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The effect of long-term lactobacilli (lactic acid bacteria) enteral treatment on the central nervous system of growing rats

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Abstract

The aim of this study was to explore the relationship between consumption of large doses of lactic acid bacteria (LAB) and the behaviour and brain morphobiochemistry of normal growing rats. Four groups of rats were treated with LAB cultures twice daily for 6 months. The control group received 1 ml of saline per treatment, while two experimental groups received 1 ml of living bacteria (*Lactobacillus plantarum* and *Lactobacillus fermentum*, respectively) and the remaining group received a heat-treated (inactivated) *L. fermentum* culture. After 2 and 6 months of treatment, respectively, eight animals from each group were sacrificed, and specimens were taken for further analyses. The behaviour of the rats was evaluated five times in an open-field test at monthly intervals throughout the study. Lactobacilli treatment for 2 months induced changes in the motoric behaviour of the rats. The concentration of the astrocytesoluble and filament glial fibrillary acidic protein (GFAP) decreased in the posterior part of the hemispheres, including the thalamus, hippocampus and cortex of the rats treated with *L. fermentum*. A greater decrease in filament GFAP (up to 50%) was shown in the group receiving the live form of *L. fermentum*. In contrast, the GFAP in the live *L. plantarum*-treated group increased, showing elevated levels of the soluble and filament forms of GFAP in the posterior part of the hemispheres.

A 60–66% decrease in the amount of the astrocyte-specific Ca-binding protein S-100b was shown in the posterior parts of the hemispheres and in the hindbrain of rats given LAB for 2 months.

Prolonged feeding with LAB for 4 months up to full adulthood led to a further decrease in astrocyte reaction, reflected as an additional decrease in the amount of soluble GFAP and locomotor activity in all experimental groups. The changes in filament GFAP and S-100b appeared to disappear after prolonged feeding (total of 6 months) with LAB.

In summary, LAB dietary treatment affected the ontogenetic development of the astrocytes, with the highest intensity observed in the early stages of rat development. It can be postulated that LAB treatment may play a preventive role in neurological diseases by decreasing astrocyte reaction and, consequently, lowering locomotor activity.

Keywords: Lactic acid bacteria (LAB); Behaviour; Brain; N-CAM; GFAP; S-100b

1. Introduction

Lactic acid bacteria (LAB) are important members of the microflora in the gastrointestinal tract. The interactions in the gastrointestinal microflora community are poorly understood; however, these interactions, especially those of the microflora and host, play a key role in human and animal health. These bacteria are part of a complex balanced ecosystem in the gastrointestinal tract that includes a few hundred species belonging to over 200 genera [1]. The inhibitory effect of lactobacilli on other microorganisms, including pathogens, is well known [2]. Moreover, lactobacilli are involved in the protection of the intestinal barrier

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defence system [3], production of short-chain fatty acids [4], vitamin synthesis [5], reduction of the levels of neurotoxic compounds such as ammonia and indoles [6], and stimulation of the immune system [7]. However, the mode of action of lactobacilli at the cellular level is only beginning to be studied. It has been recently shown that extracts of LAB stimulate nitric oxide [8] and cytokine production [9]. While lactobacilli form a normal part of the diet and indeed are necessary for gastrointestinal health, supplementary LAB are often ingested as probiotics to aid in the maintenance of health and to reduce gastrointestinal problems. However, little is known about their actual effects on a normal system [especially on the central nervous system (CNS)] or on normal growing animals/humans.

The nervous system (enteric and central) influences all gastrointestinal functions. There is some information available describing the effect of gut bacteria on the enteric nervous system [10], but there are no available data about the effect of gut bacteria and probiotics on the neurons or glia of the CNS.

In the CNS, neural cell adhesion molecules (N-CAM) are well known for their involvement in many critical events of the nervous system: cell migration, differentiation [11], synaptogenesis [12] and synapse transmission [13]. The last decade of research has challenged the traditional view of N-CAM as being homophilic cell adhesion molecules that only regulate cell attachment and has suggested the existence of much wider N-CAM functions. These functions included acting as a signalling receptor that provides both homophilic and heterophilic contacts and plays a regulatory role in transcriptional activation, cytoskeletal dynamics, neurite outgrowth and memory formation [14]. The three major isoforms of N-CAM (N-CAM-120, cytoplasmic form; N-CAM-140 and N-CAM-180, transmembrane forms) are products of alternative exon usage downstream of a single gene.

The glial reaction to nervous system damage, often termed gliosis, represents a hallmark for all types of nervous system injuries. Astrogliosis is accompanied by an increased expression of intermediary filament protein. Glial fibrillary acidic protein (GFAP) is found only in the astroglial cells of the CNS [15] and is a monomeric molecule of 40–53 kDa representing the major part of the astrocyte cytoskeleton. It polymerises spontaneously to fibrils with a length of 0.8–1.06 μm under physiological conditions. It is well known that astroglial GFAP can serve as a most useful biomarker of neurotoxicity.

Much data indicate that neurotoxicity or brain damage also induces elevation of the S-100b protein [16]. Since ischemia induces an increase in the protein S-100b in the absence of signs of brain cell death and probably reflects a specific cell response to ischemia, Buyukuysal [17] has proposed that the protein S-100b could be a more valuable biological marker of pathogenicity than lactate dehydrogenase. S-100 protein is a calcium-binding protein found predominantly in the cytosol of glial cells in all parts of the CNS. Three different subtypes (S-100a, S-100b and S-100a0) are known, and S-100b is specific for astrocytes [18]. An increase in the concentration of S-100 in the CSF and blood has been found to be a sensitive indicator of nervous system damage due to various neurological disorders [19], CNS tumours [20], acute ischemic stroke [21], multiple sclerosis [22] and postoperative pain [23].

The main aim of the present study was to investigate the effect of long-term exposure of the gut to enteral LAB diet supplementation on CNS function and morphology in a normal growing animal model. The behaviour of the normal growing rat and the level of some major proteins reflecting the adhesion capacity of nerve cells, astrocyte cytoskeleton plasticity, calcium-binding capacity in the brain and the morphology of the brain have been studied after long-term supplementation with LAB.

2. Materials and methods

Male rats (*Rattus norvegicus*) of the Sprague–Dawley stock (Mol:SPRD Han; Taconic M&B A/S, Ry, Denmark) weighing 100 g each were used in this experiment. The animals were housed in a room in separate animal quarters maintained at 20±1°C and illuminated for 12 h (0700–1900 h). Water and standard feed were freely available. All treatments and experiments were conducted in accordance with the European Community regulations concerning the protection of experimental animals, and the Lund University Ethical Review Committee on Animal Experiments approved the study.

2.1. Animal model

The rats were adapted to the environment for 2 weeks and then divided into four groups (n=16 per group): Group I — control group, treated with 0.9% NaCl; Group II — group treated with inactivated *Lactobacillus fermentum*; Group III — group treated with live *L. fermentum*; Group IV — group treated with live *Lactobacillus plantarum*. All treated animals were given the test solutions intragastrically using a feeding tube (0.96 mm OD; PE_{50}; Becton Dickinson, Sparks, MD) every day for a 2-month period and every other day for the subsequent 4 months. The total daily volume given per animal was 2 ml (1 ml in the morning and 1 ml in the evening), containing 10^9 cfu/ml LAB resuspended in saline.

2.2. Open-field exploratory behaviour

The body weight of the rats was checked every 12–14 days.

The activity of the rats was tested in the middle of the day in an open field at 1, 2, 3, 4 and 6 months after treatment, respectively, using a white plastic box (100×100×40 cm^3) where the bottom of the box was divided into 25 squares of the same size, marked with black lines [24]. The number of lines crossed by the rat during the 3-min test time was registered to assess horizontal activity.

The number of times they reared up on their hind legs with their forepaws pressed against the walls or the number of times they carried out this activity in the central area of the box characterised vertical activity and was noted as such. In addition, activities such as self-grooming, urination and defecation were noted and studied. This test was carried out with the observer present in the room. All behaviour investigations were performed under normal light and temperature conditions for 3 min per rat, starting from the positioning of the animal in the central square. The same test was repeated on the following day, and the mean value for each parameter was recalculated for each animal.

2.3. Brain samples and measurements

Each treatment group was divided into two subgroups. After 2 months of LAB treatment, the rats in one subgroup were decapitated under isoflurane anaesthesia, and the remaining subgroup was sacrificed after 6 months. The brain was quickly removed and divided sagittally into two halves. One part of the brain was fixed in Bouin solution for morphological and immunohistochemical investigations, and the second part was divided into three sections: Section 1 — the anterior part of the hemisphere (motor and somatosensory regions); Section 2 — the posterior part of the hemisphere (thalamus, hypothalamus, hippocampus and cortex); and Section 3 — the hindbrain, including the cerebellum. The tissues were dissected and homogenised in 10 vol of cold buffer containing 25 mM Tris–HCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.2 mM phenylmethanesulphonylfluoride and 0.01% merthiolate (pH 7.4). The suspensions were centrifuged at 100,000×*g* for 60 min, and the supernatants containing the water-soluble and cytosol proteins were collected. After the pellets had been washed, they were extracted with the same buffer by adding 2% Triton X-100 for membrane protein extraction or 4 M urea for cytoskeletal protein extraction and centrifuged at 100,000×*g* for 60 min. All procedures were conducted at 4°C.

Specific protein quantification was performed by a competitive ELISA procedure using polyclonal rabbit antiserum against rat brain N-CAM, S-100b (Sigma) and GFAP (Sigma), respectively. Polyclonal antibodies against N-CAM were produced in our laboratory as described in detail previously [25,26]. The results were expressed as micrograms of specific protein per milligram of total protein. The total protein content was determined according to Bradford [27], with bovine serum albumin as standard.

Fixed brain samples were embedded in paraffin in accordance with standard histological techniques, cut into 7-μm-thick sections and processed for histological and immunochemical studies.

2.4. Statistical analyses

The results were expressed as mean±S.E.M. For statistical evaluation of the data, Student's *t* test and Mann–Whitney test (SigmaStat 2.0 programs) were used to compare the variables between the groups to determine whether they were homogeneous or not. $P<.05$ was established as the significance level. Two-tailed power analysis was performed on all end-point variables. Different letters or stars were used for marking significant differences between groups, as indicated.

3. Results

The control animals and those receiving LAB were weighed every 12–14 days. As shown in Fig. 1, the LAB treatments did not significantly affect body weight. The increase in body mass began slowly in all groups during the 6-month period. Treatment with inactivated *L. fermentum* and live *L. plantarum* led to a slight increase in body weight (103% and 107%, respectively) as compared to that of control rats during the period from 3 to 6 months.

Evaluation of intestinal permeability in vitro using Ussing chambers did not show any changes in the macromolecular permeability of the gut; thus, there was neither transfer of LAB over the intestine in the treated animals nor changes due to their presence. The hematoxylin/eosin-stained histological samples, taken from the middle part of both the proximal and the distal small intestines, have not shown any differences in villi or crypt morphology for all rats after 2 months of LAB treatment from those of controls.

3.1. Open-field behaviour test

In all groups, the locomotor activity of the growing rats — expressed as the number of crossed squares (Fig. 2A) — decreased with time. After 3 months of treatment, the treated groups showed a decrease in activity with respect to that of the control group, with the greatest decrease being found for the group receiving live *L. fermentum* (Group III). Activity

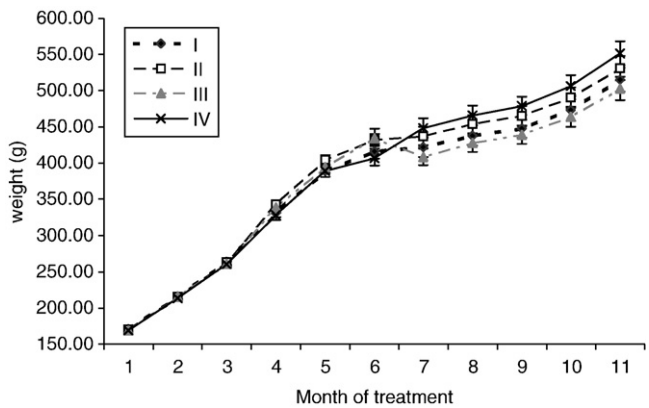

Fig. 1. Growth curve of experimental rats during LAB supplementation. Group I, control; Group II, inactivated *L. fermentum*; Group III, live *L. fermentum*; Group IV, live *L. plantarum*. Each group was fed a saline solution (2 ml/day) containing 10^9 cfu/ml of a LAB strain via a stomach tube, where controls only received saline. n=16 per group for the first 2 months; n=8 per group for the subsequent 4 months. Results are presented as mean±S.E.M.

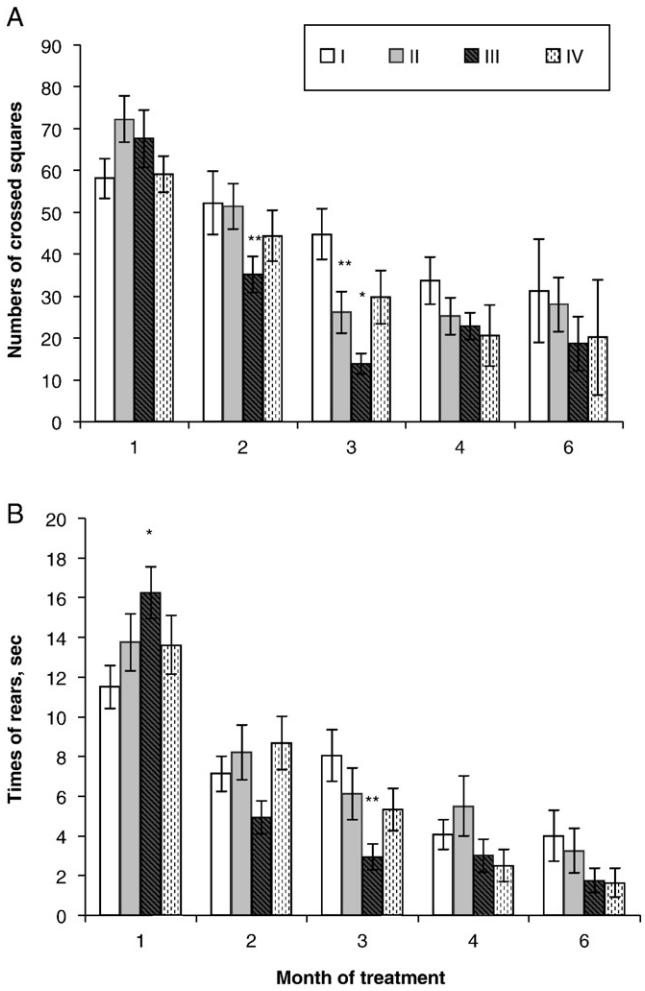

Fig. 2. The horizontal (A) and vertical (B) motoric activities of the rats during 6 months of experimentation. Group I, control; Group II, inactivated *L. fermentum*; Group III, live *L. fermentum*; Group IV, live *L. plantarum* (Groups II–IV: groups fed 2 ml/day saline solution containing 10^9 cfu/ml LAB via a stomach tube). n=16 per group for the first 2 months; n=8 per group for the subsequent 4 months. Results are presented as mean±S.E.M., where statistically significant differences from controls were indicated as *P<.001 and **P<.05.

— expressed as the number of times the animals reared up on their hind legs (Fig. 2B) — showed an increase only after the first month of treatment for Group III, but then decreased somewhat with time, presenting a pattern slightly different from that found for the locomotor activity in these animals. After supplementation for 6 months, however, these activities were lowest in the groups receiving the live bacteria (Groups III and IV).

3.2. Distribution of neuron and astroglia-specific proteins

The distribution of the soluble and membrane N-CAM within the different regions of the brains of control and LAB-treated rats after 2 months of treatment are presented in Fig. 3. In controls, the highest level (19.44±0.81 μg/mg total protein) of membrane N-CAM was found in the anterior part of the hemisphere (motor and somatosensory cortices) and in the hindbrain, including the cerebellum (12.7±1.0 μg/mg total protein). The concentrations of the soluble form of N-CAM varied in the range of 1.1–1.6 μg/mg total protein. No statistically significant differences, however, were found between the observations for the control group and the observations for the treatment groups. There were no differences in the levels of N-CAM molecules between the rats treated with LAB for 2 months and the rats treated with LAB for 6 months (data not shown).

The contents of the astrocyte-specific protein GFAP and the Ca^{2+}-binding protein S-100b in the anterior part of the hemisphere (including the motor and somatosensory cortices of all treated animals), and of GFAP in the hindbrain of rats receiving either heat-inactivated or live *L. fermentum* did not change after 2 months of treatment. The concentrations of soluble and filament GFAP were significantly decreased in the posterior part of the hemisphere, including the thalamus, hippocampus and cortex of the rats given *L. fermentum* (Fig. 4A), in comparison to those of the control rats. The greatest decrease in filament GFAP (up to 50%) was found for the group given the live form of *L. fermentum* (Fig. 4B). The astrocyte reaction to *L. plantarum*, however, was the

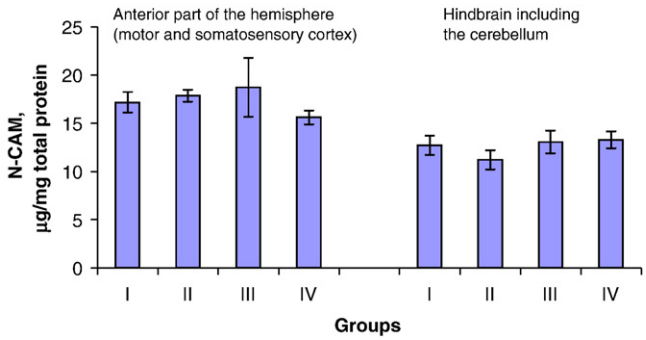

Fig. 3. Contents of N-CAM in the different rat brain regions after 2 months of LAB supplementation. Group I, control; Groups II–IV, treated with LAB (2 ml/day of 10^9 cfu/ml bacteria in saline via a stomach tube) for 2 months; Group II, inactivated *L. fermentum*; Group III, live *L. fermentum*; Group IV, live *L. plantarum* (n=8). Results are presented as mean±S.E.M.

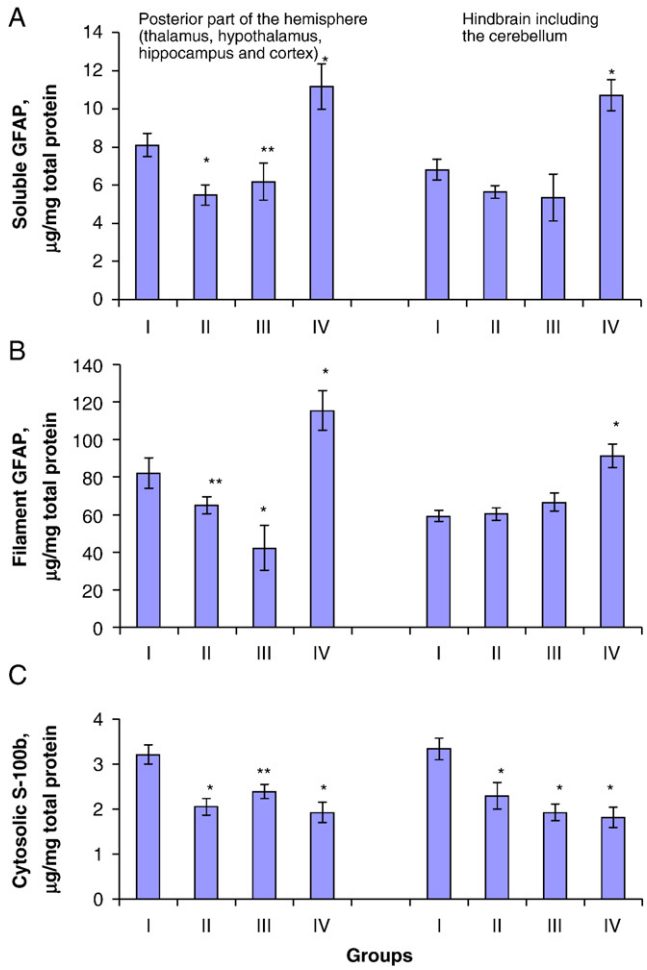

Fig. 4. Contents of soluble GFAP (A), filament GFAP (B) and cytosolic S-100b (C) proteins in the different rat brain regions after 2 months of LAB supplementation. Group I, control; Groups II–IV, treated with LAB (2 ml/day bacterial culture containing 10^9 cfu/ml; fed via a stomach tube) for 2 months; Group II, inactivated *L. fermentum*; Group III, live *L. fermentum*; Group IV, live *L. plantarum* (n=8). Results are presented as mean±S.E.M., where statistically significant differences from controls were indicated as *P<.001 and **P<.05.

reverse of that for *L. fermentum*. An elevated level of the soluble and filament forms of GFAP was detected in the posterior part of the hemisphere of *L. plantarum* rats.

A significant decrease (about 60–66%) in the amount of the astrocyte-specific Ca-binding protein S-100b was found in the posterior part of the hemisphere and hindbrain of rats fed the LAB for 2 months compared to that of the control group (Fig. 4C). No difference between the treatment groups was found.

The distribution of astrocyte proteins was down-regulated to normal conditions by 6 months of treatment. However, the level of the soluble form of GFAP was still significantly decreased in all groups receiving the LAB (Fig. 5A), with the groups receiving live bacteria showing the greatest decrease. No differences were detected for filament GFAP, with the exception of the group receiving *L. plantarum* for the hindbrain and cerebellum, which had amounts significantly lower than those of the control rats (Fig. 5B). A lower concentration of S-100b was found in the posterior part of the hemisphere and in the hindbrain of rats treated with the live form of *L. plantarum* as compared with that of the control animals (Fig. 5C).

There was a strong correlation (.7; P>.001) between the decreasing level of soluble GFAP and the decreasing motoric activity of LAB-treated rats in all the time periods tested.

3.3. Immunohistochemical data

No morphological changes in the brain could be detected in any of the rat groups studied. Astrocyte density was determined in accordance with the staining of GFAP-positive cell bodies. The GFAP-stained astrocytes were observed to originate in the same manner from different brain regions for both control and LAB rats. The most GFAP-immunoreactive star-like cells, identified as mature astrocytes, were present in

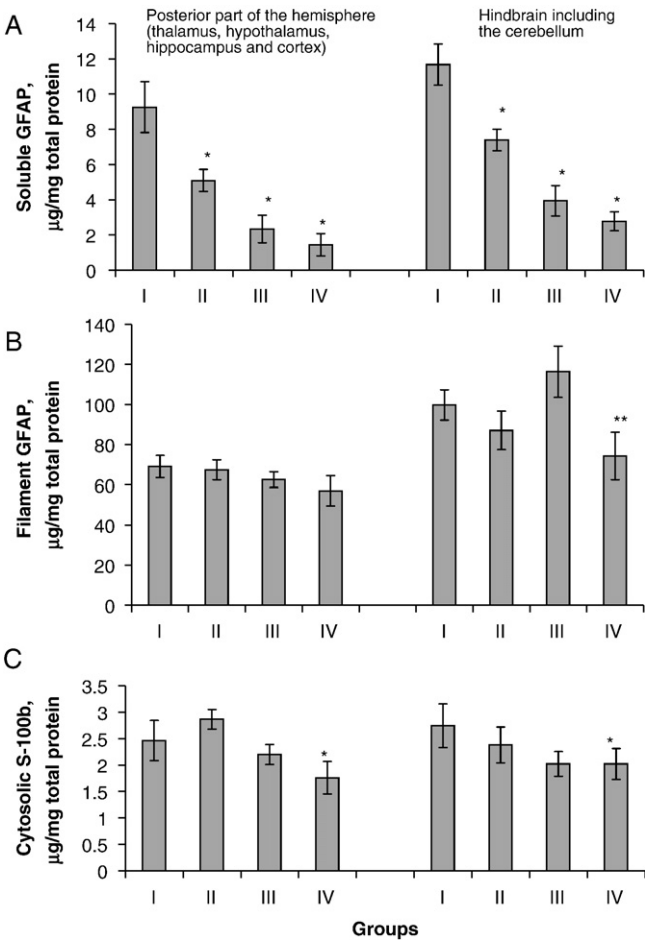

Fig. 5. Contents of soluble GFAP (A), filament GFAP (B) and cytosolic S-100b (C) proteins in the different rat brain regions after 6 months of LAB supplementation. Group I, control; Groups II–IV, treated with LAB (2 ml/day bacterial culture containing 10^9 cfu/ml; fed via a stomach tube) for 6 months; Group II, inactivated *L. fermentum*; Group III, live *L. fermentum*; Group IV, live *L. plantarum* (n=8). Results are presented as mean±S.E.M., where statistically significant differences from controls were indicated as *P<.001 and **P<.05.

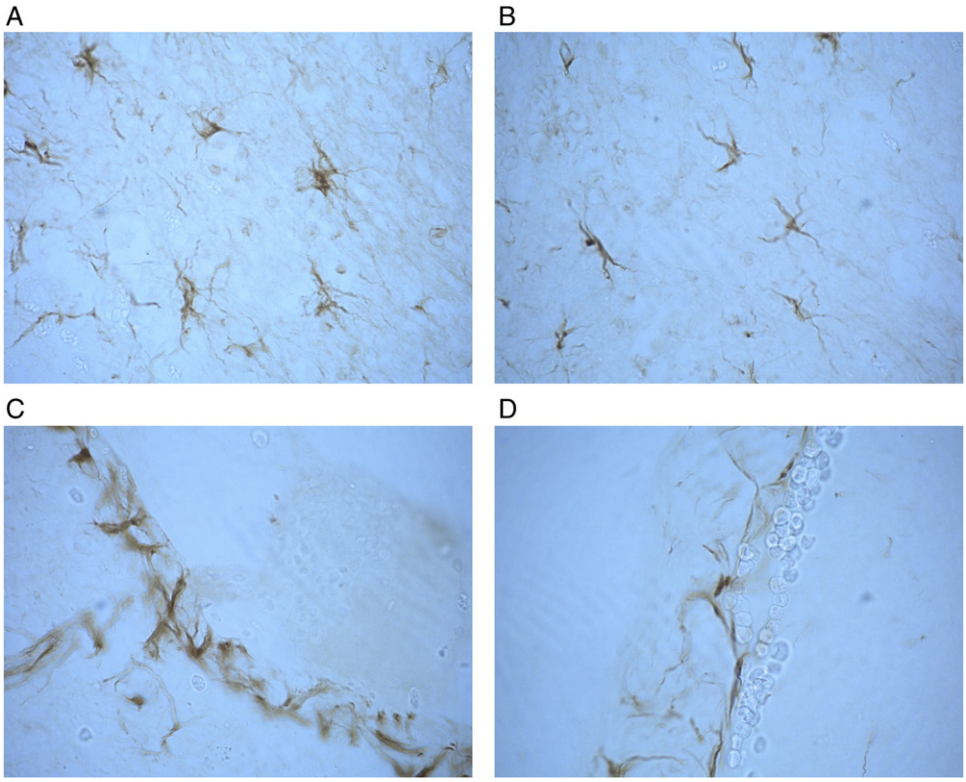

Fig. 6. GFAP immunostaining of the sagittal brain section of control rats (A and C) and rats supplemented with live *L. fermentum* (2 ml/day bacterial culture containing 10^9 cfu/ml; fed via a stomach tube) for 2 months (B and D). (A and B) Internal granular cell layer of the cerebellum (GFAP-positive staining of star-like astrocytes). (C and D) GFAP immunostaining of astrocytes located around the lateral ventricles. Original magnification, ×1150.

the internal granular cell layer of the cerebellum (Fig. 6A). A high amount of immunostaining for GFAP was also indicated around the ventricles (Fig. 6C). The treatment with the live form of LAB for 2 months led to a decrease in the GFAP immunoreactivity of the astrocytes, especially in the glial processes running from the ventricles (Fig. 6B and D). No differences were found between GFAP-stained astrocytes of the control rats and GFAP-stained astrocytes of the rats treated with LAB for 4–6 months.

4. Discussion

Different life situations with stress or disharmony create conditions that lead to gastrointestinal disorders. Similarly, a great number of medical drugs, especially enteric antibiotics, inhibit and modify the composition and function of the beneficial gut flora. Environmental toxins (or a different climate with a variety of bacterial cultures) can also provoke these intestinal disorders. Susceptibility to developing inflammation or appetite suppression is important in many clinical settings in which nutritional factors may have an effect on long-term prognosis.

LAB cultures, recognised as probiotics, have been widely used in many countries as an important category of food supplements. Questions about their biological nature, available products, positive effect on human health and molecular mechanisms of action are important, but answers still remain unclear or unavailable, especially for normal situations or growing humans.

Evidence suggests that probiotics have the following beneficial effects: normalisation of the intestinal microflora [28], ability to block the invasion of potential pathogens in the gut [29] (as treatment for several types of diarrhoea) [30], relief of the symptoms of irritable bowel syndrome [31] and inflammatory bowel disease [32], modulation of immune function [33], inhibition of *Helicobacter pylori* [34], possible enhancement of calcium absorption [35], reduction of blood cholesterol levels [36] and prevention of colon cancer [37]. Molecular mechanisms for the above benefits have been proposed, but none has been established. Moreover, there is little information available about the adequate level of viable bacteria in a probiotic product, and an appropriate daily dose is critical to achieving health benefits.

The aim of this work was to study the interactions of long-term LAB enteral treatment with behaviour in normal growing rats and to relate them to the distribution of neurospecific proteins. This could help to develop new functional foods that mediate intestinal integrity without subsequent CNS disturbances.

Treating rats with *L. fermentum* (heat-inactivated or live) and live *L. plantarum* in a daily dose of 10^9 cfu/ml for 1 month did not affect neural and astrocyte plasticity or the behaviour of the rats. Continued treatment with the indicated

lactobacilli for 2–3 months induced a statistically significant decrease in astrocyte reaction in areas of the hypothalamus, hippocampus and hindbrain, including the cerebellum, while no changes in other brain regions and in the neural cell adhesion plasticity of different areas of the rat brain could be found. According to the biochemical and immunohistochemical data obtained in these experiments, it could be suggested that long-term supplementation (2–3 months) with live *L. fermentum* and *L. plantarum* could decrease astrocyte reaction by reducing S-100b and GFAP protein syntheses in the brain. These observations were accompanied by a decrease in the locomotor activity of the treated rats; none of the other behavioural activities tested appeared to be affected. However, it should be noted that this experiment was performed on growing animals, and the effect of LAB was most evident while they were in the growing phase.

The nervous system and the immune system mutually cooperate via the release of mediators of both neurological and immunological derivations. The cytokines — released by monocytes, macrophages and lymphocytes upon antigenic stimulation — are able to cross the blood–brain barrier, thus modulating nervous functions (e.g., thermoregulation, sleep and appetite). However, the cytokines are also locally produced in the brain, especially in the hypothalamus, and thus may contribute to the development of anorexic, pyrogenic, somnogenic and behavioural effects [38]. Neurological problems such as phobic disorders, migraine, chronic fatigue syndrome, autism and others are examples of stress-related disorders in which phagocytic immune deficits, increased oxidative stress, endotoxemia, exaggerated levels of proinflammatory cytokines and altered gastrointestinal function have been detected [39,40]. As a supplement to the traditional treatment of neurological diseases, probiotic diet may elevate the efficiency of detoxification, immunomodulation and prevention of astrogliosis. Moreover, Logan and Katzman [41] have shown that a probiotic diet given to decrease lipid peroxidation and antioxidant supplementation can prevent the negative influence of saturated fats on brain-derived neurotrophic factor. An enteral formula containing glutamine and probiotics decreases brain-injury patients' infection rate and shortens their stay in the intensive care unit [42].

Previously, it was reported that ingestion of milk fermented with *Lactobacillus helveticus* decreases blood pressure in humans and rats [43]. Tanida et al. [44] showed that *Lactobacillus johnsonii La1* or its metabolites might also lower blood pressure by changing autonomic neurotransmission via the central histaminergic nerves and the hypothalamic suprachiasmatic nucleus. Angiotensinogen (AGT) is widely expressed in astrocytes [45], which could then be secreted into the extracellular fluid. There, AGT could be processed by renin and angiotensin-converting enzyme to form angiotensin II and to control blood pressure by regulating sympathetic outflow and baroreceptor reflexes [46]. It was interesting to note that astrocytic AGT mRNA has been found in areas of the medulla, midbrain, hypothalamus, thalamus and hippocampus [47]. In the present study, these brain sections have shown decreased astrocytic reaction after 2–3 months of lactobacilli supplementation.

It should be noted that the application of lactobacilli supplementation to treat neurological diseases is recommended for use for only 2–4 months because longer treatment (6 months) might abolish the beneficial effects obtained during treatment in the first months. It could be speculated that 6 months of LAB supplementation would not have an effect on patients with neurological conditions because of a common adaptation of the gastrointestinal, immunological and nervous systems. Moreover, the times of adaptation to a LAB diet can be different for children and adults.

With respect to the effect of the different stains of *Lactobacillus* on astrocyte reaction and on the behaviour of the rats, it was shown that live *L. fermentum* was more effective than the heat-inactivated *L. fermentum* and live *L. plantarum*. The reason for these differences was probably strain-dependent. Apparently, the effect of lactobacilli depends on the strain and whether it was live or not.

In conclusion, this study showed that daily stomach feeding of 10^9 cfu of LAB for 2–4 months effectively decreased astrocyte reaction and locomotor activity in normal growing rats. This was a demonstration of the effect of *Lactobacillus* on normal growing subjects and indicated that the probiotic effect of lactobacilli was not confined to the gastrointestinal tract. From the results presented here, it could be postulated that LAB treatment might play a preventive role for neurological diseases by decreasing astrogliosis reaction and locomotor activity.

Acknowledgments

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