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Brief communication

Glyphosate based- herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice

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ABSTRACT

Recently, a number of studies have demonstrated the profound relationship between gut microbiota (GM) alterations and behavioral changes. Glyphosate-based herbicides (GBH) have been shown to induce behavioral impairments, and it is possible that they mediate the effects through an altered GM. In this study, we investigated the toxic effects of GBH on GM and its subsequent effects on the neurobehavioral functions in mice following acute, subchronic and chronic exposure to 250 or 500 mg/kg/day.

The effect of these acute and repeated treatments was assessed at the behavioral level using the open field, the elevated plus maze, the tail suspension and splash tests. Then, mice were sacrificed and the intestinal samples were collected for GM analysis.

Subchronic and chronic exposure to GBH induced an increase of anxiety and depression-like behaviors. In addition, GBH significantly altered the GM composition in terms of relative abundance and phylogenic diversity of the key microbes. Indeed, it decreased more specifically, *Corynebacterium*, *Firmicutes*, *Bacteroidetes* and *Lactobacillus* in treated mice.

These data reinforce the essential link between GM and GBH toxicity in mice and suggest that observed intestinal dysbiosis could increase the prevalence of neurobehavioral alterations.

1. Introduction

Emerging behavioral and molecular evidence from Germ Free mice reveals a relationship between the gut microbiota and brain disorders, and provides support for the idea that normal healthy gut bacteria may influence the development of the central nervous system and thereby its function (Wiley et al., 2017). In this regard, GF mice exhibited pronounced cognitive deficits and expressed higher anxiety and depression-like behaviors, paralleled with lower turnover rates of monoamines in several brain regions (Nishino et al., 2013).

Pesticide residues are a persistent and serious environmental problem; they are ubiquitous in food materials, water and soil. Because of their antimicrobial activity, pesticides have the potential to change the gut microbiota leading to disorders of energy metabolism, immune system function and psychoaffective functions (Jin et al., 2015). Glyphosate-based herbicide (GBH), the active ingredient present in Roundup® (Monsanto Company, St. Louis, MO), is the most heavily used organophosphate herbicide worldwide (Powles et al., 1996). It has been found that glyphosate's mechanism of action in plants is related to the disruption of the shikimate pathway, which is involved in the synthesis of the essential aromatic amino acids (Herrmann and Weaver, 1999). The currently accepted dogma is that glyphosate (Gly) is not harmful to humans or to any mammals because of the absence of the shikimate pathway in mammals (Samsel and Seneff, 2013). However, this pathway is present in gut bacteria, which plays an important and heretofore largely overlooked role in human physiology (Moco et al., 2012).

Because GBH-exposure has been shown to impact the neurobehavioral functions, and that altered gut mirobiota (GM) profiles have been associated with anxiety and depressive-like behavior (Bercik et al., 2011a, 2011b), it can be hypothesized that GBH-induced GM alterations may contribute in mediating behavioral changes. Thus, the purpose of the present study was to evaluate the impact of GBH on a healthy microbiota–gut–brain axis by investigating the gastrointestinal microbiota alterations and their subsequent effects on the neurobehavioral functions in mice.

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2. Material and methods

2.1. Pesticide

Roundup herbicide (glyphosate concentration 360 g/l in the form of glyphosate isopropylamine salt 486 g/l) with molecular formula $C_6H_{17}N_2O_5P$, molecular weight of 228.183 g/Mol, melting point 200 °C and density 1.218 g/cm^3 was used in the liquid commercial form supplied by Monsanto Company (St. Louis, MO, USA).

2.2. Animals and treatment

Male Swiss mice (1-month-old) were obtained from the animal husbandry of the Faculty of Sciences, Cadi Ayyad University, Marrakech, Morocco. The animals were housed in Plexiglas cages (30 cm × 15 cm × 12 cm) under standard conditions of temperature (22 ± 2 °C) and photoperiod 12 h/12 h (lights on at 08:00 h). Food and water were available *ad libitum*. All procedures were conducted in accordance with approved institutional protocols, and with the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive: EU2010/63. All efforts were made to minimize any animal suffering. The study was approved by the Council Committee of Research Laboratories of the Faculty of Sciences, Cadi Ayyad University, Marrakech.

Mice were subdivided to three experimental groups (acute, subchronic and chronic: $n = 18$/group), and each group was subjected either to orally gavages by NaCl 0.9% (control $n = 6$), by 250 mg/kg/day (n = 6) or 500 mg/kg/day (n = 6) of GBH. These doses were selected on the basis of Gly no-observed adverse effect level (NOAEL) of 500 mg/kg/day for subchronic toxicity (EPA, 1993). The mice assigned to the acute group received one administration of 0.3 ml of GBH, while the subchronic and chronic groups were treated daily by this volume for 6 and 12 weeks, respectively. Then, they were submitted to behavioral testing. On the last day of the experiment, the treated animals were sacrificed for gut microbiota analysis.

2.2.1. Behavioral assessments

2.2.1.1. Open field test. This test is the commonly used to assess locomotor activity and emotional reactivity in rodents placed into novel environments (Wilson et al., 1976). The apparatus was a square field (50 × 50 × 50 cm), within each mouse was placed, and allowed to move freely for 20 min. The time spent in the center area (15 × 15 cm) as an index of anxiety behavior was recorded using a video camera (JVC), and analyzed by Ethovision XT Noldus 8.5 video tracking program (Noldus Information Technology b.v., Wageningen, Netherlands).

2.2.1.2. Elevated-plus maze test. This test is used to assess anxiety-like behavior in rodents (Handley and Mithani, 1984). The apparatus elevated to a height of 45 cm above the floor, comprised two opposing open arms (OA) (50 × 5 cm) and two closed arms (CA) (50 × 5 × 15 cm), which joined at a central square area (5 × 5 cm) to form a plus sign. Animals were tested individually for 5 min, by placing them in the center of the maze platform, with the head facing an open arm. The time spent in the OA and CA as well as the number of entries to each arm were quantified using Ethovision XT Noldus 8.5 video tracking program, allowing us to evaluate the anxiety index, according to Cohen et al. (2013) method, expressed as: anxiety index = 1 − [([open arm time/total time] + [open arm entries/total number of entries])/2]).

2.2.1.3. The tail suspension test. This is one of the accepted behavioral tests used to evaluate potential antidepressant-like effects in rodents (Cryan and Holmes, 2005). The mice were individually suspended by the tail above the ground, with adhesive tape placed about 40 cm from the floor. A single 6 min session was recorded for each animal. The total time spent immobile during the last 4 min of a session was scored.

2.2.1.4. Splash test. This test assess grooming behavior, defined as cleaning of the fur by licking or scratching, after vaporization of 10% sucrose solution onto the mouse's dorsal coat (David et al., 2009). The latency to initiate a grooming behavior as well as the duration of grooming was recorded during 5 min after the vaporization of sucrose solution. Previous works in mice have shown that in the splash test, chronic stress decreases grooming behavior, a form of motivational behavior considered to parallel indifferent behavior as a symptom in depression (Isingrini et al., 2010).

2.2.2. Sample collection and abundances of intestinal microbiota determination

In mammals, the lower gastrointestinal tract (the small intestine, caecum and large intestine) contains a variety of distinct microbial habitats, in which physiological variations along its lengths include chemical and nutrient gradients, as well as compartmentalized host immune activity, known to influence bacterial community composition (for review see Gregory et al., 2016). Thus, to examine the effects of glyphosate-based herbicide on the composition of bacterial communities, intestinal samples were obtained from control and GBH-exposed mice at euthanasia, after behavioral tests completion. For each animal, sample of the intestinal tract starting from the duodenum to the end of the large intestine was collected directly into sterile tube, diluted ten times in sterile physiological water (NaCl 9 g/l), homogenized by vortexing for 10–15 min. Bacterial strains were counted using dilution / spreading method after the incubation at 37 °C for 72 h.

2.2.3. Phoenix system identification method

The Phoenix identification method uses modified conventional, fluorogenic, and chromogenic substrates. Combination panels for investigational use only (PMIC/ID-33, catalog no. 448587) for both identification and susceptibility testing were used for this comparison. Software V7. 00A/V5.91A was used for this study. The ID side contains 45 wells with dried biochemical substrates and 2 fluorescent control wells. The ID broth was inoculated with bacterial colonies adjusted to a 0.5 McFarland standard by using a Crystal Specnephelometer (BD Diagnostics), according to the manufacturer's recommendations. The specimen was logged and loaded into the instrument within the specified timeline of 30 min. Quality control and maintenance were performed according to the manufacturer's recommendations.

3. Statistical analysis

To compare data from behavioral tests and gut microbiota analysis between groups (control and treated), a statistical analysis of the different independent variables was performed by two-way ANOVA (treatment and treatment duration), using the Sigma Plot software 11.0. *Post hoc* analysis was performed using Holm-Sidak *post hoc* test. Results are presented as mean ± standard error of the mean (S.E.M). The significance threshold was set at $p < 0.05$.

4. Results

4.1. Behavioral changes after GBH exposure

Repeated exposure to GBH elicited evident emotional behavioral alterations in mice, as assessed by various behavioral tests: open field, elevated plus-maze, tail suspension and splash test.

Our results indicated that the control groups of each test showed no significant difference as a function of treatment duration, except for the controls of the open field test. In fact, we observed a significant increase in controls of subchronic (81.77 ± 5.15 s) and chronic treatment groups (76.63 ± 5,19 s) compared to the acute control group (34.68 ± 3.13 s) ($t = 8.47$ and $t = 7.56$ respectively, with 10 degrees

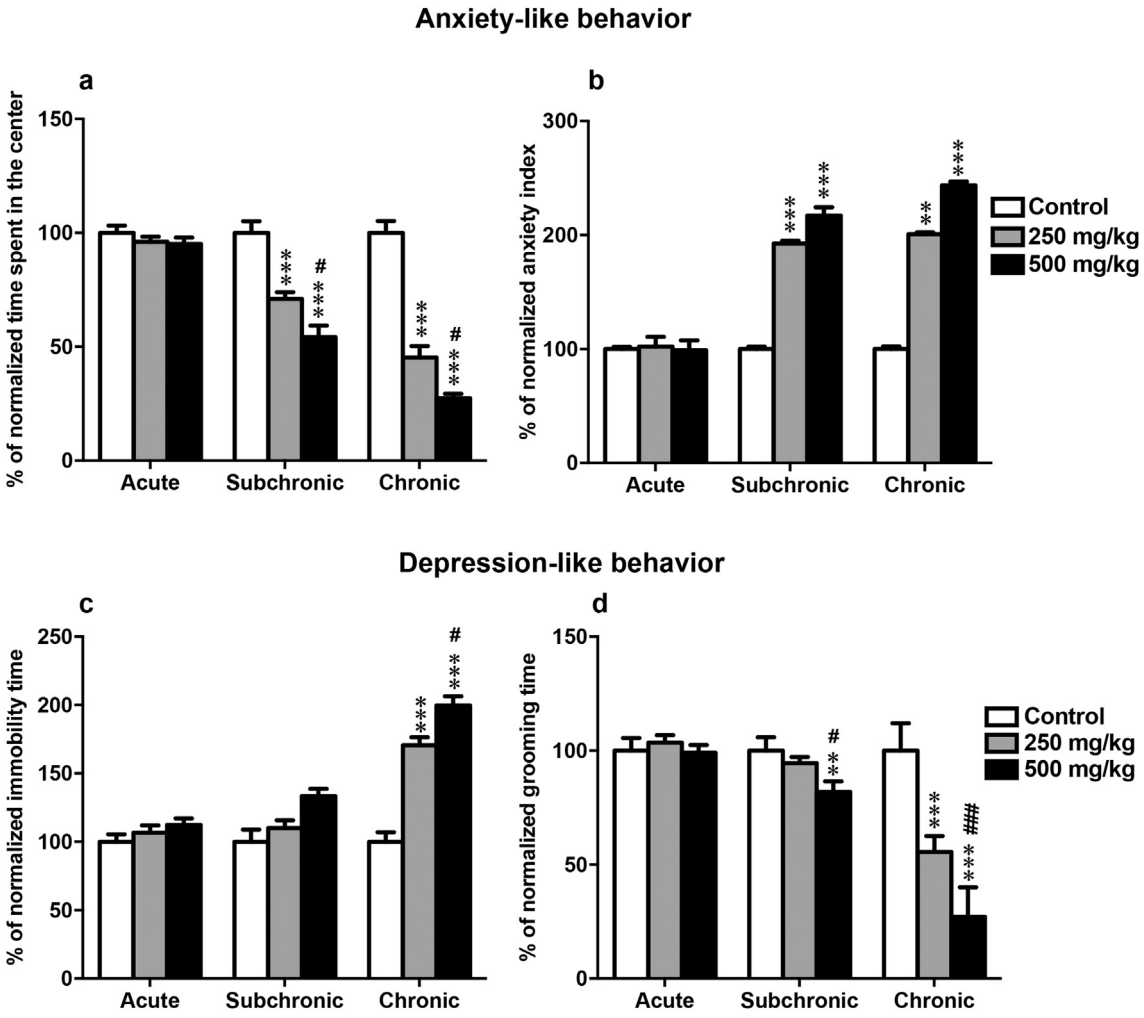

Fig. 1. Effect of GBH on the anxiety and depression like-behaviors. (a): Percentage of the time spent in the center of open field test normalized to control. (b): Percentage of the anxiety index in the elevated plus-maze test normalized to control. (c): Percentage of the immobility time in the tail suspension test normalized to control. (d): Percentage of the grooming time in the splash test normalized to control. $^{\#}P < 0.05$, ** and $^{\#\#}P < 0.01$, $^{***}P < 0.001$. The "$_{*}$" refers to the control *vs.* 250 mg/kg and 500 mg/kg group comparison, and the "#" refers to the 250 mg/kg *vs.* 500 mg/kg group comparison.

of freedom, $p < 0.001$).

In order to circumvent that the difference between the control groups could influence the observation of GBH effects, and to highlight the impact of the dose and the duration of the treatment, we expressed the results in percentage normalized to control groups as shown in Fig. 1a–d. Indeed, two-way ANOVA analysis of the normalized percentage of the time spent in the central zone of the open field and the anxiety index recorded during the elevated plus-maze test revealed significant differences among the factors of treatment ($F_{(2.17)} = 51.18$, $p < 0.001$; $F_{(2.17)} = 17.75$, $p < 0.001$, respectively), treatment duration ($F_{(2.17)} = 38.04$, $p < 0.001$; $F_{(2.17)} = 8.77$, $p < 0.001$, respectively), as well as the interaction of treatment × treatment duration ($F_{(2.17)} = 13.22$, $p < 0.001$; $F_{(2.17)} = 4.53$, $p < 0.001$, respectively). The *post hoc* comparisons using Holm-Sidak test confirmed that subchronic and chronic treated groups (both 250 and 500 mg/kg) showed a significant decrease in the time spent in the center, and an important increase of the anxiety index ($p < 0.001$) with respect to control (Fig. 1a–b). On the other hand, the statistical analysis of the immobility and grooming time respectively recorded in the tail suspension and splash tests, revealed a significant differences among the factors: treatment ($F_{(2.17)} = 20.12$, $p < 0.001$; $F_{(2.17)} = 39.39$, $p < 0.001$, respectively) and treatment duration ($F_{(2.17)} = 42.02$, $p < 0.001$; $F_{(2.17)} = 92.41$, $p < 0.001$, respectively), as well as in the interaction treatment × treatment duration ($F_{(2.17)} = 7.31$, $p < 0.001$; $F_{(2.17)} = 19.89$, $p < 0.001$, respectively). The *post hoc* comparisons using Holm-Sidak test confirmed that the treated groups showed a significant increase in the immobility time only following chronic treatment ($p < 0.001$) and a decrease in the grooming time ($p < 0.01$) after both subchronic and chronic exposure (Fig. 1c–d).

4.2. Gut microbiota changes after GBH exposure

Our results showed that the repeated exposure to GBH acted significantly on GM profile of exposed mice. Indeed, two-way ANOVA analysis of the normalized percentage of the intestinal bacteria abundance revealed significant effects for treatment ($F_{(2.11)} = 30.17$, $p < 0.001$), treatment duration ($F_{(2.11)} = 71.41$, $p < 0.001$), as well as for the interaction between treatment and treatment duration ($F_{(2.11)} = 12.68$, $p < 0.001$)(Fig. 2a). The *post hoc* comparisons using Holm-Sidak test confirmed that the treated groups showed a significant decrease in the total bacterial count following both subchronic and chronic treatments ($p < 0.001$) compared to their corresponding control groups (Fig. 2a). Moreover, the *post hoc* analysis showed no difference of GM between acute control (211.50 ± 13.23) and subchronic control groups (230.00 ± 16.83) ($t = 1.13$ with 8 degrees of freedom, $p = 0.26$). However, GM decreased significantly in the control of chronic treatment group (145.00 ± 9.98) compared to both acute control group ($t = 3.19$ with 8 degrees of freedom, $p < 0.01$) and to

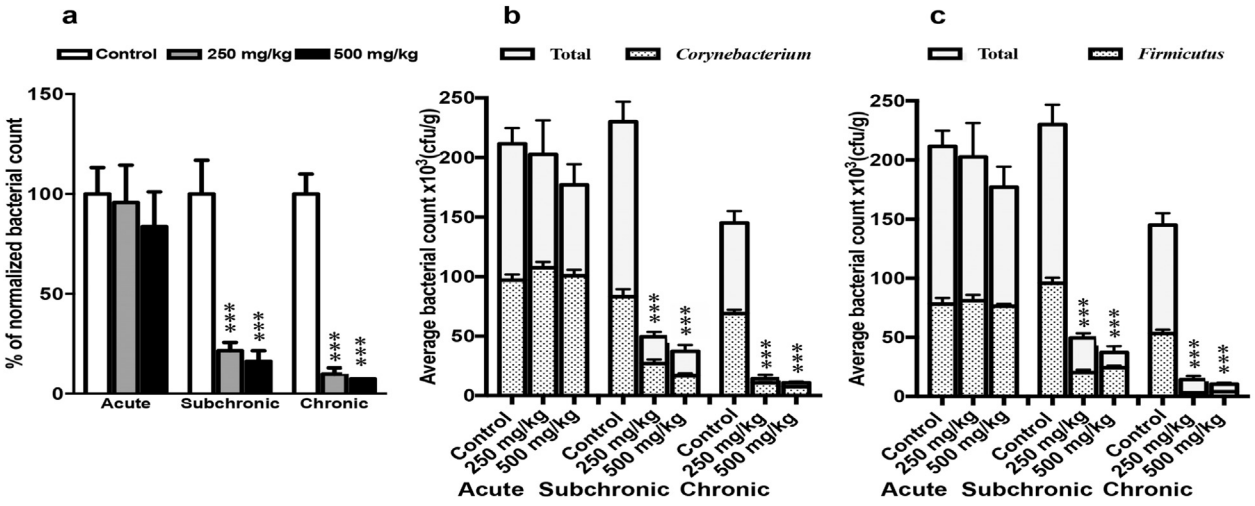

Fig. 2. Effect of GBH on the intestinal bacteria abundance. (a): Percentage of the total bacterial count normalized to control group. (b): *Corynebacterium* strain count. (c): *Firmicutus* strain count. ***$P < 0.001$. "*" refers to the control *vs*. 250 mg/kg and 500 mg/kg group comparison.

subchronic treatment control group ($t = 4.46$ with 8 degrees of freedom, $p < 0.001$).

4.3. Gut microbiota diversity after GBH exposure

In this study, BD Phoenix™ detection was used to evaluate the changes of gut microbiota community in mice following GBH exposure. The identified gut bacteria assigned at the intestine level showed that control mice were dominated by *Firmicutes*, *Bacteroidetes*, *Corynebacterium* and *Lactobacillus* type of bacteria, while the GBH-treated mice were dominated only by *Firmicutes* and *Corynebacterium* species following subchronic and chronic exposure (Table 1).

Furthermore, the abundance of *Corynebacterium* was significantly affected under GBH exposure. Two-way ANOVA analysis of the bacterial average count revealed significant differences among the factors: treatment ($F_{(2.11)} = 43.65$, $p < 0.001$), and treatment duration ($F_{(2.11)} = 197.30$, $p < 0.001$), as well as the interaction of treatment × treatment duration ($F_{(2.11)} = 22.97$, $p < 0.001$)(Fig. 2a). Likewise, two-way ANOVA analysis of *Firmicutus* abundance revealed significant effects of treatment ($F_{(2.11)} = 76.85$, $p < 0.001$) and treatment duration factors ($F_{(2.11)} = 159.03$, $p < 0.001$), as well as for the interaction between treatment x treatment duration ($F_{(2.11)} = 30.04$, $p < 0.001$) (Fig. 2b–c). The *post hoc* test showed that *Corynebacterium* and *Firmicutus* decreased significantly ($p < 0.001$) for both treated groups (250 and 500 mg/kg), compared to their controls within subchronic and chronic treatments. However, no significant difference between the treated groups was noted ($p > 0.05$).

5. Discussion

There are fundamental emerging data on the human gut-microbiota-brain connection which suggest that there is a link between changes in the gut microbiota and neurological diseases and disorders (Lyte, 2013). Based on these findings, we wanted to know whether behavioral impairments reported in our recent study, where repeated exposure to GBH elicits evident emotional behavioral alterations in male mice paralleled by the serotoninergic system impairment (Ait Bali et al., 2017), were associated with gut microbiota abnormalities.

Consistent with the potential of pesticide-contaminated foods to induce gut microflora dysbiosis in rodent models (Nasuti et al., 2016), we found that the anxiogenic and depressive-like behaviors observed in the present work paralleled by an altered gut microbiota in GBH-treated mice in term of abundance and bacteria species. Indeed, the oral exposure to GBH decreased the gut microbiota abundance of *Firmicutes*, *Corynebacterium*, *Bacteroidetes* and *Lactobacillus*. This alteration of the gut microbiota composition by decreasing the abundance of *Firmicutes and Bacteroidetes* was also described in animals exposed to other pesticides such as Chlorpyrifos (Joly Condette et al., 2015) and Permethrin (Nasuti et al., 2016).

Otherwise, the ratio of *Firmicutes/Bacteroidetes* has already been used as an effective indication for the status of human gut microbes (Mariat et al., 2009), and it was reported that the balance between these predominant phyla might impact host physiology (Bruce-Kellera et al., 2015). In the present study, our findings showed that the relative proportion of *Bacteroidetes* and *Firmicutes* substantially decreased under GBH-exposure. Beside the fact that the Bacteroidetes could contribute to the behavioral changes observed, our results are consistent with previous work indicating that Firmicutes are important for behavioral function. (Nasuti et al., 2016). This suggestion of beneficial roles for representatives of the *Firmicutes* phylum is in agreement with the known ability of *Firmicutes* to produce butyrate as a byproduct of fermentation (Macfarlane and Macfarlane, 2003). Butyrate is a major energy source for colonic epithelium (Shoaie et al., 2013), and more importantly, butyrate-producing bacteria play a vital role in

Table 1
Effect of GBH on taxonomic diversity of gastrointestinal microbiota.

Type of bacteria acute treatment				Subchronictreatment			Chronictreatment		
	Control	250 mg/kg	500 mg/kg	Control	250 mg/kg	500 mg/kg	Control	250 mg/kg	500 mg/kg
Firmicutes spp.	+	+	+	+	+	+	+	+	+
Coryenobacterium spp.	+	+	+	+	+	+	+	+	+
Bacteroidetes spp.	+	+	+	+	−	−	+	−	−
Lactobacillus spp.	+	+	+	+	−	−	+	−	−

(+): presence, (−): absence.

maintaining the integrity of the intestinal epithelial barrier (Canani, 2011). Dysbiotic states with reduced butyrate production could facilitate translocation of intestinal antigens, which could disrupt brain function through multiple local or systemic pathways (El Aidy et al., 2016). Thus, the relative abundance of supposedly beneficial and harmful species of each group is capable of producing neurobehavioral consequences.

Other studies demonstrated also that the perturbation of gut microbiota by non-absorbable antimicrobials was associated with the changes of central nervous system (CNS) activities and behaviors (Bercik et al., 2011a, 2011b). In line with this, antibiotics are known to cause metabolic acidosis through several mechanisms. One mechanism by which antibiotics can cause acidosis is by selectively killing bacteria in the intestines, thereby causing an imbalance in the microbiota (Coronado et al., 1995). A second possible mechanism is through inhibition of mitochondrial protein synthesis, or otherwise disrupting the mitochondrial metabolism process (Palenzuela et al., 2005). Direct evidence from the literature described on the one hand that glyphosate is a patented chelator, antibiotic and biocide (Abraham, 2010) and on the other hand, that acidosis/mitochondrial dysfunction can cause a multitude of symptoms and diseases including neurological disorders similar to those reported for chronic glyphosate poisoning (Samsel and Seneff, 2013).

In the present study, repeated exposures to GBH affected the microbiota composition in exposed groups, which express at the same time anxiety and depression phenotype, with the absence of the *Lactobacillus* and *Bacteroidetes* bacteria. This result is consistent with previous work demonstrating that *Lactobacillus* phylum has shown psychoactive and neuroactive properties, by reducing anxiety and depression-like behaviors in healthy mice *via* the vagus nerve (Bravo et al., 2011). The same study showed that chronic consumption of the *Lactobacillus rhamnosus JB-1* probiotic strain lowered the stress-induced corticosterone secretion and decreased the level of anxiety-like behavior in specific pathogen free BALB/c mice (Archibald and Duong, 1984).

A possible explanation for the effect of GBH on *Lactobacillus* abundance is the manganese (Mn) chelation. *Lactobacillus* utilize Mn for protection from oxidation damage, and as a consequence, their requirements for Mn are more important than those of other species (Archibald and Duong, 1984).Therefore, Mn chelation by glyphosate could lead to the reduction of these essential bacteria in the gut, leading directly to neurological symptoms such as anxiety (Cryan and Dinan, 2012). Moreover, as the pH increases gradually from 6 to 7.4 in the terminal ileum (Fallingborg, 1999), the Mn bioavailability can be expected to be reduced by 50% as a result of glyphosate chelation (Lundanger Madsen et al., 1978).

Another explanation is the main potential of gut bacteria to interact with host nervous systems through neurotransmitters or their metabolic precursors (Sampson and Mazmanian, 2015) such as GABA and tryptophan. Tryptophan is a central precursor of serotonin or 5-hydroxytryptamine (5-HT), an important neurotransmitter involved in multiple physiological processes, such as psychoaffective functions (Fukumoto et al., 2003). Previous studies have shown that gut microbiota participate in 5-HT turnover modulation (Yano et al., 2015). Indeed, tryptophan is generated by the gut microbiota and then can cross the blood brain barrier to be converted to 5-HT (O'Mahony et al., 2009). In light of these latter findings, a study has reported that glyphosate suppresses 5-enolpyruvyl shikimic acid-3-phosphate synthase, the rate-limiting step in the synthesis of the aromatic amino acids, tryptophan, tyrosine, and phenylalanine, in the shikimate pathway of bacteria (Samsel and Seneff, 2013). This mode of action is unique to glyphosate among all emergent herbicides. Humans do not possess this pathway, and therefore depend upon ingested food and gut microbes to provide these essential nutrients.

Glyphosate, patented as an antimicrobial (Monsanto Technology LLC, 2010) has been shown to disrupt gut bacteria in animals, preferentially killing beneficial forms and causing an overgrowth of pathogens (Shehata et al., 2013). Nevertheless, it is important to highlight that a large array of crucial molecules with neuroactive functions are produced by microbes (Cryan and Dinan, 2012). Indeed, several neurotransmitters such as GABA, serotonin, catecholamines and acetylcholine are produced by bacteria (Cenit et al., 2017). Collectively, these data suggest that GBH-induced perturbation of gut microbiota might disturb the normal metabolism of neurotransmitters or related peripheral precursors in the gut, which could further interfere with normal gut-brain interactions.

Recent data suggest a key role for intestinal microbiota in the development of the CNS, especially serotoninergic system (Tognin, 2017). In regards with this, the male mice born from germ-free dams show increased 5-HT and decreased BDNF in the hippocampus, altered anxiety, and increased plasma levels of tryptophan (Cenit et al., 2017).These additional evidences support our previous findings describing the emotional disturbances accompanied by altered 5-HT homeostasis in the dorsal raphe nucleus, amygdala and prefrontal cortex in the same groups of animals treated from adolescence to adult age (Ait Bali et al., 2017). These data suggest that microbiota could regulate neurobehavior by modulating the development of CNS serotonergic neurotransmission.

In this study, an exacerbation of anxiety and depression-like behaviors and significant perturbations in relative abundance and phylogenic diversity of gut microbiota in mice were induced by GBH exposure. Our findings may provide novel insights into the mechanism underlying GBH-induced health risk, particularly mood disorders.

Conflict of interest

The authors declare that there are no conflicts of interest.

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