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Research report

# Bifidobacteria modulate cognitive processes in an anxious mouse strain

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## HIGHLIGHTS

- *B. longum 1714* improves cognition in BALB/c mice.
- *B. breve 1205* had little or no positive effects on memory.
- Neither of the bacteria had an effect on visceral sensitivity.
- The effects of bacteria on cognition are strain-dependent.

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## ABSTRACT

Increasing evidence suggests that a brain–gut–microbiome axis exists, which has the potential to play a major role in modulating behaviour. However, the role of this axis in cognition remains relatively unexplored. Probiotics, which are commensal bacteria offering potential health benefit, have been shown to decrease anxiety, depression and visceral pain-related behaviours. In this study, we investigate the potential of two *Bifidobacteria* strains to modulate cognitive processes and visceral pain sensitivity. Adult male BALB/c mice were fed daily for 11 weeks with *B. longum 1714*, *B. breve 1205* or vehicle treatment. Starting at week 4, animals were behaviourally assessed in a battery of tests relevant to different aspects of cognition, as well as locomotor activity and visceral pain. In the object recognition test, *B. longum 1714*-fed mice discriminated between the two objects faster than all other groups and *B. breve 1205*-fed mice discriminated faster than vehicle animals. In the Barnes maze, *B. longum 1714*-treated mice made fewer errors than other groups, suggesting a better learning. In the fear conditioning, *B. longum 1714*-treated group also showed better learning and memory, yet presenting the same extinction learning profile as controls. None of the treatments affected visceral sensitivity. Altogether, these data suggest that *B. longum 1714* had a positive impact on cognition and also that the effects of individual *Bifidobacteria* strains do not generalise across the species. Clinical validation of the effects of probiotics on cognition is now warranted.


## 1. Introduction

Increasing evidence suggests that a brain–gut–microbiome axis exists and that it plays a key-role in regulating emotional functions, brain and behaviour [1–3]. Notably, disruption of the microbiota has been linked to gastrointestinal (GI) disorders following antibiotic treatment or infection [4–7], as well as stress-related disorders and alterations in behaviour [8]. Indeed, mice allowed to grow up in a germ-free environment showed altered anxiety behaviour [9–11], impaired stress axis [12] and deficits in sociability and social cognition [13]. These mice also displayed changes in the serotonergic system [14] and in brain-derived neurotrophic factor (BDNF)

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expression, one of the key molecules involved in memory functions [15,16]. Moreover, BDNF expression was also increased following enteric microbiota manipulation in healthy rats through prebiotics feeding [17]. Gut bacterial infection also induced increased anxiety [18,19] and such infection followed by stress, could induce memory impairments [20]. Conversely, social stress, or stress early in life, can also alter the enteric microbiota [21–23]. Thus, regulating the enteric microbiota may be an interesting strategy for targeting new treatments for cognitive deficits, either related to stress [24–26] or to neurodegenerative disorders such as Alzheimer's disease for which there is still no satisfactory treatment [27].

Probiotics, which are commensal bacteria offering potential health benefit to the host, when provided in adequate amount, actively interact with the endogenous microbiota [28]. Among these bacteria, certain *Lactobacilli* and *Bifidobacteria* spp have been shown to improve gut health, as well as mood disorders and stress-induced alterations such as impaired colonic microbiota [26,29]. Some *Lactobacilli* strains also normalised corticosterone release, reversed stress-induced colonic alterations [30] and improved anxiety associated with chronic fatigue syndrome [31]. Moreover, we have recently shown that *L. rhamnosus* was able to improve the naturally anxious phenotype of healthy BALB/c mice by decreasing their anxiety [32] and it has been shown that a *B. longum* decreased anxiety in both healthy and DSS-induced colitis AKR mice or mice infected with *T. muris* [33]. Thus it is of high relevance to investigate whether gut bacteria would also improve cognition. To this aim, Gareau and colleagues [20] found that the stress-induced cognitive impairments induced by gut bacterial infections could be reversed by ingestion of probiotics.

*Bifidobacteria* spp, which are amongst the main components of human and animal GI tracts, are of high health benefit to the host and are used as beneficial food supplements in dairy products [34–36]. *B. infantis 35624* was shown to have potential therapeutic effects on GI disorders and associated symptoms [37,38], as well as depression [39,40]. Also, *B. breve NCIMB 702258* showed a therapeutic potential for inflammatory and neurodegenerative diseases via its modulation of fatty acids composition [41], whereas *B. breve 6330* positively modulated BDNF expression in the hippocampus [42]. Moreover, *B. longum NCC3001* decreased the anxiety of both healthy and DSS-induced colitis AKR mice [33].

We have recently shown that two different *Bifidobacteria* strains, *B. longum 1714* and *B. breve 1205*, improved the anxious phenotype of BALB/c mice by reducing their anxiety [43]. Interestingly, the pattern of behavioural effects induced by both strains was different. Indeed, *B. longum 1714* reduced stress and anxiety of mice in the stress-induced hyperthermia and marble burying tests and reduced the latency to the anxiogenic inner zone of the open field, whilst also reducing depression-like parameter in the tail suspension test. On the contrary, *B. breve 1205* reduced rather various forms of anxiety solely by having a positive effect in the marble burying test and the elevated plus maze. However, it is unclear if either or both of the bacteria can also modify cognitive processes. As a result, we assessed in this study the effects of *B. longum 1714* and *B. breve 1205* on various aspects of cognition. Importantly, cognitive deficits have been associated with a myriad of diseases, and notably with the functional gastro-intestinal disorder irritable bowel syndrome (IBS) [44,45]. Probiotics, and especially *Bifidobacteria* spp, have shown particular efficiency against one of the core symptoms of IBS, visceral pain [37,46,47]. This latter has been shown in our laboratory to be associated with the activation of different regions of the prefrontal cortex and amygdala [48], which we suspected to be positively modulated by the two *Bifidobacteria* strains we are testing here following a study on anxiety [43]. Therefore in the present study, we also assessed the effects of *B. longum 1714* and *B. breve 1205* on visceral sensitivity.

## 2. Material and methods

### 2.1. Animals

Forty-eight male BALB/cOlaHsd (BALB/c) mice, 7–8 weeks old (Harlan Laboratories, UK), were used and remained housed in groups of 4 in plexiglas cages (33 cm × 15 cm × 13 cm, L × H × W) under standard controlled laboratory conditions (22 ± 1 °C, humidity 55 ± 5%) on a 12-h light/dark cycle (lights on 7.30 a.m.). Mice were provided with standard laboratory diet and water *ad libitum* throughout. Animals were housed in a separated room from other animals and treatments groups were separated from each other to avoid cross contamination. For each treatment group, mice were issued from 3 different litters. All mice were evenly distributed regarding treatment groups, order of feeding, order of testing, day and time of testing. The sex of the mice was specifically chosen to compare with our previous studies in BALB/c mice investigating the effects of the same *Bifidobacteria* strains [43] and of other potential probiotics [32]. BALB/c mice were chosen for their innate anxiety [49], as they are fundamental to model stress-related disorders, and their associated impaired cognitive processes [32,50–53] and as they have been used to characterise the in vivo effects of probiotics [54]. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

### 2.2. Bacteria treatment

*B. longum 1714* and *B. breve 1205* were kindly donated by Alimentary Health Ltd. (Cork, Ireland) from freeze-dried stocks (−80 °C). Bacteria were reconstituted in sterile phosphate buffered saline (PBS) so that the final concentration ingested by mice was $1 \times 10^9$ CFU mL$^{-1}$. This dose was selected based on previous studies showing a reduction in visceral pain following treatment with *B. infantis 35624* [37]. Vehicle-treated animals received PBS only. All treatments were given orally.

### 2.3. Study design

The experiment design is presented in Fig. 1. After a 5-day habituation to the animal facility, mice were fed daily (6–7 p.m.) with *B. longum 1714*, *B. breve 1205* or vehicle treatment, using sterile gavage needles, for 11 weeks. Bodyweight was monitored throughout. Behavioural testing was conducted (Fig. 1) from week 4 onward, from the least to the most stressful task [55], including resting days between tests. Animals were tested one at a time in a counterbalanced fashion regarding cage, treatment and time of the day and under the same conditions with an experimenter blind to conditions. All apparatus were cleaned between animals with 70% ethanol to remove odours. Starting at 10–11-week old, mice ($n$ = 12 per group) were tested in a battery of cognitive tasks, the object recognition test for short term/episodic memory, the Barnes maze for spatial learning and memory and the fear conditioning for Pavlovian conditioning, memory and extinction. Locomotor activity was also assessed in the two first tests. At 17-week old (10-week feeding), animals underwent colorectal distension test (CRD) for visceral sensitivity. All animals were sacrificed 5–7 days following last test (18-week old, 11-week feeding) and blood was collected for measure of basal corticosterone levels in the plasma.

### 2.4. Behavioural testing

#### 2.4.1. Object recognition

This test presents the advantages of not requiring an extensive training or aversive conditions (fearful environment, food

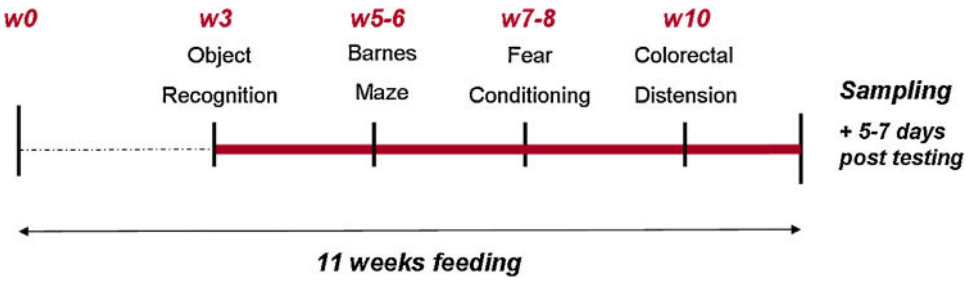


**Fig. 1.** Representation of the study design. BALB/c mice were fed for a total period of 11 weeks with *B. longum 1714*, *B. breve 1205* or vehicle (PBS). All groups were weighed daily. After 3-week feeding, animals underwent a battery of testing relevant to learning and memory and visceral sensitivity. All groups were sacrificed on the same day, +5 to 7 days post test. Blood was harvested for further physiological analysis.

deprivation, etc.) and is based on the natural attraction mice display for novelty [56,57]. After investigation of two identical objects, mice are presented with an old and a new object; a mouse remembering the old object spends more time investigating the new one. The protocol used was based on literature [58,59] and optimised in-house for BALB/c mice (Savignac and Cryan, unpublished).

*2.4.1.1. Set up and experimental conditions.* Mice were tested after 3-week feeding. Experiments occurred between 9 a.m. and 2 p.m., under red light (5–10 lux) and experimenter left the room. The object recognition arena was made of a plastic white-painted open field (40 cm × 30 cm × 25 cm, L × W × H) without bedding and was placed under a ceiling infra-red camera wired to the computer for animal movements tracking using Ethovision software (3.1 version Noldus, TrackSys, Nottingham, UK). The objects used were of different shape, texture and colour but of equivalent volume (size~2–3 × 5–4 cm): two grey and red polish tubes, two green plastic toy shoes and two round and yellow plastic tube caps. The sets of objects used were randomised so that each cage and treatment group received all categories of objects. Data were further analysed either manually or with Ethovision.

*2.4.1.2. Habituation.* To decrease stress related to testing, animals were daily handled and transported from the breeding to the experimental room for 3 days. On day 4 and 5, animals were habituated to the object recognition arena for 15 min after an initial 2-h acclimatisation to the experimental room.

*2.4.1.3. Training.* The following day, after 1-h acclimatisation to the experimental room, mice were individually placed in the arena, facing the wall opposite to the two identical objects A. These latter were placed on one side of the arena, at equal distance from the back (4 cm) and side walls (8 cm). Mice were allowed 6-min free exploration and returned to their home cage thereafter.

*2.4.1.4. Test.* One hour post training, mice were placed again in the arena with two objects, the old (familiar) one A and a new one B. Mice were allowed 5-min free exploration. For both training and test, the time spent investigating each object within a 2-cm area was measured. An equal time investigating the two identical objects during the training reflects no object or place preference; a higher time spent investigating the new object over the old one during the test reflects good recognition memory. Locomotor activity was also monitored for each session and the number of faecal pellets produced was also counted during the habituation for novelty-induced stress state [60].

*2.4.2. Barnes maze*

This test is based on the natural fear and avoidance mice display for brightly lit open areas in favour of dark and safe places [61,62]. Animals are placed in the centre of a fearful wide open arena, from which they try to escape by learning to locate a safe hidden box. Good learning and memory skills are reflected by fewer errors to remember and find the hidden box. The test was conducted mainly as previously described [63] and the protocol used was optimised for BALB/c mice (Savignac and Cryan, unpublished).

*2.4.2.1. Set up and experimental conditions.* Mice were tested after 5–6-week feeding. Experiences occurred under dim light (~10 lux for room, ~20 lux for maze centre); experimenter remained in the room. The apparatus (MED Associates, St. Albans, VT, USA) consisted of an elevated rotatable composite white platform (1.3 m height × 1.2 m diameter) comprising 40 holes along the periphery (~5.5 cm from edges). A black plastic box (23 cm × 5.3 cm × 9 cm, L × W × H) was hidden under one of the holes and constituted the escape box mice had to learn to locate. The maze was rotated between trials to avoid odour cues. The box was randomly placed in different areas of the maze across groups to avoid place preference from the animals but remained at the same place throughout testing for a given mouse. Additionally to the experimental room cues, distal cues were placed on the walls (cross, rounds). Mice were habituated to travelling to the experimental room 1 day prior to pre-training. For pre-training and training, mice were tested 10 min after arrival in the experimental room.

*2.4.2.2. Pre-training.* Mice were placed into the escape box for 2 min for habituation to the maze and box. Mice were then placed in the centre of the maze and guided to the escape box where they remained for 2 min. Afterwards animals were placed into a transparent glass chamber outside of the escape box for 3 min before remaining in the escape box for 2 min.

*2.4.2.3. Training.* Mice received 4 trials per day for 4 days, as follows. The day following the pre-training, mice were placed in the centre of the maze under a glass chamber before being allowed 3 min to find the escape box. A cut-off was established for the mice who did not find the box; these were gently guided to the box where they remained for 2 min. Mice were returned to their home cage after each trial and the inter-trial interval lasted 15 min.

*2.4.2.4. Probe trial.* On day 5, the escape box was rotated 90 degrees from its original place. Animals were placed in the centre of the maze under the glass chamber and allowed a single 3-min exploration before being returned to their home cage.

The latency to reach the escape box and the number of errors (wrong hole pokes) before finding the escape box was measured for both training and probe trial. Stress-induced defaecation due to a novel environment [60] was also monitored on the pre-training day.

*2.4.3. Fear conditioning*

This test is based on the learning of the association of an initially neutral and non-aversive stimulus, such as light cue (conditional stimulus, CS), with an aversive one, such as foot shock (unconditional stimulus, US) [64,65]. The fear to receive the aversive stimulus is translated by a freezing response (absence of movement except for breathing) in response to the presentation of the cue (CS) or context. A good CS-US association reflects a good learning and higher freezing rate from mice. The repeated presentation of the CS alone over time induces a reduction, or, *in fine*, absence of freezing response from the mice and induces an extinction of the CS-US association [66]. Failing to forget or erase the CS-US association, as assessed by a maintained freezing rate in mice, constitutes a pathological process, notably observed in post-traumatic stress disorder [67]. The protocol used was based on a paradigm combining context and cue, allowing to differentiate the behavioural responses to each of these components [68] and optimised for BALB/c mice [32]. Prior control studies confirmed that the repeated CS alone did not induce any freezing response from the mice. The full procedure lasted 3 days: 1 day of training (learning) and 2 days of memory/extinction assessment.

*2.4.3.1. Set up and experimental conditions.* Mice were tested after 7–8-week feeding. All equipment used was purchased from MED Associates, St. Albans, VT, USA. The conditioning chambers (32 cm × 26 cm × 25 cm, L × W × H) comprised aluminium walls, a black frame insert, a transparent plexiglas door for video recording by infra-red camera, a 19-stainless-steel-rod floor connected to a generator and delivering electric shock and auditory and lighting apparatus to deliver the cues (CS, tone + light). These chambers were inserted into a soundproof isolation cubicle connected to an interface transmitting information between the conditioning chambers and the computer and Video Freeze software. The background noise from the set up was of 60 dB. The CS was constituted of the combination of an auditory tone lasting 20 s, 70 dB, 10 KHz and a light stimulus; the US was an electric foot shock (0.4 mA, 2 s) paired to the last 2 s of the CS presentation.

*2.4.3.2. Day 1: Training, assessment of learning skills.* Upon arrival in the experimental room, mice were immediately placed into the conditioning chambers and allowed a 180-s acclimatisation period to the chambers, which constituted the movements' baseline and initial response to context. Thereafter, mice received 6 pairings of the CS-US, separated by a 60-s interval. After the last pairing, mice remained 120 s in the conditioning chamber before being returned to their home cage.

*2.4.3.3. Day 2 and 3: Test of memory and extinction.* Mice underwent the same procedure as day 1, in the same chambers (i.e. same context) 24 h (day 2) and 48 h (day 3) later but were presented with the CS only. Hence, mice had to learn that both the CS and context no longer predicted a foot shock.

The procedure for each day lasted 12 min per mouse. The freezing response was measured as % freezing for every component (cue and context), as previously described [68]. The context component was assessed during the periods without cue presentation (acclimation, 60-s intervals and the last 120-s period in the chamber); the cue component was assessed during each of the 20-s CS presentation.

*2.4.4. Colorectal distension*

This test is commonly used to assess visceral pain or hypersensitivity, one of the core symptoms of IBS. Higher visceral pain is reflected by a lower threshold tolerance to colonic pain and higher pain behaviours [23]. It is assessed in mice by measuring the visceromotor response (VMR) to increasing pressures of a balloon inserted in the colon. The procedure was conducted as previously described in our laboratory [69–71].

*2.4.4.1. Set up and experimental conditions.* Mice were tested after 10-week feeding. Experiences occurred under normal light (100 lux at 1 m above the floor). CRD-system was composed of a barostat (Distender Series II, G&J Electronics, Toronto, ON, Canada) and a transducer amplifier (LabTrax4, World Precision Instruments, Sarasota, FL, USA). The barostat was used to control air inflation and pressure during the CRD procedure and Protocol Plus TM software (G&J Electronics, Toronto, ON, Canada) was used to control the barostat. A custom-made balloon (2 cm length × 1 cm inflated diameter) prepared from a polyurethane plastic bag (GMC Medical, Denmark) was tied over a PE60 catheter with silk 4.0. Before securing the balloon to the catheter, several holes were punched in the distal 20 mm of the tubing with a 27-gauge needle to allow the balloon to inflate.

*2.4.4.2. Protocol.* On the experimental day, mice were lightly anesthetised with isoflurane (IsoFlo®, Abbott, UK) and a balloon with a connecting catheter was inserted into the colon, 0.5 cm proximal to the anus. The catheter was fixed to the base of the tail with tape to avoid any displacement. After 10-min recovery for the mice, the balloon was connected to the barostat system and subsequent pressure changes within the distending balloon, observed in response to a distension paradigm, were monitored and recorded using Data Trax 2 software (World Precision Instruments, Sarasota, FL, USA). Ascending phasic distension paradigm (from 10 to 80 mmHg) was used, consisting of 3 × 20 s pulses at each pressure and 5 min interpulse intervals. Raw data of the traces were further analysed by using DataTrax2 software. VMR were determined for the 5 s period before and after each pulse (baseline activity) and over pulse. In all cases, VMR were established for each 3 consecutive pulses as a group.

*2.5. Plasma collection*

Animals were sacrificed in a random fashion regarding treatment and test condition, 5–7 days following the last test; sampling occurred between 8:30 a.m. and 1 p.m. Trunk blood was collected in potassium EDTA (Ethylene Diamine Tetra Acetic Acid) tubes and spun for 15 min at 5000 rpm. Plasma was isolated and stored at −80 °C for further corticosterone analysis as germ-free mice have been shown to display altered HPA-axis [12] and as a combination of *L. rhamnosus* (*R0011*) and *L. helveticus* (*R0052*) has been demonstrated to normalise the elevated corticosterone release induced by maternal separation in rats [30]. In addition, as we showed that the two *Bifidobacteria* strains used in the present study reduced stress and anxiety [43] and these latter are associated with cognitive impairment, we also scored additional routine stress-sensitive physiological parameters [50]. Measuring these may give first valuable insights into the systemic mechanisms involved behind bacteria effects on the brain, as part of the microbiome–brain–gut axis. The colon was removed, mechanically cleaned and its length measured to 0.1 cm precision, as colon length reduction is observed in case of colonic inflammation following stress [72]; thymus, heart, spleen, and adrenals were also weighed as thymus and adrenals hypotrophy, heart hypertrophy and splenomegaly are observed following chronic stress due to stress impact on the immune system, immune cells survival, as well as interactions with the autonomic nervous system and metabolic pathways [73–76].

*2.6. Corticosterone assay*

Corticosterone levels were measured using an Enzyme Immunoassay Kit (Assay Designs, Inc., MI, U.S.A.) according to

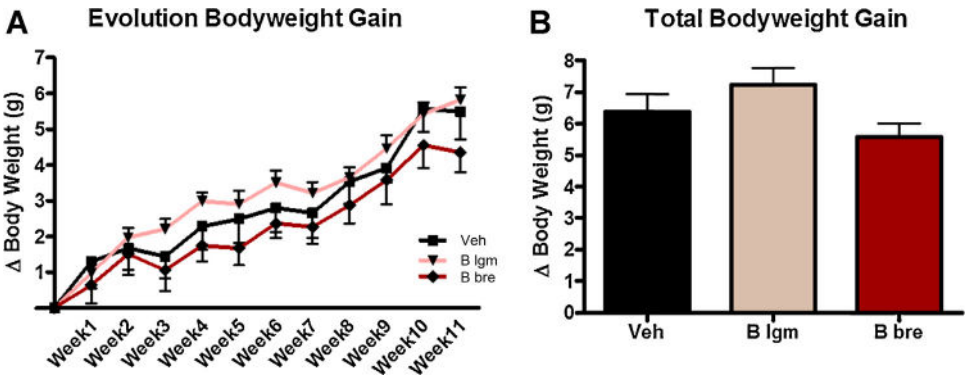


**Fig. 2.** Effect of the two *Bifidobacteria* strains on bodyweight gain. There was no difference between groups for bodyweight gain, over time (A) or in total (B). $n = 9–11$ per group. Veh = vehicle, B lgm = *B. longum 1714*, B Bre = *B. breve 1205*. Data are expressed as means ± SEM.

the manufacturer's instructions. Samples were analysed in duplicate in a single assay, using 20 μL of plasma per sample; the threshold detection was less than 32 pg/mL; coefficient of variation limit = 20%; the concentrations are expressed in ng/mL.

### 2.7. Data analysis

Data were analysed using SPSS software (version 15). Data normality was assessed using Shapiro–Wilk test; for data non-normally distributed, non-parametric statistics were conducted. For repeated data, a two-way ANOVA with matching sample was conducted with factor 1 = the treatment and factor 2 = the parameter that was repeated, i.e. week for bodyweight, time for object recognition, context and cue for fear conditioning. Where there was an overall significant effect, one-way ANOVA (or Kruskal–Wallis test) was further conducted for within time-points comparison, followed by Fisher (or Dunn's) post hoc test for multiple group comparison. For object recognition, two-way ANOVA was conducted on the total time of the test with treatment and object as factors, followed by post hoc paired Student's *t*-test (or Wilcoxon test) on every time-point measured for object effect within treatment group. All other data, including other object recognition parameters, were analysed using a one-way ANOVA followed by Fisher post hoc test for multiple group comparison. Statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

## 3. Results

### 3.1. Effect of the two Bifidobacteria strains on bodyweight gain

Fig. 2 shows the cumulative evolution (Fig. 2A) or total bodyweight gain (Fig. 2B). There was an effect of time on bodyweight gain (two-way ANOVA, $F(10,280) = 80.32$, $p < 0.0001$), without effect of treatment ($F(2,280) = 1.16$, $p = 0.328$) or time × treatment interaction ($F(20,280) = 0.76$, $p = 0.759$). Although on the graph, the curve of bodyweight gain for *B. breve 1205* animals is constantly under the one of vehicle animals, post hoc analysis on individual days did not reveal any differences between groups ($p > 0.05$ all days). There was also no significant difference between treatments for the total bodyweight gain (week 1–11) (one-way ANOVA, $F(2,26) = 2.86$, $p = 0.075$).

### 3.2. Effect of the two Bifidobacteria strains on object recognition

#### 3.2.1. Habituation

Table 1 shows locomotor activity for day 1 (day 2 was similar, data not shown). There was no difference between groups in the distance travelled or stress-induced defaecation (one-way ANOVA, $F(2,29) = 0.069$, $p = 0.933$; $F(2,31) = 2.53$, $p = 0.096$, respectively). Thus, none of the treatments altered the basal activity of mice.

#### 3.2.2. Training

Table 1 shows the time of investigation (sniffing) of the two identical objects. The animals did not display any object preference as there was no effect of object (two-way ANOVA, $F(1,31) = 0.02$, $p = 0.877$), treatment ($F(2,31) = 2.90$, $p = 0.07$), or object × treatment interaction ($F(2,31) = 0.77$, $p = 0.47$). *B. breve 1205*-fed animals tended to explore the two objects less than vehicle-treated group (Table 1, 11.1 ± 2.2 object 1 vs. 17.7 ± 3.9 control animals and 9.8 ± 1.8 object 2 vs. 20.7 ± 4.3 control animals, a priori *t*-test, $p = 0.161$ object 1, $p < 0.05$ object 2). Thus, locomotor activity was also assessed and there was no difference between groups (one-way ANOVA, $F(2,31) = 0.43$, $p = 0.655$).

#### 3.2.3. Test

Some animals were freezing and investigated the objects for less than ~2 s; these were excluded from the analysis (3 from vehicle group and 1 from *B. breve 1205* group). Overt freezing is often seen in BALB/c mice [77]. Data are presented in Fig. 3.

**Table 1**
Effect of the two *Bifidobacteria* strains on object recognition. There was no difference between treatments for the habituation phase in the locomotor activity or stress-induced defaecation (faecal outputs). There was also no difference in the locomotor activity during the training or the time spent investigating the two objects. For the test, *B. breve 1205* induced a lower motor activity than vehicle treatment. $n = 10–11$ per group.

| | Parameter | Vehicle | *B. longum 1714* | *B. breve 1205* |
|---|---|---|---|---|
| Habituation (15 min) | Locomotor activity (cm) | 4027 ± 322.5 | 3928 ± 359.5 | 4093 ± 266.3 |
| | Faecal output (*n*) | 12.1 ± 0.8 | 11.8 ± 0.9 | 14.2 ± 0.8 |
| Training (6 min) | Locomotor activity (cm) | 2370 ± 218.8 | 2301 ± 156 | 2135 ± 169.6 |
| | Object 1 time sniffing (s) | 17.7 ± 3.9 | 15.0 ± 2.2 | 11.1 ± 2.2 |
| | Object 2 time sniffing (s) | 20.7 ± 4.3 | 14.0 ± 1.9 | 9.8 ± 1.8* |
| Test (5 min) | Locomotor activity (cm) | 1903 ± 123.5 | 1669 ± 114.8 | 1418 ± 127.7* |

* $p < 0.05$, treatment vs. vehicle groups. Data are expressed as means ± SEM.

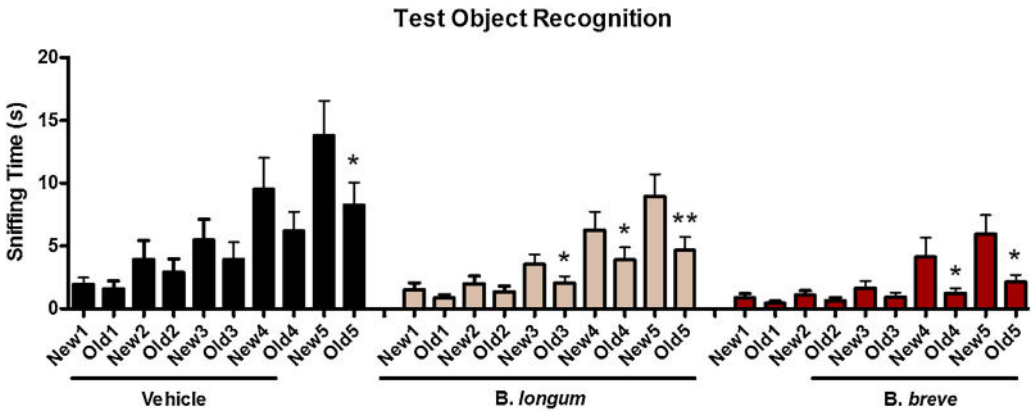


**Fig. 3.** Effect of the Two *Bifidobacteria* strains on Object Recognition. The two *Bifidobacteria* induced a significant recognition of the familiar from the new object at an earlier stage than vehicle treatment; this was more pronounced in *B. longum 1714*-fed animals (3 min vs. 5 for vehicle). $n = 9$–12 per group. Veh = vehicle, *B longum* = *B. longum 1714*, *B Breve* = *B. breve 1205*. $^{*}p < 0.05$, $^{**}p < 0.01$, new vs. old object. Data are expressed as means ± SEM.

When comparing groups at the total time of investigation of the objects (i.e.; time-point 5 min), there was an overall object effect (two-way ANOVA, $F(1,28) = 29.71$, $p < 0.0001$) and a treatment effect ($F(2,28) = 5.08$, $p < 0.05$) but no object × treatment interaction ($F(2,28) = 0.36$, $p = 0.702$). Post hoc analysis showed that all animals remembered the old object as the new object was significantly more investigated than the old (familiar) one (vehicle and *B. breve 1205* groups, $p < 0.05$, *B. longum 1714* group, $p < 0.05$); however, there was no statistical difference in the time of investigation of the objects between groups. As it has been previously shown that analysing a test minute per minute, as opposed to only for the total time, highlights better differences between groups which may not raise in the total time (open field test) [78], we decided here to split the data and analysis per min as well (represented in Fig. 3 as cumulative time of sniffing). Two-group comparison within treatments groups showed that differences raised early in the test but for the two *Bifidobacteria* strains; indeed, there was no statistical difference before min 5 for vehicle animals, whereas *B. longum 1714* group discriminated between the two objects at the 3- ($p < 0.05$), 4- ($p < 0.05$) and 5-min ($p < 0.01$) time-points and *B. breve 1205* group, at 4- and 5-min ($p < 0.05$ each).

Although in the post hoc analysis conducted above, *B. breve 1205*-fed animals did not differ statistically from the vehicle group in their time spent exploring the two objects, they still seemed to display lower time of investigation (Fig. 3, time-point 5, new object, 13.8 ± 2.8 vehicle vs. 5.9 ± 1.5 *B. breve 1205*, old object 8.2 ± 1.8 vehicle vs. 2.1 ± 0.6 *B. breve 1205*). Therefore, locomotor activity was assessed (Table 1). There was a significant difference between treatments (one-way ANOVA, $F(2,31) = 3.85$, $p < 0.05$), confirmed by post hoc test as *B. breve 1205* group travelled less distance than vehicle group ($p < 0.05$). This difference in locomotor activity may explain the trend towards lower object exploration. As *B. breve 1205*-fed animals were not freezing, did not differ in their locomotor activity in the training from vehicle animals and displayed lower anxiety than them in another study we conducted [43], we cannot discount the fact that mice fed with *B. breve 1205* might have been less anxious, which altered their behaviour during testing. This did not interact with their memory skills as they still discriminated the two objects in the test phase and at an earlier stage than vehicle mice. The data may also suggest that *B. longum 1714* group may have displayed better working memory skills than vehicle group as it was the first group to discriminate significantly more the two objects, at 3-min, followed by *B. breve 1205*-fed animals.

### 3.3. *Effect of the two* Bifidobacteria *strains in the Barnes maze*

#### 3.3.1. *Pre-training*

For stress-induced defaecation (Table 2), there was an effect of treatment (one-way ANOVA, $F(2,30) = 4.54$, $p < 0.05$), confirmed by post hoc analysis as *B. longum 1714*-fed animals defaecated significantly less than vehicle-treated animals ($p < 0.05$).

#### 3.3.2. *Training*

For the number of errors over the 3 days of training (day 2, 3, 4, Fig. 4A), there was no effect of treatment (two-way ANOVA, $F(2,73) = 0.68$, $p = 0.51$), days ($F(2,73) = 1.17$, $p = 0.316$) and no treatment × days interaction ($F(4,73) = 0.17$, $p = 0.953$).

For the latency to find the escape box (Fig. 4B), there was an effect of days as expected (two-way ANOVA, $F(2,60) = 3.81$, $p < 0.05$), but no effect of treatment ($F(2,60) = 0.04$, $p = 0.963$) and no treatment × days interaction ($F(4,60) = 0.75$, $p = 0.563$). Further post hoc analysis on individual days did not reveal any group differences.

#### 3.3.3. *Probe trial*

Some animals, which were freezing, were excluded from the analysis (3 for vehicle group, 1 for *B. longum 1714* group and 2 for *B. breve 1205* group).

Regarding the number of errors (Fig. 4C), there was a trend towards a significant effect of treatment (Kruskal–Wallis, $H(df = 2) = 5.813$, $p = 0.055$), with post hoc analysis revealing that *B. longum 1714* group made fewer errors to find the escape box than vehicle group ($p < 0.05$).

**Table 2**
Effect of the two *Bifidobacteria* strains on cognitive performance in the Barnes maze. For the pre-training, *B. longum 1714* induced a lower stress-induced defaecation number (faecal outputs) than vehicle treatment. For the probe trial, there was no difference between treatments in the time spent in the centre of the maze; however, *B. breve 1205* induced a lower locomotor activity than vehicle treatment.

| | Parameter | Vehicle | *B. longum 1714* | *B. breve 1205* |
|---|---|---|---|---|
| Pre-training | Faecal output ($n$) | 7.7 ± 0.6 | 6.0 ± 0.4* | 8.3 ± 0.6 |
| Probe trial | Time in centre (s) | 99.3 ± 18.5 | 118.1 ± 22.3 | 98.9 ± 20.3 |
| | Distance travelled (cm) | 745.5 ± 105.8 | 694 ± 60.8 | 468.7 ± 56.6* |

* $p < 0.05$, treatment vs. vehicle groups. Data are expressed as means ± SEM.

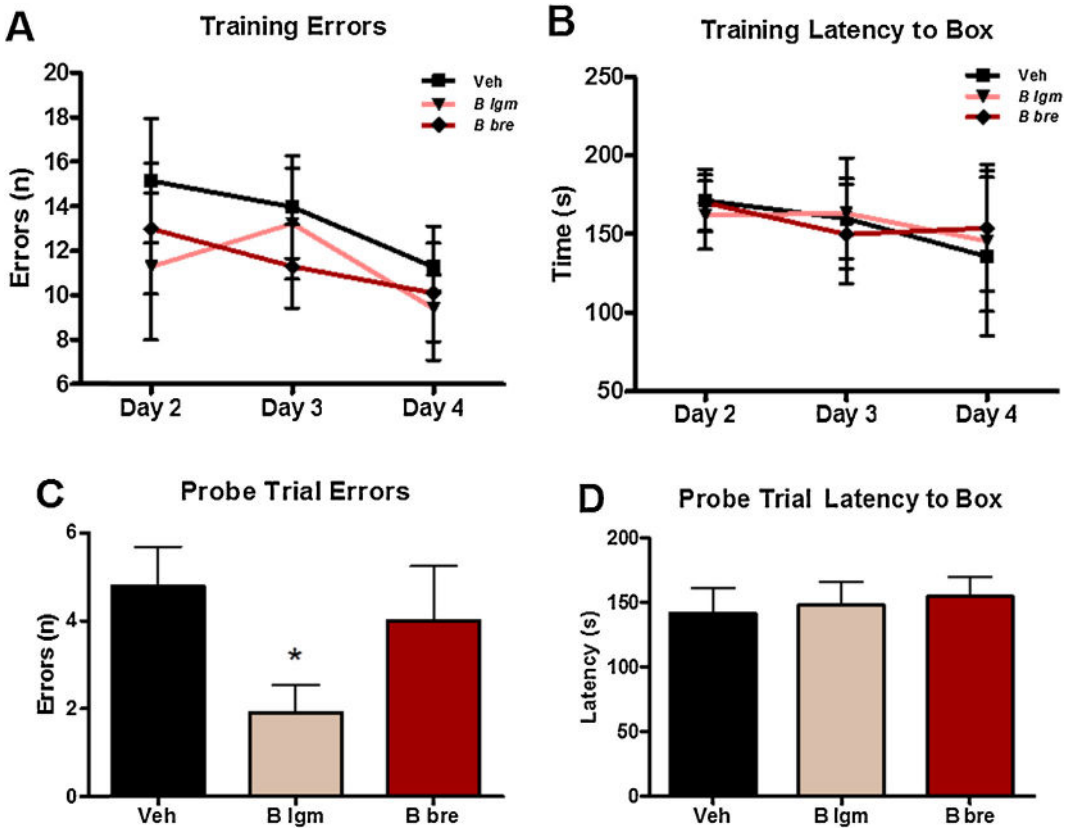


**Fig. 4.** Effect of the two *Bifidobacteria* strains on cognitive performance in the Barnes maze. Although there was no difference between treatments for the number of errors to find the escape box during the training (A), *B. longum 1714* induced a lower number of errors than vehicle treatment for the probe trial (C). However, there was no difference between groups in the latency to find the escape box for both the training (B) and the probe trial (C). $n = 7$–11 per group. Veh = vehicle, B lgm = *B. longum 1714*, B Bre = *B. breve 1205*. $^{*}p < 0.05$, treatment vs. vehicle groups. Data are expressed as means ± SEM.

For the latency to find the escape box (Fig. 4D), there was no difference between groups (Kruskal–Wallis, $H(df = 2) = 0.171$, $p = 0.918$).

As the latencies to find the box did not differ, the distance travelled was measured (Table 2). There was an overall difference between groups (one-way ANOVA, $F(2,30) = 3.595$, $p < 0.05$) and post hoc analysis revealed that *B. breve 1205* group travelled less distance than vehicle group ($p < 0.05$).

Further, the time spent in the centre of the maze (starting point of the trials) was also measured to assess freezing behaviour of mice (Table 2). There was no difference between groups (Kruskal–Wallis, $H(df = 2) = 1.12$, $p = 0.571$). Altogether, these data suggest that *B. longum 1714* – fed animals displayed better memory of the place of the hidden box.

### 3.4. *Effect of the two* Bifidobacteria *strains on fear conditioning*

#### 3.4.1. *Overall freezing response*

Regarding the total freezing response over the 3 days of testing (Fig. 5), there was an effect of day (two-way ANOVA, $F(2,60) = 15.04$, $p < 0.001$) and a trend towards a significant interaction effect treatment × day ($F(4,60) = 2.38$, $p = 0.061$) but no overall effect of treatment ($F(2,60) = 2.02$, $p = 0.151$). Post hoc analysis revealed that *B. longum 1714* induced a higher freezing response than vehicle on day 1 ($p < 0.001$ and $p < 0.05$, respectively). The analysis was further split into cue/context component to better characterise the freezing response from animals to each component.

#### 3.4.2. *Freezing response to the context*

On day 1 (Fig. 5A), there was an effect of context (two-way ANOVA, $F(7,217) = 321.38$, $p < 0.0001$) and a treatment × context interaction ($F(14,217) = 2.23$, $p < 0.001$), but no overall effect of treatment ($F(2,217) = 2.87$, $p = 0.072$), on the freezing response. Post hoc analysis revealed that *B. longum 1714*-fed mice displayed significantly higher freezing response than vehicle-treated animals for context 3 ($p < 0.05$), 4 ($p < 0.01$), 5 ($p < 0.05$) and 6 ($p < 0.05$) and *B. breve 1205*-treated mice on context 4 ($p < 0.05$).

On day 2 (Fig. 5A), there was an effect of context (two-way ANOVA, $F(7,210) = 14.11$, $p < 0.0001$), treatment ($F(2,210) = 5.28$, $p < 0.05$) and a treatment × context interaction ($F(14,210) = 2.37$, $p < 0.01$) on the freezing response. Post hoc analysis revealed that *B. longum 1714*-fed mice displayed a significant 2-fold increase (40% more) in the freezing response than vehicle-treated animals for context 2 ($p < 0.05$), 3 ($p < 0.01$), 4 ($p < 0.05$) and 5 ($p < 0.05$) and *B. breve 1205*-treated mice displayed a lower freezing response than vehicle group on context 8 ($p < 0.05$).

On day 3 (Fig. 5A), there was an effect of context (two-way ANOVA, $F(7,210) = 11.95$, $p < 0.0001$) but no effect of treatment and no treatment × context interaction in the freezing response. Further analysis on individual context events did not reveal any difference between groups.

#### 3.4.3. *Freezing response to the cue*

On day 1 (Fig. 5B), there was an effect of cue (two-way ANOVA, $F(5,155) = 174.5$, $p < 0.0001$), treatment ($F(2,155) = 7.46$, $p < 0.01$), and a treatment × cue interaction ($F(10,155) = 3.57$, $p < 0.0001$) on the freezing response. Post hoc analysis revealed that *B.*

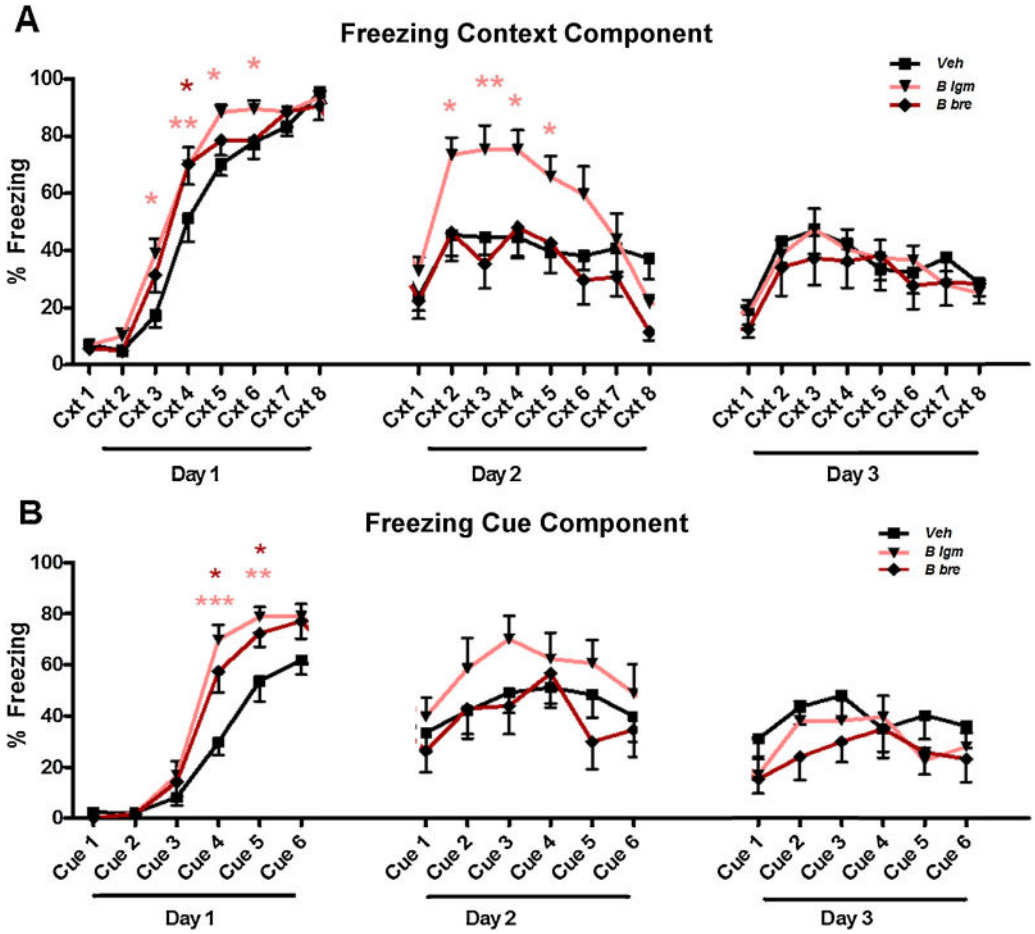


**Fig. 5.** Effect of the two *Bifidobacteria* strains on fear conditioning. *B. longum 1714* treatment induced a significant higher % freezing to the context (A) on day 1 and 2, to the cue (B) on day 1 and a non-significant 1.5–2-fold increase on day 2. *B. breve 1205* induced a significant higher % freezing to the context (A) and cue (B) on day 1. $n = 11–12$ per group. Veh = vehicle, *B lgm* = *B. longum 1714*, *B Bre* = *B. breve 1205*. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, treatment vs. vehicle groups. Data are expressed as means ± SEM.

*longum 1714*- and *B. breve 1205*- fed mice displayed significantly higher freezing response than vehicle-treated animals, for cue 4 ($p < 0.001$ and $p < 0.05$, respectively) and 5 ($p < 0.01$ and $p < 0.05$, respectively).

On day 2 (Fig. 5B), there was an effect of cue (two-way ANOVA, $F(5,150) = 4.31$, $p < 0.01$), but no effect of treatment or treatment × cue interaction in the freezing response. Of note, although not significant, *B. longum 1714* induced 20% more freezing response than vehicle.

On day 3 (Fig. 5B), there was an effect of cue (two-way ANOVA, $F(5,150) = 3.90$, $p < 0.01$), but no effect of treatment or treatment × cue interaction in the freezing response. Of note, although not significant, *B. breve 1205* induced 10–20% less freezing than vehicle group.

These results overall indicate that *B. longum 1714*-fed mice displayed a better learning than vehicle animals (day 1), and a better memory (day 2), on both cue and context components, although this was not significant for day 2. However, groups did not significantly differ in their extinction ability (day 3, i.e. the ability to re-learn that the cue does not predict a shock anymore), although *B. breve 1205* group tended to display a lower freezing rate than other groups.

### 3.5. *Effect of the two* Bifidobacteria *strains on colorectal distension*

There was an overall effect of increasing pressure on viscero-motor response as expected (Fig. 6, two-way ANOVA, $F(4,92) = 1049.65$, $p < 0.0001$), but no treatment effect ($F(2,92) = 0.64$, $p = 0.534$) and no treatment × pressure interaction ($F(8,92) = 0.58$, $p = 0.788$).

### 3.6. *Effect of the two* Bifidobacteria *strains on tissue weight, colon length, corticosterone levels*

Table 3 shows there was no difference between treatments in thymus weight ($F(2,26) = 0.927$, $p = 0.409$), spleen weight ($H(df = 2) = 4.082$, $p = 0.130$), left adrenal ($F(2,21) = 0.215$, $p = 0.809$), right adrenal weights ($F(2,18) = 0.103$, $p = 0.903$) and colon length ($F(2,26) = 0.524$, $p = 0.599$).

There was also no statistical difference between groups in basal corticosterone levels ($H(df = 2) = 3.693$, $p = 0.158$). Altogether, these data show that neither commensal bacteria altered stress-sensitive physiological parameters at the time-point assessed post-stress termination.

## 4. Discussion

An increasing body of data indicates that probiotic consumption has positive effects on behaviour and stress-related disorders [2,79–81]. We have previously shown in the innately anxious BALB/c mice, that two *Bifidobacteria* strains, *B. longum 1714* and *B. breve 1205*, decreased anxiety [43] and that a *Lactobacillus*, *L. rhamnosus*, decreased both anxiety and stress response, along with changes in central gamma-aminobutyric acid (GABA) receptors expression [32]. In the present study, we also show that certain

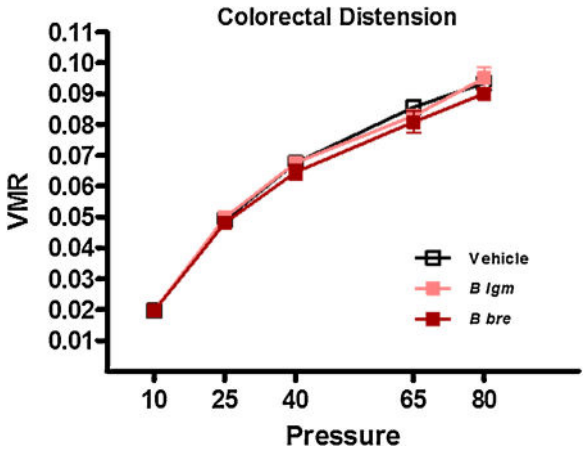


**Fig. 6.** Effect of the two *Bifidobacteria* strains on colorectal distension. There was no difference between groups in the colorectal distension test. $n = 11–12$ per group. Veh = vehicle, *B lgm* = *B. longum 1714*, *B Bre* = *B. breve 1205*. Data are expressed as means ± SEM.

*Bifidobacteria* strains and *B. longum 1714* in particular, are able to induce some positive effects on cognition (summary in Table 4) in fear-related cognitive tasks, possibly by decreasing anxiety in mice. To the best of our knowledge, this is one of the first studies showing that commensal bacteria can benefit cognition in healthy animals (i.e. without previous physiological intervention such as gut bacterial infection or stress event).

*Bifidobacteria* spp were chosen for their reported high beneficial effects on host health, gastrointestinal disorders and their suspected therapeutic effects on psychiatric disorders [26,34,82]. Thus, we show here a new potential health benefit from certain bacteria, which fits with recent studies showing that gut microbiota can impact on key components of anxiety and memory functions, such as BDNF and N-Methyl-D-Aspartate (NMDA) receptors expression [11,12,16,42,17]. It was also shown that a *Lactobacillus* improved memory processes that had been impaired by stress and infection in tasks that are hippocampal-dependent, the T-maze and object recognition [20]. Moreover, combinations of *Lactobacilli* and *Bifidobacteria* spp were shown to decrease acute stress and depression [83,84] and *Mycobacterium vaccae* improved anxiety and learning in a zero-maze task [85]. We also showed that two *Bifidobacteria* strains and *L. rhamnosus* reduced anxiety [32,43], which is linked to changes in the amygdala. Thus, altogether, this literature, added to our data, converge to show that hippocampus and amygdala are two structures which are positively impacted by probiotics. Interestingly, it has been recently shown that the effects of a *Lactobacillus* on behaviour correlated with the microbiome profile and was dependent on the diet and genotype of mice [86]. This shows that the effects of bacteria on behaviour are highly dependent on a wide range of complex factors and reinforces the fact that there is a need for customised individual therapies.

The battery of tests we conducted here aimed at characterising the effects of the two *Bifidobacteria* strains on different aspects of cognition and thus brain functions. In the object recognition test, we aimed at assessing short-term/episodic memory [87]. We choose to conduct the test with a one hour delay, as it has been shown that this is one of the best inter-trial intervals to assess short-term memory processes in BALB/c mice [58]. At the end of the 5-min test, all animals discriminated between the two objects, but this was more significant in *B. longum 1714* group. As the animals fed with the two *Bifidobacteria* strains displayed a trend of lower exploration levels than vehicle animals, we decided to conduct a min-per-min analysis of the test in order to assess if changes in mice behaviours had occurred along the test but would have been masked by the total time of exploration at the end. This idea was motivated by our own experience [88] based on an innovative analysis from Van Heerden and colleagues [78] which showed that measuring the total time of investigation from the animals may mask differences which may rise during the test when analysed minute-per-minute. As a result, using this method in the present study, it appeared that animals fed with both bacteria, and in particular *B. longum 1714* – fed animals,

**Table 3**
Effect of the two *Bifidobacteria* strains on basal corticosterone levels, tissue weight and colon length. Tissue weight data are expressed as % bodyweight. Colon length is expressed in cm. There was no statistical difference between groups for any of the stress-sensitive parameters measured. $n = 7–11$ per group. Data are expressed as means ± SEM.

| % body weight | Vehicle | *B. longum 1714* | *B. breve 1205* |
|---|---|---|---|
| Thymus | 0.190 ± 0.028 | 0.214 ± 0.030 | 0.240 ± 0.023 |
| Spleen | 0.338 ± 0.022 | 0.378 ± 0.035 | 0.362 ± 0.010 |
| Right adrenal | 0.0092 ± 0.0014 | 0.0088 ± 0.0017 | 0.0098 ± 0.0016 |
| Left adrenal | 0.0093 ± 0.0018 | 0.0099 ± 0.0011 | 0.0105 ± 0.0010 |
| Colon length (cm) | 9.5 ± 0.3 | 9.0 ± 0.3 | 9.4 ± 0.3 |
| Corticosterone (ng/mL) | 5.02 ± 1.67 | 5.03 ± 1.23 | 10.33 ± 2.35 |

**Table 4**
Summary of the effects of the two *Bifidobacteria* strains on cognition and visceral sensitivity compared to control group. Study = 11-week feeding, behaviour started at 3-week feeding; cognition and visceral sensitivity; *B. longum = 1714*; *B. breve = 1205*; $n = 9–12$ for short-term spatial memory and locomotor activity (object recognition), $n = 7–12$ for spatial learning and long-term memory (Barnes Maze), $n = 11–12$ for fear learning, memory and extinction (fear conditioning), visceral sensitivity (colorectal distension), $n = 8–12$ for secondary stress-sensitive physiological parameters (bodyweight gain, 9–11 and tissue weight, 7–11) and HPA-axis activity (corticosterone levels in the plasma).

| Feeding time | Test | *B. longum 1714* | *B. breve 1205* |
|---|---|---|---|
| 3 weeks | Object recognition (OR) | Exploration, memory OK<br>Fastest recognition | Exploration, memory OK<br>Faster recognition than controls |
| 3 weeks | Locomotor activity OR | =activity | Decreased activity |
| 4/5 weeks | Barnes maze | =learning<br>Increased memory<br>Decreased faecal output<br>=activity | =learning<br>=memory<br>=faecal output<br>Decreased activity |
| 6/7 weeks | Fear conditioning | Increased learning and memory<br>=extinction | =learning, memory<br>=extinction |
| 9/11 weeks | Colorectal distension | =sensitivity | =sensitivity |
| 11 weeks | Body weight gain (bwg) | =bwg | =(trend -) |
| 11 weeks | Corticosterone | =basal levels | =basal levels |
| 11 weeks | Tissue weight | =weight | =weight |

recognised the new object at an earlier stage than vehicle group. As early exploration of a new object over an old one has been associated with better recognition and thus, increased memory [59], our data indicate that the two *Bifidobacteria* strains may have improved memory processes. Thus, they possibly affected episodic memory and the perirhinal cortex and the hippocampus [58], although there is a discrepancy in the literature about the brain regions involved in this test [89]. Noteworthy, this idea of faster early exploration is also related to the length of the test we used, 5-min, as opposed to 6-min as we did for the training. Indeed, if the test lasts too long in comparison to the training (such as equal time or more), the animals are reported to get too familiar with the new object and start to explore again the old one, masking any difference occurring during the test [59]. However, we cannot rule out the fact that a decrease in anxiety from *Bifidobacteria*-fed mice may be a reason for an earlier exploration. Indeed, we previously showed that the two *Bifidobacteria* strains decreased stress and anxiety [43] and a decreased latency to perform a task can also be related to decreased anxiety. Thus, the positive effects of *B. longum 1714* are likely to be related to their positive action on anxiety, making the interpretation of the object recognition difficult and warranting the use of a battery of tests.

In the Barnes maze, *B. longum 1714* also reduced the number of errors to find the hidden platform compared with vehicle treatment. This indicates an improvement in hippocampal-dependent spatial learning and long-term memory [61,62], thus broadening *B. longum 1714* effects on cognition. When analysing other parameters, locomotor activity during both the training and the probe test and learning during the training were similar between groups. Thus, it is possible that in this test, *B. longum 1714* rather affected retrieval processes of a memory trace, which is thought to be both hippocampal and prefrontal cortex-dependent rather than the encoding, which is thought to be mainly hippocampal-dependent [61,90]. Nonetheless, spatial memory encoding is source of discrepancy as it was before solely thought to be hippocampal and NMDA receptor-dependent, but recent evidences show that other brain structures – and neurotransmitters systems – are involved, in a task- and reference strategy-dependent manner [91–93]. Retrieval of this memory trace, as involving higher cortical structures, would be less dependent on NMDA receptors [90]. Thus, it is possible that *B. longum 1714* had predominantly effects on the prefrontal cortex (PFC), non-NMDA-dependent processes. Further, fitting with the observation in the object recognition that cognitive performance may have been influenced by a decrease in anxiety due to a lower latency to explore the new object, in the Barnes maze, stress novelty-induced defaecation was reduced with *B. longum 1714*. Thus, this gives further confirmation that this bacterium may have decreased stress or anxiety in the present study as well, as we previously showed [43]. Hence, as the learning construct of the Barnes maze has a fear component – animals have to slightly fear the wide open arena to be motivated to explore the maze and find the escape box [63] – it is possible that the improvement in memory was related to, and dependent on, a decrease in anxiety induced by *B. longum 1714* rather than being solely due to better cognitive skills. Thus, altogether, it is possible that *B. longum 1714* did not directly improve hippocampal function but rather induced either a reduced amygdala activity which thus prevented an subsequent inhibition of the hippocampus, or that prefrontal cortex (PFC) functions were improved and exerted a stronger inhibition on the amygdala [94,95]. Finally, the latency to find the target box did not differ between groups. A possible explanation for this is that BALB/c mice are naturally explorative when not stressed [58,96], thus *B. longum 1714*-fed mice might have travelled distance to explore the maze before reaching the escape box, while vehicle group was travelling and making more errors.

One challenge which had to be overcome in the present study was the widely known high anxiety of BALB/c mice [49,51]. This strain is rather avoided in cognitive studies due to their low or variable cognitive skills and high freezing behaviour. We chose this strain as it constitutes excellent models for investigating stress-related disorders and conduct genetic studies comparisons with other mouse strains less anxious [88,96,97]. Moreover, as stress and anxiety and associated with lower cognitive skills [98,99], we were interested in trying to improve cognition in naturally anxious mice. This said, BALB/c mice are either completely freezing when frightened or highly explorative in the absence of a perceived threat [51,58,96]. In this latter case, they still may not perform a given task and explore the behavioural set up instead of learning. Moreover, BALB/c mice differentially respond to different types of stress, showing either better or lower learning skills than other mouse strains [51,52], making it difficult to predict their behaviour in a given task and what the testing conditions and protocol should be. Thus, we conducted extensive pilot studies in order to find the appropriate conditions and protocols for which BALB/c mice would not display confounding overt fear-related behaviour. On the other hand, in the Barnes maze, as the principle of the maze is to display a mild fear in order to be motivated to find the escape box, it is necessary to set up the exact conditions that will induce mice to be motivated but not too scared. In the present study, although some animals displayed freezing and had to be excluded from the analysis (up to 40%) for some parameters, the test still worked and the majority of the animals could learn. As a result, all animals that were not freezing were kept in data analysis. As a result, our data prove that it is possible to conduct cognitive tasks with BALB/c mice and that they are able to learn, provided the right experimental conditions and protocols are found.

In fear conditioning, our data suggest that *B. longum 1714* improved the learning and memory of an aversive event (day 1 and 2) without impairing extinction processes (day 3). As the two *Bifidobacteria* strains have been shown to improve stress and anxiety in a previous study [43] and as stress, anxiety and stress hormones are known to impair cognition [100], it is possible that memory retrieval functions were enhanced by *B. longum 1714* by their action on stress and anxiety. However, it is difficult to conclude on this point as it has been shown that corticosterone, the main stress hormone, facilitates fear extinction in BALB/c mice while it reinforces it in the normo/non-anxious C57BL/6 [68]. Nonetheless, our data suggest that *B. longum 1714* had an effect on the hippocampus (context component) and on the amygdala (context and cue component); but also, amongst others, the periaqueductal grey (freezing behaviour) and hypothalamus (autonomic response), the thalamus (encoding of the stimulus) [101,102]. *B. breve 1205* improved learning on day 1 only without further improvement on memory processes. Interestingly, using the same fear conditioning protocol as in the present study, *L. rhamnosus* increased memory on day 2 only [32]. Thus, it seems like each bacteria impacted on different brain structures and/or sub-regions of the amygdala and hippocampus and also that they affected different memory encoding/retrieval processes. This confirms that commensal bacteria induce highly strain-specific effects and warrants further complex mechanistic studies. Thus, data from the object recognition and Barnes maze suggest that *B. longum 1714* rather impacted on PFC, amygdala, enterhinal and perirhinal cortex (better retrieval processes) and to a milder extent on the hippocampus but data from the fear conditioning suggest that this bacterium did have an impact on the hippocampus, as well as amygdala and perhaps not so much on the PFC, which is involved in extinction [90,95,100]. Another hypothesis leaving apart the hippocampus, as emitted for the Barnes maze, is that *B. longum 1714* possibly improved ventromedial PFC inhibition of the amygdala by modulation of the serotonin (5-HT) feedback loop coming to the PFC or by acting on

GABA interneurons inhibition to the amygdala, therein modulating synaptic plasticity and NMDARs expression in the amygdala [94,95,102]. A lower amygdala activity would then induce a cascade of mechanisms with lower autonomic response and hypothalamus stimulation, decrease stress response and decreased hippocampal inhibition by stress hormones [100,102,103]. This is also confirmed by the fact that *L. rhamnosus* decreased stress response and anxiety via changes in GABAergic activity in key brains areas of the limbic system [32]. Finally, *B. longum 1714* may have had an impact on many more brain regions and systems such as acetylcholine, therein modulating spatial inputs and theta waves to the hippocampus, thus promoting memory [104,105], or dopamine and the mesolimbic pathway, also involved in memories related to fear [106–108]. Further complex lesion and molecular studies would shed light on the exact processes, molecules and brain regions involved behind bacteria effects. Of note, mice from all groups still displayed a residual freezing response on day 3 which was higher than the initial freezing response on day 1 to the context and first cues before presentation of the shock. Thus, it is arguable that extinction processes did not fully occur with possibly either a partial inhibition only of the initial fear trace by the new one or an absence of strong re-learning of the new memory trace. As a result, it is also possible that there was a reinstatement of the freezing response [109]. To assess whether a full extinction could have occurred and the freezing response totally disappear, a possibility would have been to keep testing the animals on consecutive days, or test them in a new context to assess whether renewal processes occurred, or to assess spontaneous recovery after several days of rest [66]. Nevertheless, and importantly, we found that the freezing response on day 3 is still much lower than on previous days, showing that learning and extinction did occur [109,110].

As probiotics have been foremost studied for GI disorders including IBS [47,54] and as IBS has been associated with cognitive impairments [44], we have conducted tests to assess one of the core symptoms of IBS, visceral sensitivity. However, this latter was not affected by either *Bifidobacteria* strains. This was surprising as *Lactobacilli* and *Bifidobacteria* genus have been widely shown to improve gut health and visceral pain [26,38,46,111] and modulate colonic neurons that may be associated with pain perception [112,113]. Moreover, we have previously shown that *B. infantis 35624* reversed visceral sensitivity in normal and viscerally hypersensitive Wistar Kyoto rats [37]. This said, we have recently found in our laboratory that BALB/c mice did not display higher visceral sensitivity than C57BL/6 mice (unpublished observations), although these two mouse strains are known to differ in anxiety and stress-related parameters [96,114–116]. Thus, BALB/c mice may as well not be particularly prone to visceral pain, making it difficult for improvement from probiotics *per se*. Visceral sensitivity is under a complex regulation of mechanisms and its perception in the brain would notably be mediated via the vagus nerve [117]. Moreover, it has been shown that visceral pain is associated with an increased activity in parts of the PFC (cingulate cortex) and notably an absence of inhibition of the pain networks [48,118,119]. These data, put in relation with the comorbidity between IBS, cognitive impairment and stress-related disorders suggest a strong link between visceral pain and parts of the limbic system. Moreover, neurotransmitters involved in anxiety and cognition (such as 5-HT, GABA, acetylcholine (Ach)) have been involved in the aetiology of visceral pain and treatments targeting these molecules are also used in the treatment of IBS [120,121]. Thus, the absence of effects from the two *Bifidobacteria* strains used in our study on visceral pain is somewhat surprising but is in line with the lack of effects of these strains on secondary physiological parameters.

Indeed, these results fit with our previous findings were we showed that *B. longum 1714* and *B. breve 1205* decreased anxiety in BALB/c mice without affecting either basal or stress corticosterone levels, or other secondary stress parameters [43]. However, data related to stress hormones are controversial in the literature as *Bifidobacteria* spp are rather reported to induce no change in rodents [37,39,40] or humans [122], whereas certain *Lactobacilli* strains have been shown to reduce corticosterone stress levels in rodents [20,30,32]. Thus, commensal bacteria effects, again, appear to be highly strain-dependent and preferentially impact on stress response rather than basal levels. However, of note, it has been suggested that corticosterone levels differences between groups may rather rise when samples are harvested in the evening, as opposed to mornings as done so in most studies, due to circadian rhythms of the hormone [76]. Therefore, it is possible that these two *Bifidobacteria* strains induced changes in stress hormones that our time of harvesting did not allow to monitor; it will be interesting to assess this hypothesis in future studies.

So far, the molecular mechanisms underlying bacteria action on brain and behaviour remain unclear. Changes occurring in the brain following gut microbiota manipulation may mainly come, notably, from a humoral, hormonal or neuronal route; and this, via action directly within the gut, via signals transmitted up to the brain (via nerves, second messengers), or through molecules directly travelling up to the brain and exerting their action there (via the blood stream) [1,26] However, at that stage of knowledge in the literature, it is impossible to emit an assertive statement on the exact mechanisms behind a given bacterium's effects or to conduct a full mechanistic study answering all the questions at once, as so many various systems and molecules may be involved. As mentioned before, commensal bacteria actively interact with gut cells, enteric microbiota and the immune system [8,123], in a bacterial strain dependent-manner [54,124]. As immune system activation is also linked to stress-related disorders and cognitive deficits [125–127], in the present study, one first hypothesis is that *Bifidobacteria* effects on memory may be due to differential interactions with the immune system, either via indirect stimulation from the enteric immune system or via direct signalling to the brain [127]. Thus, *B. longum 1714* and *B. breve 1205* may have impacted on the immune system as well, as they decreased stress and anxiety [43] and other studies showed differential activations of key immune cells from *Bifidobacteria* of different strain/genera [82,124,128]. Centrally, this immune signalling may modulate the microglia and brain structures such as the nucleus accumbens and induce noradrenergic system changes, autonomous response modulation and involve further brain regions [127,129]. Such modifications may have then reduced amygdala activation while reinforcing hippocampal and PFC functions, amongst other possibilities, inducing therein both reduced stress and anxiety and improved learning and memory. However, others suggest that certain *Bifidobacteria* strains exert anxiolytic properties rather through enteric neurons and the vagus nerve [33].

A second hypothesis, thus, is that commensal bacteria may also modulate various neurotransmitters implicated in cognitive, emotional and GI functions, locally in the gut [26,130]. Indeed, enterochromaffin cells contains 95% of 5-HT stocks and enteric cells can release neurotransmitters such as glutamate, GABA, Ach, 5-HT, which can themselves, or via secondary messengers, signal to the enteric and central nervous system (brain stem, periaqueductal grey, thalamus, limbic system) a via somatosensory, spinal or vagal afferences [1,81]. Moreover, studies report an impact of both *Bifidobacteria and Lactobacilli* spp consumption on the serotonergic and GABAergic systems [32,39]. However, our present data warrant investigations of other molecules involved in fear and memory such as Ach, glutamate or even noradrenaline and also, specifically, of the differential mechanisms behind *B. longum 1714* and *B. breve 1205* effects.

Further, it is also possible, directly or indirectly, that *B. longum 1714* induced the expression of various neurotrophic factors, such

as BDNF, which was increased in the hippocampus, along with memory improvements, following probiotics consumption [20,42]. However, this effect is not systematic as the fermentation products of a *B. longum* (*NCC3001*) had no effect on BDNF mRNA expression in neuroblastoma cell cultures [33].

It is also possible that *B. longum 1714* effects were mediated via changes in the indigenous enteric microbiota and subsequent alterations in the resulting degradation products following food digestion. Indeed, probiotics may induce different indigenous bacteria to grow by altering the luminal content and conditions (acidity, pH etc.) [7,28]. Such products may cross the epithelial cell walls and travel up to the brain via the blood stream, transporters at the blood brain barrier, or induce locally a cascade of signalling, thus affecting molecules involved in cognition [17,41].

*B. longum 1714* may have also exerted its positive effects via gut hormones signalling. Indeed, in a previous study [43], *B. breve* induced bodyweight gain reduction in mice; although not significant in the present study, the same trend was observed. A link has been established between obesity and lower *Bifidobacteria* spp content in the indigenous microbiota and probiotics, and notably *Bifidobacteria*, have been shown to have positive effects in obesity [131,132]. Obesity has been linked to changes in hormonal signalling from the gut to the brain [133]. Thus, although the effects observed in bodyweight gain were related to *B. breve 1205*, which did not induce cognitive improvements in the present study, it is still possible that the effects of *B. longum 1714* were mediated via gut hormones. To correlate with this, the positive effects of prebiotics on BDNF increase in the hippocampus have been shown to be related to the gut hormone peptide YY [17].

As a result, altogether, all of these hypotheses show that the mechanisms of action of probiotics appear to be very complex and under a wide range of molecules and systems, strengthening the now well-established linked between the enteric microbiota and the brain–gut axis. Data from our studies and the literature also confirms that the effects are highly bacterial strain-dependent. The complexity of all of the possibilities emerging regarding the potential mechanisms involved and candidate molecules warrant further mechanistic studies.

## 5. Conclusions

*B. Longum 1714* induced overall a positive modulation of memory processes in cognitive tasks comprising a fear component, in the innately anxious BALB/c mice, whereas *B. breve 1205* had a lower impact. There is thus a strong possibility that *B. Longum 1714* positively modulated BALB/c behaviour by decreasing their anxiety. These data confirm that commensal bacteria effects are highly strain-specific but above all that bacteria and the enteric microbiota can also have an impact on cognition, in an unperturbed healthy mouse. These findings show a new role for the enteric microbiota and suggest a potential therapeutic approach for treating cognitive deficits associated with stress-related or neurodegenerative diseases, above all as we found evidences of positive impact from *B. Longum 1714* in the hippocampus, one of the first brain regions impacted in Alzheimer's disease. Therefore, it will be of high interest in further studies to investigate and characterise the molecular mechanisms behind these commensal bacteria's action on behaviour.

## Competing interests

The Centre has conducted studies in collaboration with several companies including GSK, Pfizer, Alimentary Health, Cremo, Suntory Wellness, Danone-Nutricia, Wyeth and Mead Johnson. The authors have spoken at meetings sponsored by food and pharmaceutical companies.

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## References

[1] Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain–gut–enteric microbiota axis. Nat Rev Gastroenterol Hepatol 2009;6(5):306–14.
[2] Collins SM, Surette M, Bercik P. The interplay between the intestinal microbiota and the brain. Nat Rev Microbiol 2012;10(11):735–42.
[3] Dinan TG, Cryan JF. Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology. Psychoneuroendocrinology 2012;37(9):1369–78.
[4] O'Mahony SM, Savignac HM, O'Brien T, Scully P, Quigley EM, Marchesi J, et al. Early-life dysbiosis-induced visceral hypersensitivity in adulthood. Gastroenterology 2010;138(5 (Suppl. 1)). S-1–S-906-.
[5] Jeffery IB, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, et al. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. Gut 2012;61(7):997–1006.
[6] Carroll IM, Ringel-Kulka T, Keku TO, Chang YH, Packey CD, Sartor RB, et al. Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2011;301(5):G799–807.
[7] Lutgendorff F, Akkermans LM, Soderholm JD. The role of microbiota and probiotics in stress-induced gastro-intestinal damage. Curr Mol Med 2008;8(4):282–98.
[8] Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 2012;13(10):701–12.
[9] Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, et al. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A 2011;108(7):3047–52.
[10] Collins SM, Kassam Z, Bercik P. The adoptive transfer of behavioral phenotype via the intestinal microbiota: experimental evidence and clinical implications. Curr Opin Microbiol 2013;16(3):240–5.
[11] Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterol Motil: Off J Eur Gastrointest Motil Soc 2011;23(3):255–64, e119.
[12] Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu X, et al. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J Physiol 2004;558(Pt 1):263–75.
[13] Desbonnet L, Clarke G, Shanahan F, Dinan TG, Cryan JF. Microbiota is essential for social development in the mouse. Mol Psychiatry 2014;19(2):146–8.
[14] Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, et al. The microbiome–gut–brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. Mol Psychiatry 2013;18(6):666–73.
[15] Tyler WJ, Alonso M, Bramham CR, Pozzo-Miller LD. From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn Mem 2002;9(5):224–37.
[16] Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 2011;141(2):599–609, 609 e1–3.
[17] Savignac HM, Corona G, Mills H, Chen L, Spencer JP, Tzortzis G, et al. Prebiotic feeding elevates central brain derived neurotrophic factor: N-methyl-D-aspartate receptor subunits and D-serine. Neurochem Int 2013;63(8):756–64.

[18] Lyte M, Li W, Opitz N, Gaykema RP, Goehler LE. Induction of anxiety-like behavior in mice during the initial stages of infection with the agent of murine colonic hyperplasia *Citrobacter rodentium*. Physiol Behav 2006;89(3):350–7.
[19] Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology 2010;139(6):2102–12, e1.
[20] Gareau MG, Wine E, Rodrigues DM, Cho JH, Whary MT, Philpott DJ, et al. Bacterial infection causes stress-induced memory dysfunction in mice. Gut 2011;60(3):307–17.
[21] Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun 2011;25(3):397–407.
[22] Bailey MT, Coe CL. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol 1999;35(2):146–55.
[23] O'Mahony SM, Marchesi JR, Scully P, Codling C, Ceolho AM, Quigley EM. Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. Biol Psychiatry 2009;65(3):263–7.
[24] Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain: behaviour and cognition. Nat Rev Neurosci 2009;10(6):434–45.
[25] Jia W, Li H, Zhao L, Nicholson JK. Gut microbiota: a potential new territory for drug targeting. Nat Rev Drug Discov 2008;7(2):123–9.
[26] Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. Biol Psychiatry 2013;74(10):720–6.
[27] Park SY. Potential therapeutic agents against Alzheimer's disease from natural sources. Arch Pharm Res 2010;33(10):1589–609.
[28] Shanahan F, Dinan TG, Ross P. Probiotics in transition. Clin Gastroenterol Hepatol 2012;10(11):1220–4.
[29] Eutamene H, Bueno L. Role of probiotics in correcting abnormalities of colonic flora induced by stress. Gut 2007;56(11):1495–7.
[30] Gareau MG, Jury J, MacQueen G, Sherman PM, Perdue MH. Probiotic treatment of rat pups normalizes corticosterone release and ameliorates colonic dysfunction induced by maternal separation. Gut 2007;56(11):1522–8.
[31] Rao AV, Bested AC, Beaulne TM, Katzman MA, Iorio C, Berardi JM, et al. A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome. Gut Pathog 2009;1(1):6.
[32] Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A 2011;108(38):16050–5.
[33] Bercik P, Park AJ, Sinclair D, Khoshdel A, Lu J, Huang X, et al. The anxiolytic effect of *Bifidobacterium longum* NCC3001 involves vagal pathways for gut–brain communication. Neurogastroenterol Motil: Off J Eur Gastrointest Motil Soc 2011;23(12):1132–9.
[34] Ishibashi N, Yaeshima T, Hayasawa H. Bifidobacteria: their significance in human intestinal health. Malays J Nutr 1997;3:149–59.
[35] Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D. Getting better with bifidobacteria. J Appl Microbiol 2005;98(6):1303–15.
[36] Camfield DA, Owen L, Scholey AB, Pipingas A, Stough C. Dairy constituents and neurocognitive health in ageing. Br J Nutr 2011:1–17.
[37] McKernan DP, Fitzgerald P, Dinan TG, Cryan JF. The probiotic *Bifidobacterium infantis* 35624 displays visceral antinociceptive effects in the rat. Neurogastroenterol Motil 2010;22(9):1029–35, e268.
[38] O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 2005;128(3):541–51.
[39] Desbonnet L, Garrett L, Clarke G, Bienenstock J, Dinan TG. The probiotic *Bifidobacteria infantis*: an assessment of potential antidepressant properties in the rat. J Psychiatr Res 2008;43(2):164–74.
[40] Desbonnet L, Garrett L, Clarke G, Kiely B, Cryan JF, Dinan TG. Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. Neuroscience 2010;170(4):1179–88.
[41] Wall R, Ross RP, Shanahan F, O'Mahony L, Kiely B, Quigley E, et al. Impact of administered bifidobacterium on murine host fatty acid composition. Lipids 2010;45(5):429–36.
[42] O'Sullivan E, Barrett E, Grenham S, Fitzgerald P, Stanton C, Ross RP, et al. BDNF expression in the hippocampus of maternally separated rats: does *Bifidobacterium breve* 6330 alter BDNF levels? Benef Microbes 2011;2(3):199–207.
[43] Savignac HM, Kiely B, Dinan TG, Cryan JF. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. Neurogastroenterol Motil 2014;26(11):1615–27.
[44] Kennedy PJ, Clarke G, Quigley EM, Groeger JA, Dinan TG, Cryan JF. Gut memories: towards a cognitive neurobiology of irritable bowel syndrome. Neurosci Biobehav Rev 2012;36(1):310–40.
[45] Kennedy PJ, Clarke G, O'Neill A, Groeger JA, Quigley EM, Shanahan F, et al. Cognitive performance in irritable bowel syndrome: evidence of a stress-related impairment in visuospatial memory. Psychol Med 2014;44(7):1553–66.
[46] Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, et al. Lactobacillus acidophilus modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 2007;13(1):35–7.
[47] Clarke G, Cryan JF, Dinan TG, Quigley EM. Review article: probiotics for the treatment of irritable bowel syndrome – focus on lactic acid bacteria. Aliment Pharmacol Ther 2012;35(4):403–13.
[48] Felice VD, Gibney SM, Gosselin RD, Dinan TG, O'Mahony SM, Cryan JF. Differential activation of the prefrontal cortex and amygdala following psychological stress and colorectal distension in the maternally separated rat. Neuroscience 2014;267:252–62.
[49] Belzung C, Griebel G. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 2001;125(1–2):141–9.
[50] Savignac HM, Hyland NP, Dinan TG, Cryan JF. The effects of repeated social interaction stress on behavioural and physiological parameters in a stress-sensitive mouse strain. Behav Brain Res 2011;216(2):576–84.
[51] Brinks V, van der Mark M, de Kloet R, Oitzl M. Emotion and cognition in high and low stress sensitive mouse strains: a combined neuroendocrine and behavioral study in BALB/c and C57BL/6J mice. Front Behav Neurosci 2007;1:8.
[52] Palumbo ML, Zorrilla Zubilete MA, Cremaschi GA, Genaro AM. Different effect of chronic stress on learning and memory in BALB/c and C57BL/6 inbred mice: Involvement of hippocampal NO production and PKC activity. Stress 2009;12(4):350–61.
[53] Cryan JF, Sweeney FF. The age of anxiety: role of animal models of anxiolytic action in drug discovery. Br J Pharmacol 2011;164(4):1129–61.
[54] Dunne C, Murphy L, Flynn S, O'Mahony L, O'Halloran S, Feeney M, et al. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek 1999;76(1–4):279–92.
[55] Crawley JN. Behavioral phenotyping strategies for mutant mice. Neuron 2008;57(6):809–18.
[56] Dere E, Huston JP, De Souza Silva MA. Episodic-like memory in mice: simultaneous assessment of object, place and temporal order memory. Brain Res Brain Res Protoc 2005;16(1–3):10–9.
[57] Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 1988;31(1):47–59.
[58] Brooks SP, Pask T, Jones L, Dunnett SB. Behavioural profiles of inbred mouse strains used as transgenic backgrounds. II: Cognitive tests. Genes Brain Behav 2005;4(5):307–17.
[59] Bevins RA, Besheer J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nat Protoc 2006;1(3):1306–11.
[60] Barone FC, Barton ME, White RF, Legos JJ, Kikkawa H, Shimamura M. Inhibition of phosphodiesterase type 4 decreases stress-induced defecation in rats and mice. Pharmacology 2008;81(1):11–7.
[61] Sharma S, Rakoczy S, Brown-Borg H. Assessment of spatial memory in mice. Life Sci 2010;87(17–18):521–36.
[62] Barnes CA. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J Comp Physiol Psychol 1979;93(1):74–104.
[63] Dawood MY, Lumley LA, Robison CL, Saviolakis GA, Meyerhoff JL. Accelerated Barnes maze test in mice for assessment of stress effects on memory. Ann N Y Acad Sci 2004;1032:304–7.
[64] Maren S. Neurobiology of Pavlovian fear conditioning. Annu Rev Neurosci 2001;24:897–931.
[65] Domjan M. Pavlovian conditioning: a functional perspective. Annu Rev Psychol 2005;56:179–206.
[66] Quirk GJ, Paré D, Richardson R, Herry C, Monfils MH, Schiller D, et al. Erasing fear memories with extinction training. J Neurosci 2010;30(45):14993–7.
[67] Heim C, Nemeroff CB. Neurobiology of posttraumatic stress disorder. CNS Spectr 2009;14(1 (Suppl. 1)):13–24.
[68] Brinks V, de Kloet ER, Oitzl MS. Corticosterone facilitates extinction of fear memory in BALB/c mice but strengthens cue related fear in C57BL/6 mice. Exp Neurol 2009;216(2):375–82.
[69] O'Mahony SM, Tramullas M, Fitzgerald P, Cryan JF. Rodent models of colorectal distension. Current protocols in neuroscience/editorial board. Curr Protoc Neurosci 2012, http://dx.doi.org/10.1002/0471142301.ns0940s61. Chapter 9:Unit 9.40.
[70] Tramullas M, Finger BC, Moloney RD, Golubeva AV, Moloney G, Dinan TG, et al. Toll-like receptor 4 regulates chronic stress-induced visceral pain in mice. Biol Psychiatry 2014;76(4):340–8.
[71] Tramullas M, Dinan TG, Cryan JF. Chronic psychosocial stress induces visceral hyperalgesia in mice. Stress 2012;15(3):281–92.
[72] Reber SO, Obermeier F, Straub RH, Falk W, Neumann ID. Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. Endocrinology 2006;147(10):4968–76.
[73] BartolomucciA, Palanza P, Sacerdote P, Panerai AE, Sgoifo A, Dantzer R, et al. Social factors and individual vulnerability to chronic stress exposure. Neurosci Biobehav Rev 2005;29(1):67–81.
[74] Engler H, Engler A, Bailey MT, Sheridan JF. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. J Neuroimmunol 2005;163(1–2):110–9.
[75] Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 2007;131(2):391–404.
[76] Reber SO, Birkeneder L, Veenema AH, Obermeier F, Falk W, Straub RH, et al. Adrenal insufficiency and colonic inflammation after a novel chronic psychosocial stress paradigm in mice: implications and mechanisms. Endocrinology 2007;148(2):670–82.

[77] O'Connor RM, Thakker DR, Schmutz M, van der Putten H, Hoyer D, Flor PJ, et al. Adult siRNA-induced knockdown of mGlu7 receptors reduces anxiety in the mouse. Neuropharmacology 2013;72:66–73.
[78] van Heerden JH, Russell V, Korff A, Stein DJ, Illing N. Evaluating the behavioural consequences of early maternal separation in adult C57BL/6 mice; the importance of time. Behav Brain Res 2010;207(2):332–42.
[79] Kantak PA, Bobrow DN, Nyby JG. Obsessive–compulsive-like behaviors in house mice are attenuated by a probiotic (*Lactobacillus rhamnosus* GG). Behav Pharmacol 2014;25(1):71–9.
[80] Dinan TG, Cryan JF. Melancholic microbes: a link between gut microbiota and depression? Neurogastroenterol Motil: Off J Eur Gastrointest Motil Soc 2013;25(9):713–9.
[81] Forsythe P, Kunze WA. Voices from within: gut microbes and the CNS. Cell Mol Life Sci 2013;70(1):55–69.
[82] O'Mahony C, Scully P, O'Mahony D, Murphy S, O'Brien F, Lyons A, et al. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. PLoS Pathog 2008;4(8):e1000112.
[83] Arseneault-Bréard J, Rondeau I, Gilbert K, Girard SA, Tompkins TA, Godbout R, et al. Combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model. Br J Nutr 2012;107(12):1793–9.
[84] Ait-Belgnaoui A, Durand H, Cartier C, Chaumaz G, Eutamene H, Ferrier L, et al. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 2012;37(11):1885–95.
[85] Matthews DM, Jenks SM. Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. Behav Processes 2013;96:27–35.
[86] Ohland CL, Kish L, Bell H, Thiesen A, Hotte N, Pankiv E, et al. Effects of *Lactobacillus helveticus* on murine behavior are dependent on diet and genotype and correlate with alterations in the gut microbiome. Psychoneuroendocrinology 2013;38(9):1738–47.
[87] Ennaceur A. One-trial object recognition in rats and mice: methodological and theoretical issues. Behav Brain Res 2010;215(2):244–54.
[88] Savignac HM, Dinan TG, Cryan JF. Resistance to early-life stress in mice: effects of genetic background and stress duration. Front Behav Neurosci 2011;5:13.
[89] Oliveira AM, Hawk JD, Abel T, Havekes R. Post-training reversible inactivation of the hippocampus enhances novel object recognition memory. Learn Mem 2010;17(3):155–60.
[90] Abel T, Lattal KM. Molecular mechanisms of memory acquisition, consolidation and retrieval. Curr Opin Neurobiol 2001;11(2):180–7.
[91] Bannerman DM, Bus T, Taylor A, Sanderson DJ, Schwarz I, Jensen V, et al. Dissecting spatial knowledge from spatial choice by hippocampal NMDA receptor deletion. Nat Neurosci 2012;15(8):1153–9.
[92] Taylor AM, Bus T, Sprengel R, Seeburg PH, Rawlins JN, Bannerman DM. Hippocampal NMDA receptors are important for behavioural inhibition but not for encoding associative spatial memories. Philos Trans R Soc Lond B Biol Sci 2014;369(1633):20130149.
[93] Wolbers T, Wiener JM. Challenges for identifying the neural mechanisms that support spatial navigation: the impact of spatial scale. Front Hum Neurosci 2014;8:571.
[94] Aznar S, Klein AB. Regulating prefrontal cortex activation: an emerging role for the 5-HT(2)A serotonin receptor in the modulation of emotion-based actions? Mol Neurobiol 2013;48(3):841–53.
[95] Sotres-Bayon F, Quirk GJ. Prefrontal control of fear: more than just extinction. Curr Opin Neurobiol 2010;20(2):231–5.
[96] Savignac HM, Finger BC, Pizzo RC, O'Leary OF, Dinan TG, Cryan JF. Increased sensitivity to the effects of chronic social defeat stress in an innately anxious mouse strain. Neuroscience 2011;192:524–36.
[97] Palumbo ML, Canzobre MC, Pascuan CG, Ríos H, Wald M, Genaro AM. Stress induced cognitive deficit is differentially modulated in BALB/c and C57Bl/6 mice: correlation with Th1/Th2 balance after stress exposure. J Neuroimmunol 2010;218(1–2):12–20.
[98] Buwalda B, Kole MH, Veenema AH, Huininga M, de Boer SF, Korte SM, et al. Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning. Neurosci Biobehav Rev 2005;29(1):83–97.
[99] McEwen BS, Sapolsky RM. Stress and cognitive function. Curr Opin Neurobiol 1995;5(2):205–16.
[100] McEwen BS. Glucocorticoids, depression, and mood disorders: structural remodeling in the brain. Metabolism 2005;54(5 (Suppl. 1)):20–3.
[101] Maren S. Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. Eur J Neurosci 2008;28(8):1661–6.
[102] Johansen JP, Cain CK, Ostroff LE, LeDoux JE. Molecular mechanisms of fear learning and memory. Cell 2011;147(3):509–24.
[103] Lyons DM, Lopez JM, Yang C, Schatzberg AF. Stress-level cortisol treatment impairs inhibitory control of behavior in monkeys. J Neurosci 2000;20(20):7816–21.
[104] Barry C, Heys JG, Hasselmo ME. Possible role of acetylcholine in regulating spatial novelty effects on theta rhythm and grid cells. Front Neural Circuits 2012;6:5.
[105] Hasselmo ME. The role of acetylcholine in learning and memory. Curr Opin Neurobiol 2006;16(6):710–5.
[106] Zhang L, Zhou FM, Dani JA. Cholinergic drugs for AIzheimer's disease enhance in vitro dopamine release. Mol Pharmacol 2004;66(3):538–44.
[107] Myers KM, Davis M. Mechanisms of fear extinction. Mol Psychiatry 2007;12(2):120–50.
[108] Nestler EJ, Carlezon Jr WA. The mesolimbic dopamine reward circuit in depression. Biol Psychiatry 2006;59(12):1151–9.
[109] Quirk GJ, Mueller D. Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 2008;33(1):56–72.
[110] Amano T, Unal CT, Pare D. Synaptic correlates of fear extinction in the amygdala. Nat Neurosci 2010;13(4):489–94.
[111] Brenner DM, Moeller MJ, Chey WD, Schoenfeld PS. The utility of probiotics in the treatment of irritable bowel syndrome: a systematic review. Am J Gastroenterol 2009;104(4):1033–49, quiz 1050.
[112] Kunze WA, Mao YK, Wang B, Huizinga JD, Ma X, Forsythe P, et al. Lactobacillus reuteri enhances excitability of colonic AH neurons by inhibiting calcium-dependent potassium channel opening. J Cell Mol Med 2009;13(8B):2261–70.
[113] Wang B, Mao YK, Diorio C, Wang L, Huizinga JD, Bienenstock J, et al. Lactobacillus reuteri ingestion and IK(Ca) channel blockade have similar effects on rat colon motility and myenteric neurones. Neurogastroenterol Motil 2010;22(1):98–107, e33.
[114] Browne CA, Clarke G, Dinan TG, Cryan JF. Differential stress-induced alterations in tryptophan hydroxylase activity and serotonin turnover in two inbred mouse strains. Neuropharmacology 2011;60(4):683–91.
[115] Jacobson LH, Cryan JF. Feeling strained? Influence of genetic background on depression-related behavior in mice: a review. Behav Genet 2007;37(1):171–213.
[116] O'Mahony CM, Sweeney FF, Daly E, Dinan TG, Cryan JF. Restraint stress-induced brain activation patterns in two strains of mice differing in their anxiety behaviour. Behav Brain Res 2010;213(2):148–54.
[117] Bravo JA, Julio-Pieper M, Forsythe P, Kunze W, Dinan TG, Bienenstock J, et al. Communication between gastrointestinal bacteria and the nervous system. Curr Opin Pharmacol 2012;12(6):667–72.
[118] Bonaz B. Visceral sensitivity perturbation integration in the brain–gut axis in functional digestive disorders. J Physiol Pharmacol 2003;54(Suppl. 4):27–42.
[119] Gibney SM, Gosselin RD, Dinan TG, Cryan JF. Colorectal distension-induced prefrontal cortex activation in the Wistar-Kyoto rat: implications for irritable bowel syndrome. Neuroscience 2010;165(3):675–83.
[120] Kuiken SD, Tytgat GN, Boeckxstaens GE. Review article: drugs interfering with visceral sensitivity for the treatment of functional gastrointestinal disorders – the clinical evidence. Aliment Pharmacol Ther 2005;21(6):633–51.
[121] Clarke G, Quigley EM, Cryan JF, Dinan TG. Irritable bowel syndrome: towards biomarker identification. Trends Mol Med 2009;15(10):478–89.
[122] Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A. Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. Br J Nutr 2011;105(5):755–64.
[123] Forsythe P, Kunze WA. Voices from within: gut microbes and the CNS. Cell Mol Life Sci: CMLS 2013;70(1):55–69.
[124] Ng S, Hart AL, Kamm MA, Stagg AJ, Knight SC. Mechanisms of action of probiotics: recent advances. Inflamm Bowel Dis 2009;15(2):300–10.
[125] Mandolesi G, Grasselli G, Musumeci G, Centonze D. Cognitive deficits in experimental autoimmune encephalomyelitis: neuroinflammation and synaptic degeneration. Neurol Sci 2010;31(Suppl. 2):S255–9.
[126] Dinan TG. Inflammatory markers in depression. Curr Opin Psychiatry 2009;22(1):32–6.
[127] Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 2008;9(1):46–56.
[128] Lyons A, O'Mahony D, O'Brien F, MacSharry J, Sheil B, Ceddia M, et al. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. Clin Exp Allergy 2010;40(5):811–9.
[129] Hăulică I, Bild W, Boişteanu D, Ioniţă T, Mihăilă C. Actual data concerning the brain–immune system interface. Roum Arch Microbiol Immunol 2002;61(3):141–57.
[130] Spiller R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: alterations in 5-HT signalling and metabolism in human disease. Neurogastroenterol Motil 2007;19(Suppl. 2):25–31.
[131] Million M, Angelakis E, Maraninchi M, Henry M, Giorgi R, Valero R, et al. Correlation between body mass index and gut concentrations of *Lactobacillus reuteri*: *Bifidobacterium animalis*, *Methanobrevibacter smithii* and *Escherichia coli*. Int J Obes (Lond) 2013;37(11):1460–6.
[132] Delzenne NM, Neyrinck AM, Bäckhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 2011;7(11):639–46.
[133] Schellekens H, Dinan TG, Cryan JF. Ghrelin at the interface of obesity and reward. Vitam Horm 2013;91:285–323.