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ANXIOGENIC EFFECTS OF A LACTOBACILLUS, INULIN AND THE SYNBIOTIC ON HEALTHY JUVENILE RATS

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Abstract—Gut microbiota interventions, including probiotic and prebiotic use can alter behavior in adult animals and healthy volunteers. However, little is known about their effects in younger individuals. To investigate this, male Sprague–Dawley rats (post-natal day 21, PND21) received *Lactobacillus casei* 54-2-33 (10^4 cfu/ml), inulin as prebiotic (16 mg/ml), or both together (synbiotic) via drinking water for 14 days. Control rats received water alone. Open field (OF) and elevated plus maze (EPM) behaviors were evaluated at PND34 and 35, respectively. 30 min after EPM, brains and trunk blood were collected to evaluate hippocampal 5-HT_{1A} (mRNA and protein) and plasma corticosterone (CORT). *Lactobacillus*, inulin and synbiotic-treated rats had fewer entries to the OF's center and spent more time in its periphery than controls. Synbiotic-fed rats explored the EPM's open arms longer than probiotic and inulin-fed rats. Synbiotic, but not *Lactobacillus* nor inulin-fed rats had lower levels of EPM-evoked CORT than controls. Basal CORT levels, evaluated in a naïve cohort, were higher in *Lactobacillus*- and inulin-fed rats than controls. In naïve synbiotic-fed rats, 5-HT_{1A} mRNA levels were higher in dentate gyrus and cornus ammonis 1 layer (CA1), than in all other naïve groups, while hippocampal 5-HT_{1A} protein levels were lower in bacteria-fed rats than controls. 5-HT_{1A} mRNA changes suggest complex effects of gut microbes on hippocampal gene expression machinery, probably involving endogenous/exogenous bacteria and prebiotics interactions. Importantly, age might also influence their behavioral outcomes. Together, these data suggest that interventions in young rat microbiota evoke early behavioral changes upon stress, apparently in a hypothalamus–pituitary–adrenal axis independent fashion.

Key words: lactobacillus, prebiotic, synbiotic, 5-HT_{1A}, hippocampus, gut-brain axis.

INTRODUCTION

The intestinal microbiota has gained importance as it has been thoroughly described as a major modulator of the central nervous system (CNS) establishing what is now recognized as the microbiota-gut-brain axis, a bidirectional communication system comprising neural connections, endocrine and immune signaling (Mayer, 2011). Moreover, alterations in gut microbiota composition affect gastrointestinal and CNS functions. For instance, germ-free rodents (born and raised under sterile conditions) have reduced anxiety-like behaviors, even though the plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone evoked by an acute stress are exaggerated in comparison to conventionally reared mice (Sudo et al., 2004). In addition, germ-free mice have higher plasma tryptophan; a precursor of serotonin (5-HT), suggesting that the gut microbiota may also influence the CNS through a humoral route (Clarke et al., 2013). Also, rodents treated with broad-spectrum non-absorbable antibiotics display reduced anxiety-like traits, an effect that disappears after a 10-day washout period (Bercik and Collins, 2014), and it has been shown that probiotics (Bravo et al., 2011) and acute infections (Lyte et al., 2006); (Goehler et al., 2008) induced by exogenous bacteria also affect behavior (for a review see Bravo et al., 2012). All of these findings have been obtained in adult individuals, however little has been done in younger animals, at critical stages of development when the brain is still establishing connections (Stiles and Jernigan, 2010) and the gut is adjusting to changes in diet and luminal composition, including gut symbionts.

Preclinical studies have shown that microbiota alterations promote behavioral changes associated with anxiety disorders and depression. For example, healthy adult BALB/c mice fed daily for three weeks with *Lactobacillus rhamnosus* JB-1 display

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Abbreviations: ACTH, adrenocorticotropic hormone; CA, central area; CA1, cornus ammonis 1 layer; CNS, central nervous system; CORT, corticosterone; DIG, digoxigenin; DSS, dextran sodium sulfate; ELISA, enzyme-linked immunosorbent assay; EPM, elevated plus maze; GPCR, G protein-coupled receptors; IL, interleukin; OF, Open field; SCFA's, short chain fatty acids; SSC, saline sodium citrate.

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antidepressant-like and anxiolytic-like effects in comparison to control mice (Bravo et al., 2011). However, adult mice fed with *L. rhamnosus* JB-1 also displayed an increased freezing behavior in comparison to healthy control mice in the fear conditioning test, an unwanted side effect, considering the results observed in the forced swim test (depression-like behaviors) and elevated plus maze test (anxiety-like behaviors) (Bravo et al., 2011). In addition, data from germ-free mice studies suggest that the gut microbes are essential to brain development in early-life stages, affecting behavior (Neufeld et al., 2011); (Desbonnet et al., 2014), brain gene expression and myelinization (Neufeld et al., 2011; Diaz et al., 2012; Clarke et al., 2013; Hoban et al., 2016). It is interesting to highlight that the serotonergic system in germ-free mice is affected. For instance, germ-free mice have lower levels of 5-HT_{1A} mRNA receptor expression in the dentate gyrus of the hippocampus (Neufeld et al., 2011), a brain structure involved in memory and learning, which is highly sensitive to stress and has been involved in the development of stress-related psychiatric disorders (de Kloet et al., 2016). The serotonergic system mediates its effects in the CNS through 5-hydroxytryptamine (5-HT or serotonin) receptors, a family of G protein-coupled receptors (GPCR) (Bockaert et al., 2006). Within the family of 5-HT_1 receptors, 1A subtype is extensively distributed in the CNS, with a higher density level at the limbic system, particularly in the hippocampus (Hamon et al., 1990; Bockaert et al., 2006). It is coupled with G_i/G_o, and is located at presynaptic level (autoreceptors), in the somatodendritic region of neurons in the raphe nucleus limiting the release of 5-HT. It can also be found in the post synaptic region (heteroreceptors) of neuronal targets (Lanfumey and Hamon, 2004). 5-HT_{1A} receptor has been linked to mood disorders and anxiety disorders (Garcia-Garcia et al., 2014) suggesting that signaling through this receptor is required for normal development of circuits related to anxiety behaviors (Akimova et al., 2009). Therefore, the evidence available to date indicates that once formed, these circuits are sufficiently stable and that 5-HT_{1A} receptors play a different role in adulthood than they do in development.

All these evidences suggest that dietary interventions, which alter gut microbiota, might be able to generate improvements in mental health. Thus, the aim of this study is to evaluate if the use of a Lactobacillus, a prebiotic and the synbiotic (the mixture of bacteria and prebiotic), would affect parameters associated with stress-related behaviors, including changes in 5-HT_{1A} expression in the hippocampus. Moreover, in this work the effects of such treatments were evaluated in young animals (post-natal day 35) in order to assess whether the effects are observable at an earlier age.

EXPERIMENTAL PROCEDURES

Animals

21-day-old male Sprague–Dawley rats (n = 59) were separated into four different treatments (control n = 15, *L. casei* 54-2-33 n = 14, inulin n = 14 and synbiotic n = 16) which lasted 2 weeks. All animals were group-housed in standard conditions: room temperature of 21 °C, with a 12-h light dark cycle, access to regular chow and water with treatment for each group. Rats were of comparable weight (108–128 g) and age (5 weeks) at the end of the experiment. All procedures were carried out according to protocols similar to the standards used in the European Union (Cruelty to Animal Act 1876, Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [89/609/EEC], and approved by Pontificia Universidad Católica de Valparaíso's Bioethical Committee.

Lactobacillus casei 54-2-33 culture

L. casei 54-2-33 isolated from samples of Chilean population and previously characterized as a potential probiotic strain by Dubos et al. (2011) (Dubos et al., 2011) was used. Bacteria were grown as described elsewhere (Bravo et al., 2011) in Man-Rogosa-Sharpe (MRS) broth (Merck Millipore. Darmstadt Germany with shaking (250 rpm) at 37 °C under aerobic conditions (LSI-3016R Incubator Shaker, New Delhi, India). Bacterial growth was verified by optical density at 600 nm (Genesys Spectrophotometer 8 Spectronic, Bogota, Colombia). All culture procedures were performed in a sterile area under a Bunsen burner.

Treatments

All treatments were given in the drinking water. *L. casei* 54-2-33 was administered at a final concentration of 10^4 CFU's/mL, while inulin (Sigma–Aldrich, St. Louis, MO. USA) was used as a prebiotic at a concentration of 16 mg/mL. For the synbiotic treatment, aliquots of bacteria and inulin generated as previously mentioned were used. Each treatment was administered for 14 days. The drinking water was changed every two days and was replenished with fresh bacteria and inulin. Additionally, water consumption was measured daily as well as body weight.

Behavioral tests

Open field (OF): At postnatal day 34, rats were individually placed in a 60 × 60-cm apparatus and behavior was recorded for 5 min with a camera located 1.5 m above the test site. After each test, the apparatus was cleaned with 5% ethanol. Once all videos were acquired, manual quantification was carried out in a blind fashion. The arena was divided into 16 squares, where the inner four squares determined the central area of the arena. Then the following parameters were determined: number of entries to the center, number of entries to the peripheral zone, time spent in the center and time remained in the peripheral zone.

Elevated plus maze (EPM): This test was performed the day after the OF test. The EPM apparatus consists of two open arms and two enclosed arms (each 40 cm long × 10cm wide), extending from a common central platform. To start the test, each rat was individually placed in the central platform facing an open

arm. Behavior was then recorded for 5 min. The number of entries to closed arms, number of entries into open arms and time spent in open or closed arms were analyzed manually by an observer blinded to treatments.

Corticosterone enzyme-linked immunosorbent assay (ELISA)

Trunk blood was collected upon decapitation. A commercially available ELISA kit was used to quantify the levels of the stress hormone corticosterone in the plasma according to the manufacturer's instructions (Cortocosterone kit, Enzo LifeSciencies, NY, EE.UU).

In situ hybridization

Immediately after rapid decapitation, brains were extracted and snap frozen in isopentane kept cold with liquid nitrogen. The brains were stored at −80 °C before being processed for *in situ* hybridization and immunohistochemistry. 10-μm coronal brain slices were obtained using a cryostat (Cryostat Cryo3 +, Sakura Finetek, Torrance, CA, USA) Sections were obtained between bregma −2.80 mm to −3.80 mm according to the rat brain atlas of Paxinos and Watson (2007). The sections were mounted on super frost-plus glass slides and stored at −80 °C until used.

In situ hybridization was carried out with oligodeoxynucleotide (cDNA) probes complementary to 5-HT_{1A} receptor mRNA (NCBI NucleotideDatabase, N° AF217200), labeled with digoxigenin (DIG) oligonucleotide 3′-OH tailing kit. The hybridization was conducted as previously described (Bravo et al., 2011). Briefly, brain sections were brought back to room temperature and post-fixed in 4% paraformaldehyde made in 100 mM PBS for 30 min. Then the slides were permeabilized with proteinase K (0.5 mg/100 mL in TE buffer) and treated with acetic anhydride buffer. Next, the slides underwent dehydration through a series of ethanol dilutions (70, 95, and 100%) before being delipidated in chloroform for 5 min. The tissues were then rehydrated and placed in a humidity chamber with the hybridization solution [formamide 50%, saline sodium citrate (SSC) buffer 4×, sheared salmon DNA 6.25 mg/mL, tRNA 125 μg/mL, and cDNA probe at fixed concentration of 100 pmol/mL] and incubated overnight at 37 °C. After that, the sections were washed in ascending dilutions of SSC buffer (4, 2, 1, and 0.5×), and then equilibrated with maleic acid 0.1 M buffer before blocking for nonspecific protein binding with blocking reagent (Roche, Molecular Biochemicals). After 30 min of blocking, the DIG molecules attached to the hybridized probes were detected with an alkaline phosphatase conjugated anti-DIG antibody. Finally, a substrate for the alkaline phosphatase NBT/BCIP was added, and when a violet/blue precipitate was present on the tissues, the reaction was stopped. Sections were cleared with distilled water and coverslips were applied. Once the mounting media was dry, areas of interest were photographed (Nikon Eclipse, Tokyo, Japan). For semi-quantitative analysis, densitometric measurements of each hippocampal area were performed using Multi Gauge v2.2 software. All pictures were analyzed in gray scale and the value given by the software corresponds to the intensity of pixels (the darkest staining is the highest intensity; and the lightest staining the lowest intensity) in a given area (density of pixels). All samples were processed and evaluated blindedly.

Immunohistochemistry

Mounted brain slices were fixed in cold 4% paraformaldehyde made in 100 mM PBS for 45 min, then antigen retrieval was performed by immersion for 5 min in sodium citrate buffer (10 mM, pH 6.0) that was heated to 90 °C using a vegetable steamer. All following incubations were performed in a humidity chamber to prevent tissue from drying. Hydrogen peroxide was used at 0.5% (H_2O_2) in buffered saline with 100 mM (PBS) phosphate for 40 min to remove endogenous peroxidase activity. Samples were washed twice with PBS 1X for 10 min with shaking at 95 rpm and once with PBS-T for 10 min with shaking at 70 rpm and protein serum block solution (Dako, Carpinteria, CA, USA) was added for 15 min. Sections were incubated with mouse monoclonal anti-5-HT_{1A} (Abcam, Cambridge, United Kingdom) diluted 1:200 in protein serum block at 4 °C for 24 h. Slides were washed 3 times with PBS-T 0.4% with shaking at 50 rpm for 10 min and then 1 time with PBS 1X with shaking 50 rpm for 3 min. Samples were incubated for 2 h with biotinylated polyclonal secondary antibody (goat anti-mouse, Gaithersburg, MD, USA) diluted 1:200 in protein serum block solution respectively.

ABC amplification method was used. Tissues were incubated with the mixture for 1 h at room temperature (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). For subsequent color developing, the samples were incubated with DAB solution (Cell Signal Technology, Danvers, MA, USA) for 20 min protected from light. Finally, they were washed with PBS 1X with shaking at 50 rpm for 10 min and allowed to dry at room temperature for subsequent mounting. Samples were observed under light microscopy and the images obtained were processed and evaluated blindly by densitometry.

Statistical analysis

Results were analyzed by a one-way ANOVA using GraphPadPrism 5.0 software (GraphPad Software, La Jolla, CA, USA). Repeated measures ANOVA was used to analyze daily weight gain and water intake. All values are reported as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

RESULTS

Body weight and water intake

Repeated measures ANOVA shows that *L. casei* 54-2-33, inulin and synbiotic-treated animals had similar weight gains to control animals within the 14 days of dietary intervention (Fig. 1A) with no different statistical significance between groups ($F(1.055,7.385) = 1.154$; $p = 0.3208$). In addition, following an ANOVA with

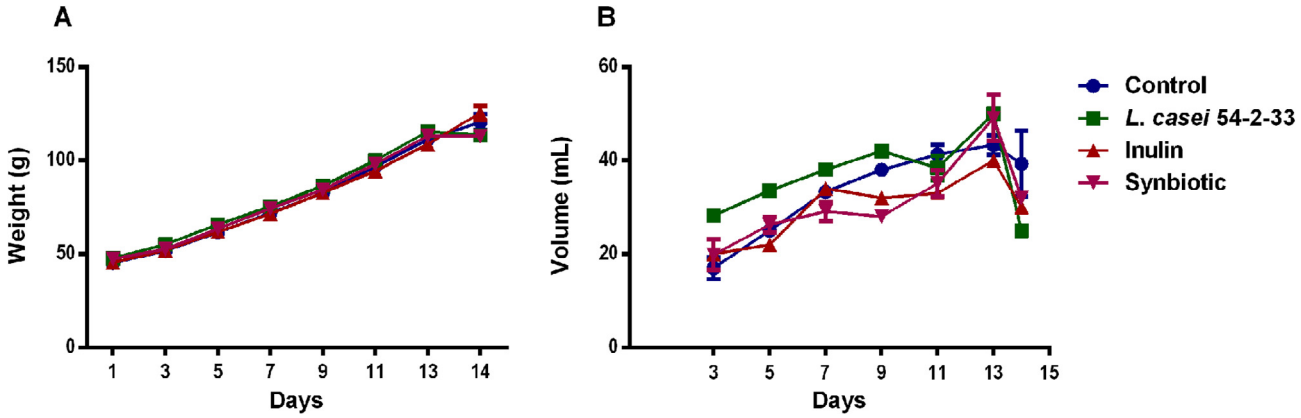

Fig. 1. Weight gain and water consumption. Daily weight gain is not affected by treatments (A), and there are no differences in water consumption (B).

repeated measures, there was no significant difference in water consumption between experimental conditions (F(1.972,11.83) = 2.851; p = 0.0981) (Fig. 1B).

Plasma corticosterone

Two cohorts of animals were evaluated. One cohort was only given the bacteria, inulin and synbiotic, but no behavioral analyses were preformed, while the second cohort was used for behavioral analyses. In the latter, blood was collected 30 min after the EPM test. Fig. 2A shows that there is an effect of dietary intervention (F(3,15) = 16.71; p < 0.0001) in animals that were not subjected to behavioral studies. Tukey's comparison test reveals that *L. casei* 54-2-33, and inulin-fed rats have significantly higher levels of plasma corticosterone (CORT) than control-fed rats (p < 0.001). In addition, plasma CORT levels of synbiotic-fed rats were no different than control animals, although they were significantly lower than plasma levels of *L. casei* 54-2-33, and inulin-fed rats (p < 0.01 in both cases). In rats exposed to behavioral studies (Fig. 2B), there is also an effect of experimental interventions (F(3,23) = 9.279; p < 0.001). Post-test shows that synbiotic-fed rats have lower plasmatic levels of CORT evoked by EPM test, in comparison to *L. casei* 54-2-33, inulin, and control rats (p < 0.01 control vs. synbiotic; p < 0.001 *L. casei* 54-2-33 vs. synbiotic; p < 0.01 inulin vs. synbiotic).

Behavioral tests

At 13 days of treatment, results indicate that there is an effect in the number of entries to the central area (CA) (F(3,32) = 9,415; p < 0.001). Post-test indicates significantly less entries to the CA for all treatments compared to control (p < 0.001 control vs. *L. casei* 54-2-33; p < 0.01 control vs. inulin; p < 0.01 control vs. synbiotic) (Fig. 3A). Moreover, the analysis of variance for time spent in CA shows that there is an effect of treatments (F(3,32) = 10.46; p < 0.001), while post-test also showed significantly lower times for all treatment groups when compared to controls (p < 0.001 control vs. *L. casei* 54-2-33; p < 0.001 control vs. inulin; p < 0.001 control vs. synbiotic) (Fig. 3B).

In the EPM test, entries to closed arms, open arms and total time spent in them were analyzed. Regarding the number of entries to the open arms, ANOVA reveals a significant effect of treatments (F(3,26) = 4.342; p < 0.05). There are a higher number of entries into the open arms for synbiotic-treated rats that do not reach statistical significance by Tukey's post-test when compared to the control group. However, the number of entries into the open arms of synbiotic-fed rats is significantly higher when compared to inulin treatment (p < 0.01) (Fig. 4A). On the other hand, there are no significant differences in *L. casei* 54-2-33 and inulin treatments when compared to control group. As for the number of entries to the closed arms, ANOVA shows that there is no effect of treatment nor there are statistically significant differences between groups (Fig. 4B). In addition, there is an effect of treatment on the time spent in the open arms (F(3,30) = 3.736; p < 0.05). Tukey's post-test indicates that synbiotic-fed animals spent significantly more time exploring the open arms than rats treated with inulin (p < 0.05), however there are no significant differences with the controls or *L. casei* 54-2-33 and inulin-fed rats (Fig. 4C). Moreover, ANOVA reveals that there is no effect of these interventions on the time spent in the closed arms (Fig. 4D).

Hippocampal 5-HT$_{1A}$ mRNA expression

To determine hippocampal 5-HT$_{1A}$ mRNA expression, *in situ* hybridization was carried out and different hippocampal zones were analyzed. Results show that in the suprapyramidal layer of the dentate gyrus (Sup DG) there is an overall effect of interventions on young rats (F(3,13) = 4.440; p < 0.05). Moreover, post-test shows significantly higher levels of 5-HT$_{1A}$ mRNA expression in synbiotic-fed animals compared to control and inulin groups (p < 0.01) (Fig. 5A). In the infrapyramidal layer of the dentate gyrus (Inf DG) there is also a treatment effect (F(3,13) = 3.817; p < 0.05), and post-test indicates that 5-HT$_{1A}$ mRNA expression is significantly higher than synbiotic and control groups (p < 0.05) (Fig. 5B). A similar treatment effect is observed in

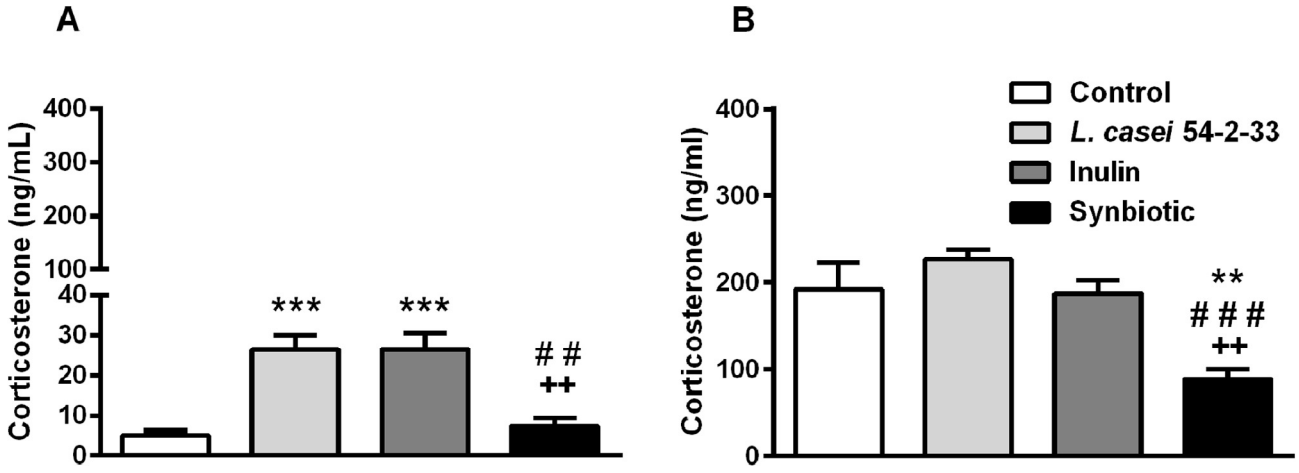

Fig. 2. Plasma corticosterone concentration. The values in (A) are plasma corticosterone concentration at the end of treatment in naïve rats (control $n = 6$; *L. casei* 54-2-33 $n = 5$; inulin $n = 5$; synbiotic $n = 7$), whereas B shows hormone levels evoked 30 min after subjecting animals to the elevated plus maze test, (control $n = 9$; *L. casei* 54-2-33 $n = 9$; inulin $n = 9$; synbiotic $n = 9$. $^{***}p < 0.0001$ when compared to control; $^{**}p < 0.001$ when compared to control; $^{\#\#\#}p < 0.0001$ when compared to *L. casei* 54-2-33; $^{\#\#}p < 0.001$ when compared to *L. casei* 54-2-33; $^{++}p < 0.001$ when compared to inulin).

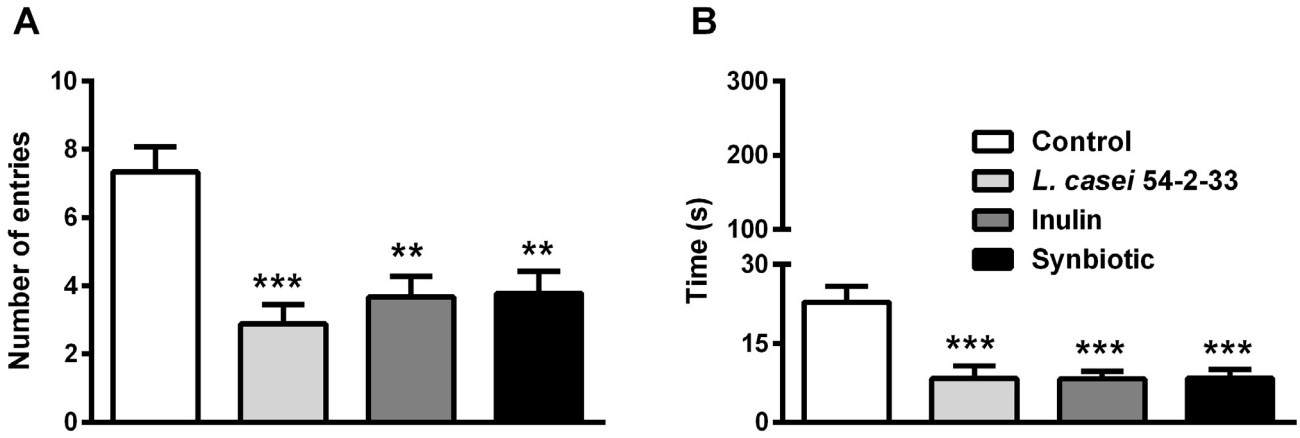

Fig. 3. Open-field test. Number of entries to central area was significantly lower for different treatments compared to control conditions ($^{**} = p < 0.01$ and $^{***} = p < 0.001$ when compared to controls) (A). Time spent in central area was significantly lower for different treatments ($^{***} = p < 0.0001$) (B). (Control $n = 9$; *L. casei* 54-2-33 $n = 9$; Inulin $n = 9$; Synbiotic $n = 9$).

cornus ammonis 1 layer (CA1) ($F(3,13) = 4.484$; $p < 0.05$), where synbiotic treatment has a higher expression of 5-HT_{1A} mRNA than control and inulin groups ($p < 0.01$) (Fig. 5C). Nevertheless, in hippocampal CA3 region no overall or individual effect was found (Fig. 5D).

Hippocampal expression of 5-HT_{1A} receptor

Immunohistochemistry was used to determine hippocampal 5-HT_{1A} protein levels in the same mentioned areas (Sup DG, Inf DG, CA3 and CA1). Results show that in Sup DG there is an overall effect of interventions on young rats ($F(3,13) = 5.325$; $p < 0.05$). Post-test shows significantly lower levels of 5-HT_{1A} protein in *L. casei* 54-2-33-fed animals compared to control group ($p < 0.05$) (Fig. 6A). In Inf DG there is also a treatment effect ($F(3,13) = 4.147$; $p < 0.05$), and post-test indicates that 5-HT_{1A} protein levels are significantly lower than control rats ($p < 0.05$) (Fig. 6B). A similar treatment effect is observed in CA1 ($F(3,13) = 7.472$; $p < 0.01$), where *L. casei* 54-2-33 treatment has a lower expression of 5-HT_{1A} protein than control rats ($p < 0.01$), while synbiotic-fed rats have significantly higher levels of 5-HT_{1A} protein than *L. casei* 54-2-33 rats ($p < 0.05$) (Fig. 6C). There is no statistically significant overall effect of treatments in the CA3 layer ($F(3,13) = 3.355$; $p = 0.0522$) (Fig. 6D). However Tukey's comparison test reveals that *L. casei* 54-2-33-fed rats have significantly lower levels of 5-HT_{1A} protein than control animals ($p < 0.05$) (Fig. 6D).

DISCUSSION

The effect of administration of *L. casei* 54-2-33, inulin and synbiotic (mixture of both) in healthy young rats and the impact of this change behaviors and brain parameters were analyzed. It was possible to determine that such

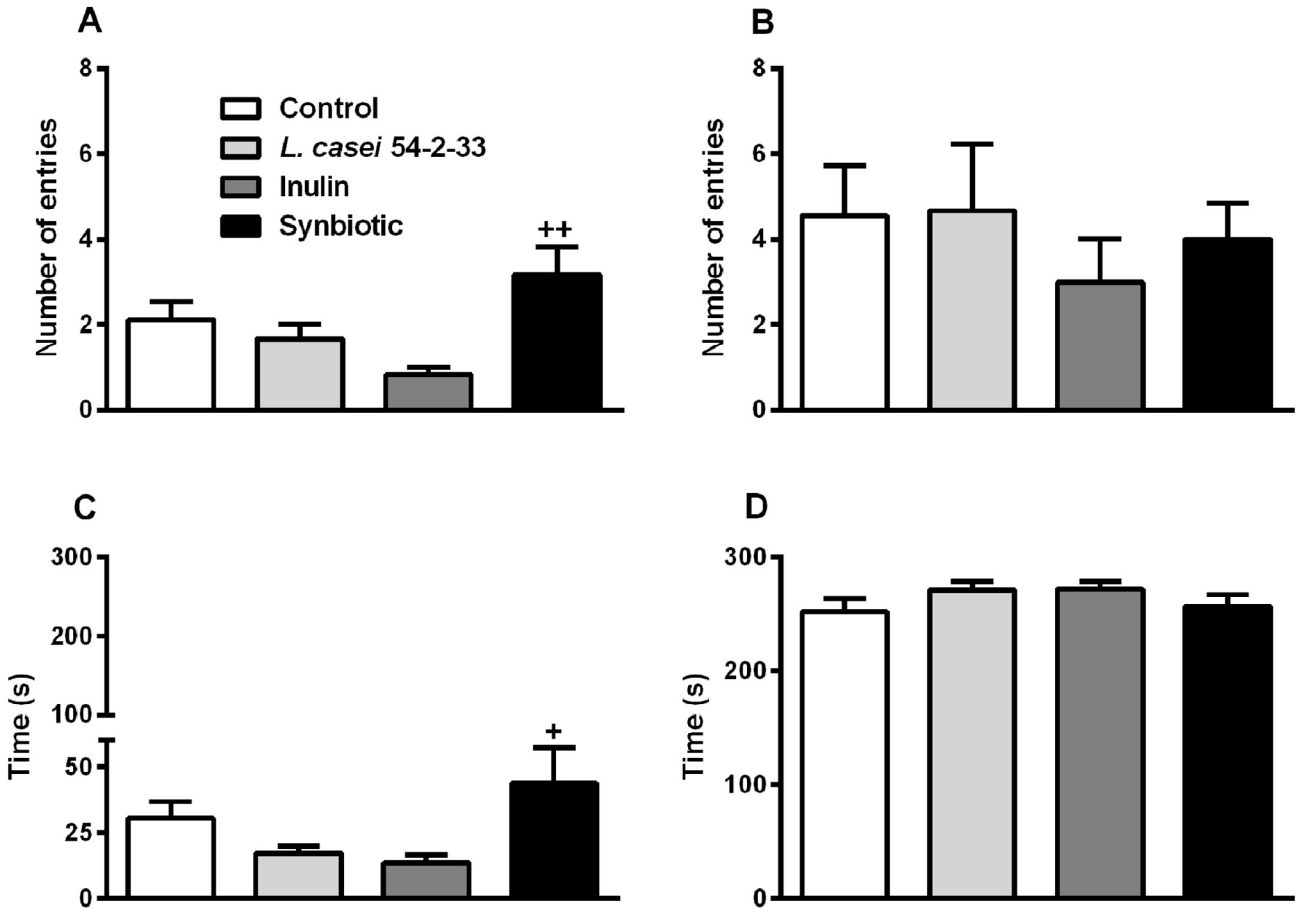

Fig. 4. Elevated plus maze. Number of entries (A) and time spent in open arms (C) show a significant difference between synbiotic and inulin treatment which shows an increase in both cases ($^{+}$ = $p < 0.01$ when compared with inulin; $^{++}$ = $p < 0.001$ when compared to inulin). The number of entries into the closed arms (B) and time remained in the closed arms (D) are similar in all experimental groups with no significant differences between treatments and control condition in both cases. (Control $n = 9$; *L. casei* 54-2-33 $n = 9$; Inulin $n = 9$; Synbiotic $n = 9$).

interventions do not affect weight gain in juvenile rats, but it does alter basal plasma corticosterone concentration, being the administration of *L. casei* 54-2-33 and inulin significantly effective in elevating plasma concentration of this hormone, when compared to control-fed rats. On the other hand, synbiotic-fed rats showed no differences in basal corticosterone levels when compared to control rats. In addition, when plasma concentrations of corticosterone are evaluated after behavioral testing, there is a different pattern than basal measurements: hormone levels in *L. casei* 54-2-33 and inulin are similar to the control group. The current findings suggest that *L. casei* 54-2-33 and inulin affect the basal tone of HPA axis function, leading to a significantly higher basal level of corticosterone. The effects of *L. casei* 54-2-33 and inulin on basal concentration of corticosterone might arise as a result of the bacterium generating an inflammatory response within the gut. Some species of *Lactobacilli* have been described to induce interleukin (IL) 1β, IL-8 and tumor necrosis factor-α expression (for a review see Gourbeyre et al., 2011). However, we have not assessed the immunomodulatory effects of this bacterium. We have previously demonstrated that *L. casei* 54-2-33 reduces the expression of urocortin 2 in Caco-2 cells *in vitro* (Gonzalez-Arancibia et al., 2016). Urocortin 2 is involved in gastrointestinal emptying and visceral pain perception (Martinez et al., 2004). With these findings, we thought that this bacterium would promote beneficial effects on live animals, but the current findings may suggest that *L. casei* 54-2-33 might have promoted an inflammatory response that is species specific, which includes an increase in basal levels of corticosterone. The effect of inulin on plasma concentration of corticosterone is a novel finding, and little can be speculated on how this stimulus provokes an increase in plasma corticosterone, particularly if inulin has been described to increase short chain fatty acids (SCFAs) such as acetic, butyric and propionic acid in colon, increase the expression of mucin 3 (MUC3), and increases colon crypt depth (Paturi et al., 2012), while promoting an anti-allergic effect in the respiratory tract (Gourbeyre et al., 2011; Verheijden et al., 2015). Future studies will address this finding. In addition, EPM-evoked levels of corticosterone in *L. casei* 54-2-33 and inulin-fed rats are similar to control, which suggest that HPA axis regulation has reached a maximum ceiling effect that is not further affected by either *L. casei* 54-2-33 or inulin. In synbiotic-fed rats, basal secretion of corticosterone is similar to control rats, and EPM-

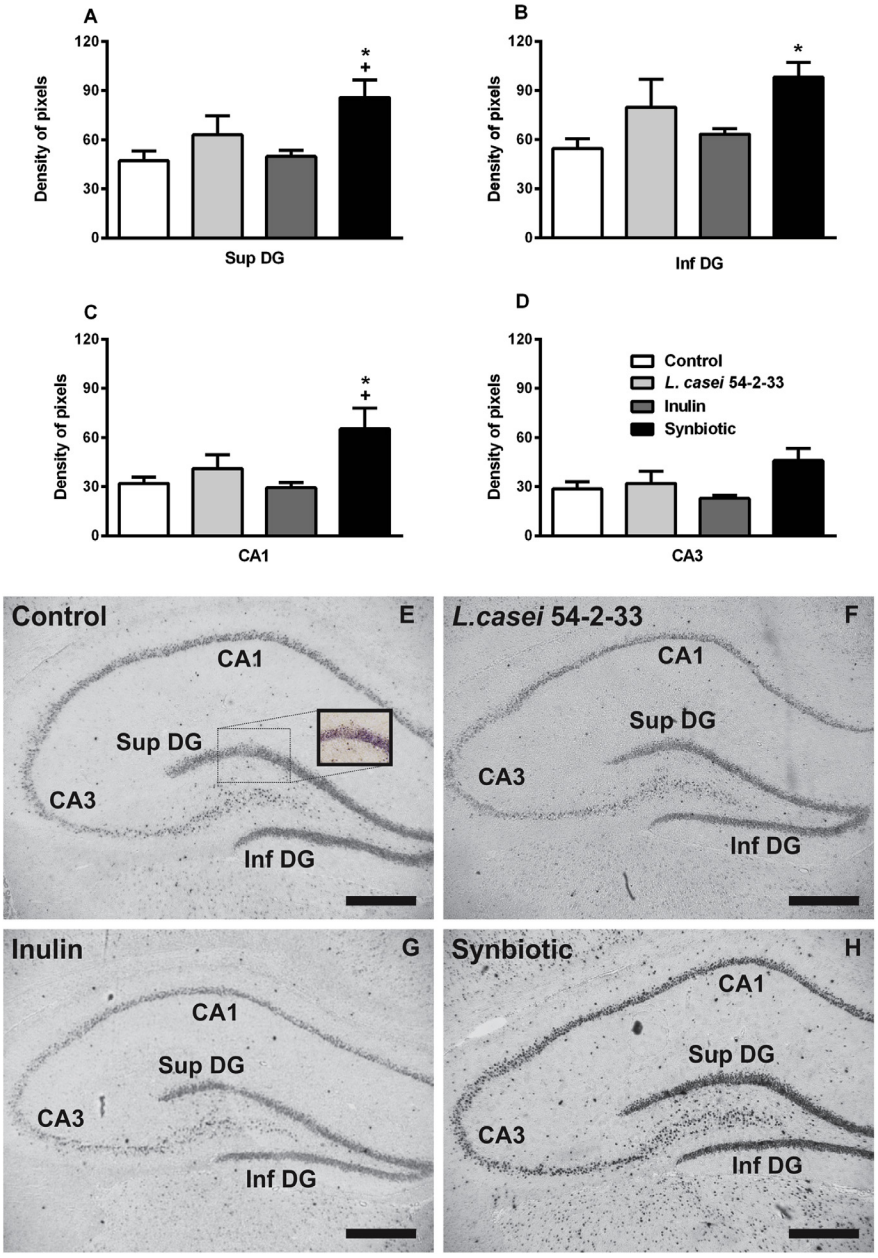

Fig. 5. Densitometric analysis of hippocampal 5-HT_{1A} mRNA expression. Synbiotic treatment shows an increase in 5-HT_{1A} mRNA expression compared to control and inulin group (A) (* = $p < 0.05$ when compared to control; $^{+}$ = $p < 0.05$ when compared to inulin). The same effect can be observed in CA1 (C) (* = $p < 0.05$ when compared to control; $^{+}$ = $p < 0.05$ when compared to inulin) and also there is significant difference between synbiotic and control group in Inf DG (B) (* = $p < 0.05$ when compared to control). There is no effect of treatment in the CA3 region (D). (Control $n = 6$; *L. casei* 54-2-33 $n = 5$; Inulin $n = 5$; Synbiotic $n = 5$). Panels E to H are representative microphotographs of each condition. Bar represents 500 μm.

evoked corticosterone secretion is significantly lower in comparison to control and *L. casei* 54-2-33 or inulin-fed rats. At 35 days of age, rats are periadolescent (Sengupta, 2013), a stage of development characterized by substantial changes in stress reactivity (Romeo et al., 2006), which impacts brain plasticity. For example, after a brief stressor, young rats show a prolonged ACTH and corticosterone response taking 45–60 min to return to baseline, while adult rats at that time point have already reached basal levels of both hormones (Romeo et al., 2006). This suggests that the HPA axis in puberty is still undergoing maturation, and its responses may affect glucocorticoid-sensitive regions of the brain such as the hippocampus (de Kloet et al., 2016). Here we explored whether HPA axis activity was susceptible to modification by *L. casei* 54-2-33, inulin and the mixture of both. This seems to be the case with synbiotic feed, which decreases corticosterone secretion in juvenile rats, suggesting a dampening effect on HPA axis response to acute stress. Interestingly, a similar effect has been previously demonstrated by Bravo et al. (2011) where the oral administration of the probiotic *Lactobacillus rhamnosus* JB-1 was able to reduce plasma corticosterone levels induced by stress in healthy adult mice (Bravo et al., 2011). However, in the present work this effect is achieved through a mixture of *L. casei* 54-2-33 and inulin (synbiotic) but not with the bacteria alone. Also, the findings by Bravo et al. (2011) were carried out in healthy adult male BALB/c mice, while the current findings were observed in healthy juvenile male Sprague–Dawley rats, which suggests that the effects of *L. casei* 54-2-33, inulin and synbiotic are not only species-specific, but also age-specific.

There were also effects on behavior. In the OF test, animals given *L. casei* 54-2-33, inulin and synbiotic had significantly less entries into central area in comparison to control rats. In terms of time spent: animals fed with *L. casei* 54-2-33, inulin and synbiotic spent less time in the center area of the arena. Overall, these results suggest that *L. casei* 54-2-33, inulin and synbiotic generate anxiogenic effects in juvenile rats, as they avoided the central area. However, this anxiogenic effect is not entirely replicated the EPM test, where the entries and time spent in the closed arms were similar between experimental groups, and not different to control rats. Moreover, the number of entries to the open arms and time spent there was lower for *L. casei* 54-2-33 and inulin-fed rats in comparison to control-fed animals, although this effect was not statistically significant. Furthermore, synbiotic-fed rats had significantly higher entries to the open arms and spent more time in them than inulin-fed rats, with

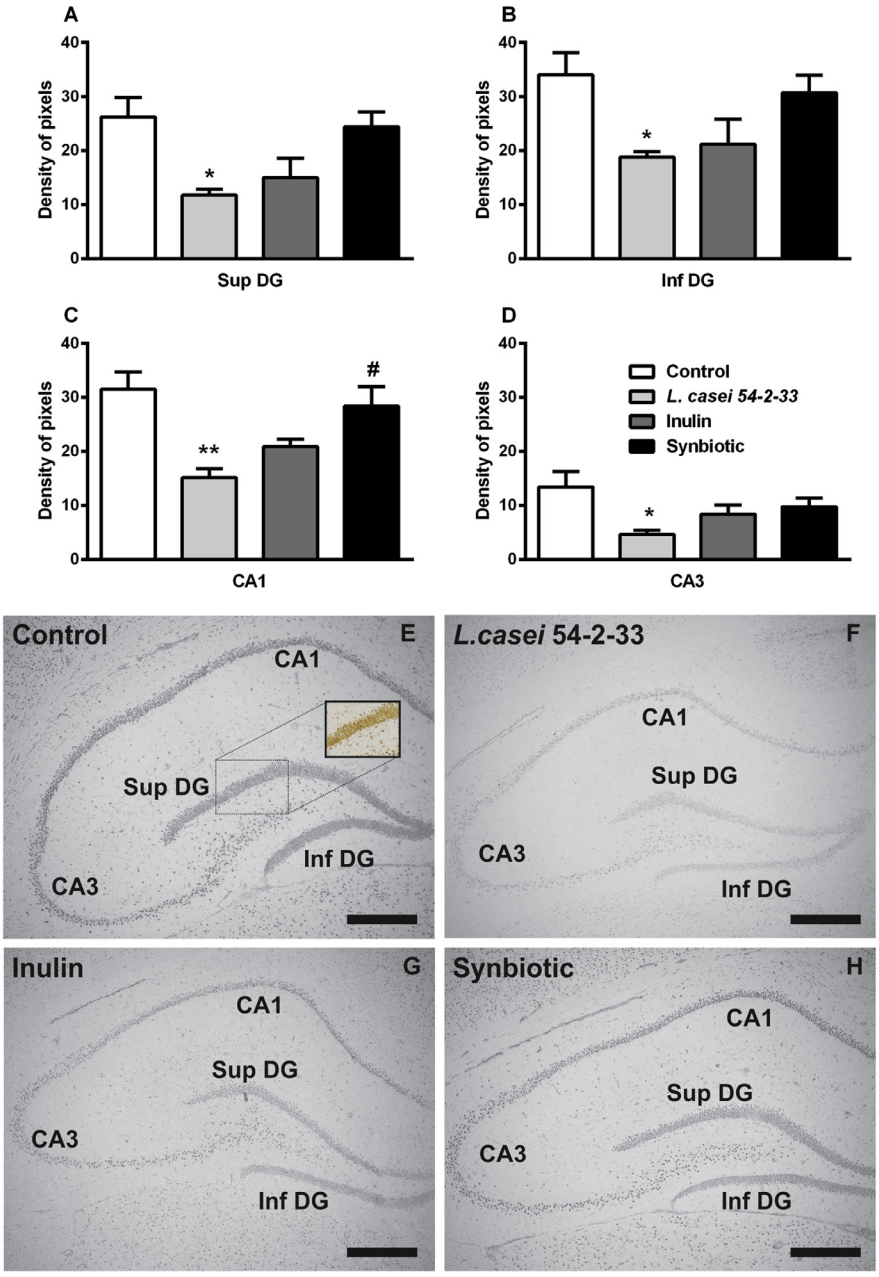

Fig. 6. Densitometric analysis of hippocampal 5-HT$_{1A}$ protein expression. *L. casei* 54-2-33 treatment reduces 5-HT$_{1A}$ protein expression compared to control rats in Sup DG (A), Inf DG (B) and CA1 (C) (* = $p < 0.05$, ** = $p < 0.01$ in comparison to control). There is also a significant difference between synbiotic-treated animals and *L. casei* 54-2-33-fed rats at CA1 ($^{\#}$ = $p < 0.05$) (C). There is no effect of treatments on the CA3 region (D). (control $n = 6$; *L. casei* 54-2-33 $n = 5$; inulin $n = 5$; synbiotic $n = 5$). Panels E to H are representative microphotographs of each condition. Bar represents 500 μm.

no statistical difference with control animals. This could be a consequence of how the tests were performed, as the first test (OF) was done on postnatal day 34, followed by EPM test on postnatal day 35. It has been shown that repeated testing affects behavioral outputs in young rats (Belviranli et al., 2012), suggesting that the anxiogenic effects seen in naïve rats subjected to the OF test might not necessarily replicate in another test that also measures anxiety-like behaviors. Nonetheless, other investigations have carried out repeated behavioral testing in the same animals, including tests for fear and depression-like behaviors over longer periods of time (Bravo et al., 2011). The latter suggests that, although both OF and EPM tests have been widely used to test anxiety-like traits in rodents, the context in each of the tests might have triggered different behavioral responses in these animals. However, changes in behavior due to differences in context are often observed in conditioned fear tests (Maren, 2008), but not in the tests carried out in the present work. Additionally, the differences between OF and EPM could also arise as a result of the animal's young age, as the CNS including serotonergic circuits and receptors is still undergoing maturation. (Loizou, 1972; Murrin et al., 2007). For instance, it has been observed that SSRI antidepressant's effectiveness is different between children and adults (Murrin et al., 2007). This evidence suggests that interventions affecting CNS gene expression (i.e.: changes in 5-HT receptors as a result of oral administration of *L. casei* 54-2-33, inulin and synbiotic) in brain areas related to anxiety behaviors (i.e.: hippocampus and amygdala), during a stage of development where the brain is still undergoing changes, might induce different behavioral outcomes than those observed at a later age. In addition, studies in young rats have found differences in age-related behavior during early development, which are manifested through variations in scanning mechanisms, particularly as an increased drive to explore new places, and a reduced capacity for risk assessment (Doremus et al., 2006). Furthermore, age significantly affects brain gene expression, a condition that is also affected by probiotic administration. Distrutti et al. (2014) compared an array of genes in the brain cortex of young (3 month old) and aged (20–22 months old) male Wistar rats fed with a mixture of eight probiotic bacteria (Distrutti et al., 2014). The authors found changes in several genes as a result of this dietary intervention, some of which only occurred in aged animals fed with the probiotics. Moreover, Distrutti et al. (2014) found that aged rats fed with the probiotic mixture had an enhancement in hippocampal long-term potentiation in comparison to aged rats fed with vehicle. All of this evidence suggests that changes in behavior obtained after

administration *L. casei* 54-2-33, inulin and synbiotic in juvenile animals may be age-dependent, and also that the effects on the CNS are specific depending on the type of bacteria, prebiotic and the mixture of both.

In relation to the latter, another contributing factor to the behavioral differences observed between OF and EPM tests could be the nature of the treatments, as *L. casei* L-54-2-33, inulin and synbiotic promote specific effects on behavior, corticosterone secretion (basal and EPM-evoked levels of the hormone) and changes in hippocampal 5-HT_{1A} mRNA expression. This has been reported elsewhere: for example, oral administration of a single strain of *Lactobacilli* to healthy adult mice induces anxiolytic and antidepressant-like effects, while increasing fear behavior, and reducing stress induced plasma corticosterone levels (Bravo et al., 2011). Prebiotics on the other hand, such as 3′sialyllactose and 6′sialyllactose affect anxiety-like behaviors in adult C57BL/6 mice, but without changes in plasma corticosterone levels (Tarr et al., 2015). Moreover, 3′sialyllactose and 6′sialyllactose prevent stress-induced reduction of hippocampal neurons, which suggests that prebiotics affect hippocampal neuronal plasticity even in adult animals, a finding that is also supported by others (Savignac et al., 2013). Interestingly, the works by Tarr et al. (2015) and Savignac et al. (2013) use prebiotics of different chemical nature and different animal models: Savignac et al. (2013) use fructooligosaccharide, and galacto-oligosaccharides in rats, while Tarr et al. (2015) use 3′sialyllactose and 6′sialyllactose in mice. As for the mixture of *L. casei* L-54-2-33 and inulin, the results shown here are the first to demonstrate that such combination affects stress-related behaviors, and that this effect is different from those generated by the components of the synbiotic mixture on their own. However, the mechanisms of this synbiotic effect on behavior are still unknown. Inulin is a known substrate for several bacteria, including *Lactobacilli*. Therefore, it cannot be ruled out that putting *L. casei* L-54-2-33 and inulin in the drinking water might have produced an interaction that generated the observed results. One such possibility is that *L. casei* L-54-2-33 used inulin in the water to generate butyrate, propionate and acetate. It has been shown that oral administration of butyrate prevents the detrimental effects of dextran sodium sulfate (DSS, administered in the drinking water) on C57Bl/6 mice (Vieira et al., 2012). Moreover, DSS is known to generate anxiety-like behaviors (Reichmann et al., 2015; Emge et al., 2016), while probiotic bacteria prevents DSS-induced alterations including behavioral ones (Bercik and Collins, 2014; Emge et al., 2016). Therefore, SCFA might induce the improvements on behaviors observed in synbiotic-fed rats. However, in the present work the concentration of SCFAs in the drinking water was not evaluated. Therefore, we can only speculate about the interaction between *L. casei* L-54-2-33 and inulin in this medium, and the possible outcome of such mixture (i.e.: increased SCFA concentration in the water). Moreover, and in order to explore this hypothesis other strategies should be followed, like determination of SCFA concentration in synbiotic drinking water.

Regarding the effects of each intervention on hippocampal 5-HT_{1A} mRNA expression, rats fed with the synbiotic had higher levels of the transcript in most hippocampal areas, however at the protein level, there was no difference in the amount of 5-HT_{1A} receptor, indicating that even though the synbiotic may alter hippocampal transcriptional machinery in the dentate gyrus and CA1 areas, this does not reflect on changes in protein expression. This finding suggests that receptor levels are not only subject to changes in transcription, but also to other stimuli within each specific brain area. Such discrepancies between mRNA expression and protein levels are not uncommon (Bravo et al., 2009). It has been shown that 5-HT_{1A} mRNA expression is repressed by glucocorticoids, as its promoter bears a negative glucocorticoid response element (Ou et al., 2001). Therefore, in animals fed with *L. casei* L-54-2-33 and inulin, which had higher levels of basal plasma corticosterone in comparison to control-fed rats, lower levels of transcript to 5-HT_{1A} were expected. However, transcript levels for this receptor were no different than in controls, but synbiotic-fed animals, which had significantly lower basal and stress-evoked levels of plasma corticosterone displayed an enhanced hippocampal 5-HT_{1A} mRNA expression, which seems to be in line with the findings by Ou et al. (2001). This difference might arise as a compensating mechanism due to higher levels of corticosterone, as this hormone displays an inverted U-shaped dose–effect curve (Baldi and Bucherelli, 2005). Therefore, the high levels of corticosterone observed in *L. casei* L-54-2-33 and inulin-fed rats may have not modified 5-HT_{1A} mRNA expression to the extreme of blunting its expression. In addition, our data suggest that 5-HT_{1A} mRNA expression is modulated by other transcriptional regulatory pathways that go beyond the scope of the present study. Similarly, hippocampal protein levels of 5-HT_{1A} are also subject to a plethora of pathways regulating its expression. For example, chronic exposure to corticosterone has been shown to modulate the expression of other neurotransmitter receptors and transporters in several brain areas. Daily subcutaneous injections of corticosterone for 29 days in adult male Wistar rats induce anxiety and depression-like behaviors, along with a reduction in $GABA_{A\alpha2}$ protein in the prelimbic cortex (Skorzewska et al., 2014). On the other hand, oral administration of corticosterone increases protein levels of the noradrenalin transporter in the *locus coeruleus* of adult male Fischer 344 rats (Fan et al., 2014). However, neither Skorzewska et al. (2014) nor Fan et al., (2014) suggest a mechanism explaining corticosterone affects protein levels. One alternative is that corticosterone activates the ubiquitin–proteasome system (Braun and Marks, 2015), which could allow us to suggest that the lower levels of 5-HT_{1A} protein observed in *L. casei* 54-2-33-fed animals is due to an increase in the activity of this protein degradation system induced by higher levels of corticosterone. However, activation of the ubiquitin–proteasome system leads to a loss in skeletal muscle mass (Braun and Marks, 2015), and there is no observed weight loss in *L. casei* 54-2-33-fed animals in comparison to

control rats that could further support this suggestion. Despite the lack of a mechanism describing how protein changes occur in this experiment, the current findings open the possibility to analyze mechanisms of protein level regulation in specific brain areas of animals fed with a bacterium (probiotic or not), a prebiotic and the mixture of both, which would strengthen the idea of a highly integrated microbiota-gut-brain axis.

In addition, the hippocampus is anatomically and functionally segregated along its longitudinal axis into dorsal and ventral regions in rodents, whereby the dorsal hippocampus plays a preferential role in spatial learning and memory, whereas the ventral hippocampus is predominantly involved in the regulation of anxiety and the stress response (Moser and Moser, 1998; Bannerman et al., 2004; Fanselow and Dong, 2010). However, previous studies have shown that 5-HT_{1A} mRNA is highly expressed in dorsal hippocampus (Bravo et al., 2014), and moreover it has been shown that intestinal microbiota is involved in hippocampal neurogenesis in both dorsal and ventral hippocampus (Ogbonnaya et al., 2015). Hippocampal neurogenesis has been linked to the effects of antidepressants, including anxiolytic effects (Sun et al., 2017). In addition, 5-HT_{1A} expression in forebrain areas is required for adequate anxiety-like behaviors during development in mice (Gross et al., 2002). Therefore, analysis of 5-HT_{1A} mRNA and protein levels in the dorsal hippocampus of these animals contributes to the understanding of a stimulus such as a bacterium, a prebiotic and the mixture of both on a brain area that is key to stress-related psychiatric disorders. In future studies, ventral hippocampus would also be included in the analyses.

In relation to behavior, there is evidence suggesting that most of the observed changes in 5-HT_{1A} expression are consistent with the current behavioral findings. For example, it has been shown that rats with high levels of anxiety-like behaviors have reduced 5-HT_{1A} mRNA levels in the CA1 (Keck et al., 2005). Also, mice with null expression of 5-HT_{1A} receptor display anxiety-like traits (Heisler et al., 1998), whereas overexpression of the receptor in early stages of development, induces an anxiolytic-like effect in adult stages (Kusserow et al., 2004). On the other hand, 5-HT_{1A} knockout mice, in which the receptor's expression was recovered only in forebrain areas, displayed anxiety-like behaviors similar to wild-type mice (Gross et al., 2002). Moreover, the findings by Gross et al. (2002) show that 5-HT_{1A} expression is important during early-life development, as the absence of the receptor during embryonic and early postnatal periods is sufficient to generate the 5-HT_{1A} knockout phenotype in these animals, which cannot be reverted by expression of the receptor later in life. This emphasizes the significant participation of this receptor during early-life to the development of anxiety behaviors in adulthood. Also, these findings suggest that interventions in early-life affecting forebrain expression of 5-HT_{1A} expression, whether these are pharmacological, dietary or environmental, might impact the subject's ability to cope with stressful situations later in life. There is plenty of evidence demonstrating 5-HT_{1A} receptor participation in the development of anxiety behaviors, thus suggesting potential therapeutic strategies (Heisler et al., 1998; Cryan and Leonard, 2000; Gross et al., 2002; Kusserow et al., 2004; Keck et al., 2005; Parracho et al., 2005; Lyte et al., 2006; Whitaker-Azmitia, 2010). It is interesting that most studies have focused largely on the early events that increase vulnerability to stress-related psychiatric disorders, therefore the present results suggest that achieving a complete sense of the pathophysiology of anxiety not only requires an understanding of the molecules involved within the CNS, but also of the various stimuli that lead to alterations in genes like 5-HT_{1A}, including alterations in the composition of the gut microbiota during early stages of development.

CONCLUSION

Treating rats with *L. casei* 54-2-33, inulin or synbiotic during their juvenile life affects plasma levels of corticosterone, induces changes in anxiety-like behaviors and changes in brain gene expression. The alterations in plasma corticosterone verify that these treatments have an impact on the HPA axis. Moreover, *L. casei* 54-2-33 generates an anxiogenic-like effect, and should therefore not be considered as potential probiotic. In a novel finding, inulin also promoted anxiogenic-like effects, but the opposite response was observed when this prebiotic is administered in conjunction with *L. casei* 54-2-33 (synbiotic). Therefore, the effects here observed are specific to the age of intervention, the species of bacteria used, and to whether this bacterium is accompanied by prebiotic compounds.

Finally, these treatments generate changes in 5-HT_{1A} expression in brain areas such as the hippocampus that might partly explain the effects on anxiety-like behaviors. Therefore, administration of a bacterium, a prebiotic or the mixture of both in infanto/juvenile stages of life may have important consequences for the individual's endocrine, neurochemical and behavioral response when exposed to stress even before it reaches adulthood.

CONFLICT OF INTEREST

The authors of the present work have no conflict of interests.

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