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Probiotics modify body weight together with anxiety states via pro-inflammatory factors in HFD-treated Syrian golden hamster

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Abstract

Emerging studies are beginning to suggest that emotional states together with healthful measures constitute pertinent features of our lifestyle in which bad eating habits but more importantly what our gut has to host are causing great concern. It is well known that humans have established mutual relationships with a wide array of colonized microbes (collectively called gut microbiota) consisting of bacteria, fungi, eukaryotic parasites and viruses. The gut microbiota has exhibited a notable ability of communicating with the brain via a two-way system that includes the vagus nerve, immune sites, and a number of neurotransmitters. Interestingly, stressful along with obesity, cognitive, and brain developmental states are strongly influenced by microbiota homeostatic conditions. It was our aim to investigate behavioral and obesity effects evoked by treatment with probiotics via neuroinflammatory

factors and namely IL-1β, NLRP3, Caspase-1 and NF-kB levels in the Syrian golden hamster. Following treatment with a high-fat diet (HFD), in the presence or absence of a multi-species probiotic formulation, hamsters were exposed to an unpredictable chronic mild stress (UCMS) test for 4 weeks. Independently of the diet, probiotics treatment markedly reduced stress-like behaviors in the different mazes. Moreover, probiotics decreased hypothalamic expression levels of the pro-neuroinflammatory factors like IL-1β, NLRP3, Caspase-1 and NF-kB, whereas HFD increased them. Contextually, they decreased plasmatic levels of IL-1β, NLRP3 and caspase-1 but not NF-kB. Our findings clearly support probiotics as a potentially valuable treatment strategy in obesity and anxiety, thereby proposing them for clinical treatments in patients with metabolic and mood disorders.

Key words: Obesity, Neuroinflammation, Probiotics, Anxiety

1. Introduction

Obesity is now recognized as a worldwide health issue and has reached epidemic proportions affecting both developed and developing countries. Far from being limited to weight gain, obesity is generally associated with a cluster of disorders collectively known as metabolic syndrome. The etiology of this metabolic disorder stems from an interplay between genetic predispositions and environmental factors resulting in an immune response and subsequent low-grade inflammation that affects numerous tissues including the liver, adipose tissue, and central nervous system (CNS) [1]. In this regard, the hypothalamus (HTH) has naturally attracted great attention because of one of its main site and namely the arcuate nucleus contains two distinct neuronal populations (NPY/AgRP neurons and POMC/CART neurons) that are involved with the regulation of body weight and energy balance

[2]. The interrelations between inflammation, HTH, and metabolic disorders have been extensively studied [3,4] since such a condition has shown to be involved with the onset and maintenance of obese phenotypes [2].

The immune system generates high levels of pro-inflammatory interleukins IL-1 and IL-6 via a pro-inflammatory reaction [5]. It is noteworthy that individuals with major depression have significantly higher concentrations of IL-6 in comparison with controls, as demonstrated by a meta-analysis of 16 studies [6]. From another meta-analysis, it appears that antidepressant therapies are capable of reducing IL-1β serum level and simultaneously alleviating depressive symptoms [7]. This is not at all surprising since gut microbiota is a rich source of inflammatory molecules that can contribute to inflammation and metabolic diseases [8]. Other studies have consistently demonstrated a shift in the microbiota profile from a "lean" (anti-inflammatory) toward an obese phenotype in animals and humans [9,10]. Interestingly NLRP3, a multi-protein formation leads to autocatalysis and activation of Caspase-1, which in turn converts IL-1β and IL-18 into their respective biological active forms prior to secretion [11]. The importance of IL-1β has been highlighted together with NLRP3 inflammasome, which seem to induce the development of several common human diseases, i.e. gout, type 2 diabetes, non-alcoholic steatohepatitis, atherosclerosis and Alzheimer's disease [12,13]. Additionally, NLRP3 inflammasome has shown to be a potential target for the treatment of depression because it is involved with stress-related depressive moods [14]. More and more convincing evidences point to gut microbiota and their genes (collectively called gut microbiome) as a major element cross-talking with the brain and vice versa [15,16]. In this regard recent studies have also pointed to bacteria of the gastrointestinal (GI) tract, including commensal probiotic, and pathogenic bacteria, as major causes of altered central nervous system (CNS) signaling pathways [17,18].

A growing number of diseases have now been reported to be associated with altered gut flora [19]. The prevalence of metabolic diseases, particularly obesity and obesity-related disorders have drastically increased as a result of changes in diet and dietary habits, lifestyle and environment factors

like pollution [20]. An imbalance of gut microbiota, particularly regarding microbial diversity, has caused the predisposition to a variety of other diseases including malnutrition, asthma, anxiety, depression, allergies and metabolic diseases [21]. Anxiety has shown to strongly alter gut barrier function, thereby leading to the passage of various molecules, and perhaps bacteria, from the gut into the bloodstream [22]. As a consequence, body weight increase has been linked to increased rates of dysmetabolic conditions, including hypertension and diabetes mellitus type II [23,24], and this association may be partly caused by an unhealthy diet [25,26]. Furthermore "Western pattern" high-fat low-fiber diets have been demonstrated to be associated with marked changes in gut microbiota composition and poor microbial diversity [27]. In this context the modulation of microbiota results to be a key regulator of behaviors like stress, anxiety, depression, autism and mood. In particular probiotics via the activation of neural pathways and SNC signaling system exert their effects on gut microbiota that seem to have a positive impact on the management of psychological disorders such as depressive-like behaviors [28,29].

Based on these indications, it was the intention of the present work to address the effect of probiotic treatment on obesity and anxiety, since chronic stressful conditions appear to account for increased neuroinflammatory events. Mood-congruent judgment biases for ambiguous information have been reported in a wide range of animal species of which the hamster is considered a valuable rodent for testing anxiety states [30]. For this purpose, the expression of inflammation markers in HTH, blood and their possible involvement with the maintenance of anxiety states and obesity via brain neurosignals were considered. Consequently, the expression differences of IL-1β, NLRP3, Caspase-1 and NF-kB in HTH and blood of hamsters exposed to unpredictable chronic mild stress (UCMS) and high fat diet (HFD), may constitute novel molecular approaches supporting probiotics treatment as a potential strategy in patient suffering from metabolic and mood disorders.

2. Materials and methods

2.1. Animals

In the present study, male Syrian golden hamsters (*Mesocricetus auratus*) weighing 130-160 g (6 months old) were purchased from Charles River (Como, Italy) and brought into the laboratory 2 weeks before starting the experiments. This rodent model was mainly chosen for its valuable ability to supply distinctly specific signs of anxiety when tested in behavioral analysis such as UCMS [31]. Forty-two hamsters were randomly chosen for UCMS protocols, of which 12 served as unstressed controls with normal chow diet i.e. unstressed (unstressed + probiotic). Six groups of hamsters all received probiotics (Biocult Strong, Homesyn Italy) in their water-bottle (3 g/200 ml for each animal per day). Three g of probiotics contained: 1.5×10^{10} colony-forming unit CFU of *Streptococcus thermophilus* (CNCM strain number I-1630), 1.5×10^{10} colony-forming unit CFU of *Lactobacillus bulgaricus* (CNCM strain numbers I-1632 and I-1519); 1.5×10^{10} colony-forming unit CFU of *Lactococcus lactis subsp. lactis* (CNCM strain number I-1631); 1.5×10^{10} colony-forming unit CFU of *Lactobacillus acidophilus*; 1.5×10^{10} colony-forming unit CFU of *Streptococcus thermophiles*; 1.5×10^{10} colony-forming unit CFU of *Lactobacillus plantarum*; 1.5×10^{10} colony-forming unit CFU of *Bifidobacterium lactis* (CNCM I-2494); $1.5 \times 1\ 10^{10}$ colony-forming unit CFU of *Lactobacillus reuteri* (DSM 17938), maltodextrin from corn, anticaking agent (silica), casein, lactose, and gluten < 3 ppm LLOQ (lower limit of quantitation). For this study the hamsters of either the unstressed+probiotics group or of only the unstressed group showed similar behavioral effects and so only the group unstressed+probiotics was chosen as our control.

Subsequently hamsters were divided as follows: 6 animals HFD+probiotics, 6 animals HFD (without probiotics), 6 animals stressed+probiotics (UCMS+probiotics), 6 animals UCMS (without probiotics), 6 animals UCMS+HFD+probiotics, 6 animals UCMS+HFD (without probiotics). Room temperature was set at 22°C, and hamsters were housed one per cage unless grouping was applied during the protocol. A 12-h light/dark cycle was maintained except when a specific condition was required during the course of stressor schedule. Food and water were freely available. Control animals had no contact with stressed or HFD animals and were fed with normal chow diet, they were not treated, except for water and food deprivation, for 18 hours. HFD hamsters were fed with high fat

pellet during the entire 28 days of UCMS protocol. Animal maintenance and experimental procedures were carried out in compliance with ethical provisions for Care and Use of Laboratory Animals reported in the legislative law n°116 (27-01-1992) and authorized by the National Committee of the Italian Ministry of Health. Efforts were made to minimize animal suffering and reduce the number of experiments.

2.2. Experimental procedures

Four of the above animal groups (HFD+probiotics, HFD, UCMS+probiotics, UCMS, HFD+UCMS+probiotics and HFD+UCMS) were exposed to UCMS for 28 days. During the behavioral protocol, HFD group was fed with a high fat concentration (HFD; 60% energy from fat, Envigo Lab. USA) ad libitum during the entire 28 days of UCMS together with a stressful injury [32], while probiotics were added to the water every day. Control of HFD group was fed with a normal chow diet as were controls for probiotic and UCMS treatments. Every evening at the end of behavioral sessions, food consumption and animal body weight were measured. After UCMS protocol, hamsters were subjected to light-dark test (LDT) and elevated plus maze (EPM, on the same day but at different time periods) for 3 consecutive days in morning, noon and afternoon as indicated in a previous study [33]. At the end of LDT and EPM, animals were subjected to conditioned place preference (CPP) and novel object for 7 days before being sacrificed, in which blood and HTH were collected for western blot analysis. Blood was collected immediately using heparin as anticoagulant after the animal was decapitated. Plasma for western blot analysis was obtained by centrifugation (4000 g for 15 min at 4 °C).

2.3. Chronic mild stress protocol

Experimental groups defined as stressed were exposed to a variety of external relevant stressors over a period of 4 weeks. The stressor procedure was performed according to an optimized procedure of UCMS protocol [34] to achieve anxiety symptoms. At the age of 5 months ± 1 week, animals were

ear-punched for identification and assigned to 1 of 2 treatment groups, controls and UCMS. UCMS treatment started at this point and continued during the behavioral testing phase. Care was taken not to apply stressors just before a behavioral test. The initial tests took place 2 weeks after the beginning of the stress procedure. Because of the nature of UCMS procedure, stressed and control animals were kept in separate, but otherwise identical holding rooms. The protocol consisted of a series of mild unpredictable stressors: food deprivation, swimming test, cage tilted, inversion of the light/dark cycle, lights on for a short period of time during the dark phase and switching cages. Effects of all stressors lasted for at least 14 h with the exception of swimming, which was conducted for 5-10 minutes. On the average, two of these stressors were applied daily at different times and following a random 2-weeks schedule. The stress procedure lasted for 4 weeks prior to behavioral tests. Stressors continued to be applied during the testing phase, with at least 6 h of rest being allowed between each testing session. Due to the similar values of controls for the different treatment groups for all behavioral evaluations only one representative control value, which derived from the media of all controls, was used for the different behavioral analyses.

2.4. LDT

At the end of UCMS sessions, hamsters were exposed to LDT apparatus in order to determine the effective anxiety conditions. LDT consisted of a box with two compartments: a first arena composed of a small and dark plastic compartment (16×16×16 cm); a second arena containing a large translucent and white illuminated compartment (25×25×30 cm), which was considered the unfamiliar environment. For the different behavioral evaluations, a camera (Logitech QuickCam Pro5000) was positioned 1 m above the apparatus, connected to a computer so that it was possible to record behaviors during the testing sessions. All observations plus behavioral analysis was conducted in a same manner to that previously described [35].

2.5. EPM test

Together with LDT, animals were also exposed to an EPM apparatus that allowed us to evaluate stress responses in a more specific manner as previously described [33]. EPM is a wooden maze consisting of four arms arranged in a cross-shaped design. The apparatus was elevated to a height of 50 cm above the floor plus being illuminated by white lamps (4x60 W). After UCMS session, hamsters were tested with LDT and with EPM (on the same day but at different time periods) for 3 consecutive days in morning, noon and afternoon as indicated in previous studies [36,37].

2.6. CPP procedure

The apparatus consisted of two Plexiglas chambers divided into two main compartments (60 x 50 x 25 cm) that were separated by a smaller compartment (10 and 20 cm floor size). The smaller compartment had vertical sliding opaque black floor and walls that were coated with clear lacquer. One of the main compartments had a floor with a rough plywood surface, and both the floor and the walls were painted with black paint. The other main compartment had a smooth black plastic floor, and its walls were also painted with black paint. However, at the bottom of its long walls, four white rectangles were painted, each with a height of 4 cm and 10, 5.5, 5.5 and 8 cm in length, respectively (listed in order of proximity to the door). Each rectangle was spaced 2 cm apart. CPP test was performed in a 4.4~2.9 m room with ceiling and walls painted white according to previous works plus modifications [38]. The walls were lit with four reflectors (each facing a different wall) that were placed centrally and 48 cm below the ceiling. Each wall was equipped with an incandescent 40W matte white light bulb. Two CPP apparatuses were used simultaneously and were thoroughly cleaned after each behavioral session. The apparatuses were separated with sound-attenuating wall made of a 2 cm thick particleboard with black veneer on both sides. The sound-attenuating wall was tested to ensure that it blocked vocalizations emitted by hamster in CPP apparatus behind the wall so that other hamsters were not able to detect them. The behaviors in non-preferred and preferred areas were recorded with a high resolution Waterproof Action Camera (DBPOWER- SJ4000 SPORTS HD DV)

that was connected to a PC equipped with the EthoVision XT VideoTracking System v.10 (Noldus Information Technology B.V., Wageningen, The Netherlands). Initial place preference was determined in a 15-minute pretest. Four days later, hamsters were trained for CPP test. On the first day of training, hamsters were instantly confined to the preferred section of the apparatus for a 40 minutes session. On the next day of training, animals were immediately confined to the non-preferred section of the apparatus for another 40 minutes session. The training procedure was repeated for two other consecutive days. Six days after the initial training day, all animals were given access to the open CPP apparatus for 15 min to assess place preference. After the test, animals were given probiotics every day and were then confined to the non-preferred section of the apparatus for another 40-minute session. After the 8th probiotics dose, animals were subjected to a 14-day withdrawal period. Subsequently after the last probiotics dose (17^{th} day), all hamsters were tested in the CPP apparatus to assess the place preference.

2.7. Novel object test

An object recognition test was performed in order to evaluate novelty preferences according to previous works plus modifications [38]. Hamsters were individually habituated to a dim-lit black plexiglass box (20 cm) for 10 minutes on the day before the test. On the first day of testing, a session of two trials was conducted. In particular, hamsters were exposed to two novel yellow plastic cylinders A-A (approximately 8 cm tall, 2 cm in diameter) symmetrically placed 6 cm from the nearest walls. The animals were placed equidistant from the two objects and allowed to explore them for 5 minutes. After 1 h, configuration A–A was changed to configuration A–B so that it was possible to assess short-term memory ability by replacing one of the novel plastic cylinders with a purple plastic figure (approximately 8 cm in height). To minimize potential confusion of spatial memory with respect to object recognition, animals were placed in the same corner as in the previous trial and their behavior performances was recorded for 10 minutes. Time spent exploring familiar (F) and new (N) objects were recorded for 10 minutes and assessed by an independent grader. Discrimination

index was calculated (N − F /N + F) for intergroup comparisons. Exploration behavior was defined as sniffing or touching either object at a distance of <2 cm from the snout, while sitting on the object was not considered exploration. Care was taken to avoid olfactory stimuli by cleaning the apparatus and target objects with 70% alcohol between trials. After 24 hours, animals were tested for long-term memory. The novel object (B) from the short-term memory trial was replaced with a red triangular wooden block (4-8 cm in height) so that the apparatus displayed an A–C configuration. All sessions were video recorded with a camera positioned above arena and analyzed by a blind experimental condition. The same procedure was utilized to record and calculate the discrimination index.

2.8. Western blotting

Hamsters belonging to the above groups were sacrificed after the last behavioral session. Their brains were removed and the HTH was dissected out, homogenized on ice in a lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, plus 1% nonidet P-40] containing a cocktail of proteinase inhibitors (Roche) and a phosphatase inhibitor (Sigma). Samples were centrifuged at 12500 rpm at 4°C for 30 min and the supernatant was collected, stored at -80ºC for future immunoblotting analyses. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein per sample (20 μg) were separated by electrophoresis on 8% and 10% Tris-glycine gels with 4% stacking gels, then they were transferred to PVDF membranes and the membranes were blocked with 5% serum albumin for 1 hr. The primary antibodies anti-IL-1β, Caspasi-1, NF-kB (p50 subunit), NLRP3 and anti-β-actin were used for HTH and plasma (1:1000; Cell Signaling Technology, Danvers, MA). The secondary antibodies (all 1:7000 diluted in blocking solution) were horseradish peroxidase conjugated anti-rabbit IgG (Chemicon International, Inc., Temecula). A horseradish peroxidase chemiluminescence kit with enhanced luminol and oxidizing reagents (Bio-Rad) were used to visualize chemiluminescent signals. These enhanced blot signals were photographed using a CCD camera (Molecular Imager Gel Doc XR System; Bio-Rad) in a darkroom. The volume of the bands (i.e. area-intensity) was quantified using

Quantity One Software (Bio-Rad).

2.9. *Statistical Analysis*

Data expressed as mean ± SEM were evaluated and compared among the different conditions by using a two-way ANOVA followed by adequate *post hoc* test (GraphPad InStat 3.0 for MacIntosh, La Jolla, CA). Statistical significance was considered when $p<0.05$. The number of animals used in the present study was justified using a free online statistical program (http://stat.ubc.ca/~rollin/stats/ssize/n2.html; Department of Statistics of the University of British Columbia-Canada). Efforts were made to reduce the number of animals used where possible.

3. Results

3.1. Anxiolytic-like behaviors induced by probiotics treatment

Animals that received a probiotics treatment exhibited anxiolytic-like behaviors. In particular, such probiotics exerted moderate ($p<0.05$) locomotor activities as indicated by HFD+probiotics-, UCMS+probiotics- and HFD+UCMS+probiotics-treated hamsters having spent from a moderate (60%, 34%) to a greater (75%; $p<0.01$) amount of time in open arms, respectively, when compared to their corresponding treatment without probiotics (Fig. 1A, D, G). Contextually, this trend was still conserved for open arms entries in which notably increased movements were detected in HFD+probiotics (75%) and UCMS+probiotics (79%) with respect to HFD and UCMS without probiotics (Fig. 1B, E). It was worthy to note that the difference between all groups considered for time spent in open arms and for open arms entries, was of a very notable entity ($p<0.001$) with respect to that of the control group (Fig. 1AB, DE, GH). On the other hand, HFD and UCMS appeared to enhance locomotor activities as displayed by HFD, UCMS and HFD+UCMS hamsters having spent from a very notable (90%), to a notable (69%) and moderate (38%) amount of time moving from open to closed arms, respectively, when compared to their control (Fig. 1C, F, I). Even in this case, treatment with probiotics exerted anxiolytic effects especially for HFD and UCMS hamsters, as

indicated by a notable (-61%) and very notable (-94%) decrease in locomotor activity being detected, respectively, (Fig. 1C, F). Interestingly, very evident exploration activities appeared to be induced by HFD+UCMS ($p<0.001$) during light/dark test, despite HFD and UCMS alone displayed a moderate (40%) and greater (69%) amount of time, respectively, in the dark room (Fig. 1L, M, N). Such probiotics-dependent permanence in the dark room turned out to be notably decreased in all groups when compared to HFD, UCMS and HFD+UCMS (Fig. 1L, M, N) groups.

- please insert Figure 1 here -

3.2. Novel Object Recognition and CPP

For the short-term memory test, HFD, UCMS and HFD+UCMS hamsters showed a significantly lower discrimination index compared to the control (check *) and to the same groups with probiotics (Fig. 2A, E, I; check letters). Both HFD ($p<0.001$) and HFD+UCMS ($p<0.01$) groups explored familiar object at a significantly greater frequency than the novel object as compared to groups treated with probiotics (Fig. 2B, L). For long-term memory test, the discrimination index (Fig. 2C, G, M) of HFD, UCMS and HFD+UCMS hamsters proved to be the same with respect to that of short-term memory**.** Indeed, hamsters treated with probiotics explored the novel object significantly longer than the familiar object, while surprisingly for this task, HFD and UCMS hamsters explored the novel object in the same manner (Fig. 2D, H, N). For CPP procedures, all hamster groups treated with probiotics spent significantly more time in the grid floor than HFD (91%, Fig. 2O), UCMS (78%, Fig. 2P), HFD+UCMS (92%, Fig. 2Q) with respect to their corresponding controls (90%, 99%, 95%).

- please insert Figure 2 here -

3.3. Food and body weight regulated by probiotics

Treatment of the probiotics mix in HFD, and HFD+UCMS but not in the UCMS proved to notably modify food and body weight of the hamster. HFD hamsters treated with probiotics consumed notably ($p<0.01$) elevated quantities of food (78%) with respect to controls (Fig. 3A; check *). This activity was further strengthened by a moderate increase in body weight (30%) with respect to control but rather turned out to be of a greatly decreased body weight (-73%) compared with HFD (Fig. 3A;

check letters). Similar quantities of food consumed were reported for HFD+UCMS states, despite these probiotics mix accounted for a moderate reduction of food consumption (-49%) when compared to HFD+UCMS (Fig. 3C). Even body weight variations reflected substantial feeding changes as displayed by a notable decrease in body weight (-72%) in animals treated with probiotics mix with respect to HFD+UCMS group (Fig. 3C).

- please insert Figure 3 here -

3.4. IL-1β, Caspase-1, NLRP3 and NF-kB signaling in HTH

In order to further delineate the downstream signaling involved with neuroinflammatory effects of the different groups, protein expression differences of IL-1β, Caspase-1, NLRP3 and NF-kB in the HTH of HFD, UCMS plus HFD+UCMS with and without probiotics, demonstrated overlapping protein expression differences. In the case of IL-1β protein, this cytokine resulted to be extremely increased in HTH of the HFD (91%) and HFD+UCMS (94%) without probiotics with respect to controls (Fig. 4A,C) while it extremely decreased in HFD (-89%) along with a moderate reduction in HFD+UCMS (-48%) with probiotics compared with HFD and HFD+UCMS alone (Fig. 4A,C). Such a trend was also observed for the expression of Caspase-1 and NLRP3 but not for NF-kB in which only moderately increased levels were reported for these anti-inflammatory elements in HFD (40%) and HFD+UCMS (36%) without probiotics with respect to controls (Fig. 4L,N). In addition, Caspase-1 resulted to be extremely greatly and moderately ($p<0.05$) increased in all groups with respect to controls, while, extremely notable and notable reductions were instead observed in hamsters treated with probiotics when compared to HFD (-92%), UCMS (-67%) and HFD+UCMS (-74%; Fig 4D-F). Even in this case increased levels of NLRP3 were reduced following treatment with probiotics when compared with HFD, UCMS and HFD+UCMS (Fig. 4G-I) groups.

- please insert Figure 4 here -

3.5. IL-1β, Caspase-1, NLRP3 and NF-kB signaling in blood

It was worthy to note that hematic protein differences of the above neuroinflammatory factors in the same groups ± probiotics supplied comparable trends. In the case of IL-1β protein, notable (76-79%)

increases were estimated in HFD, and in HFD+UCMS (79%) whereas moderate values were reported for UCMS (42%) with respect to controls. Conversely, when HFD and UCMS hamsters were compared with the same groups plus probiotics treatment, only moderately decreased levels were observed in UCMS with probiotics (-34%) compared with UCMS groups without probiotics (Fig. 5 A-C). This same trend was also observed for Caspase-1 levels in which very great increases (87%) in HFD+UCMS while only notable increases was typical of UCMS (79%) and HFD (62%) with respect to controls (Fig. 5D-F). Moreover, animals treated with probiotics displayed a decreased expression of Caspase-1 in particular in HFD+UCMS with probiotics (-75%) when compared with HFD+UCMS without probiotics (Fig. 5D-F). Interestingly, NLRP3 but not NF-kB hematic levels displayed very notable (-94%), notable (-76%) and moderate (-49%) decreases after probiotics treatment in HFD, UCMS and HFD+UCMS, respectively, when compared with the same groups without probiotics (Fig. 5 G-I).

- please insert Figure 5 here -

4. Discussion

The results of the present study pointed to probiotics treatment exerting a very strong reducing influence on stress-like behaviors in the different mazes in which they were evaluated. It is widely known that neuroinflammatory-related anxiety states are associated with environmental, genetic and psychological factors and so it is of no surprise to observe an obesity and chronic inflammation state in HFD animals. In this context, gut microbiota has shown to mediate many effects of HFD state as indicated by epidemiological data supporting the great risks of developing mood disorders and anxiety states, which represents the most prevalent link with depression in obesity patients [39]. Interestingly, the relationship between obesity and anxiety/depression is bi-directional since individuals with anxiety and depression have a 50% higher risk of developing obesity and, conversely, people with obesity have an increased risk of developing depressive symptoms and manic episodes [40]. Despite the introduction of new drugs for anxiety states, many obese patients treated

for depression respond poorly to therapy, suggesting that obesity states may interfere with the efficacy of the pharmacological treatment [41]. Our results represent a good start for proposing the use of probiotics as valuable therapeutic prospects for treating mood disorders-related obesity conditions. The application of such pharmacological preparations has shown to be responsible for diminished anxiety states together with body weight in hamsters thereby sustaining the key role played by gut microbiota on the regulation of brain homeostatic conditions as shown recently in other works [42,43].

At the cognitive level, novel object test performances clearly pointed to HFD as a major cause of reduced exploratory bouts in hamsters to explore new objects as suggested. This relationship is strongly supported in HFD hamsters associated with anxiety states and above all accentuated cognitive impairments due to a reduced attraction towards novel objects [38]. Hence it should not be of any surprise if CPP like that of novel object supplied a diminished preference score in HFD hamsters, since obesogenic conditions by influencing the preference for the chamber lacking food lead to decreased mnemonic abilities and thus animals were unable to remember the rewarding event [38]. As far as CPP and novel object discrimination tests, it seems that probiotic treatments in all groups were able to invert the negative effects typical of anxiety states and HFD in hamsters. Such indications are in good agreement with others that demonstrated the effect of probiotics interfering with anxiety states but not on body weight [8,42] and this emphasizes a direct relationship between gut microbiota and brain neuronal activities [44].

Recent studies indicate that chronic inflammation caused by HFD may play a major role in the induction of anxiety states through the variations of neuroinflammatory agents. Treatment of our animal with probiotics reduced, not only hematic circulating levels of Caspase-1, IL-1β, NF-kB and NLRP3 but also the expression of these neuroinflammatory factors in HTH. In this context it appears as if obesity, which is associated with chronic low-grade peripheral inflammation [45], alters normal body states and above all encephalic neuronal circuits that are involved with stressful behavioral conditions. This association was previously reported in relation with elevated levels of the pro-

inflammatory cytokine TNF-α in the blood and adipose tissues of obese individuals [46]. In this case, treatment with probiotics tend to reduce inflammatory conditions through the synthesis of cerebral anti-inflammatory factors, which is line with increasing levels of fat mass enhancing the secretion of pro-inflammatory cytokines (or adipokines) such as TNF-α and IL-6 from adipocytes [47]. One evident cause of inflammation in HFD is the diet itself. Indeed, a very short term HFD appeared to be capable of evoking hypothalamic neuroinflammatory conditions in the absence of obesity-dependent features [48]. In this regard other studies have shown that increased fatty acid intake induces the activation of immune cells and consequently the onset of a series of inflammatory responses in many organs including adipose tissue, liver, pancreas, and muscle [1]. On the contrary, adipocytes of lean individuals secrete higher amounts of anti-inflammatory adipokines such as adiponectin, which increases insulin sensitivity and protects against type 2 diabetes mellitus and cardiovascular diseases [49]. It is known that fatty acids are able to activate the innate immune system through Toll-like receptors (TLRs) [50]. For instance, binding of fatty acids to TLR4 activates two different transcription factors (NF-kB and activator protein 1, AP-1) that in turn up-regulate the expression of pro-inflammatory mediators such as cytokines IL-1β, IL-6, and TNF-α at both the brain level as well as peripheral organs [51,52,53]. However, the most important mechanism for obesity-induced inflammation relies on the ability of a HFD to modulate the gut microbiota [54,55].

5. Conclusion

Overall, results of the present work point to a switching on/off mechanism of microbiota activation as key factors operating towards the regulation of pro-inflammatory molecules in the brain and in the blood. In particular treatment of HFD hamsters with probiotics promptly reduced inflammatory factors in both the HTH and in the blood, which were tightly linked to reduced stressful-related anxiety plus mood disorders. In previous works high levels of IL-1β together with NF-kB, Caspase-1 and NLRP3 in HFD exposed to stressful injuries, tend to alter the equilibrium of gut microbiota with consequently variations in HTH homeostasis [56,57]. Moreover, elevated levels of pro-

inflammatory factors, seem to favorably maintain evident anxiety conditions plus obesity. Nonetheless neuronal mechanisms involved with low inflammation and gut/brain microbiota connection still remain to be elucidated, our data might pave the way for novel therapeutic alternatives consisting in probiotics administration as a potentially valuable treatment strategy in patients with metabolic disease and mood disorders.

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Figure Legends

Figure 1. Effects of probiotics on anxiety-like behaviors of hamsters

Effects of treatments (n=6/treatment groups) on anxiety-like behaviors were checked in animals receiving HFD+probiotics, HFD, UCMS+probiotics, UCMS, UCMS+HFD+probiotics, UCMS+HFD with respect to unstressed controls (*) during EPM (A-I) plus LDT (L-N). Animals treated with probiotics received a single day dose directly dissolved in water (3 g/200 ml to each hamster per day). Each bar represents % change (± S.E.M) with respect to unstressed controls of time spent in open arms (A, D, G), open arms entry (B, E, H), back- and forward locomotor activities (C, F, I) along with time spent in the dark box (L, M, N) by treated hamsters. Behavioral changes were determined by ANOVA plus a *post hoc* Newman–Keuls test when $*^{a}p<0.05$, $**,^{b}p<0.01$ and $***,^{c}p<0.001$.

Figure 2. Effects of probiotics on memory performances of hamsters.

Effects of treatments n=6/treatment groups) on memory performances were evaluated in animals receiving HFD+probiotics, HFD, UCMS+probiotics, UCMS, UCMS+HFD+probiotics, UCMS+ HFD with respect to unstressed control (*) during Novel Object (A-N) plus Conditioned Place Preference Tests (O-Q). Animals treated with probiotics received a single day dose directly dissolved in water (3 g/200 ml to each hamster per day). Each bar represents % mean ± S.E.M. of discrimination index (A, C, E, G, I, M) and object exploration time (B, D, F, H, L, N; short and long term memory) along with mean time on grid floor box (O, P, Q). Behavioral changes were determined by ANOVA plus a *post hoc* Newman–Keuls test when $p<0.05$, $*,^{a}p<0.05$, $**,^{b}p<0.01$ and $***,^{c}p<0.001$.

Figure 3. Effects of probiotics on food intake and body weight. Effects of treatments n=6/treatment groups) on food intake and body weight were checked in hamsters receiving HFD+probiotics, HFD, UCMS+probiotics, UCMS, UCMS+HFD+probiotics, UCMS+HFD with respect to unstressed control. Even for this part, hamsters received a single day treatment of probiotics (3 g/200 ml to each hamster per day). Each bar represents % mean ± S.E.M. of food intake and body weight variations with respect to controls. Differences were evaluated by ANOVA plus a *post hoc* Newman–Keuls test, $*,^{a}p<0.05$, $**,^{b}p<0.01$ and $***,^{c}p<0.001$.

Figure 4. Effects of probiotics on hypothalamic expression of IL-1β, Caspase-1, NLRP3 and NF-kB. Immunoblotting was used to detect IL-1β (A, B, C), Caspase-1 (D, E, F), NLRP3 (G, H, I) and NF-kB (L, M, N) expression in the HTH of HFD+probiotics, HFD, UCMS+probiotics, UCMS, UCMS+HFD+probiotics, UCMSd+HFD with respect to unstressed controls. Quantification of immunoblotting results was expressed as ratio of IL-1β/β–Actin, Caspase-1/β–Actin, NLRP3/β–Actin and NF-kB/β–Actin ± s.e.m.. Immunoblotting differences in animal groups (n=6/group) were evaluated using a two-way ANOVA followed by a *post hoc* Newman-Keuls multiple range test when *p*-value $*,^{a}p<0.05$, $**,^{b}p<0.01$ and $***,^{c}p<0.001$.

Figure 5. Effects of probiotics on blood expression of IL-1β, Caspase-1, NLRP3 and NF-kB. Immunoblotting was used to detect IL-1β (A, B, C), Caspase-1 (D, E, F), NLRP3 (G, H, I) and NF-kB (L, M, N) expression in the plasma of HFD+probiotics, HFD, UCMS+probiotics, UCMS, UCMS+HFD+probiotics, UCMS+HFD with respect to unstressed controls. Quantification of immunoblotting results was expressed as ratio of IL-1β/β–Actin, Caspase-1/β–Actin, NLRP3/β–Actin and NF-kB/β–Actin ± s.e.m.). Immunoblotting differences in animal groups (n=6/group) were evaluated using a two-way ANOVA followed by a *post hoc* Newman-Keuls multiple range test when *p*-value *,[a]p<0.05, **,[b]p<0.01 and ***,[c]p<0.001.

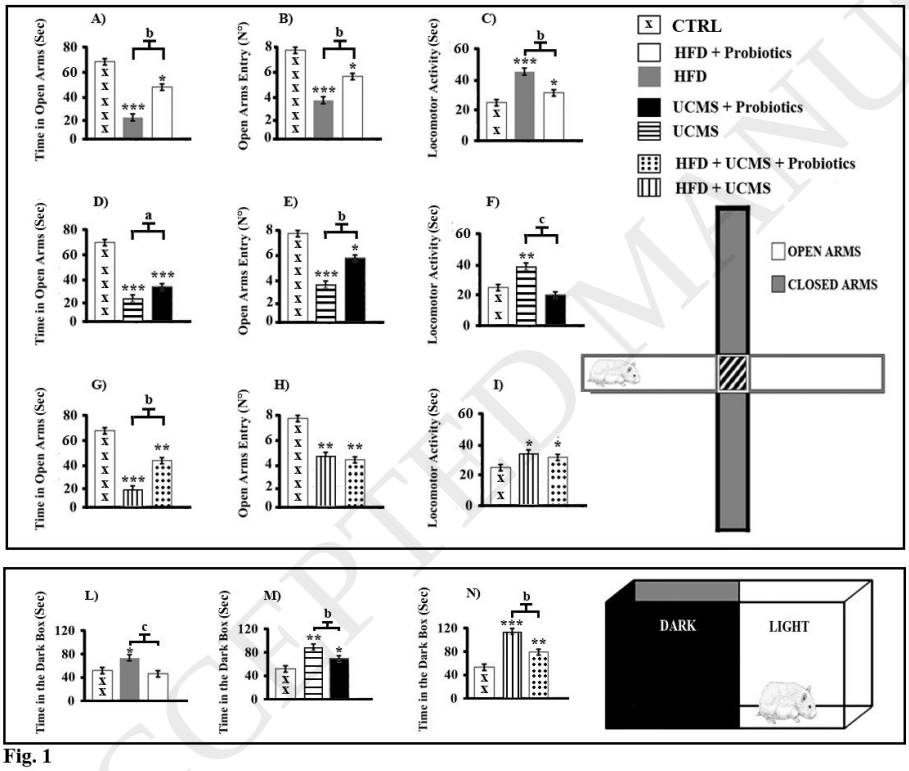

Fig. 1

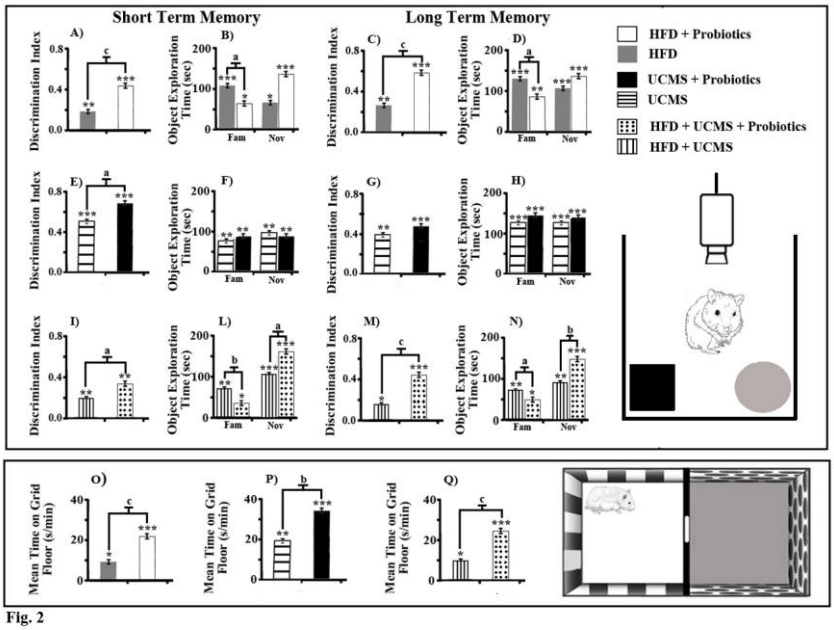

Fig. 2

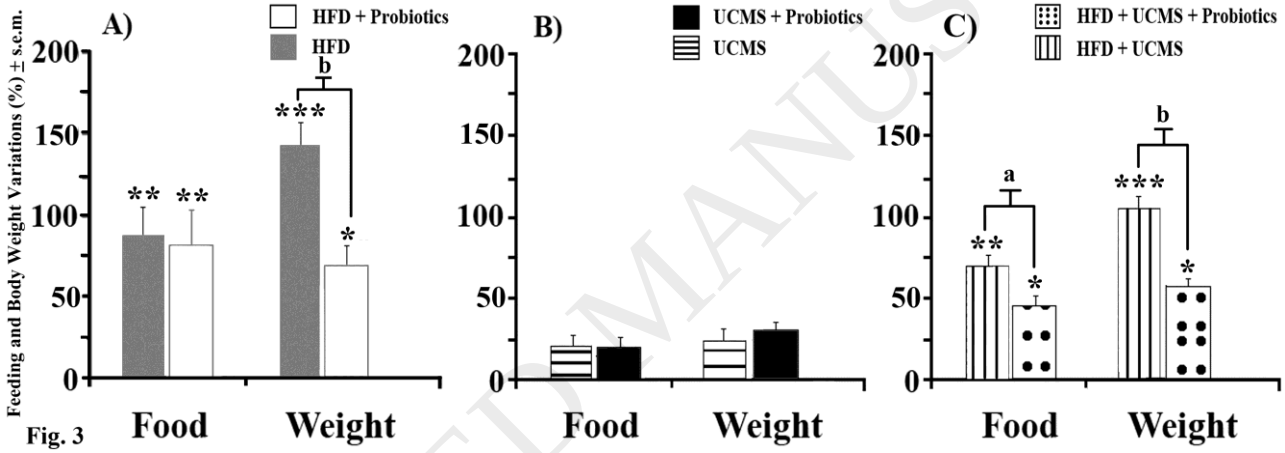

Fig. 3

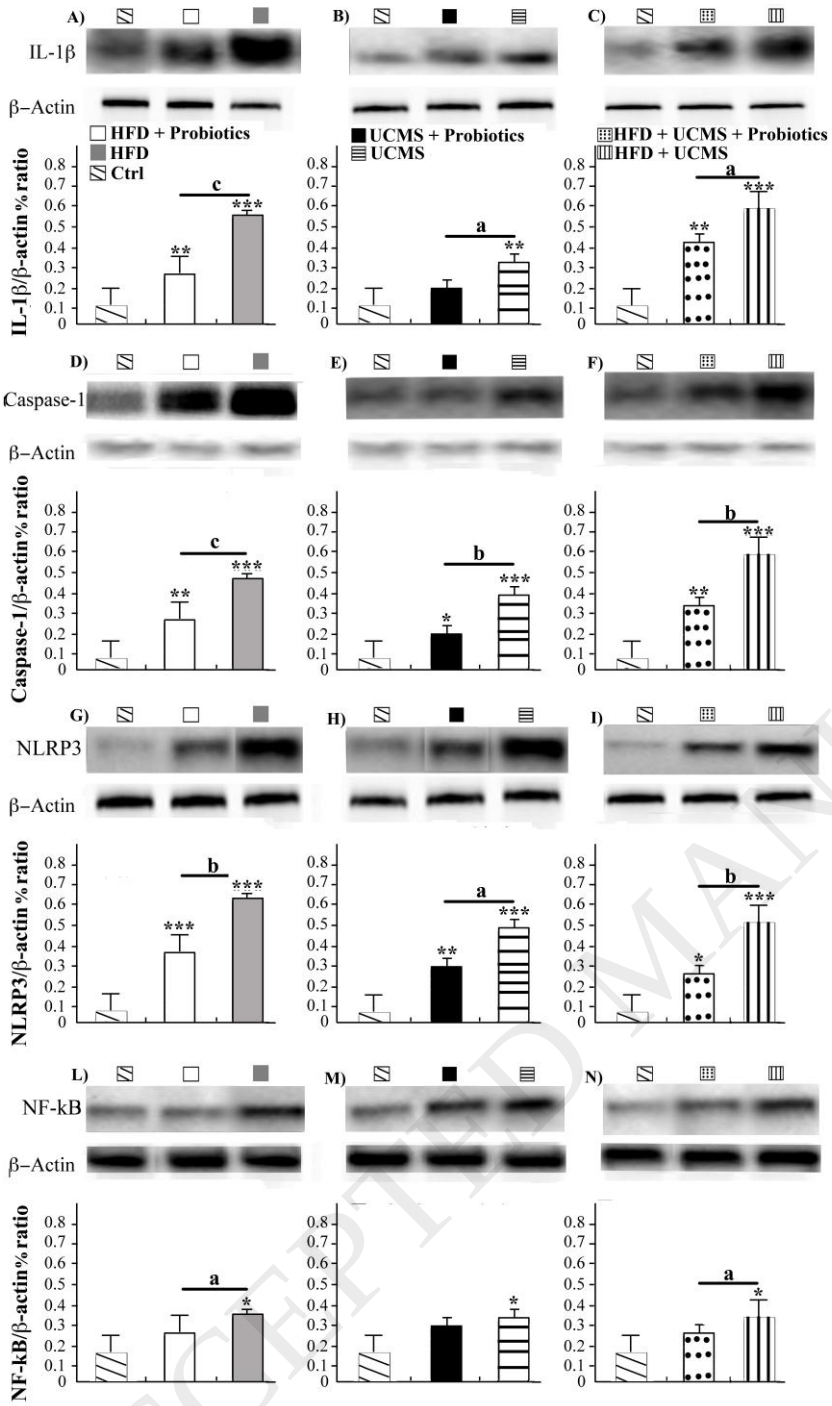

Fig. 4

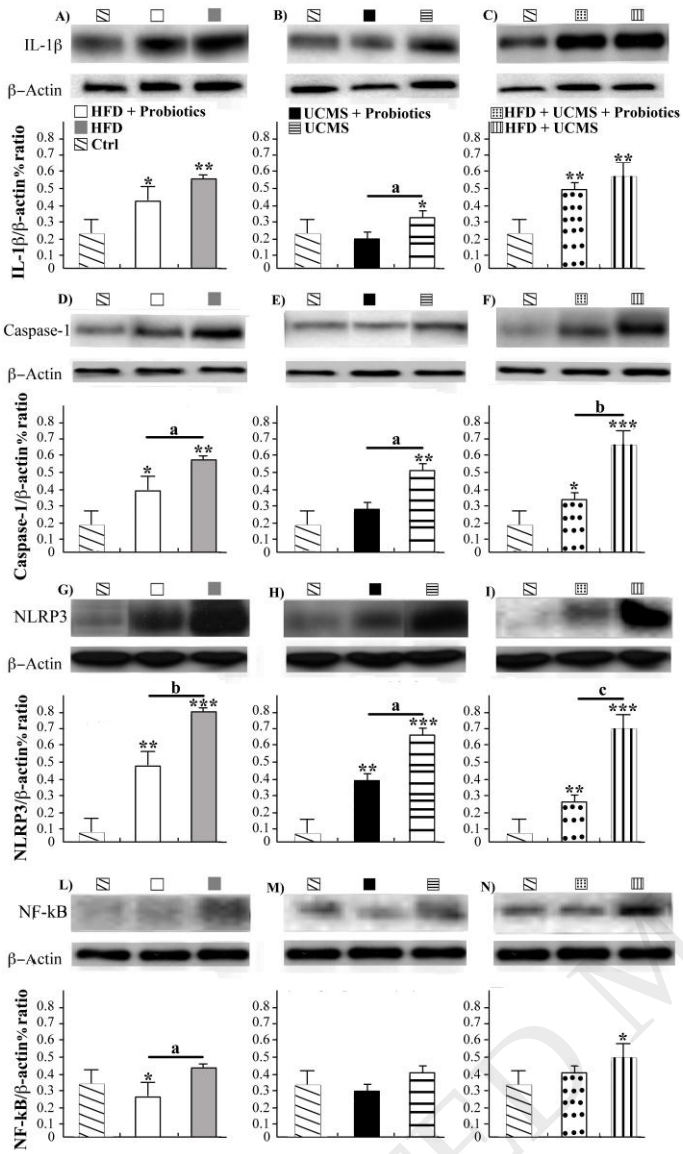

Fig. 5