as modulators of humoral, cellular, and nonspecific immunity [9, 10], such as decreased proinflammatory cytokines [11]. In addition, several studies have reported that probiotics also produce antioxidants and reduce lipid peroxidation [12].

On the other hand, the gut microbiota has the function of producing metabolites that can have positive effects on the host, including anti-inflammatory and antioxidant activity, regulating the intestinal barrier

function, in addition to participating in the development and maintenance of the immune and sensory functions of the gut [13]. The literature reports a series of evidence that the intestinal microbiota communicates with the central nervous system (CNS) through the gut-brain axis, possibly through neural, endocrine and immune pathways [14], where interactions change gut-brain are associated with intestinal inflammation, chronic abdominal pain syndromes and eating disorders [15], and their modulation is associated with stress response and behavior [16].

Thus, the present study evaluated the effects of supplementation of three different probiotic microorganisms *B. lactis, L. rhamnosus*, and *L. reuteri* on intestinal health, anti-inflammatory activity, antioxidant, and anxiety in an animal model of LPS-induced inflammation.

Materials And Methods

Animals

The experimental procedures involving animals were performed in accordance with the Brazilian law of animal welfare and with the approval of our institutional ethics committee (protocol number: 22/2021. Adult male mice (Balb/C), 60 days old, weighing between 20-30g were used. The mice were kept in light-dark cycles of ± 12 hours (7:00 am to 7:00 pm) at a temperature between 18 and 22 ° C, relative humidity between 55 and 65%. The animals had free access to water and food.

Probiotic strains

The probiotic microorganisms used in this study were supplied by the company Gabbia Biotechnology: *B. lactis* (LACT GB^{TM}), *L. rhamnosus* (RHAM GB^{TM}) and *L. reuteri* (REUT GB^{TM}).

Experimental design

The animals were divided into five groups, each group consisting of 10 animals:

1. Group Control
2. Group LPS
3. Group LPS + *B. lactis* (LACT GB^{TM})
4. Group LPS + *L. rhamnosus* (RHAM GB^{TM})
5. Group LPS + *L. reuteri* (REUT GB^{TM})

After the acclimation period, treatment started. Treated groups were supplemented, via gavage, once a day with their respective probiotics for 30 days, at a concentration of 1x10^{9} CFU/animal/day.

After 30 days of supplementation, the animals received an inflammatory insult by LPS at a dose of 15mg/kg. On the 32nd day, a behavioral test was performed, and then animals were euthanized to collect serum, whole brain, and intestine samples for subsequent analyses.

Behavior test- Elevated plus maze

The elevated plus-maze test was performed to assess anxiety-like behavior [17]. The equipment consists of two open arms (50x10 cm) and two closed arms (50 x 10 x 40 cm) arranged perpendicularly forming a central platform (5 x 5 cm). The experiments were conducted in a dark room with a red light positioned 50 cm high from the central platform. The animals were placed on the central platform and had 5 minutes to explore the device. The parameters evaluated were: length of stay in the closed and open arms and the total number of entries into both.

Cytokines levels

The concentrations of IL-1β, IL-6 and IL-10 were determined in serum, brain, and intestine using the enzyme immunoassay technique (ELISA) in a microplate reader using a commercial kit (R&D System - USA). Results were expressed as pg/ml.

Myeloperoxidase Activity

Myeloperoxidase activity is indicative of tissue neutrophil infiltration. Tissue was homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide and centrifuged. The suspension was sonicated and an aliquot of the supernatant was mixed with 1.6 mM TMB and 1 mM H_2O_2 solution. MPO activity was measured spectrophotometrically at 650 nm at 37^{o}C. Results were expressed as mU/mg protein [18].

Nitrite/nitrate

The nitrite/nitrate concentration is indicative of the amount of nitric oxide (NO) present in the samples. Concentration was measured by the Griess reaction, reading at absorbance of 550 nm using a microplate reader. Results were expressed as nmol/mg protein [19].

Lipid Oxidative Damage (TBARS)

TBARS is a technique that assesses lipid damage through the reaction to thiobarbituric acid. Briefly, the samples were mixed with 1 ml of 10% trichloroacetic acid for deproteinization and then incubated with 1 ml of 0.67% TBA. Afterwards, samples were heated in a boiling water bath for 30 min. Equivalents to malondialdehyde (MDA) were determined by absorbance at 532 nm, using 1,1,3,3-tetramethoxypropane as external standard. Results were expressed as MDA equivalents (nmol/mg protein) [20].

Histology for evaluation of gut inflammation

Immediately after death, samples of the brain and terminal ileum were removed. The samples were washed with saline solution and immediately immersed in 4% paraformaldehyde and remained for 48 hours; after this period the tissues were removed, placed in different concentrations of 70%, 80% and 90% ethanol and embedded in paraffin. Longitudinal sections (5 µm) of colon and brain tissue were cut and stained with hematoxylin and eosin (HE). Digital micrographs were taken with an inverted Nikon microscope. Inflammatory alterations in the tissue were evaluated independently by a researcher and a pathologist blinded to information on treatment. Semiquantitative scoring was performed according to Erben et al. (2014) [21].

Statistical analysis

Variables are presented as mean ± standard deviation and compared using one-way analysis of variance (ANOVA) followed by Tukey's test when F is significant. All tests were performed using SPSS version 21 and/or GraphPad Prism 7.0. In all analyses, a p-value < 0.05 was adopted as a level for statistical significance.

Results

Elevated plus-maze test was performed in order to assess anxiety-like behavior in animals. As expected LPS induced an anxiety-like behaviour (Figure 1). The animals supplemented with *B. lactis* had more entries in the open arms when compared to the LPS group (Figure 1).

In order to assess the profile of inflammation and the potential immunomodulation of probiotic microorganisms, the quantification of cytokines in serum, brain and gut was performed. The results are shown in figure 2. In serum, interleukin IL-1β and IL-6 levels increased in the LPS group, and were not affected by any probiotic. There were no statistically significant differences in serum IL-10 levels. In the brain the levels of interleukin IL-1β were lower in the groups supplemented with *L. reuteri* and *B. lactis*, after an inflammatory insult. IL-6 interleukin levels were reduced in all supplemented groups. In the gut, the levels of interleukin IL-1β and IL-6 were reduced in all groups supplemented after inflammation. In the LPS group there is a reduction in IL-10 levels, and the *L. reuteri* group IL-10 levels were similar to the control group.

As a more general marker of tissue inflammation, myeloperoxidase activity was measured in gut and brain (Figure 3). There was a statistically significant reduction in myeloperoxidase activity in the groups supplemented with probiotics only in the brain.

Oxidative stress was also measured in gut and brain using nitrite/nitrate concentration and TBARS technique. In both, there was a statistically significant reduction in nitrite/nitrate concentration in all supplemented groups. In the gut, there was a statistically significant reduction in lipid peroxidation in all supplemented groups. In the brain, this reduction was verified only in the group supplemented with *L. Reuteri*. (Figure 4).

Finally, histological analysis of the gut and brain were performed (Figure 5). Semiquantitative scoring was performed in the gut. The effect of LPS on tissue integrity was verified as well as inflammatory infiltrate. In supplemented animals, the villi remain intact, as well as the absence of inflammatory infiltrate. In addition, the *B. lactis* group had a higher number of goblet cells per villi, indicating greater efficiency in mucus production ($p<0.005$). In the brain tissue there is an increase in the number of perivascular inflammatory cell infiltrates in the LPS group (only qualitative analysis). This condition was not verified in the groups supplemented with probiotic microorganisms.

Discussion

Our study shows the bidirectional relationship between the intestine-brain axis and the role of different probiotic strains in the presence of inflammatory insult, improving anxiety-like behavior, reducing inflammation, oxidative stress and tissue damage.

Bidirectional communication between brain and gut has long been recognized. There is a growing body of evidence documenting the ability of prebiotics, probiotics, synbiotics and other diets to normalize dysbiosis associated with psychological disorders [22, 23]. Numerous works focus on the impact of the microbiota on behaviors such as anxiety or depression [24, 25, 26]. Anxiety and depressive episodes are associated with dysregulation of the HPA axis [27]. Evidence from experiments carried out in animals with altered intestinal microbiota, whether GF mice or conventionally animals treated with antibiotics and/or probiotics or infected, all indicate that rodent behavioral responses are impacted when the bacterial status of the gut is manipulated [28, 29, 30].

Probiotics play a key role in the balance of the gut flora, restoring the composition of the microbiota [31]. Our results indicate immunomodulatory activity by probiotics, since they were able to reduce the inflammatory response through the reduction of different proinflammatory players (See Graphical abstract). LPS produced by Gram negatives bacteria enters in the circulation through intestinal permeability, activating the immune response and TLR4/NF-kB signaling pathway [32].

This result contributes to the gut-brain axis relationship. Some studies suggest that an improvement in the symptoms associated to psychiatric and neurological disorders, as well as the oxidative stress, inflammatory biomarkers and metabolic state in general, through the probiotic effects on CNS bidirectional circuits are mediated by the gut-microbiota-brain axis [33, 34]. Different probiotics have been investigated for psychiatric and neurological disorders; however, Bifidobacterium and Lactobacillus have been shown to be more effective [35]. Literature evidence shows that increased inflammation is associated with anxiety-like behavior [28–30, 36–38]. In general, the mechanisms underlying the effects of the microbiota on the CNS are multifactorial (immunologic, endocrine and neural), but these effects are believed to principally occur via the generation of bacterial metabolites [39]. The mechanisms of action of probiotics involve colonization of intestinal microbial; competitive exclusion of pathogens and bacteriocin production; modulation of enzymatic activities and production of volatile fatty acids. In addition, probiotics increase mucin production and cell adhesion in the gut [40]. Thus, probiotic metabolites are able to interact with the brain-gut axis and play a role in behavior [40].

The decrease in oxidative stress in animals supplemented with probiotics indicates immunomodulatory and antioxidant activity provided by probiotic supplementation. Thus, probiotics provide health benefits, mainly by maintaining intestinal integrity. This assertion can be supported by the results obtained in this study, the reduction of inflammation and oxidative stress, the preservation of intestinal villi, the better behavioral response as well as the reduction of brain inflammation confirm the interaction between the gut-brain bidirectional axis, demonstrating how the maintenance of intestinal integrity provided by probiotic microorganisms prevented inflammation in brain tissue, ensuring greater health and homeostasis.

Conclusion

The probiotic microorganisms B. lactis (LACT GBTM), L. rhamnosus (RHAM GBTM) and L. reuteri (REUT GBTM) showed efficient immunomodulatory activity, verified through the anti-inflammatory and antioxidant activity. In addition, probiotic supplementation was able to guarantee the integrity and the intestinal barrier function, even after the severe insult by LPS, where the modulation of the inflammatory process was verified, avoiding systemic inflammation. Finally, supplementation with the probiotic B. lactis (LACT GBTM) had an effect on the behavior of the animals, being able to reduce anxiety related to the bidirectional interaction of the intestine-brain axis.

Declarations

Funding: GABBIA Biotechnology and UNESC

Data availability: Data will be made available on reasonable request

Conflicts of interest/Competing interests: Gabbia Biotechnology are developing probiotics strains for commercial purposes. Gabriel Jesus, Fernanda Ramlov, Marina Rosseto and Ana Paula Voytena are members of Gabbia Biotechnology. The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Consent to participate: All authors listed have contributed sufficiently to the review to be included as authors, and all those who are qualified to be authors are listed in the author byline.

Consent for publication: The manuscript has been read and approved by all named authors and we confirm that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions, and final approval of proofs.

Authors' contributions: MM, GFAJ - conceptualization and design of the study; Data curation; Formal analysis and Methodology; Roles/Writing - original draft.MRA, EC, LC - conceptualization and design of the study; Methodology**.** HMB, NSM, LBR, RD - conceptualization and design of the study; Methodology, Data curation**.** CSS, APV, MR, FR - Data curation; Formal analysis and Methodology. FDP - conceptualization and design of the study; Project administration; Supervision; Funding acquisition; Writing - review and editing. All authors approved the final version submitted.

Acknowledgements: GABBIA Biotechnology and UNESC

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Figures

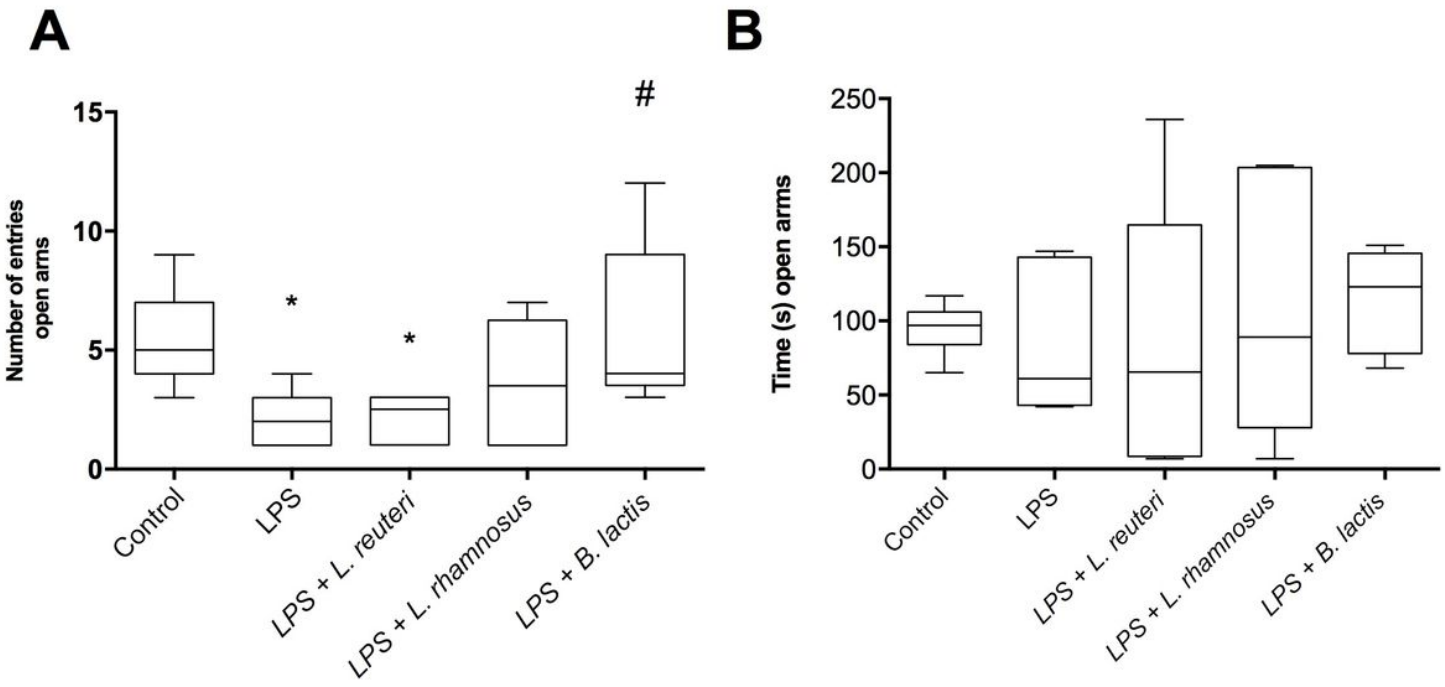

Figure 1

Behavioral elevated plus maze test in mice supplemented with probiotics that received an inflammatory insult by LPS. (A) Number of entries in open arms; (B) Time in open arms [seconds]. mean±SD. ANOVA test. * different of control group; # difference of LPS group. n=10. $p<0.05$.

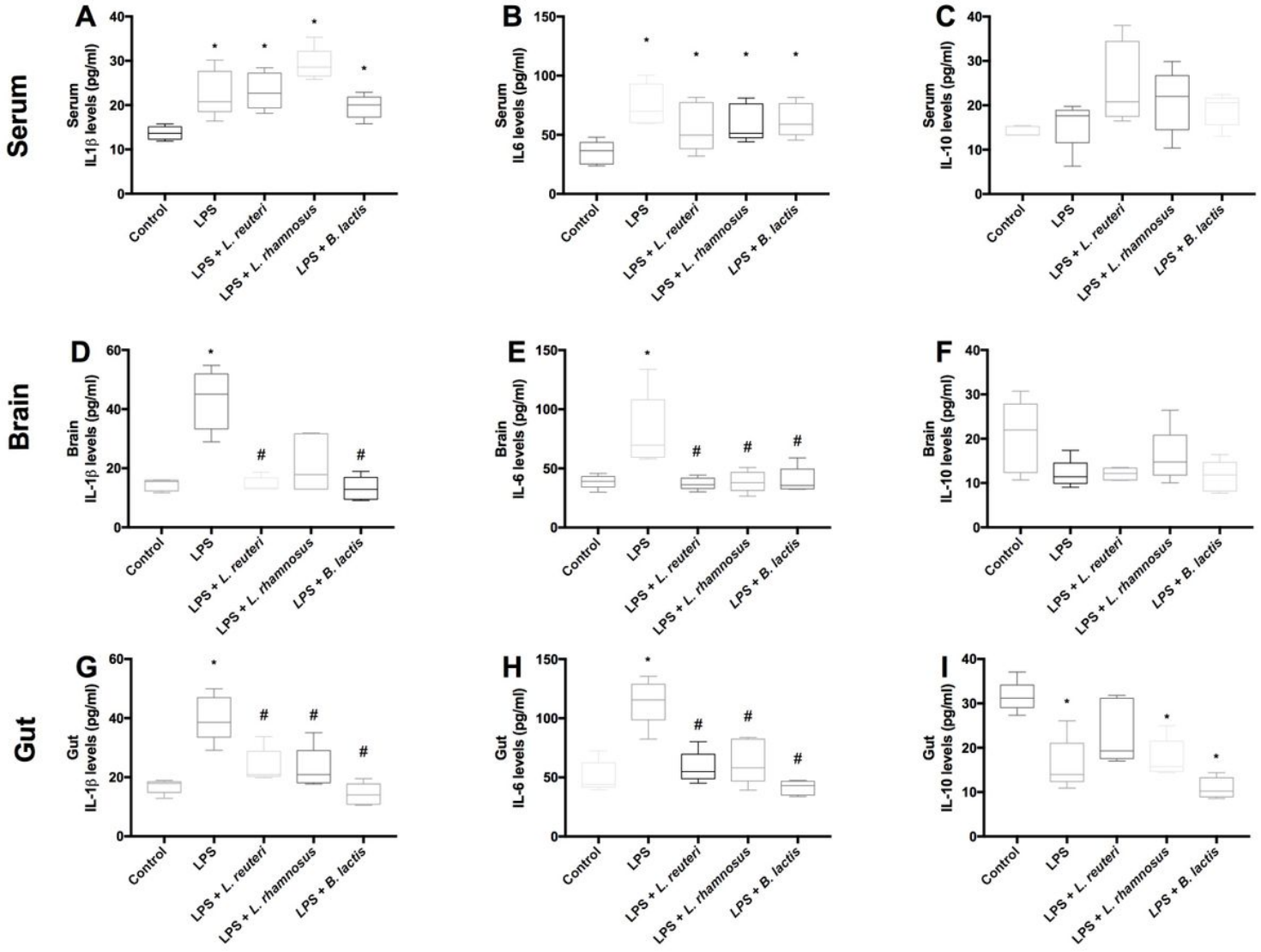

Figure 2

Cytokines levels IL-1, IL-6 and IL-10 in serum (A, B, C), brain (D, E, F) and gut (G, H, I) in mice supplemented with probiotics that received an inflammatory insult by LPS. mean±SD. ANOVA test. * different of control group; # difference of LPS group. n=6-8. $p<0.05$.

Figure 3

Myeloperoxidase activity in gut (A) and brain (B) in mice supplemented with probiotics that received an inflammatory insult by LPS. mean±SD. ANOVA test. * different of control group; # difference of LPS group. n=6-8. $p<0.05$.

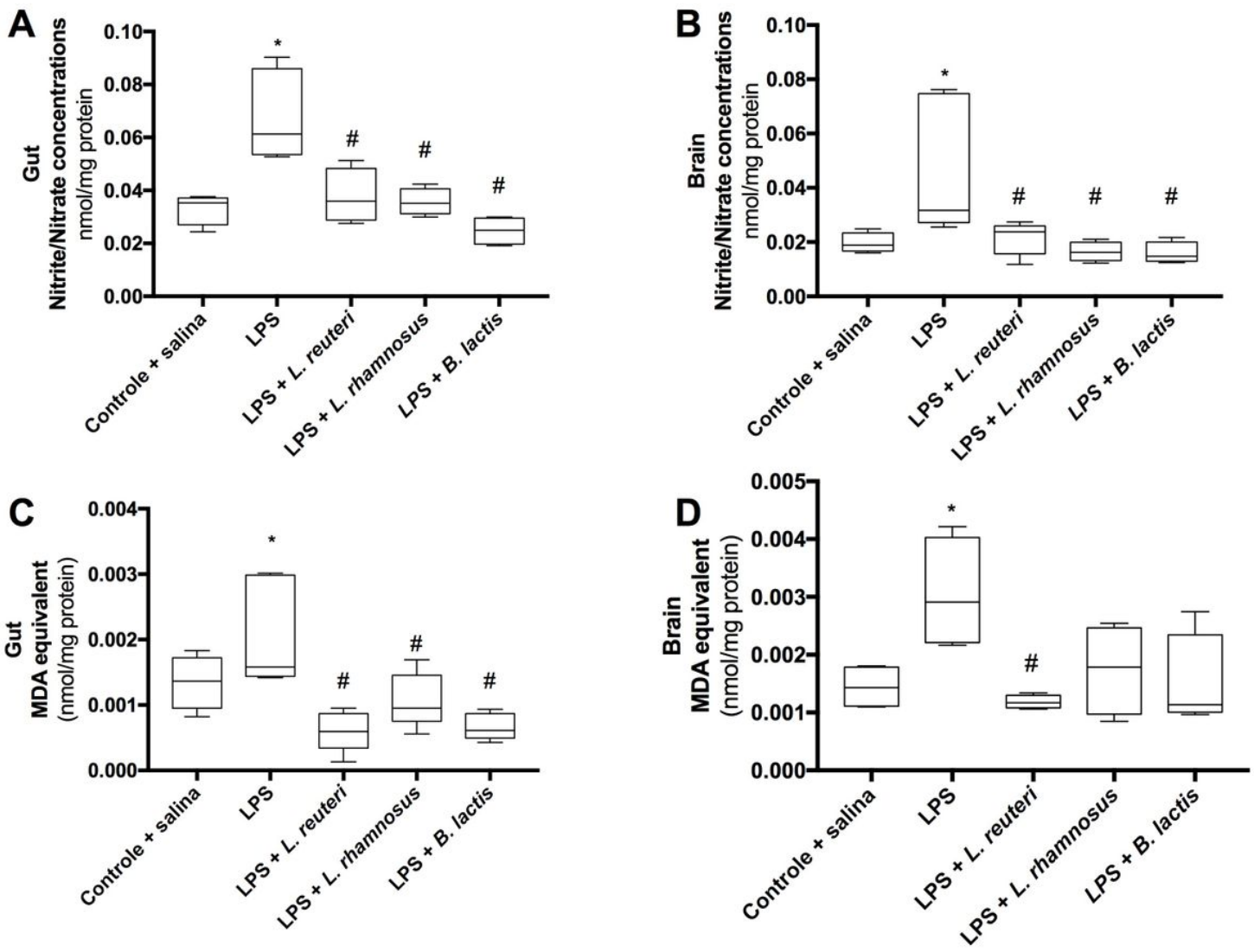

Figure 4

Nitrite/nitrate concentrations (A, B) and lipid peroxidation (C, B) in gut and brain of mice supplemented with probiotics that received an inflammatory insult by LPS.mean±SD. ANOVA test. * different of control group; # difference of LPS group. n=6-8. $p<0.05$.

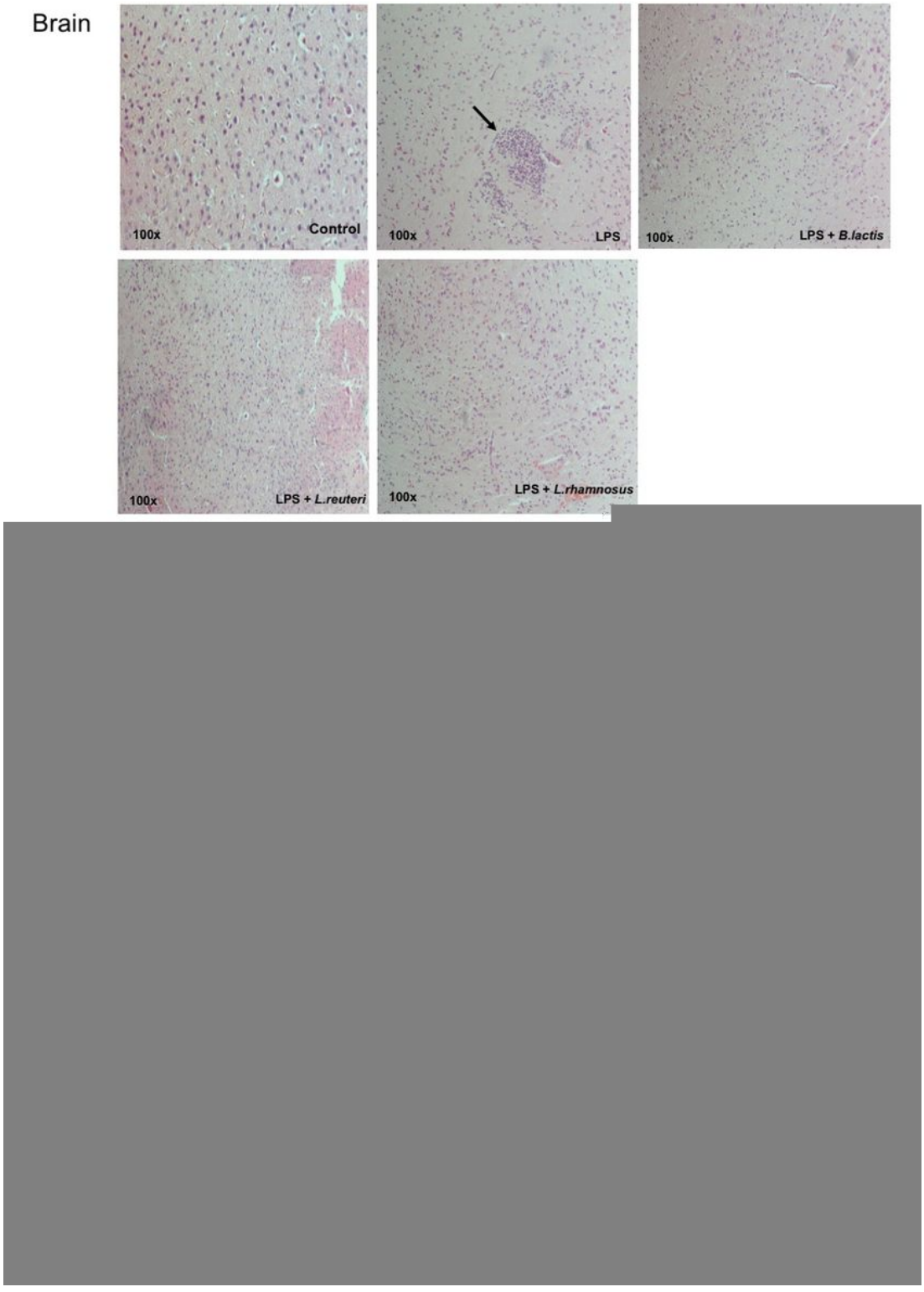

Figure 5

Representative histological images of gut and brain from mice supplemented with probiotics that received an inflammatory insult by LPS. (A) Gut architecture score; (B) Inflammatory cell infiltrade score in gut.

Supplementary Files

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- Graphicalabstract.png