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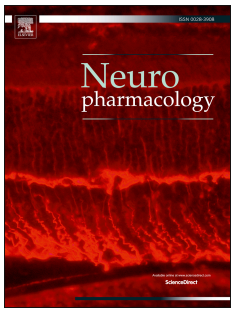

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Author Contributions

Emilio Russo, Paolo Mainardi, Pasquale Striano, Giovambattista De Sarro and Andrew Constanti contributed in the conceptualization; Antonio Leo, Carmen De Caro, Emilio Russo, Nadia Marascio and Rita Citraro contributed to data curation and formal analysis; Rita Citraro, Emilio Russo, Carmen De Caro and Giovambattista De Sarro participated to funding acquisition; Antonio Leo, Carmen De Caro, Nadia Marascio, Valentina Nesci, Martina Tallarico contributed to investigation, methodology; Antonio Leo, Carmen De Caro, Emilio Russo contributed to the original draft; Andrew Constanti, Antonio Leo, Pasquale Striano, Emilio Russo and Rita Citraro contributed to the final version of the manuscript.

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Abstract

The Microbiota-Gut-Brain axis (MGBA) is a bidirectional communication pathway between gut bacteria and the central nervous system (CNS) (including the intestine) that exerts a profound influence on neural development, neuroinflammation, activation of stress response and neurotransmission, in addition to modulating complex behaviours, such as sociability and anxiety. Several MGBA modulating approaches are possible, such as probiotic administration. A reasonable pharmacological approach would also be the contemporarily administration of both prebiotics *and* postbiotics. To test this hypothesis, we probed the effects of α-lactalbumin (ALAC; a prebiotic in the dose range of 125-500 mg/kg) and sodium butyrate (NaB; a postbiotic in the dose range of 30-300 mg/kg) alone and in combination. We used two animal behavioural models of idiopathic autism, (BTBR mice) and anxiety/depression (chronic unexpected mild stress - CUMS mice) respectively, using several standard behavioural paradigms such as Three-chamber social interaction test, Marble burying assay, depression-, anxiety- and memory-tests. In BTBR autistic mice, we found that both ALAC and NaB improve animal sociability, and memory in the passive avoidance (PA); drug combination was more effective in almost all tests also reducing immobility time in the forced swimming test (FST), which was not affected by single drug administration. Similarly, in the CUMS mice, single drug administration was effective in improving: 1) depressive-like behaviour in the FST and sucrose preference test; 2) memory and learning in the PA, novel object recognition and Morris water maze tests. Drug combination was again more effective than single drug administration in most cases; however, in the CUMS model, neither single drug or combination was effective in the elevated plus maze test for anxiety. Our results suggest that in both models, ALAC and NaB combination is more effective in improving some pathological aspects of animal behaviour than single administration and that the prebiotic/postbiotic approach should be considered a reasonable approach for the manipulation of the MGBA to improve efficacy.

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Abstract

The Microbiota-Gut-Brain axis (MGBA) is a bidirectional communication pathway between gut bacteria and the central nervous system (CNS) (including the intestine) that exerts a profound influence on neural development, neuroinflammation, activation of stress response and neurotransmission, in addition to modulating complex behaviours, such as sociability and anxiety. Several MGBA modulating approaches are possible, such as probiotic administration. A reasonable pharmacological approach would also be the contemporarily administration of both prebiotics *and* postbiotics. To test this hypothesis, we probed the effects of α-lactalbumin (ALAC; a prebiotic in the dose range of 125-500 mg/kg) and sodium butyrate (NaB; a postbiotic in the dose range of 30-300 mg/kg) alone and in combination. We used two animal behavioural models of idiopathic autism, (BTBR mice) and anxiety/depression (chronic unexpected mild stress - CUMS mice) respectively, using several standard behavioural paradigms such as Three-chamber social interaction test, Marble burying assay, depression-, anxiety- and memory-tests. In BTBR autistic mice, we found that both ALAC and NaB improve animal sociability, and memory in the passive avoidance (PA); drug combination was more effective in almost all tests also reducing immobility time in the forced swimming test (FST), which was not affected by single drug administration. Similarly, in the CUMS mice, single drug administration was effective in improving: 1) depressive-like behaviour in the FST and sucrose preference test; 2) memory and learning in the PA, novel object recognition and Morris water maze tests. Drug combination was again more effective than single drug administration in most cases; however, in the CUMS model, neither single drug or combination was effective in the elevated plus maze test for anxiety. Our results suggest that in both models, ALAC and NaB combination is more effective in improving some pathological aspects of animal behaviour than single administration and that the prebiotic/postbiotic approach should be considered a reasonable approach for the manipulation of the MGBA to improve efficacy.

Keywords: Alpha-lactalbumin; Sodium butyrate; BTBR mice; autism; anxiety/depression; behaviour.

1. Introduction

Over the past decade, it has become clear that the Microbiota-Gut-Brain axis (MGBA) is a bidirectional communication pathway between gut bacteria and the central nervous system (CNS) (including the intestine) that exerts a profound influence on neural development, neuroinflammation, activation of stress response and neurotransmission, in addition to modulating complex behaviours, such as sociability and anxiety (Iannone et al., 2019). This increasing knowledge has opened new research perspectives in the neurological and psychiatric area with the promise, among others, of future innovative treatment options (Iannone et al., 2020, 2019; Long-Smith et al., 2020). One possible approach resides in the definition of probiotics (Reid et al., 2019), while it would also be possible to intervene by using a combination of prebiotics *and* postbiotics (defined as a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host)(Salminen et al., 2021). The former would act on gut microbiota *composition* favouring a therapeutically positive communication between the gut and the brain while the latter would be expected to have a *direct* effect on the brain (but not only) (Iannone et al., 2020, 2019; Long-Smith et al., 2020; Stilling et al., 2016). Several molecules and mediators have attracted interest in the past; among these, we have previously focused our attention on two candidates [*i.e.*, alpha-lactalbumin (ALAC) and sodium butyrate (NaB)]. We have explored their efficacy and potential mechanisms of action, linking both also to intestinal inflammation (Citraro et al., 2020, 2011; De Caro et al., 2019; Russo et al., 2012).

ALAC is a whey protein rich in tryptophan, and is the major protein in human colostrum (20-25% of total protein *vs* 4% of total protein in milk). Besides its peripheral activity on the gut, including its recognized prebiotic role (Brück et al., 2006), it has surprisingly promising protective effects against seizures in both preclinical and clinical studies (Citraro et al., 2011; Errichiello et al., 2011; Mainardi et al., 2008; Russo et al., 2012) but also on other brain

functions (Markus et al., 2000; Nielsen et al., 2020; Vekovischeva et al., 2013). Recently, we demonstrated that the effects of ALAC on seizures are linked to its ability to restore normal intestinal functions in a model of gut inflammation (De Caro et al., 2019). We have also previously linked its antiepileptic effects to tryptophan and serotonin in the brain (Citraro et al., 2011; Russo et al., 2012); it is now evident that this effect may also be linked to the gut, considering the role of the kynurenine pathway as one of the major communication systems between the gut and the brain (O'Mahony et al., 2015).

On the other hand, NaB is considered a major mediator of gut-brain communication in the MGBA (Stilling et al., 2016); NaB is a short-chain fatty acid (SCFA) which represents a major microbial metabolite in the gut with important pleiotropic effects, and NaB has been implicated in various important physiological functions including energy homeostasis, diet control and obesity, immune system regulation, cancer and various brain functions (*e.g.*, brain metabolism, neurogenesis) (Stilling et al., 2016). However, its exact mechanism of action in these areas is not yet completely known, involving among others, receptor signalling, enzymatic inhibition and epigenetic modulation through histone deacetylases (HDAC) (Canani et al., 2011; Stilling et al., 2016) further than modulation of hypothalamic–pituitary–adrenal (HPA) axis in mice and humans (Dalile et al., 2020; van de Wouw et al., 2018a). The beneficial effects of NaB on the brain has been demonstrated in several animal models including epilepsy and its comorbidities (Citraro et al., 2020), pain (De Caro et al., 2020; Russo et al., 2016) and in particular, improved social and repetitive behaviour in the BTBR mouse model of idiopathic autism (Kratsman et al., 2016). Moreover, similarly to ALAC, NaB has been shown to modulate brain excitability in the pentylenetetrazole (PTZ) seizure model by reducing intestinal inflammation (De Caro et al., 2019).

A reasonable pharmacological approach would be the manipulation of the MGBA by contemporarily administering both prebiotics *and* postbiotics; as such, the right combination

would be one in which the two agents would not interfere or completely overlap in their effects. Considering that in our previous experiments, ALAC administration did not increase NaB levels in the faeces as much as NaB administration would in control animals and above all, in the group with intestinal inflammation (De Caro et al., 2019), we hypothesized that their combination would be at least additive. To test this hypothesis, we probed the effects of ALAC and NaB alone and in combination, in two animal behavioural models of idiopathic autism, (BTBR mice) and anxiety/depression (chronic unexpected mild stress - CUMS mice) respectively, using several standard behavioural paradigms. Our results suggest that in both models, ALAC and NaB combination is very often more effective in improving behaviour than single administration in most of the tests used.

2. Material and methods

2.1 Animals

Male C57Bl/6J (B6) (control and for CUMS) and BTBR T + tf/J (BTBR, autistic) inbred strains of mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and colonies were established and maintained at the animal facility of the University Magna Graecia of Catanzaro, Italy. Animals were housed in the same room, six per cage, and kept under controlled environmental conditions (60 ± 5% humidity; 22 ± 2 °C; 12/12 h reversed light/dark cycle; lights on at 20.00), with free access to water and standard laboratory chow diet (4RF21, Mucedola srl, Italy). All experimental procedures were carried out in compliance with the international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept) and approved by Italian Ministry of Health. The experimental protocols and the procedures reported here were approved (authorization no. 177/2019-PR) by the Animal Care Committee of the University of Catanzaro, Italy.

2.2 Drugs and treatment

The study consisted of two experimental sections (see experimental scheme) evaluating the effects of alpha-lactalbumin (ALAC), sodium butyrate (NaB) and their combination on autistic-like behaviour, anxiety- and depressive-like behaviour and learning and memory in BTBR mice (experiment #1), a validated animal model of idiopathic autism (Meyza and Blanchard, 2017) and on animal behaviour in the chronic unexpected mild stress (CUMS) model in B6 mice (experiment #2) (Russo et al., 2013a).

In experiment #1, mice (8 weeks of age) were randomly divided into twelve groups (n = 10 per group). ALAC (125, 250 and 500 mg/kg; Kolfarma srl, Italy), NaB (30, 100, 300 mg/kg; Sigma-Aldrich, Milan, Italy) and the co-administration of ALAC (125, 250 and 500 mg/kg) with a fixed NaB dose (100 mg/kg; also chosen according to Kratsman et al., 2016) were administered in drinking water for 15 days (De Caro et al., 2019; Russo et al., 2012) and the animals then subjected to different behavioural tests.

In experiment #2, at the end of CUMS, all animals were divided into nine groups (n = 30 per group) and ALAC, NaB and their combination were administered in drinking water for 15 days using the same doses reported for experiment #1. After treatment, mice were divided into three subgroups and subjected to different behavioural tests.

2.3 Chronic unexpected mild stress (CUMS) protocol

C57BL/6J (B6) mice (8 weeks of age) were subjected to CUMS for a period of 4 weeks, as previously described (Russo et al., 2013a). Mice were subjected daily to one unpredictable stressful stimulus (stressor) such as a wet cage, food deprivation, restraint stress, a period of stroboscopic illumination (150 flashes/min for 60 min), a tilted cage (45°), or loud noise (109

dB for 2 min). All stressors and/or sequences were applied at different time points to avoid habituation and to add an element of unpredictability to the stressor (Russo et al., 2013a).

2.4 Behavioural tests

All behavioural tests were performed under controlled environmental conditions and standardized procedures for our laboratory, including temperature, humidity and light intensity (dim illumination), and with the support of video-tracking software (EthoVision XT15; Noldus Information Technology, Wageningen, the Netherlands) (Citraro et al., 2017; Leo et al., 2017).

2.4.1 Three-chamber social interaction test

The three-chamber social interaction test consisted of three equally sized rooms (20 cm × 45 cm each) divided by clear Plexiglas, and with an access door between each compartment. The test occurred in three distinct stages: (1) acclimatization phase (baseline), where the test animal was placed in the central compartment and allowed to freely explore the entire empty maze for 10 min; (2) sociability phase, where an unfamiliar male mouse was contained within a wire mesh container in an outer chamber of the maze and an identical clean and empty container was placed in the chamber at the opposite side of the arena (Silverman et al., 2010).

2.4.2 Marble burying assay

20 glass marbles (1.5 cm in diameter) were arranged in five rows of four in a Plexiglas cage (46 x 24 x 21 cm) filled with 5 cm of clean bedding. Each mouse was individually placed into the cage and allowed to freely explore for 30 min. When a threshold of 75% coverage for each marble was observed, it was considered buried and recorded. After the test, the marbles were thoroughly cleaned and new bedding was used for each mouse (Coretti et al., 2017).

2.4.3 Elevated Plus Maze (EPM)

The EPM consists of 2 opposing open arms and 2 opposing closed arms of the same size (45 cm × 10 cm) with 10 cm high walls connected by a central platform (10 cm × 10 cm) and elevated 80 cm above the floor. Mice were positioned in the central platform facing a closed arm and the number of entries into, time spent on each arm, and central platform was measured (Leo et al., 2019). The maze was systematically cleaned to remove olfactory cues, after each animal was tested. The shorter the time spent in open arms and central platform, the higher the anxiety was and *vice versa*. Mean velocity and total distance moved was also measured and examined for every experimental group (Leo et al., 2019).

2.4.4 Open-field test

Locomotor activity was monitored for 10 min in an open field, a white Plexiglas box (50 cm ×50 cm) with its floor divided into 9 squares. Each animal was gently placed in the centre of the box, and activity was scored, as previously described (Russo et al., 2013b, 2013a). The following parameters were considered: the time spent in the centre, the total distance moved, and the mean velocity during the 10-min test. An increase of time spent in the centre or a decrease of the latency to enter the centre are indicators of anxiolytic activity and *vice versa*.

2.4.5 Forced swimming test (FST)

The FST was used for measuring the immobility time (IT) and assess depressive-like behaviour in the mice. Briefly, mice were individually forced to swim for 6 min in a clear plastic cylinder (height: 26.5 cm, diameter: 16.5 cm) containing 15 cm of water at 22–23 °C. The total duration of immobilization, including passive swimming, was measured in the last 4 min of the test. The criterion for passive swimming was floating vertically in the water while making only those movements necessary to keep the head above the water. After the FST, animals were removed

and dried with a towel before being placed in their home cages (Citraro et al., 2017, Russo et al., 2013a).

2.4.6 Sucrose preference test (SPT)

Anhedonia is a key component of depression and can be measured in mice by their preference to consume a sweetened solution. For the SPT, mice were placed in separate cages and habituated to a 2% sucrose solution for 48 h before the test day. Mice were deprived of water and food in the 4 hours preceding the test (between 8:00 and 12:00 a.m.) and this was followed by a 1-h preference test with water and 2% sucrose delivered from identical bottles. The bottles containing water and sucrose were weighed before and after this period, and sucrose preference (%) was determined. The sum of the water plus sucrose intake was defined as the total intake, and a sucrose preference was expressed as the percentage of sucrose intake relative to the total intake (Russo et al., 2013a).

2.4.7 Passive Avoidance (PA)

Passive avoidance (PA) is a fear-motivated test used to study learning and memory in mice. In this test, mice learn to restrain their innate tendency, namely preferring a dark compartment rather than an illuminated one (Zovkic and Sweatt, 2013). Passive avoidance behaviour was assessed by a step-through type apparatus (Ugo Basile, Italy, model 40550), measuring 57 × 27 × 30 cm, which consisted of a cage divided into two compartments (light and dark) by a sliding door. The PA test was conducted over two consecutive days, as previously reported (Leo et al., 2019; Nesci et al., 2020). The latency to enter (s) in the dark compartment was recorded and analysed. The retention trial was carried out 24 h after the conditioning trial by re-introducing the mouse into the light compartment of the cage and by recording the time taken to enter the dark compartment. Retention memory is directly linked to the latency to enter

into the dark compartment: The better the memory, the greater the latency (Citraro et al., 2017; Leo et al., 2017).

2.4.8 Novel Object Recognition Test (nORT)

The novel object recognition test is based on the tendency of rodents to discover novel objects (Antunes and Biala, 2012). Mice, individually located in an open field Plexiglas box (70 × 60 × 30 cm), were trained to distinguish between different shaped objects (cubes, pyramids and cylinders) (Rispoli et al., 2013). Briefly, on day one, mice were subjected to the habituation trial, in which they could freely explore the arena for 6 min. On day two, a single session of two trials (T1 and T2), separated by a retention interval of 60 min, was performed as previously described (Leo et al., 2019).

2.4.9 Morris water maze test (MWM)

Learning and memory functions were assessed using a spatial acquisition task in the MWM test, as previously described (Citraro et al., 2019; Russo et al., 2013a). The apparatus consisted of a circular basin (diameter = 93 cm, height = 45 cm) filled with water (approximately 25 °C) to a depth of 24 cm, with a clear (invisible) escape platform (diameter = 8 cm) placed 1 cm below the water surface. Animals (n = 10 from each group) were trained for four consecutive days, with four trials on each day. During each trial, individual animals were put in a randomly chosen quadrant in the pool with the head facing the pool wall. Each animal was given 60 s to search for and locate the submerged platform. The latency time to find the platform was recorded, and the average time from four trials represented the daily result for the animal. On the fifth day of the MWM test, each animal was subjected to a probe test where no platform was present. The time of swimming in the former platform quadrant and the total time of swimming in all four quadrants were recorded for 60 s. The percentage of swimming in the

quadrant of the former platform was calculated as a measurement of spatial memory (Russo et al., 2013a).

2.5 Statistical analysis

All statistical procedures were performed using GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA 92037, USA). We used one-way ANOVA followed by Tukey's post hoc test to analyze and compare behavioral data obtained from experiments #1 and #2 with the exception of MWM in which two-way ANOVA repeated measures was used. Data obtained were expressed as mean ± S.E.M. for each tested compound or drug combination. Normality tests (i.e., Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov) were performed and although in most cases the test was passed, in some not consistent cases among tests some groups did not; all data have been presented in graphs. In all tests $P < 0.05$ was considered significant.

3. Results

3.1 Experiment #1: Effects of ALAC or NaB administered alone and in combination on autistic-like behaviour in BTBR mice

3.1.1 Three chamber social interaction test

The effect of ALAC, NaB and their combination on social behaviour in BTBR mice was evaluated by the three-chamber social interaction test. *Post-hoc* analysis revealed that compared to the BTBR mouse group, the untreated control (CTRL) B6 mouse group spent significantly more time in the novel mouse chamber than in the novel object chamber ($F_{(10, 99)} = 13.72$; $P < 0.0001$; Fig. 1a), as previously reported (Silverman et al., 2010) and in agreement with a lower sociability for BTBR mice. Interestingly, *post-hoc* analysis revealed that BTBR mice after a pre-treatment with ALAC at different doses (ranging from 125-500 mg/kg/day)

for 15 consecutive days, spent significantly more time in the novel mouse chamber than in the novel object chamber in comparison to the respective untreated mouse group ($P < 0.05$; Fig. 1a), suggesting a 'normalisation' of their behavioural deficit. Similarly, BTBR mice pre-treated with NaB at a dose of 100 mg/kg for 15 consecutive days, displayed a significant preference for the chamber containing the novel mouse compared to the chamber with the novel object ($F_{(10, 99)} = 13.72$; $P = 0.0012$; Fig. 1a). Curiously, BTBR mice treated with the lower (30 mg/kg) or higher (300 mg/kg) dose of NaB did not exhibit any significant preference for social interaction suggesting an unusual, possibly complex dose-response relationship. None of the single treatments was able to significantly increase the time spent sniffing the novel mouse in comparison to BTBR control mice.

Conversely, BTBR mice after a co-administration of ALAC, at each dose used, with NaB, at 100 mg/kg, spent significantly ($P< 0.0001$; Fig. 1a) more time in the chamber containing the novel mouse than in the chamber containing the novel object, and significantly ($P< 0.0001$; Fig. 1b) more time sniffing the novel mouse than sniffing the novel object chambers, with sociability being at a similar level to that shown by B6 control mice. In general, no significant ($P> 0.05$) difference was detected in the time spent in the centre among all groups tested except for BTBR mice remaining longer than B6 and coadministration groups (see supplementary materials).

3.1.2 Marble burying assay

The effect of ALAC, NaB and their combination on repetitive and perseverative behaviour in BTBR mice was evaluated by the marble burying assay. As previously described (Silverman et al., 2010), we observed that BTBR mice buried significantly more marbles than B6 (CTRL) mice ($F_{(10, 99)} = 29.80$ $P<0.0001$; Fig. 2a). ALAC pre-treatment, at each dose used, was able to significantly reduce the number of marbles buried by the BTBR mouse groups ($P<0.0001$;

Fig. 2a). Similarly, a pre-treatment with NaB, at a dose of 100 mg/kg, also significantly reduced the number of marbles buried by the BTBR mice ($F_{(10, 99)} = 29.80$; $P<0.0001$; Fig. 2a). In contrast, as with social interaction, NaB pre-treatment, both at 30 or 300 mg/kg/day, had no effect on repetitive behaviour in BTBR mice, with the number of marbles buried being maintained similar to untreated vehicle mice. Very interestingly, the co-administration of ALAC, at each dose tested, along with NaB at 100 mg/kg, significantly reduced the number of marbles buried by the BTBR mice in comparison to the BTBR vehicle group ($P<0.0001$; Fig. 2a). Moreover, the co-administration of ALAC, at each dose tested, together with NaB (100 mg/kg/day) was significantly more efficacious than the respective single drug administrations in reducing the number of marbles buried ($P<0.05$; Fig. 2a).

3.1.3 Effects of ALAC, NaB and their combination on anxiety-like and depressive-like behaviour in BTBR mice

Anxiety-like behaviour in control B6 (CTRL) and both vehicle and drug-treated BTBR mice was studied in the elevated plus maze (EPM) and was not influenced by treatments (Fig. 2b). Mean velocity and total distance travelled did not significantly differ ($P > 0.05$) among groups (data not shown). The forced swimming test (FST) was also performed to study depressive-like behaviour in B6 (CTRL) and both vehicle and treated BTBR mice respectively. In detail, B6 (CTRL) and BTBR mice showed no significant difference in the immobility time (IT) ($P>0.05$; Fig. 2c). Interestingly, only the co-administration of ALAC, at each dose used, with NaB (100 mg/kg) was effective in producing antidepressant-like activity, reducing significantly the IT in treated BTBR mice in comparison to vehicle group ($P<0.05$; Fig. 2c). Both results together indicate that this latter effect is not due to an increased locomotor activity by the treatment.

3.1.4 Effects of ALAC, NaB and their combination on learning and memory performance in BTBR mice

The passive avoidance (PA) test was performed to study the learning and memory performance in all groups. We show here for the first time, that BTBR mice have poorer performance in this test. In detail, *post-hoc* analysis revealed that BTBR mice enter into the dark compartment during the retention session, significantly earlier than B6 (CTRL) mice ($F_{(10, 99)} = 7.638$; $P< 0.0001$; Fig. 2d), showing a cognitive impairment. Additionally, during the retention session, a pre-treatment with ALAC, at each dose tested, significantly increased the latency to enter into the dark compartment in BTBR mice in comparison to the vehicle group ($P< 0.05$; Fig. 2d). Likewise, a pre-treatment with NaB, at 100 mg/kg/day, was able to significantly increase the latency to enter into the dark compartment in BTBR mice in comparison to vehicle group ($F_{(10, 99)} = 7.638$; $P=0.0015$; Fig. 2d). At odds, NaB pre-treatment both at 30 and 300 mg/kg/day, did not significantly modify the latency to enter in the dark compartment in comparison to vehicle group. However, the co-administration of ALAC, at each dose used, with NaB (100 mg/kg) *was* able to significantly improve cognitive performance in treated BTBR mice in comparison to the vehicle group ($P<0.0001$; Fig. 2d), with the latency to enter into the dark compartment being maintained at the B6 (CTRL) level.

3.2 Experiment #2: Effects of ALAC, NaB and their combination on depressive-like behaviour in CUMS mice

3.2.1 Forced swimming test (FST)

The FST was performed to evaluate depressive-like behaviour in B6 (CTRL) and both vehicle and treated CUMS mice. As previously reported, CUMS mice showed a significantly higher immobility time (IT), indicating depressive-like symptoms, in comparison to the vehicle-treated mice group ($F_{(8, 81)} = 18.69$; $P<0.0001$; Fig. 5A)(Russo et al., 2013a). The treatment

with ALAC, at each dose used, was able to significantly reduce the IT in treated CUMS mice in comparison to untreated CUMS mice ($P < 0.001$; Fig. 3a). Similarly, NaB, at 100 mg/kg/day was also able to significantly reduce the IT in CUMS mice ($F_{(8, 81)} = 18.69$; $P<0.0003$). Additionally, co-treatment of ALAC, at each dose used, with NaB (100 mg/kg) significantly reduced the IT in co-treated CUMS mice in comparison to untreated CUMS mice ($P<0.0001$; Fig. 3a). Moreover, the co-administration of ALAC with NaB, in CUMS mice, was able to significantly reduce the immobility time in comparison to untreated mice ($P < 0.0001$; Fig. 3a). Interestingly the co-administration of ALAC, at each dose used, with NaB was significantly more efficacious than the respective mono-administration to reduce depressive-like behaviour in CUMS mice ($P < 0.001$; Fig. 3a), with the IT being maintained similar to CTRL (vehicle) level.

3.2.2 Sucrose preference test (SPT)

The SPT was performed to evaluate anhedonia in both vehicle and treated mice. As shown in Fig. 3b, there were significant variations in sucrose preference among mice groups, with the CUMS group and control (vehicle) group having the lowest and highest percentages of sucrose preference, respectively ($F_{(8, 81)} = 4.506$; $P<0.0001$; Fig. 3b). The CUMS+ALAC (ranging from 125-500 mg/kg/day) groups as well as the CUMS+NaB (100 mg/kg/day) group showed a significant increase in sucrose preference compared to the CUMS mice group ($P<0.05$; Fig. 3b), suggesting that these treatments can reduce anhedonia. Very interestingly, the co-administration of ALAC, at each dose used, with NaB (100 mg/kg) was able to significantly rescue anhedonia in co-treated CUMS mice in comparison to untreated mice ($P<0.001$; Fig. 3b), with the sucrose preferences being maintained similar to control (vehicle) levels.

3.3 Effects of ALAC, NaB and their combination on cognitive performance in CUMS mice

3.3.1Novel object recognition test (nORT)

The nORT was performed to study working memory in both vehicle and treated mice. *Post-hoc* analysis revealed a significant difference in discrimination index (DI) between the CTRL (vehicle) group and the CUMS mouse group ($F_{(8, 81)}$ = 9.154; P<0.0001; Fig. 3c). The CUMS+ALAC (ranging from 125-500 mg/kg/day) groups as well as the CUMS+NaB (100 mg/kg) group showed a significantly higher DI in comparison to the CUMS group (P<0.05; Fig. 3c). The co-administration of ALAC, at each dose used, with NaB (100 mg/kg) significantly rescued working memory in co-treated CUMS mice in comparison to untreated mice (P<0.001; Fig. 3c), with the DI being maintained similar to control levels. No significant (P>0.05) difference was found among groups to reach 20s of exploration in the familiarization trial.

3.3.2 Passive avoidance test (PA)

The PA, a fear-motivated test, was performed to investigate learning and memory in both vehicle and treated mice. *Post-hoc* analysis revealed a significant difference in the latency to enter (s) into the dark compartment, during the retention session, between the CTRL (vehicle) group and the CUMS mouse group ($F_{(8, 81)}$ = 8.078; P<0.0001; Fig. 3d). The CUMS+ALAC (ranging from 125-500 mg/kg/day) groups as well as the CUMS+NaB (100 mg/kg) group showed significantly higher latency to enter (s) into the dark compartment, during the retention session, compared with the CUMS group (P < 0.01; Fig. 3d). Once again, the co-administration of ALAC, at each dose used, with NaB (100 mg/kg) was able to significantly rescue learning and memory performance in co-treated CUMS mice in comparison to untreated mice, with the latency time being maintained similar to control levels (P < 0.0001; Fig. 3d).

3.3.3 Morris Water Maze test (MWM)

Cognitive performances were also evaluated in the MWM task in both vehicle and treated mice. *Post-hoc* analysis revealed that there was a significant difference in the latency (s) to reach the platform ($F_{(1, 18)}$ = 41.30; P<0.0001; Fig. 4a), and the time (s) spent in the target quadrant between the CUMS group and the control (vehicle) group (P=0.0009; Fig. 4d). The CUMS+ALAC (ranging from 125-500 mg/kg/day) groups as well as the CUMS+NaB (100 mg/kg) group showed significantly shorter latency (s) to reach the platform ($F_{(5, 54)}$ = 16.93; P<0.0001; Fig. 4b) and higher time (s) spent in the target quadrant in comparison to the CUMS group (P<0.01; Fig. 4d). Additionally, the co-administration of ALAC, at each dose used, with NaB (100 mg/kg) was also able to significantly rescue learning and memory performance in co-treated CUMS mice in comparison to untreated mice, with the latency to reach the platform ($F_{(4, 45)}$ = 19.05; P<0.0001; Fig. 4c) and the time (s) spent in the target quadrant being maintained similar to control levels (P<0.01; Fig. 4d).

3.3.4 Effects of ALAC, NaB and their combination on anxiety-like behaviour in CUMS mice

Finally, the anxiety-like behaviour was evaluated in the elevated plus maze (EPM) and in the open field test (OFT) in both vehicle and treated CUMS mice. There were no significant differences in the parameters evaluated in the OFT among the tested groups (treated and untreated CUMS mice and control mice; P>0.05; Fig. 5a, b). However, there were significant differences in the EPM among groups. In detail, the CUMS mouse group spent significantly less time in the open arms in comparison to the control (vehicle) group, showing an anxiety-like behaviour ($F_{(8, 81)}$ = 8.405; P<0.0001; Fig. 5c). The CUMS+ALAC (ranging from 125-500 mg/kg/day) groups as well as the CUMS+NaB (100 mg/kg) group spent significantly more time (s) in the open arms in comparison to the untreated CUMS group (P<0.05; Fig. 5c).

Additionally, the co-administration of ALAC, at each dose used, with NaB (100 mg/kg) was able to significantly rescue anxiety-like symptoms in co-treated CUMS mice in comparison to untreated CUMS mice, with the time (s) spent in open arms being maintained similar to control levels ($P<0.0001$; Fig. 5b, c).

4. Discussion

Our results demonstrate that the combination of ALAC and NaB in the majority of tests in both the BTBR ASD model and CUMS model of depression lead to a better improvement and control of behavioural symptoms than single administration of either agent. Considering the administration protocol, it is not possible to define whether the final effect is additive or synergistic in pharmacological terms; this is also due to the peculiar pharmacological profile of both substances. More specifically, ALAC seemed to be equieffective at almost all doses tested reaching a ceiling effect and not being able to completely resolve either models' symptomatology, as we previously observed in other animal models (Citraro et al., 2011; Russo et al., 2012). On the other hand, the effect of NaB appeared to be bell-shaped in the BTBR model, showing efficacy only at the 100mg/kg dose which is the one we used in the CUMS model. While being out of the scope of this article, it should be noted that NaB is currently used in a wide range of doses (e.g., few milligrams up to grams) in several animal models and accordingly several mechanisms direct and indirect mechanisms of action have been reported (Han et al., 2014; Russo et al., 2016; Stilling et al., 2016; Sun et al., 2016; Wei et al., 2015; Yamawaki et al., 2018). Often, a ceiling effect is observed as well as lack of effectiveness at doses found to be effective in other models; in BTBR mice, it has already been reported that a 100mg/kg dose was effective while 1200mg/kg did not modify social behaviour (Kratsman et al., 2016). As suggested in this latter article, different dosages will activate different mechanisms and in some cases drug effects may be lost or even become detrimental as in the

case of an increased anxiety-like behaviour at a high dose but not at a lower dose (Gagliano et al., 2014).

Despite the confirmation of our initial hypothesis, we cannot definitely conclude that the observed effects were only due to a positive modulation of the MGBA. Indeed, both ALAC and NaB effects on the MGBA have been widely demonstrated (Boscaini et al., 2019; Burokas et al., 2017; Coretti et al., 2018; Nielsen et al., 2020; Sun et al., 2018; van de Wouw et al., 2018b; Yang et al., 2020), and it is clear that both ALAC and NaB possess other potential mechanisms of action which might have contributed to the final observed effects in our experiments.

Recently, we demonstrated the activity of ALAC and NaB in reducing intestinal inflammation induced by dextran sulfate sodium (DSS) which was linked to their anticonvulsant effects in the pentylenetetrazol (PTZ) mouse model (De Caro et al., 2019). NaB is widely considered as a histone deacetylase inhibitor (HDACi) and there is evidence that underlines the role of HDACi in neurodegenerative and neurodevelopmental diseases including epilepsy (Citraro et al., 2018; Qin et al., 2018). Moreover, it has been shown that NaB is able to ameliorate the social and repetitive behaviour in the BTBR autism mouse model (as confirmed in our experiments) through a modulation of the excitatory/inhibitory balance in the brain (Kratsman et al., 2016). The neuropharmacological actions of NaB have been recently reviewed and include a plethora of effects such as regulation of blood brain barrier (BBB) integrity, interaction with at least four different G protein-coupled receptors (GPCRs), facilitation of synaptic tagging and capturing and regulation of immune system and vagus nerve activity (Stilling et al., 2016).

ALAC has been suggested to act through an increase of 5-HT in the brain but also through the kynurenine pathway (Citraro et al., 2011; Markus et al., 2000; Russo et al., 2012). In recent years, perinatal exposure to either sub- or supra-optimal levels of 5-HT have been suggested to

be involved in the development of ASD (Garbarino et al., 2019; Pourhamzeh et al., 2021); such changes can disturb foetal growth and neurodevelopment, with impairments for the lifetime of the individual. Furthermore, it was observed that the stimulation of 5-HT release in the nucleus accumbens promotes sociability; this suggest that the involvement of 5-HT is important in the pathogenesis and treatment of neuropsychiatric disorders such as ASD (Walsh et al., 2018). Similarly, the kynurenine pathway has been indicated as part of the physiopathological mechanisms of neurodevelopmental and mood disorders (Coplan et al., 2021; Więdłocha et al., 2021), with this pathway also being strictly linked to the MGBA (O'Mahony et al., 2015; Więdłocha et al., 2021).

Regarding ALAC/NaB combination effects in the two tested models and in the various behavioural paradigms tested, we found an improvement in social interaction and repetitive behaviour in the BTBR ASD mouse model and depressive-like behaviour in the FST and sucrose preference tests in the CUMS model of depression. Regarding this latter effect, ALAC/NaB combination was also effective in increasing mobility time in BTBR mice while single administration was not effective. This may be justified by the fact that BTBR mice do not have an increased immobility time in comparison to controls and therefore do no display depressive-like behaviour. A similar effect was also observed in the EPM test for anxiety. The difference between the two models is most likely linked to the absence of this symptomatology in BTBR mice and the lack of a diseased background. This is also supported by the similar effects on memory functions in the two models when the agents are administered. Both models are accompanied by cognitive dysfunctions that can be measured in different tests and here, we demonstrated that ALAC/NaB combination was a valuable approach to improve single drug administration. Indeed, both ALAC and NaB have already been reported to improve cognitive function in other models (Kilgore et al., 2010; Markus et al., 2002; Nielsen et al., 2020; Rane et al., 2012).

4.1 Conclusions

In conclusion, our results confirm that both ALAC and NaB are effective in animal models of ASD and depression further acting on some comorbidities above all cognitive functions. More importantly, our data increase our knowledge on the possibilities of manipulating the MGBA not using probiotics alone, but by a rationale of supplementary intervention on different aspects. Finally, the use of ALAC and NaB has already been tested in humans and therefore our results could be easily transferred to clinical research in appropriate trials using the combination to possibly alleviate symptoms in a variety of neurological disorders.

Declaration of Interest

Emilio Russo has received speaker fees or fundings and has participated in advisory boards for Angelini, Arvelle Therapeutics, Eisai, Kolfarma, Pfizer, GW Pharmaceuticals, UCB, Lundbeck. Rita Citraro has received Clicon Srl, Daiichy Sankyo, Kolfarma. Antonio Leo has received research fundings from Eisai and GW Pharmaceuticals. Carmen De Caro has received research fundings from Kolfarma. Paolo Mainardi is a scientific consultant Kolfarma Srl. Pasquale Striano developed within the framework of the DINOGMI Department of Excellence of MIUR 2018-2022 (legge 232 del 2016) and received speaker fees and participated at advisory boards for Biomarin, Zogenyx, GW Pharmaceuticals, Neuraxpharma; he also received research funding by ENECTA BV, GW Pharmaceuticals, Kolfarma srl, Eisai.

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Author Contributions

Emilio Russo, Paolo Mainardi, Pasquale Striano, Giovambattista De Sarro and Andrew Constanti contributed in the conceptualization; Antonio Leo, Carmen De Caro, Emilio Russo, Nadia Marascio and Rita Citraro contributed to data curation and formal analysis; Rita Citraro, Emilio Russo, Carmen De Caro and Giovambattista De Sarro participated to funding acquisition; Antonio Leo, Carmen De Caro, Nadia Marascio, Valentina Nesci, Martina Tallarico contributed to investigation, methodology; Antonio Leo, Carmen De Caro, Emilio Russo contributed to the original draft; Andrew Constanti, Antonio Leo, Pasquale Striano, Emilio Russo and Rita Citraro contributed to the final version of the manuscript.

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Figure Legends

Experimental Scheme. In experiment #1, BTBR mice (8 weeks of age) were randomly divided into twelve groups (*n* = 10 per group). ALAC (125, 250 and 500 mg/kg), NaB (30, 100, 300 mg/kg) and the co-administration of ALAC (125, 250 and 500 mg/kg) with a fixed NaB dose (100 mg/kg) were administered in drinking water for 15 days and the animals then subjected to different behavioural tests. In experiment #2, at the end of CUMS (4 weeks), all B6 mice were divided into nine groups (*n* = 30 per group) and ALAC (125, 250 and 500 mg/kg), NaB (100 mg/kg) and their combination were administered in drinking water for 15 days. After treatment, mice were divided into three subgroups and subjected to different behavioural tests. ALAC = α-lactalbumin; B6 = C57Bl/6J mice; CUMS = chronic unexpected mild stress; NaB = sodium butyrate.

Figure 1. Effects of ALAC (from 125-500 mg/kg/day for 15 days), sodium butyrate (NaB, from 30-300 mg/kg/day for 15 days) and their co-administration on sociability in control (B6) or BTBR mice. a) Bars indicate the time (s) spent in the chamber containing novel mice and the time (s) spent in the novel object chamber in BTBR drug-treated mice and control mice of both strains. b) Bars indicate the time (s) spent sniffing the novel mice. Data are expressed as mean ± SEM (*n*=10). **P* < 0.05 vs CTRL (B6); #*P* < 0.05 vs BTBR group.

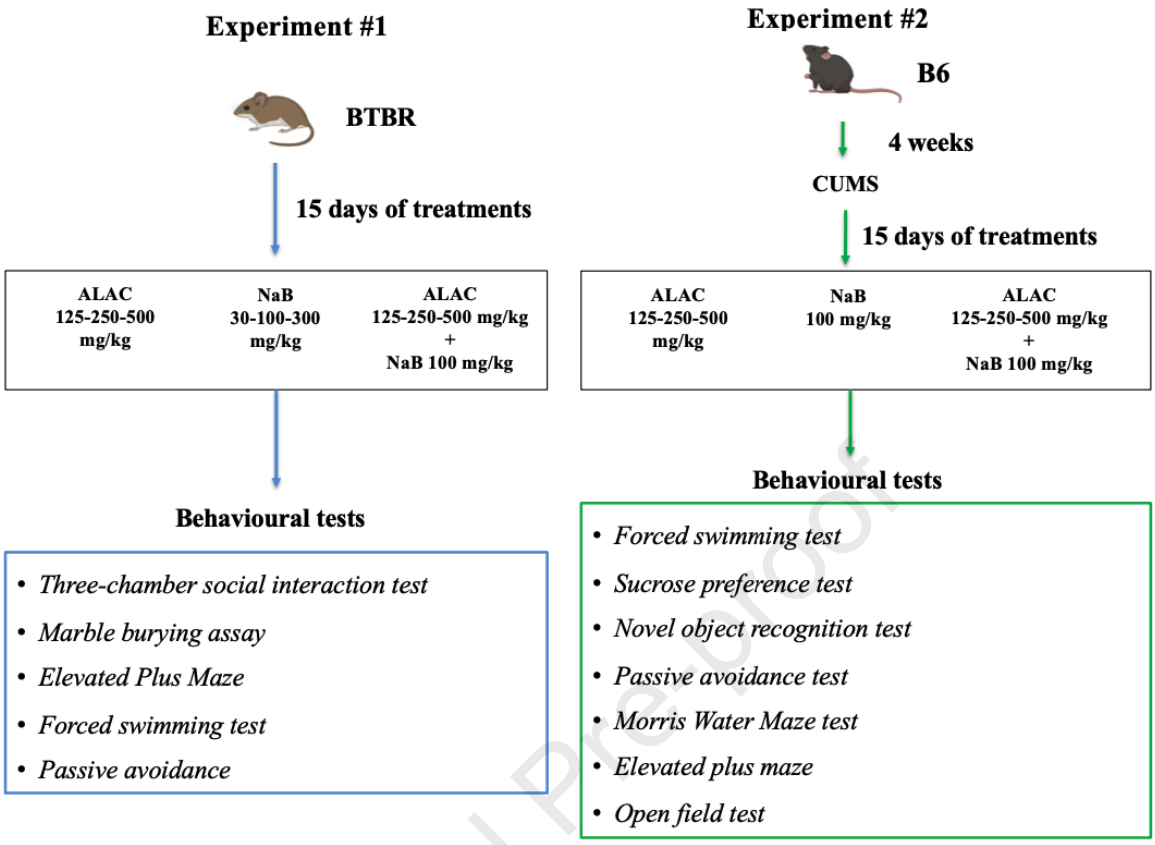
Figure 2. Effects of ALAC (from 125-500 mg/kg/day for 15 days), sodium butyrate (NaB, from 30-300 mg/kg/day for 15 days) and their co-administration on: a) repetitive and perseverative behaviour in control (CTRL; B6) or BTBR mice. Bars indicate the number of marbles buried; b) time spent (s) in open arms in the elevated plus maze; c) immobility time (s) during the forced swimming test; d) latency to enter (s) into the dark chamber, during the

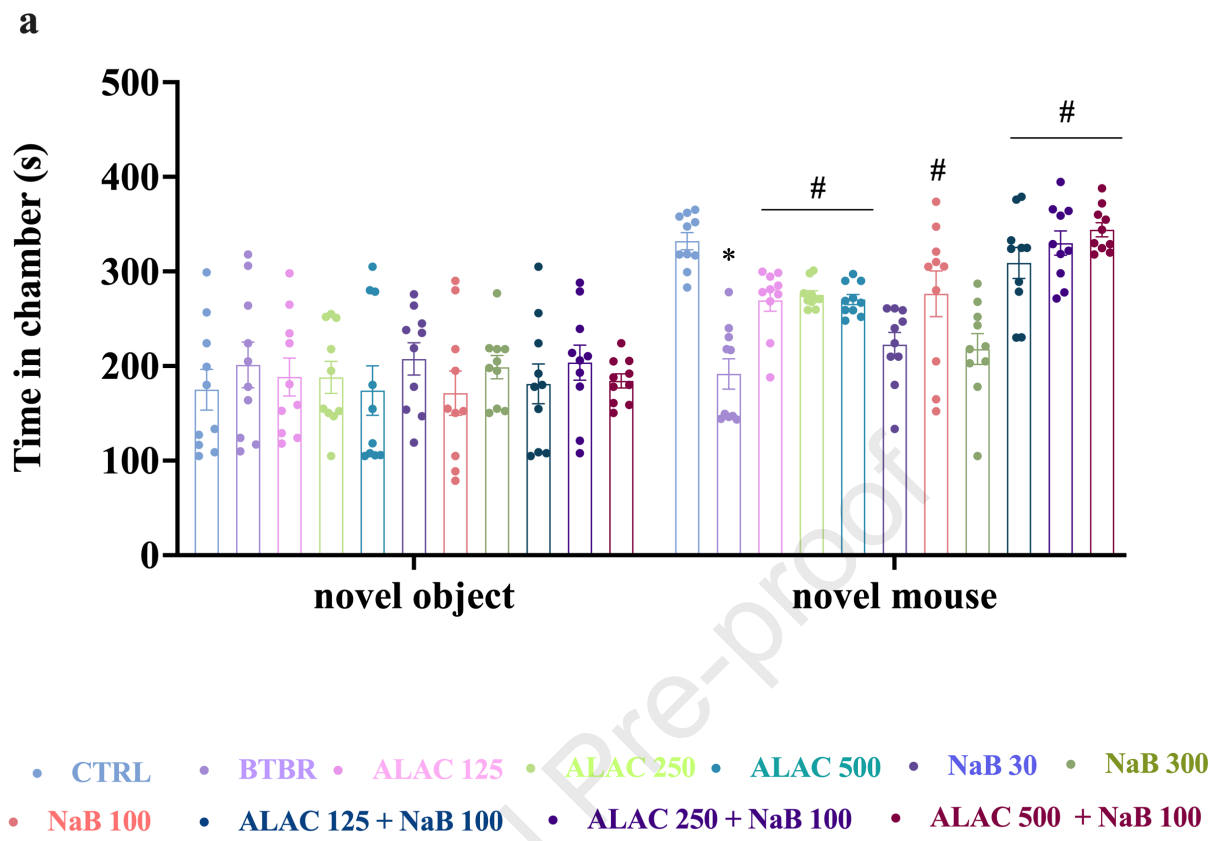
retention test, in the passive avoidance. Data are expressed as mean ± SEM (*n*=10). **P* < 0.05 vs CTRL (B6); #*P* < 0.05 vs BTBR group; °*P*< 0.05 vs single drug administration.

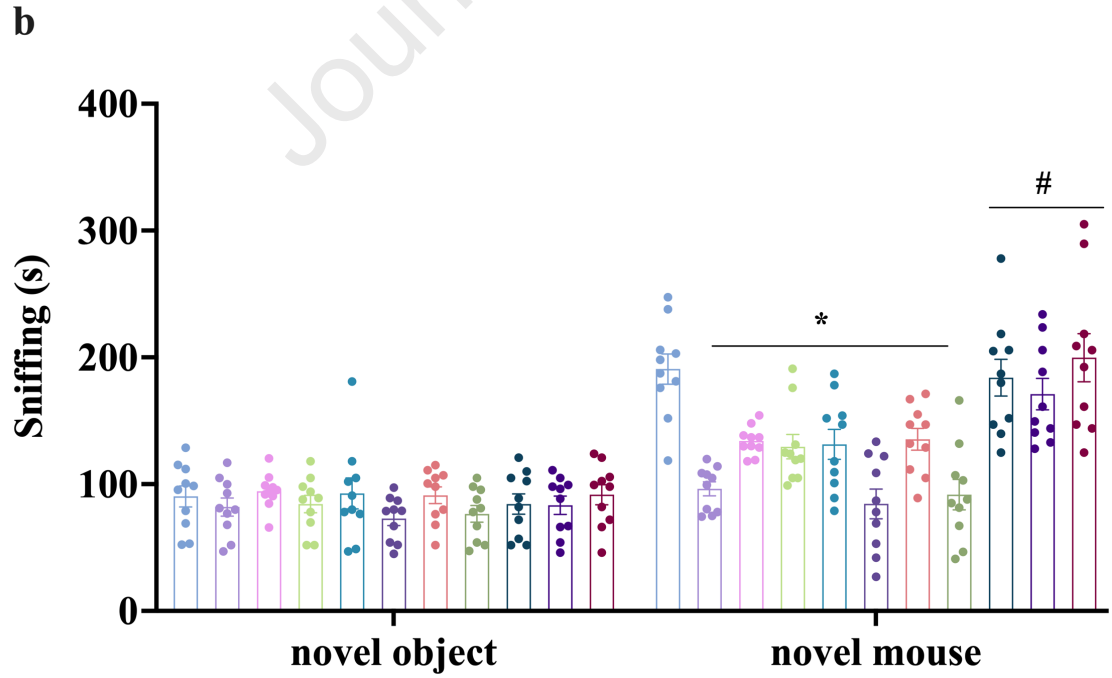
Figure 3. Effects of ALAC (from 125 up to 500 mg/kg/day for 15 days), sodium butyrate (NaB, 100 mg/kg/day for 15 days) and their co-administration on depressive-like behaviour (a and b panels) and memory and learning (c and d panels) in control and CUMS mice. **a)** Bars indicate the immobility time (s) during the forced swimming test. b) Bars indicate the sucrose consumption expressed as a percentage. c) Bars indicate the discrimination index (DI) in the novel object recognition test. d) Bars indicate the latency to enter (s) into the dark chamber, during the retention test, in the passive avoidance. Data are expressed as mean ± SEM (*n*=10). **P* < 0.05 vs CTRL (B6); #*P* < 0.05 vs BTBR group; °*P*< 0.05 vs single ALAC administration; §*P*< 0.05 vs single NaB administration.

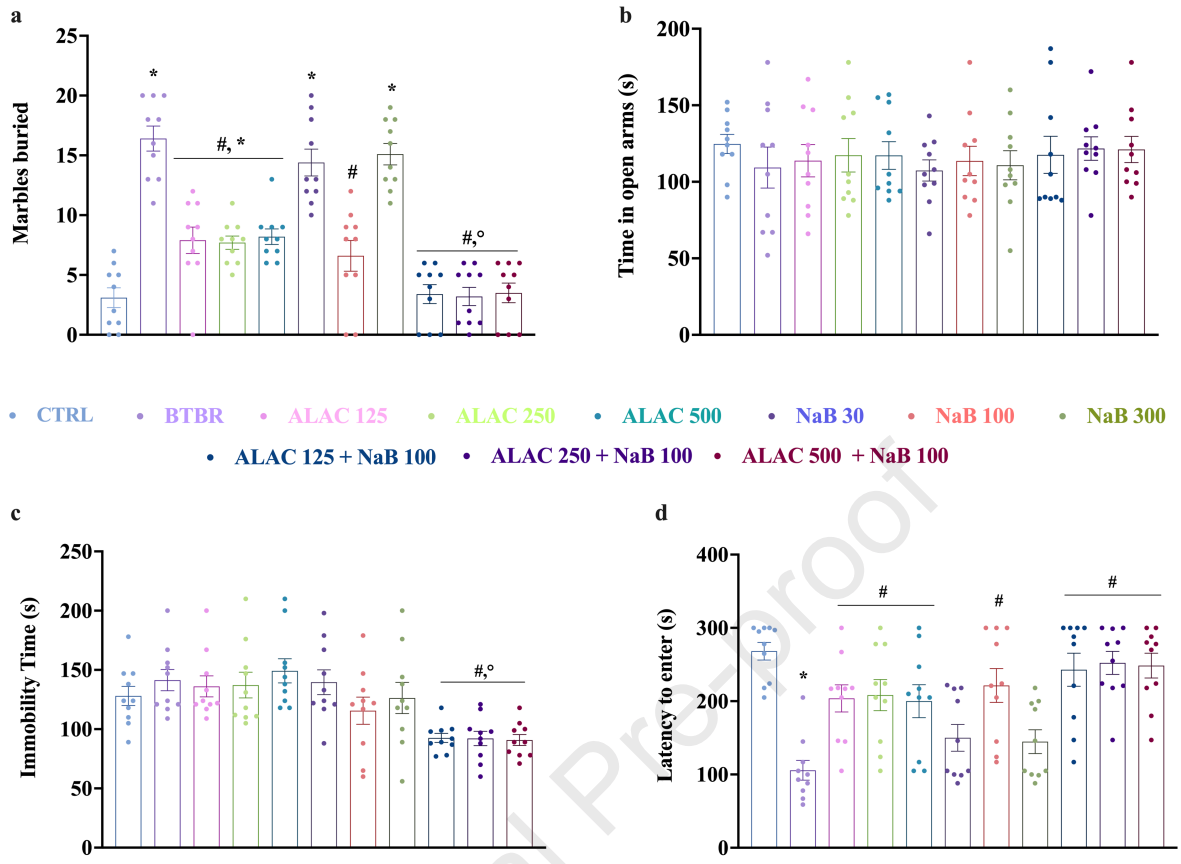
Figure 4 a, b and c) Effects of ALAC (from 125 up to 500 mg/kg/day for 15 days), sodium butyrate (NaB, 100 mg/kg/day for 15 days) and their co-administration on cognitive performance in control and CUMS mice. Points indicate the latency to platform (s), in the Morris water maze. **d)** Bars indicate performance in the probe trial on the fifth day. Data are expressed as mean ± SEM (*n*=10). *P < 0.05 *vs* CTRL; #P < 0.05 *vs* CUMS mice.

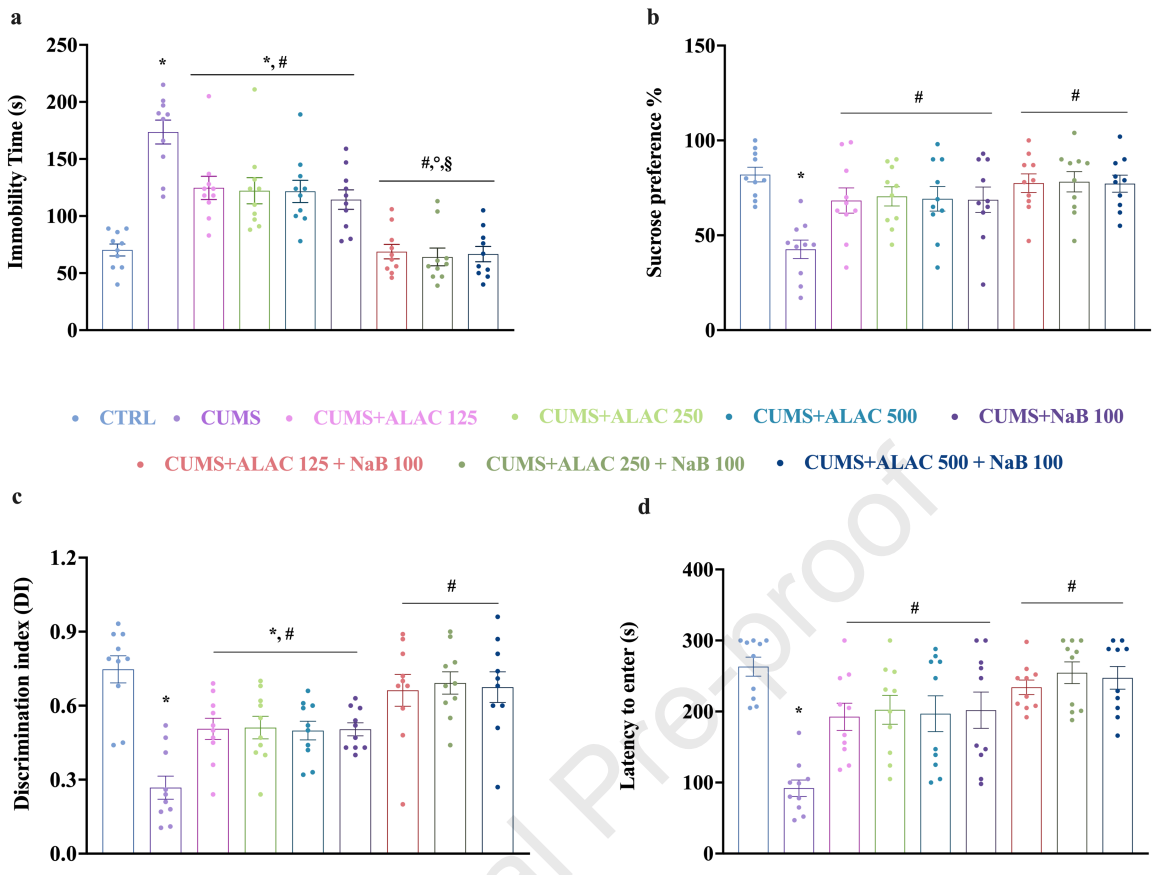
Figure 5. Effects of ALAC (from 125 up to 500 mg/kg/day for 15 days), sodium butyrate (NaB, 100 mg/kg/day for 15 days) and their co-administration on anxiety-like behaviour in control and CUMS mice. **a)** Bars indicate the time spent (s) in the center in the open field test. **b)** Bars indicate the number of entries in the center in the open field test. **c)** Bars indicate the time spent (s) in the open arms in the elevated plus maze test. Data are expressed as mean ± SEM (*n*=10). *P < 0.05 *vs* CTRL; #P < 0.05 *vs* CUMS mice.

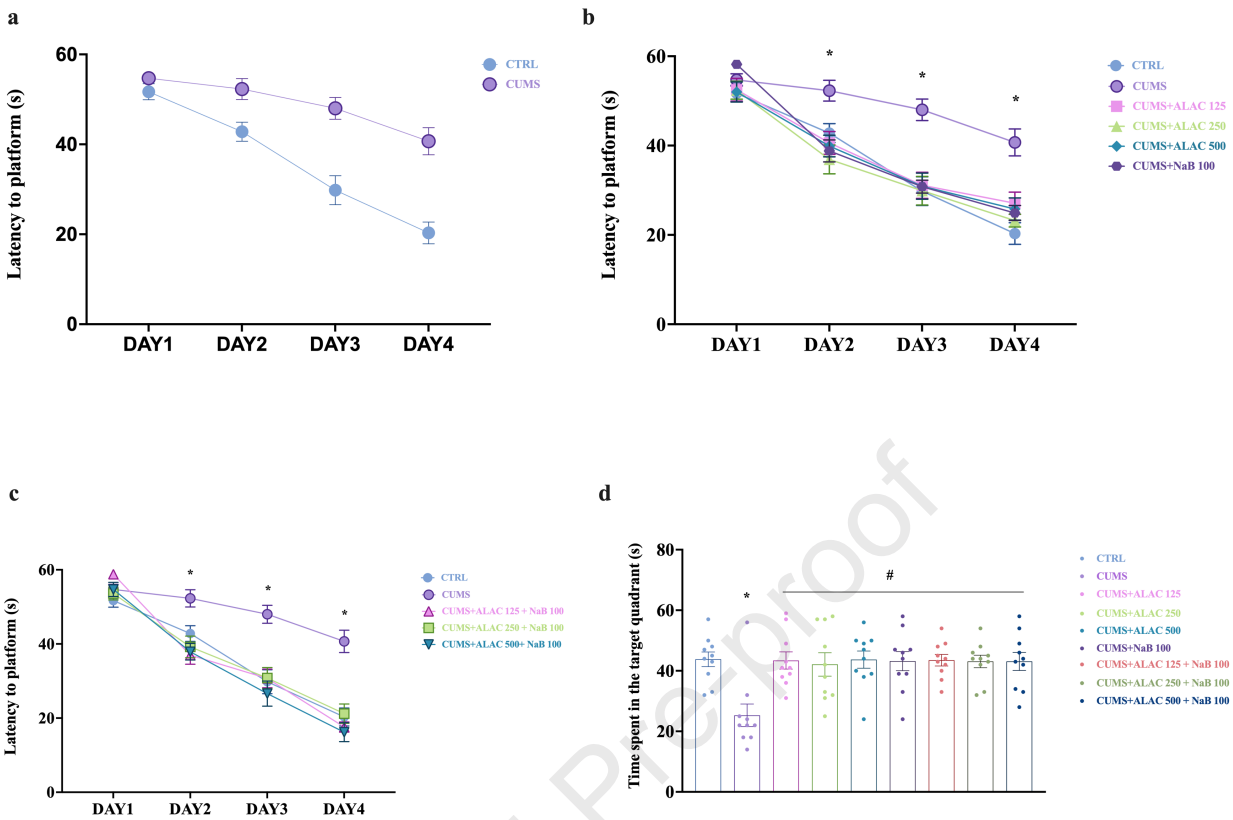


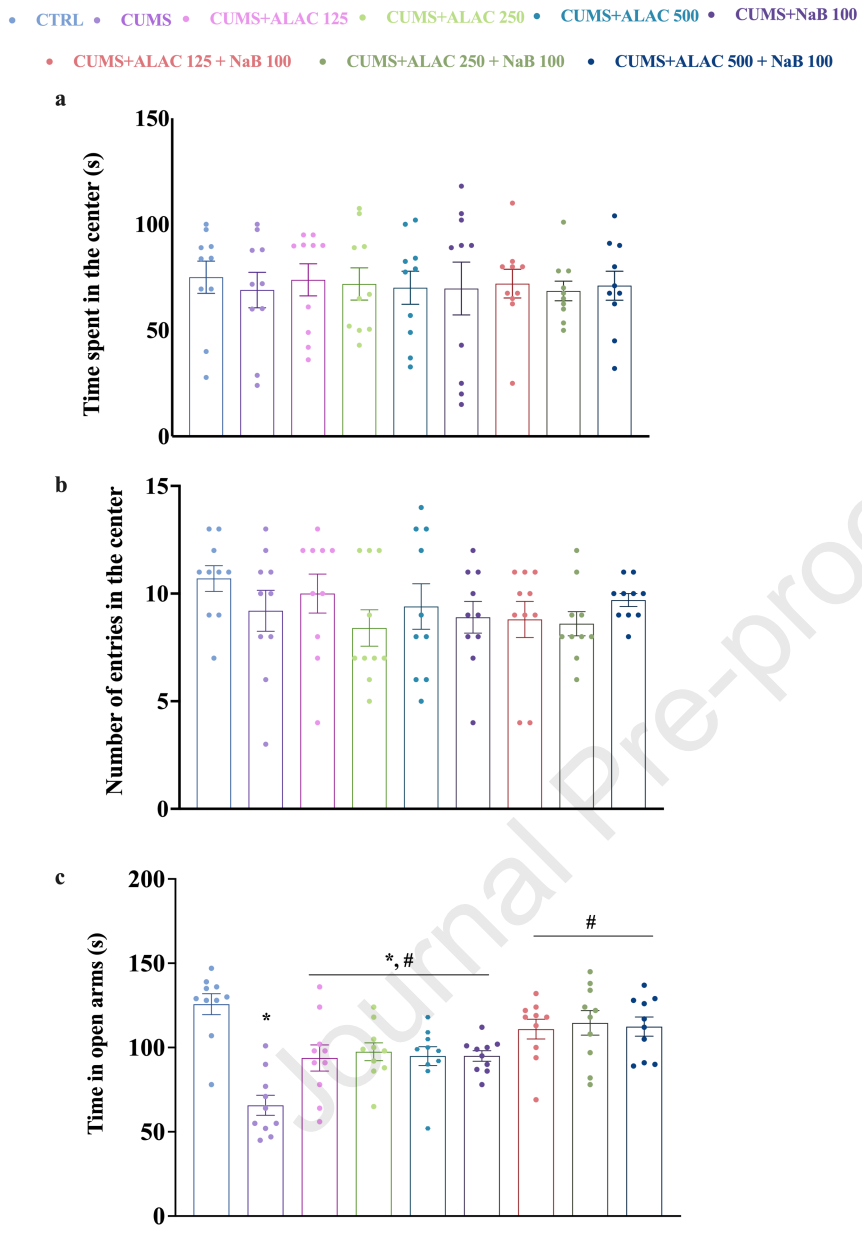












Highlights

- Prebiotic/postbiotic combination is a suitable approach in manipulating the Microbiota Gut Brain Axis
- Prebiotic/postbiotic combination is more effective than single drug administration
- α-lactalbumin/sodium butyrate combination improves animal behaviour in autistic (BTBR) mice
- α-lactalbumin/sodium butyrate combination improves animal behaviour in the depression chronic unexpected mild stress model