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Highlights

- Lactic acid bacteria (LAB) improved motor behavior in a Parkinsonism mouse model.
- LAB administration increased tyrosine hydrolase positive cells in the brain.
- LAB administration decreased the levels of pro-inflammatory cytokines in serum.
- LAB administration increased IL-10 in serum and brain.

Neuroprotective effects associated to immune modulation by selected lactic acid bacteria in a Parkinson's disease model

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Running Head: Neuroprotective effect of selected lactic acid bacteria

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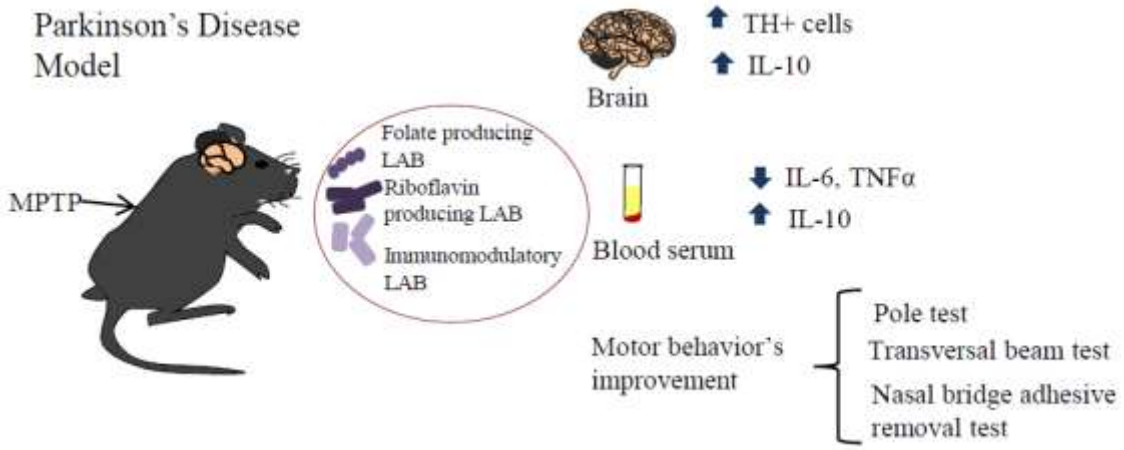
Graph abstract



Abstract

Objectives: Parkinson's Disease (PD) is a neurodegenerative process affecting motor function that involves an inflammatory response and B vitamins deficiencies. The aim of this study was to evaluate the effect of B-group vitamin producing and immunomodulatory lactic acid bacteria (LAB) in a murine model of PD.

Methods: The effect of *Lactobacillus plantarum* CRL 2130 (a riboflavin producer), *Streptococcus thermophilus* CRL 807 (an immunomodulatory strain) and *Streptococcus thermophilus* CRL 808 (a folate producer) were evaluated individually and as a mixture in mice injected with 1-methyl-4-fenil-1,2,3,6-tetrahidropiridina (MPTP). Motor capacity, tyrosine hydrolase (TH) in brain and cytokine concentrations in serum and in brain tissues were evaluated in MPTP treated mice after bacterial supplementation.

Results: Mice receiving the selected LAB showed significantly improved motor skills compared to those that did not receive bacterial supplementation. When given the mixture of all 3 strains together, animals had higher brain TH+ cell counts, decreased the inflammatory cytokines IL-6 and TNF-α in serum and increased the anti-inflammatory cytokine IL-10 in serum and brain tissues compared to animals that did not receive LAB supplementation.

Conclusions: The results showed the potential of a selected LAB mixture to improve motor behavior and neuroinflammation in PD. This probiotic mixture could be used as an adjunct treatment in the control of PD.

Keywords: Lactic acid bacteria; Parkinson's disease; probiotic; inflammation; motor behavior; animal model.

Introduction

Parkinson's disease (PD) as well as other neurological disorders has historically been studied within the central nervous system (CNS); however, peripheral influences have also been implicated in their onset and/or progression. In fact, emerging data propose bidirectional communication between the intestine and the brain; recent studies suggest that intestinal toxins can induce the formation of alpha synuclein (α-syn) aggregates in the enteric nervous system, which then can reach the CNS through the vagus nerve [1]. The intestinal microbiota has also been shown to control the differentiation and function of immune cells in the intestine, periphery and brain [2]. It has been reported that the total counts of intestinal bacteria decrease during PD progression [3]; however, it is unsure if modifications of the intestinal microbiota are the cause or consequence of this disease.

The CNS has traditionally been considered immunologically privileged due to the protection conferred by the blood-brain barrier. Neurons of the CNS are actively involved in control of the immune response by modulating the function of glial cells and T lymphocytes [4]. Pro-inflammatory cytokines produced in peripheral tissues are able to modulate neuronal circuits in the CNS through specific receptors expressed by neurons, and this response can prevent and/or induce eventual immune-mediated brain damage [4].

Researches about the modulation of the gut-brain axis via the gastrointestinal microbiota are still emerging. Probiotics, prebiotics and synbiotics can beneficially modify the gut microbiota composition and influence the gut-brain axis [5, 6]. Recently, the term "psychobiotic" has been associated to a group of probiotics that can affect the CNS [7]. Cerdó et al., (2017) showed that these supplements have been associated with benefits against different CNS disorders; however, the molecular mechanisms associated to the gut's microbial modulation and its influences on the CNS are not fully understood [8]. The need to individualize specific prebiotic compounds and probiotic strains for nutrition and lifestyle medicine practitioners has been recently described [9]. Author highlighted that simple dietary interventions as well as moderate levels of physical activity and the use of stress management techniques, pose potential benefit to management the intestinal microbiota. In this sense, current treatments for PD not only include medical and surgical therapies, but also include the management of diet and nutritional supplements.

Regarding vitamins, increases in blood homocysteine (Hcy) concentrations has been reported in PD patients, and it is known that high intakes of B vitamins, decrease the level of Hcy; however; information regarding the direct association between PD and B vitamins is still very scarce [10]. Riboflavin (vitamin B2) can be neuroprotective by decreasing oxidative stress, mitochondrial dysfunction, neuroinflammation and glutamate excitotoxicity. Another B group vitamin involved in the metabolism of Hcy is folate (vitamin B9). Patients suffering PD presented deficiency of folates [11]; and the beneficial effect of folate intake in levodopa-treated PD patients has been reported [12].

The capacity of lactic acid bacteria (LAB) to produce vitamins in novel functional foods or in situ once the microorganisms colonize the host intestine has been reported [13]. Previously, our group has demonstrated that selected LAB are able to produce B group vitamins (riboflavin and folates) and had beneficial effects against intestinal inflammatory diseases using animal models [14-16]. It has also been shown that certain immune-modulating strains of LAB also confer anti-inflammatory effects [17]. However, the potential benefits of these LAB on neurological diseases have not yet been evaluated.

Based on this information, the aim of the present work was to evaluate the neuroprotective effect associated to the anti-inflammatory properties of a LAB mixture composed of two vitamins producing strains (*Lactobacillus plantarum* CRL 2130 and *Streptococcus thermophilus* CRL808, riboflavin and folate producers, respectively) and an immune-modulator strain (*Streptococcus thermophilus* CRL807) by using an *in vivo* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced parkinsonism model.

Materials and Methods

Bacterial mixture preparation

The bacterial strains were obtained from CERELA Culture Collection (Tucumán, Argentina): *Lactobacillus* (*L.*) *plantarum* CRL 2130, a riboflavin-overproducing strain [18]; *Streptococcus* (*S.*) *thermophilus* CRL 808, a folate-producing strain [19]; and *S. thermophilus* CRL 807, a strain with immunomodulatory properties [20]. Each LAB was grown individually then mixed when required following previously described procedures [14]. Mice received 100 µl of the bacterial suspension that contain 8 ± 2 ×

108 c.f.u./ml of each strain (*L. plantarum* CRL 2130, *S. thermophilus* CRL 808, and *S. thermophilus* CRL 807), individually or as a mixture daily.

Animals

Eight-week-old male C57BL/6 mice (20–30 g) were obtained from the animal facility of CERELA and were tagged and distributed in metal housing cages at constant room temperature (20±2ºC) and relative humidity (60%), with a 12-h light-dark cycle. Food and water was provided *ad libitum*. Animals were treated according to a protocol approved by the Ethical Committee of CERELA (CRL-BIOT-LI-2008/1B).

MPTP / probenecid Parkinsonism model

Mice were divided into 8 groups (n = 7 for group). MPTP group was injected subcutaneously with 250 mg/kg of body weight of probenecid (Santa Cruz Biotechnology, Dallas, TX, USA), then intraperitoneally (i.p) with MPTP hydrochloride (Sigma, St. Luis, MO, USA) at 20 mg/kg of body weight in saline solution and received orally saline solution. MPTP/CRL 2130, MPTP/CRL 808, MPTP/CRL 807 and MPTP/MIX groups were injected with probenecid and MPTP, and orally administered *L. plantarum* CRL 2130, *S. thermophilus* CRL 808, *S. thermophilus* CRL 807 or the bacterial mixture, respectively. Control and MIX groups were injected (subcutaneously and itraperitonally) saline solution and oral administered saline solution or bacterial mixture, respectively. Probenecid was injected 30 minutes before the MPTP, in order to reduce its elimination and increase the rate of passage to the blood brain barrier [21]. Mice received a total of 5 injections of 100 µl of MPTP and 100 µl of probenecid or saline solution at the middle of the morning at an interval of 4 days. LAB (or saline solution) were administered orally by using a gavage syringe daily during the afternoon (Fig. 1).

Motor behavior

Motor behavior tests were performed during the afternoon. For each test, mice were trained for 3 consecutive days (3 tests each day) before starting the MPTP treatment. The first test recorded was the day before the first MPTP treatment (day 0) and the last

one 3 days after the last MPTP treatment (day 20). In order to avoid detect acute pharmacological effects MPTP; all behavioral tests were performed on the third day after each MPTP injection. Animals were monitored using the different tests during the experimental protocol by the same observer.

Pole test

The pole test is useful for predicting the degree of bradykinesia and the ability to balance the movement of mice [22]. Mice were placed face up on the top of a vertical wooden pole (1.3 cm of diameter and 90 cm of height) and they had to orient downwards to descent. The operator took the time (in seconds) that they reached the base. The base of the pole was placed in the home cage.

Transversal beam test

This test is used to measure motor performance [23]. It consisted of a wooden bar (1.3 cm of diameter and 90 cm of length) deposited at a height of 35 cm from the base with an angle of 15º. The animals were trained to cross the length of the bar starting at the higher section and ending in the lower section that led directly to the animal's cage. Each mouse crossed the bar in 3 attempts while the operator recording the time in seconds.

Nasal bridge adhesive removal test

This trial is a stimulation test used to motor response to sensory sensitivity [24]. Small adhesive tape (3 mm of diameter) was placed on the nasal bridge of each mouse, and the time to remove it was recorded in order to average the time in 3 consecutive attempts. All testing was performed in the animal's home cage, and cage mates were temporarily removed during testing because they can interfere with stimulus removal. If the animal did not remove the stimulus in 60 sec, the experimenter finished.

Sampling procedure

Three days after the last MPTP injection animals were euthanized by cervical dislocation after being anesthetized with a mix of ketamine hydrocholoride (König Laboratory, Buenos Aires, Argentina) and xylazine (Bayer: División Sanidad Animal, Buenos Aires, Argentina). Half of the brain was removed, immediately place on an ice-

cold surface and snap-frozen in liquid nitrogen for future determination of cytokines. The other half of the brain was stored for 24 h in 10% paraformaldehyde / PBS (pH 7.4) to be then embedded in paraffin. Blood samples were centrifuged at 1,000 x g 10 min and the serums were stored at -80 °C.

Tyrosine hydroxylase (TH) immunohistochemistry

The sections of brain tissue samples were cut at 5 µm with a microtome using positively treated slides in order to find the SNpc. After paraffin removing and rehydration, the antigenic recovery was carried out with washes in 0.1 M citrate buffer (pH 6.8 tris-HCl) at 37 °C and at room temperature. Endogenous peroxidase activity was blocked with hydrogen peroxide. The tissues were incubated with the primary antibody for TH (F-11, human, mouse monoclonal IG2a, Santa Cruz Biotechnology, CA, USA) at 4 °C overnight. Then, sections were incubated with the biotinylated antibody (Santa Cruz Biotechnology, CA, USA) at 37 °C for 1h 30 min. Samples were incubated with avidin-biotin peroxidase reactive complex (Thermo Scientific, Rockford, USA) and colored with DAB (3,3-diaminobenzidine, Thermo Scientific, Rockford, USA) in the presence of hydrogen peroxide. Subsequently, they were stained with hematoxylin and mounted with Canada balsam.

All tissue cuts were viewed using a microscope, and the images were captured by digital camera. The quantitative analysis of TH+ neurons in the SNpc was carefully delimited according to the brain atlas of Paximos et al. [25] and the cells were counted in 12 fields at 1000X of magnification.

Measurement of cytokines in mice brain and serum

Brains stored in liquid nitrogen were thawed and homogenized following a previously described protocol [26]. Cytokines were analyzed for both serum and brain samples. The concentrations of cytokines, interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), IL-6, and monocyte chemoattractant protein-1 (MCP-1) were determined by using Mouse Flex Sets (BD Bioscience, San Diego, CA, USA) following the protocols described for cytometric bead array (CBA). For the brain samples, the results were expressed in relation to the total protein concentration measured in the sample.

Statistical analysis

All data were analyzed with GraphPad Prism 6 software and ANOVA general linear model followed by Tukey's post hoc test to determine the differences between groups. The values for all measurements were expressed as the mean ± standard deviation (SD). The experiment was repeated twice; the results from both trials were analyzed together and were considered statistically significant difference when $p < 0.05$.

Results

LAB alleviated motor impairments induced by MPTP

The injections of MPTP gradually induced motor impairment starting at the third injection compared to the basal data. Mice from MPTP group delayed completing all the tests compared to the Control group. These differences were significant ($p < 0.05$) in all time points for the pole and nasal bridge adhesive removal tests, and since the 8th day for the transversal beam test (Fig. 2). The administration of LAB mixture to mice injected with MPTP significantly alleviated the motor deficiencies induced by MPTP and maintained a performance similar to the Control group and also to mice who received LAB mixture, both without neurotoxin (Fig. 2A, 2C and 2E). No significant differences were observed between MIX and Control group. For this experiment, the individual LAB were administered to mice injected with MPTP to investigate if the benefits observed with the LAB mixture could be associated to one specific strain. The results obtained did not show significant differences between the strains in most of the time points evaluated. All MPTP/LAB groups completed the three tests at the end of the experiment in a time significantly ($p < 0.05$) lower than the mice from MPTP group. The comparisons with the healthy animals (Control group) showed that for the pole and transversal beam tests, the performance was similar to those observed in mice from MPTP/MIX group, with times similar or even lower than the Control group (Fig. 2B and 2D). However, for the nasal bridge adhesive removal test, mice that received the individual LAB did not reach the average time recorded in the Control group (Fig 2F). The results showed that each individual strain exerted benefits against the motor impairment induced by MPTP, thus each one can contribute to the effect observed with

the mixture; moreover, the LAB mixture was more effective in order to improve all the tests evaluated.

Effect of LAB MIX administration on dopaminergic neuronal loss induced by MPTP

To relate the motor behavior with the loss of dopaminergic cells, TH-immunoreactivity was evaluated in the SNpc of control mice and mice injected MPTP and that received or not the LAB mixture. The number of TH+ cells in the SNpc of mice decreased significantly ($p < 0.05$) in MPTP group compared to the Control. The administration of LAB MIX in mice injected with MPTP (MPTP/MIX group) maintained the number of TH+ cells without significant differences ($p > 0.05$) compared with the Control group; similar to the results obtained in mice fromMIX group, without the neurotoxin (Fig. 3).

Effect of the LAB mixture administration and MPTP injections on serum cytokine levels

Systemically, in serum, the MPTP group showed significant increases ($p < 0.05$) of IL-6 and TNF-α compared to Control group. Instead, these pro-inflammatory cytokines decreased significantly ($p < 0.05$) in MPTP/MIX group, and maintained values similar to the Control. The administration of LAB MIX to healthy mice did not modify these cytokines in serum (Fig. 4A and 4B). On the other hand, the administration of LAB mixture to mice injected MPTP (MPTP/MIX group) or not (MIX group) showed significant increases ($p < 0.05$) for the anti-inflammatory cytokine IL-10 compared to both MPTP and Control groups (Figure 4C).

MCP-1 did not show significant differences ($p < 0.05$) between the experimental groups (Fig. 4D).

Effect of the LAB mixture administration and MPTP injections on brain cytokine levels

The analysis of cytokines in the brains showed significant increases ($p < 0.05$) for MCP-1 and IL-6 in mice from MPTP group compared to the Control group. LAB mixture administration did not modify significantly these cytokines compared to MPTP group; however, no significant differences were also observed compared to the groups without MPTP due to the variation of the values obtained between the mice from

MPTP/MIX group (Fig. 5A and 5D). TNFα increased significantly in MPTP/MIX group compared to MPTP group; moreover no significant differences were obtained compared to Control group (Fig. 5B). The highest concentrations for IL-10 were obtained in MPTP/MIX group but without significant differences ($p > 0.05$) with the other groups (Fig. 5C).

Discussion

The possibility that selected microorganisms administered orally, can beneficially affect neurological, neurophysiological and neuroimmunological aspects of the host, has aroused the interest of new studies. Although there are recent works that show the potential of certain probiotics to improve the quality of life and some physiological effects associated with PD, little is known about the possible mechanisms involved in these benefits [5, 6, 27, 28]. In addition, many studies show that dietary factors such as B vitamin deficiency are involved in the etiology of PD and that the administration of these vitamins could reverse some symptoms of this pathology [10-12]. In the present work a mixture of selected LAB (a ribloflavin producer, a folate producer and a strain with anti-inflammatory properties associated to the modulation of the host's immune response) was evaluated by using a MPTP induced parkinsonism model.

Results obtained showed that the administration of the LAB mixture improved the motor behavior altered by MPTP, and each selected strain can contribute to the benefits observed with the mixture. Behavioral tests do not detect acute pharmacological actions of MPTP/MPP+, they demonstrate neurodegenerative damage [23]. In this sense, it was reported that a probiotic mix diminished behavior disorders in MPTP- and rotenone-induced models by increasing butyrate level [29]. Likewise, SLAB51 probiotic formulation was able to counteract behavioral impairment induced by 6-hydroxydopamine (6-OHDA) inoculation in a PD mouse model, restoring the healthy control conditions. This effect was associated to the activation of peroxisome proliferator activated receptor γ (PPARγ) that may generate anti-inflammatory and antioxidant activities as well as the increase in Brain Derived Neurotrophic Factor (BDNF), involved in neuroprotection and neuronal survival [30]. Similarly to the results in animals' models, a clinical trial with PD patients demonstrated that compared to placebo group, patients who consumed a probiotic mixture showed favorable impacts

on movement parameters altered in PD [27]. Authors related these benefits with the improvement of some metabolic parameters.

Tyrosine hydroxylase is a key enzyme in the production of dopamine by dopaminergic neurons and is affected in PD as well as in MPTP induced and other animal models. Recently, it was demonstrated that long term administration of a mixture containing six probiotic strains to transgenic MitoPark PD mice significantly reduced the motor impairments and preserved TH+ cells in the SNpc [31]. Likewise, the evaluation of these cells in our model showed that motor behavior improvements observed with the LAB mixture administration to mice injected MPTP were associated to maintenance of TH+ cells' number in the SNpc. The results also showed that LAB administration by itself did not induce any effects on the number of dopaminergic neurons compared to the Control group. Similarly, other probiotic mixture showed the same protective effect against TH+ cells in a MPTP induced model; however, at difference of our model, the probiotics were administered preventively (one month before MPTP) and the neurotoxin was injected four times in one single day [29]. Our model can represent better the chronic state of PD that could be also associated with intestinal microbial dysbiosis. However, the study of the intestinal microbiota is a limitation of our work and will be analyzed in the future. In this sense, it was reported that similar to intestinal microbial dysbiosis observed in PD patients, MPTP-induced model in mice with five neurotoxin injections induced modifications of intestinal bacteria [32].

The immune response was evaluated systemically and locally in the brain of mice injected MPTP considering the anti-oxidant/anti-inflammatory effect associated to our LAB blend against other pathologies [14]. Peripheral inflammation is implicated in many neurodegenerative diseases such as in PD [33]. However, there is no general consensus whether the reactive microglia are a cause or effect of neuron loss during disease progression. In the present work, the serum concentration of IL-6 and TNF-α increased in mice from MPTP group; and LAB blend administration maintained the levels of both cytokines similar to the Control group. Elevated serum concentrations of TNF-α and IL-6 have been associated with increased risk of PD [34].Our results were similar to other described for MPTP-induced models in which the beneficial effects were related to decrease of systemic inflammation [35].

Brains from PD patients and animal models showed evidence for neuroinflammation with increased pro-inflammatory cytokines from a classical M1

activation of the microglia [36]. Similarly, our results showed that IL-6 and MCP-1 increased significantly in the brains form MPTP group, and LAB mixture administration decreased these cytokines in some animals. However, the variations for these cytokines between the mice from MPTP/MIX group, maintained the group values without significant differences with the healthy control. IL-6 is an important cytokine in the CNS, and several neurological pathologies are associated with increased expression of this cytokine in brain [37]. MCP-1 has been described as a biomarker positively correlated with PD progression [38]. In addition, LAB mixture administration to MPTP injected mice increased significantly the IL-10 concentration in brains compared to Control animals. In this sense, the neuroprotective effect in PD has been associated to an activated M2 phenotype of microglia with release of anti-inflammatory cytokines such as IL-10 and TGF-β [36]. Thus, LAB administration can balance different microglia activation phenotypes in our PD model.

Conclusions

The results obtained showed the potential of a selected LAB mixture to improve motor behavior impaired in PD. Different mechanism of action can be involved in this effect, some of them associated to the production of B vitamins and the anti-oxidant effect of them, and others are related to the modulation of the host's immune response in order to avoid an exacerbated inflammation systemically and locally in the brain. Other mechanisms such as the modulation of the intestinal microbiota could also be involved and will be evaluated in future studies.

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Conflicts of interest

The authors declare no competing financial interests or potential conflicts of interest.

CRediT author statement

Daiana Pérez VisñuK: Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Graciela Savoy de Giori**: Conceptualization, Writing - Review & Editing. **Jean Guy LeBlanc**: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - Review & Editing, Project administration, Supervision, Funding acquisition. **Alejandra de Moreno de LeBlanc**: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - Review & Editing, Project administration, Supervision, Funding acquisition.

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Figure legends

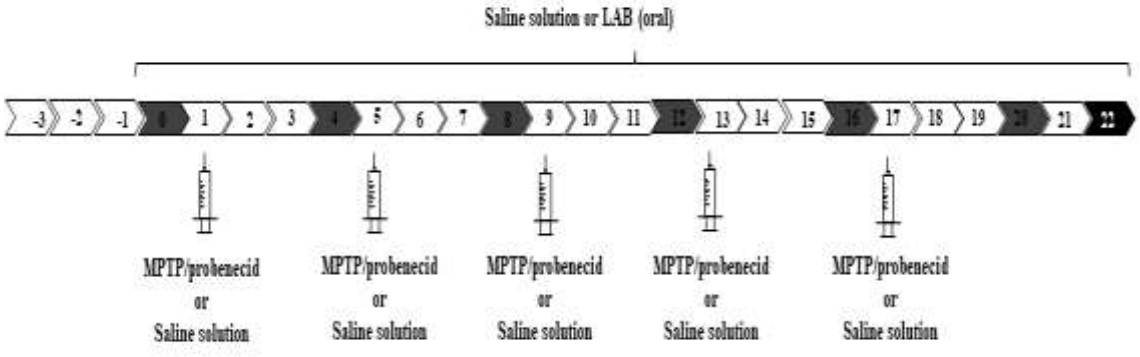

Fig. 1. Experimental model of Parkinson Disease used in this study**.** Day 0 signifies when experimental procedure starts with bacterial supplementation. Numbers in arrows show days from experimental start. Grey arrows show when motor function behavior were evaluated (days 0, 4, 8, 12, 16, 20), the needles show the days when MPTP/probenecid were injected and black arrow shows the day when mice were sacrificed and the experiment finished (day 22).

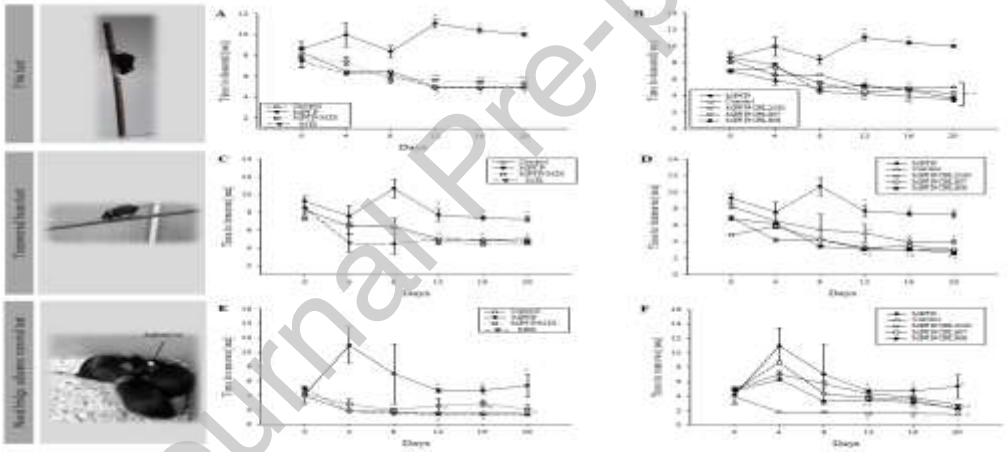

Fig. 2. Motor behavior of animals. Mice were testing for pole test (A, B), transversal beam test (C, D), and nasal bridge adhesive removal test (E, F). In all figures Control group are healthy animals and MIX group are healthy animals that received the probiotic blend; MPTP groups are animals that received the neuropathological drug MPTP: MPTP/MIX, MPTP/CRL2130, MPTP/CRL807 MPTP/CRL808 groups are MPTP treated animals that received the probiotic blend (MIX) or the individual strains. Results are the average (n=14) with the standard deviation for each group and time point. Statistical analysis is shown for the three last time points, * and # mean that the data are significantly ($p < 0.05$) different compared to Control and MPTP groups, respectively.

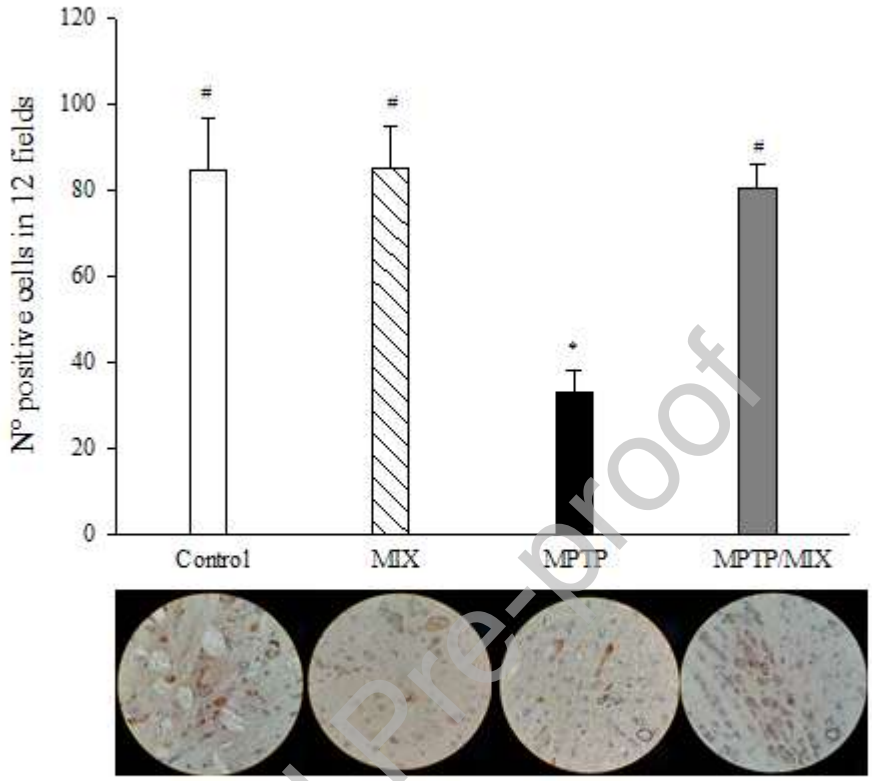

Fig. 3. Tyrosine hydroxylase (TH) positive cells in the mice brain. TH positive cells were analyzed by immunohistochemistry in the brain tissues' sections from mice. Results are the average (n=14) with the standard deviation of the number of positive cells counted in 12 fields at 1000X of magnification. * and # mean that the data are significantly ($p < 0.05$) different compared to Control and MPTP groups, respectively. Representative microphotographs (1000X) for different groups are showed at the bottom.

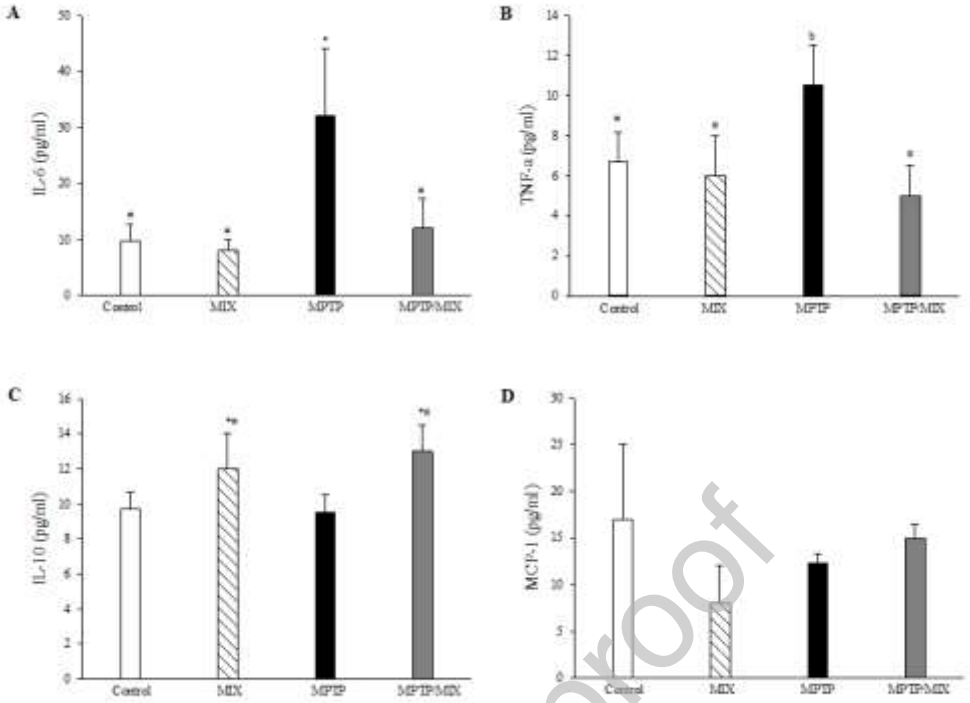

Fig. 4. Effect of LAB blend and MPTP on serum cytokines. The concentration of cytokines: Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF-α), IL-10 and monocyte chemoattractant protein-1 (MCP-1) were determined in blood serum samples obtained at the end of the experiment. Data are shown as mean ± SD (14 mice per group). * and # mean that the data are significantly ($p < 0.05$) different compared to Control and MPTP groups, respectively.

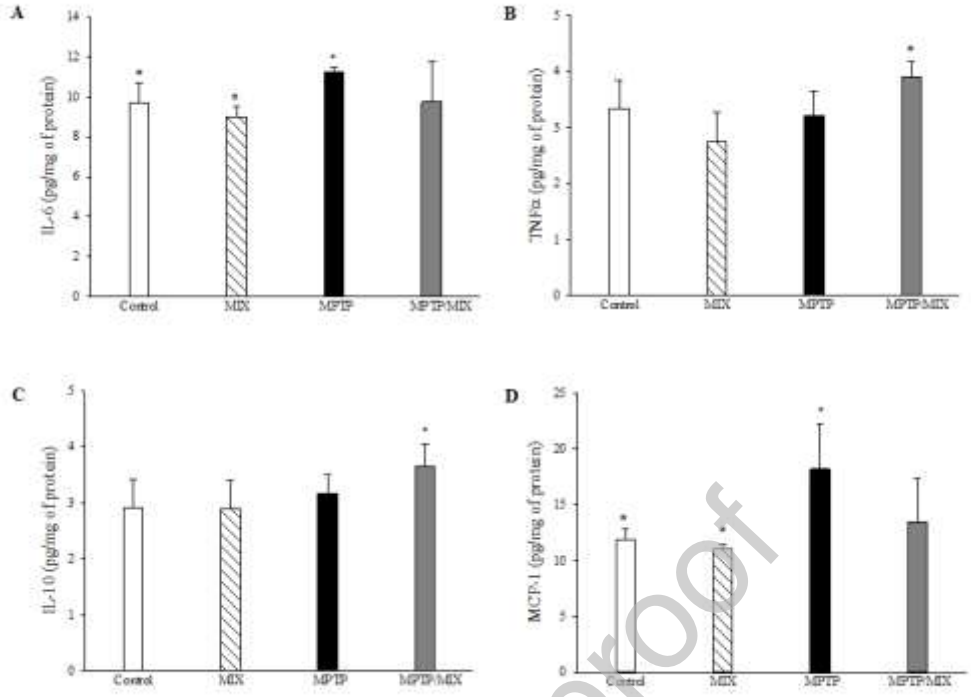

Fig. 5: Effect of LAB blend and MPTP on brain cytokines levels. The concentration of cytokines: Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF-α), IL-10 and monocyte chemoattractant protein-1 (MCP-1) were determined in brain homogenates obtained at the end of the experiment. Data are shown as mean ± SD (14 mice per group) and expressed in relation to the total protein concentration measured in the sample. * and # mean that the data are significantly ($p < 0.05$) different compared to Control and MPTP groups, respectively.