Combination of *Lactobacillus helveticus R0052* and *Bifidobacterium longum R0175* reduces post-myocardial infarction depression symptoms and restores intestinal permeability in a rat model

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Abstract

Myocardial infarction (MI) in rats is accompanied by apoptosis in the limbic system and a behavioural syndrome similar to models of depression. We have already shown that probiotics can reduce post-MI apoptosis and designed the present study to determine if probiotics can also prevent post-MI depressive behaviour. We also tested the hypothesis that probiotics achieve their central effects through changes in the intestinal barrier. MI was induced in anaesthetised rats via 40-min transient occlusion of the left anterior coronary artery. Sham rats underwent the same surgical procedure without actual coronary occlusion. For 7 d before MI and between the seventh post-MI day and euthanasia, half the MI and sham rats were given one billion live bacterial cells of *Lactobacillus helveticus R0052* and *Bifidobacterium longum R0175* per d dissolved in water, while the remaining animals received only the vehicle (maltodextrin). Depressive behaviour was evaluated 2 weeks post-MI in social interaction, forced swimming and passive avoidance step-down tests. Intestinal permeability was evaluated by oral administration with fluorescein isothiocyanate–dextran, 4 h before euthanasia. MI rats displayed less social inter-action and impaired performance in the forced swimming and passive avoidance step-down tests compared to the sham controls ($P<0.05$). Probiotics reversed the behavioural effects of MI ($P<0.05$), but did not alter the behaviour of sham rats. Intestinal permeability was increased in MI rats and reversed by probiotics. In conclusion, *L. helveticus R0052* and *B. longum R0175* combination interferes with the development of post-MI depressive behaviour and restores intestinal barrier integrity in MI rats.

Key words: Probiotics: Myocardial infarction: Depression: Intestinal barrier

After myocardial infarction (MI), 65% of patients present depressive symptoms[1], and about 20% of them incur major depression[1,2]. Evidence of a poor prognosis is particularly strong in patients with such symptoms[3–6]: the risk of cardiac death within 6 months after acute MI is approximately four times greater in patients with depression compared to non-depressed control subjects[5]. The mechanism is still hypothe-tical, but it has been postulated that the inflammatory state observed after MI is responsible for post-MI depression[7]. Elevation of pro-inflammatory cytokine levels[8,9] has been documented after myocardial ischaemia, and their attenuation is correlated with depressive behaviour reduction[10].

Our previous study indicated that MI rats present symptoms that are similar to human depression. MI rats drink significantly less sucrose and remain more immobile in the forced swimming test, indicating a state of anhedonia and behavioural despair, respectively. These behavioural signs are blocked by the tricyclic anti-depressant, desipramine, the selective serotonin reuptake inhibitor, sertraline[11,12], and the cytokine synthesis inhibitor pentoxifylline[13].

The present experimental model of post-MI depression manifests an early wave of apoptosis, with a peak at 3 d after MI, in the hippocampus and amygdala regions of the limbic system involved in the pathophysiology of depression[13,14]. We have demonstrated that this apoptosis can be blocked by antidepressant[12] or anti-inflammatory treatments[13,15], indicating a link between inflammation, apoptosis and depression. We have also shown that the

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probiotics *Lactobacillus helveticus* and *Bifidobacterium longum* can reverse MI-induced apoptosis in the limbic system[16] by a mechanism that could include a reduction of pro-inflammatory cytokines. It has been observed that *L. helveticus R0052* reduces IL-1β and IL-6, and not significantly TNF-α[17], whereas *B. longum R0175* can reduce IL-8 and TNF-α[18], indicating that each strain seems to have a specific effect on pro-inflammatory cytokine synthesis. In the present experiment, we observe that pro-inflammatory cytokine concentrations are elevated and could contribute to the depression-like symptoms[8,19]. Owing to the fact that probiotics seem to have specific effects on pro-inflammatory cytokine synthesis, we decided to use the combination of both strains in our experiments. Moreover, previous clinical and animal trials have shown these two strains to be effective in combination, and there is no indication that individual probiotic microbes have this impact on stress[20,21].

We have observed that MI induces perturbation of the blood–brain barrier[22] by an unknown mechanism. We suspect that other barriers, such as the intestinal system, could be affected as has been demonstrated with pro-inflammatory cytokines[23]. Indeed, pro-inflammatory cytokines open tight junction, increase paracellular permeability and affect the epithelial barrier integrity[23,24]. Probiotics, such as *L. helveticus R0052*, may protect the epithelial cells and thus can be helpful to maintain the intestinal barrier integrity[25,26]. In the present experiment, we investigated whether probiotics modulate intestinal barrier permeability in the present post-MI model, which could have an effect on circulating pro-inflammatory cytokines[23].

The aim of the present study was to test if probiotics can reverse the behavioural syndrome induced by MI. As an anti-apoptotic outcome may be achieved through an increase in the anti-/pro-inflammatory cytokine ratio[27,28] and changes in the intestinal barrier[23,29], another objective was to assess intestinal permeability before and after treatment with probiotics.

Materials and methods

Ethics statement

These experiments conformed to animal care guidelines published by the Canadian Council on Animal Care, and the procedures performed were approved by the local Animal Care Committee of Hôpital du Sacré-Coeur de Montréal.

Experimental groups (animals and housing)

A total of forty 12-week-old Sprague–Dawley rats (Charles River Canada, St-Constant, QC, Canada), weighing between 325 and 350 g at the beginning of the experiments, were housed individually under constant conditions (temperature: 21–22°C; humidity: 40–50%) and maintained on a 12 h light–12 h dark cycle, with lights on at 08.00 hours. Chow pellets (5075-US Charles River Rodent; Charles River Canada) and tap water were available *ad libitum* throughout the study. All animals were acclimatised to the housing conditions for

3 d after delivery by the supplier before they were randomly distributed among one of the four groups: MI rats treated with probiotics or vehicle and controls treated with probiotics or vehicle.

Probiotics treatment

The commercial probiotics given were a combination of *L. helveticus R0052* and *B. longum R0175* (Probio'Stick, provided by Institut Rosell-Lallemand, Montréal, QC, Canada). They were administered by dissolving freeze-dried cultures or vehicle (maltodextrin) in 200 ml of tap water. For 7 d before MI and between the seventh post-MI day and euthanasia, half of the MI and sham rats had access to one billion live bacterial cells of *L. helveticus R0052* and *B. longum R0175* per d dissolved in water, while the remaining animals received only the vehicle. The drinking solution was prepared every 2nd day for the duration of the experiments. Water intake was monitored throughout to ensure that enough bacteria were ingested. This mode of administration was chosen as compared to oral administration to reduce handling stress, which may influence the overall outcome of the study.

In vivo surgical procedure

The animals were anaesthetised by intramuscular ketamine or xylazine (50 and 5 mg/kg, respectively) injection. They were subsequently intubated and anaesthesia was maintained with isoflurane (1%) ventilation. Electrocardiography was undertaken and heart rate monitored throughout the procedure with electrodes placed on their paws. A left thoracotomy was performed at the fifth intercostal space, permitting occlusion of the left anterior descending coronary artery with 4-0 silk suture (Syneture; Covidien, Mansfield, MA, USA). Ischaemia was confirmed by alteration of the electrocardiographic S-T waves and myocardial surface cyanosis. After 40 min of ischaemia, the suture was removed, permitting myocardial tissue reperfusion. After the thorax was closed with 2-0 (ribs and muscle) and 3-0 (skin) silk sutures (Syneture; Covidien), the animals were injected with antibiotic (15 000 IU penicillin G; Duplocillin LA, Intervet Canada Limited, Whitby, ON, Canada) intramuscularly and analgesic (0·05 mg/kg buprenorphine; Schering-Plough Limited, Hertfordshire, UK) subcutaneously before being returned to their respective cages. They received a second analgesic injection (0·05 mg/kg buprenorphine) in the morning after surgery.

Behavioural measures

The tests employed were selected on the basis of their validity regarding behavioural depression syndrome. All tests were conducted individually, in the morning, starting 9 d after surgery. Social interaction was evaluated on day 9, the forced swimming test was performed on days 14 and 15 and the passive avoidance step-down test on day 16. All animals were killed on day 17.

Social interaction test

One pair of rats, which received the same diet (either probiotics or vehicle), was placed together in a cage for 10 min, between 09.00 and 11.00 hours. During this period, two observers blinded to the experimental condition each observed one animal, and the following dependent measures were computed: duration and number of interactions with the other rat, number of grooming events and number of rearings.

Forced swimming test

Rats were placed individually in a transparent 25-cm diameter pool filled to a depth of 30 cm with 22–25°C water, with no possible escape. Two identical chronometers were needed to time the immobile and escape trial periods of the animals. The test was conducted on 2 d: day 1 comprised 15 min of habituation and day 2 entailed the actual 5 min test. On the 2nd day, immobility time was translated into depressive symptoms by comparison to the controls. Groups of MI and control rats were tested between 09.00 and 11.00 hours.

Passive avoidance step-down test

Rats were placed individually in the passive avoidance test chamber ($14 \times 23 \text{ cm}^2$) on one platform ($14 \times 19 \text{ cm}^2$) higher than another ($14 \times 14 \text{ cm}^2$); to reach the test criterion, they needed to stay on this platform for 60 s, over three consecutive trials (inter-trial interval = 30 s). If they stepped down onto the second platform (2·5 cm lower), resting all four paws on its grid floor, they received a mild shock to the feet (1 s, 8 mA). The test was started between 13.00 and 15.00 hours. The number of trials needed to reach the test criterion and the number needed to learn the test were noted. Groups of MI and control rats were tested 1 d before they were killed.

Fluorescein isothiocyanate–dextran assessment

The animals were starved the night before euthanasia. At 4 h before being killed, they received fluorescein isothiocyanate (FITC)–dextran diluted in water (60 mg/100 g body weight) by oral administration. After killing, blood was collected and immediately centrifuged at 3000 g at 4°C for 15 min. Plasma was obtained and the sample (50 μl in triplicate) was deposited in a ninety-six-well plate for quantification against a standard curve prepared from stock solution. Measurements were performed on a Cary Eclipse spectrophotometer (Varian, Mississauga, ON, Canada), with excitation wavelength at 350 nm and emission at 515 nm.

Infarct size

After 17 d of reperfusion, the rats were restrained in a cone bag and decapitated rapidly. Blood was collected for plasma IL-1β and FITC–dextran measurements. The heart was removed and the left anterior descending coronary artery was occluded at the same site to quantify the area at risk (AR) with Evans blue (0·5 %) infusion through retrograde perfusion into the aorta. The heart was then frozen at −80°C for 5 min and sliced into four transverse sections of 2 mm. Each section was incubated for 5 min at 37°C in a triphenyltetrazolium chloride (1 %, pH 7·4) solution to distinguish the area of necrosis (I) from the AR. MI was expressed as a percentage of necrosis (I) of the AR ((I/AR) $\times$ 100). In addition, the AR was expressed as a percentage of left ventricle area.

IL-1β measurement

Blood was sampled at the onset of euthanasia and centrifuged for 15 min at 3000 g. Plasma was then obtained and frozen at −80°C until ELISA. Plasma IL-1β concentrations were quantified with commercial kits from R&D Systems Inc. (Minneapolis, MN, USA), according to the manufacturer's specifications.
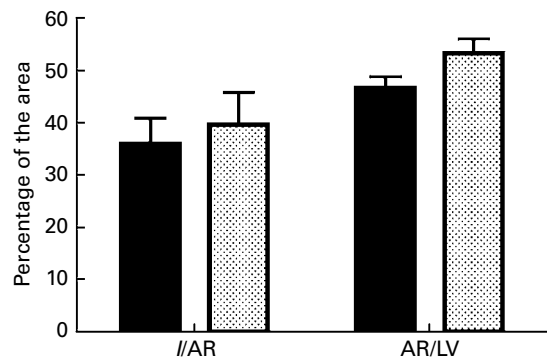
Statistical analyses

The results are expressed as mean values and their standard errors. MI size and AR were compared by Student's t test. Factorial ANOVA designs (2×2) were adopted, with MI and probiotics as factors. Simple effects were estimated according to Fields[30] whenever an interaction between factors was significant; otherwise, only main effects were reported. In the passive avoidance step-down test, the numbers of trials were evaluated by Kruskal–Wallis tests, followed by Mann–Whitney U tests, with Bonferroni correction for the number of comparisons. An α level of 0·05 was considered significant.

Results

Infarct size

The AR, expressed as a percentage of left ventricle area, was similar in both groups and represented 45–55 % of the left ventricle. After 40 min of ischaemia and 17 d of reperfusion, MI size was similar between groups (Fig. 1) and represented 35–40 % of the AR.

Fig. 1. Myocardial infarct size (I), expressed as a percentage of the area at risk (AR), shows no difference between groups. The AR, expressed as a percentage of the left ventricle (LV), is similar between groups; n 9 per group. ■, Myocardial infarction (MI) + vehicle; ▨, MI + probiotics.

Social interaction

There was a significant interaction effect between MI and probiotics ($F(1, 24) = 4.80$, $P < 0.05$; Fig. 2); analysis of simple effects indicated that probiotics did not influence interaction time in the sham controls but increased it in MI rats ($F(1, 25) = 5.92$; $P < 0.05$). No differences were detected in the number of rearings and groomings (data not shown).

Forced swimming test

We observed significant interaction between factors (MI and probiotics) in swimming time ($F(1, 31) = 5.90$, $P < 0.05$) and escape time ($F(1, 31) = 4.83$; $P < 0.05$; Fig. 3). Analysis of simple effects revealed lower values at baseline in the MI groups and improved swimming time ($F(1, 32) = 10.13$, $P < 0.05$) and escape time ($F(1, 32) = 6.47$, $P < 0.05$) with treatment. No differences were apparent in the sham groups. Immobility time showed a significant main effect on probiotics ($F(1, 31) = 5.60$, $P < 0.05$) and MI ($F(1, 32) = 10.05$, $P < 0.05$).
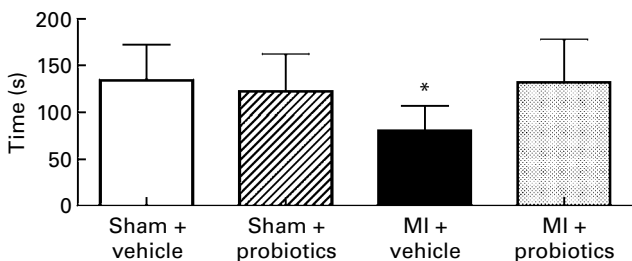
Passive avoidance step-down test

We noted significant interaction between MI and probiotics regarding time to learn the test ($F(1, 30) = 5.45$, $P < 0.05$); analysis of simple effects indicated that probiotics decreased time to learning the test in MI rats ($F(1, 31) = 4.12$; $P = 0.05$) but not in sham rats (Fig. 4). The main effects (MI or probiotics) were NS. The number of trials was significantly different between groups ($\chi^2 = 8.475$, df $= 3$; $P = 0.037$) and significantly higher in the MI v. the sham group ($Z = -2.847$; $P = 0.004$).
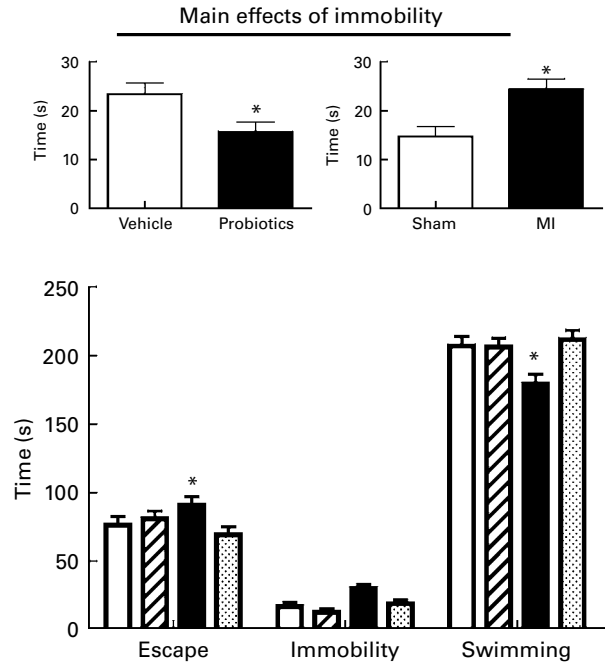
Plasma IL-1β concentrations

No significant treatment × MI interactions were evident. A main probiotics effect was evident, with probiotics significantly reducing plasma IL-1β concentrations compared to vehicle ($F(1, 18) = 5.70$, $P < 0.05$; Fig. 5). No significant MI main effect was observed.

Intestinal barrier permeability

We noted significant interaction between MI and treatment on FITC–dextran concentrations ($F(1, 16) = 8.60$, $P < 0.05$; Fig. 6). Analysis of simple effects disclosed that FITC–dextran

Fig. 3. Performance of rats from the four different groups in the forced swimming test. Escape and swimming time is less in the myocardial infarction (MI)-vehicle compared to the other groups. No interaction was found for the immobility parameter. However, the main effects (inset) indicate that probiotics decrease immobility time, whereas MI rats show more immobility than the vehicle group; n 8–10 per group. * Values were significantly different for MI-vehicle v. MI-probiotics ($P < 0.05$). Inset: * Values were significantly different between groups ($P < 0.05$). □, Sham + vehicle; ▨, sham + probiotics; ■, MI + vehicle; ▩, MI + probiotics.
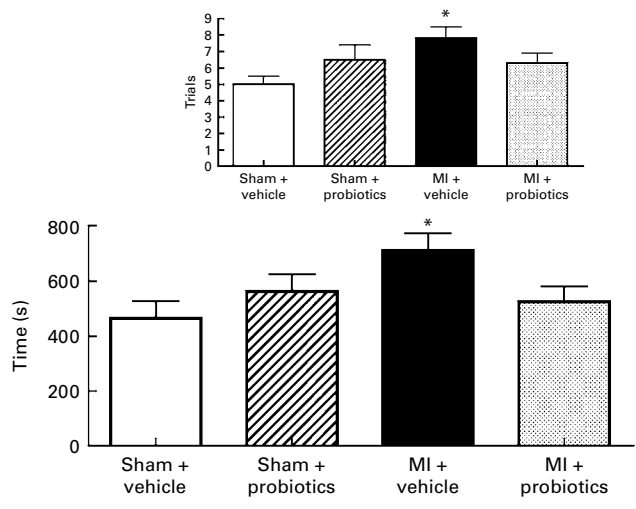
concentrations were significantly increased in MI rats treated with vehicle compared to MI rats given probiotics ($F(1, 17) = 14.92$; $P < 0.05$). No significant effects were observed in sham controls.

Discussion

We have previously shown that our model of post-MI depression replicates some of the behavioural characteristics of human depression, including anhedonia (sucrose preference test) and behavioural despair (forced swimming test)[12]. The results obtained in the present experiments constitute further converging evidence that post-MI syndrome is indeed associated with a behavioural repertoire similar to other rat models of depression, and extend behavioural markers to impaired social behaviour (social interaction test) and processing of emotional memory (passive avoidance step-down test).

The present study demonstrates that a probiotic-enriched diet with L. helveticus and B. longum can prevent behavioural despair, abnormal social behaviour and blighted processing of emotional memory. We decided to administer probiotics before MI, as reported previously, to determine if the beneficial effect that we observed on apoptosis in the limbic system[16] is reflected in depressive behaviour. We also know that the administration of probiotics before MI has no influence on infarct size, reducing potential bias in

Fig. 2. Social interaction between animals. The data indicate that myocardial infarction (MI)-vehicle rats interact socially less than the other groups. Time is expressed in s; n 6–8 per group. * Values were significantly different for MI-vehicle v. MI-probiotics ($P < 0.05$).
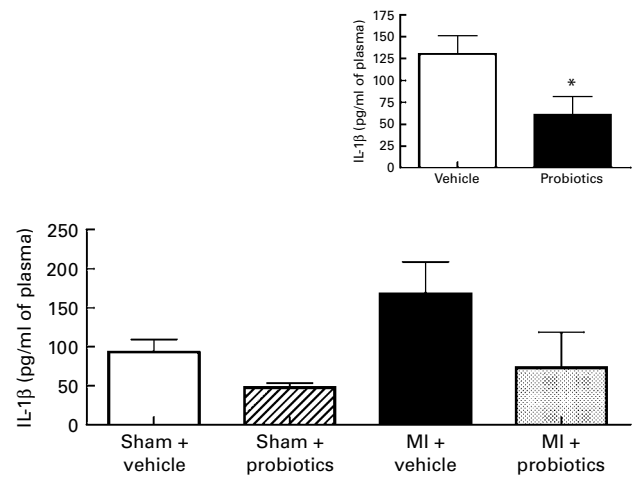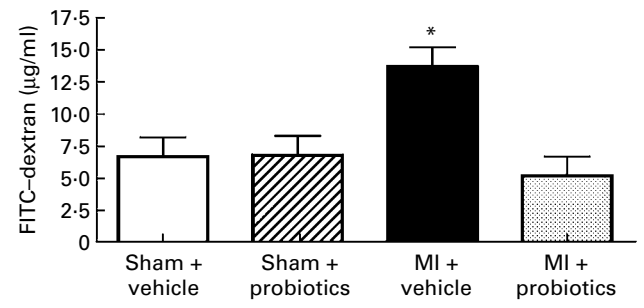
Fig. 4. In the passive avoidance step-down test, myocardial infarction (MI)-vehicle rats take more time to succeed in the test than the other groups ($P < 0.05$). Inset: The number of trials is significantly higher in the MI-vehicle than in the other groups; n 8–10 per group. * Values were significantly different for MI-vehicle v. MI-probiotics ($P < 0.05$).

Fig. 6. Fluorescein isothiocyanate (FITC)–dextran concentrations measured in µg/ml of plasma before euthanasia. The data indicate that FITC–dextran level is significantly higher in the myocardial infarction (MI)-vehicle group than in the other groups; n 5 per group. * Values were significantly different for MI-vehicle v. MI-probiotics ($P < 0.05$).

performance in the behavioural tests. However, after the surgical procedures, antibiotics were administered to all animals, and to avoid any benefit of probiotics on the potential action of antibiotics, we decide to stop the probiotics for the first 7 d of reperfusion.

Although the mechanistic relationship between MI and depression is still hypothetical, we postulate that inflammation is probably a key element. It has indeed been documented that pro-inflammatory cytokines can induce depression. For example, IL-1β and TNF-α injection can elicit behavioural changes modelling symptoms of depression, such as decreased locomotion, diminished exploration of the environment, reduced food and water intake, dwindling social interactions and impaired learning as well as memory[31,32]. IL-1β and TNF-α are also associated in animals with neuroendocrine

Fig. 5. Probiotics-treated rats present less plasma IL-1β concentrations than vehicle controls (main effect presented in the inset). No other difference was detected between groups; n 5–6 per group. * Values were significantly different for vehicle v. probiotics ($P < 0.05$). MI, myocardial infarction.

changes similar to those in depressed humans and responsible for the stress response at the hypothalamic–pituitary–adrenal axis level[33]. In addition, there is evidence that antidepressants antagonise the action of cytokines in the brain (for a review see Castanon et al.[34]), although the mechanism has not yet been elucidated. We believe that the therapeutic effects of L. helveticus and B. longum on behavioural signs of depression also involve pro-inflammatory cytokines, as we observed a significant diminution of circulating IL-1β in the probiotics groups. Interestingly, it has been demonstrated that L. helveticus R0052 and L. rhamnosus R0011 prevent stress-induced memory deficits in mice infected by pathogens[35], reinforcing the immune–gut–brain axis hypothesis.

The intestinal epithelium forms a barrier between the external environment and luminal contents in which tight junction complexes have an important regulatory role[36]. Exposure to pro-inflammatory cytokines, such as TNF-α, decreases epithelial barrier function and may thus lead to translocation of the bacterial component to the bloodstream. The present results indicate that intestinal barrier permeability may be involved, as we found it to be altered 2 weeks post-MI. As observed in chronic heart failure[37], increased intestinal permeability has been documented and said to contribute to chronic inflammation in this pathological condition. Intestinal dysfunction may add to endotoxin translocation and ultimately augment blood lipopolysaccharide concentrations. Interestingly, normal volunteers injected with lipopolysaccharide exhibited acute increases in depression and anxiety symptoms, and their severity correlated with augmented peripheral blood cytokine concentrations[38].

The presence of probiotics has a beneficial effect on intestinal barrier integrity through NF-κB pathway inhibition and intact extracellular signal-regulated kinases 1 and 2 signalling[23]. Another explanation may be related to the anti-apoptotic effect observed with these probiotics[16], although this has not been demonstrated in intestinal tissue. Indeed, it has been determined that apoptosis affects intestinal barrier integrity[39], and inhibition of apoptosis, through reduction of a cytokine-dependent mechanism, could be beneficial to intestinal integrity. The activation of toll-like receptors, a class of transmembrane pattern-recognition receptors that have a key role in microbial recognition, is

another hypothesis that could explain the beneficial effect of probiotics on intestinal barrier integrity[40]. Probiotics activate toll-like receptor, such as toll-like receptor-2, and prevent intestinal barrier impairment, indicating a link between these events[29,41]. Additional studies are needed to determine the mechanism by which *L. helveticus* and *B. longum* may be advantageous for intestinal barrier integrity.

Another possibility that could explain the beneficial effect of *L. helveticus* and *B. longum* on post-MI depression-like symptoms could be related to the perturbations that these probiotics may induce in the intestinal microbiota. According to the results obtained by Heijtz *et al.*[42], microbial colonisation process initates signalling mechanisms that may affect the anxiety behaviour. Moreover, it has been observed that altered enteric flora induced by stress are responsible for colonic pathophysiology, and probiotics improve gut dysfunction by a mechanism that could involve the normalisation of the hypothalamic–pituitary–adrenal axis activity[43]. However, as long as we ignore the effect of MI on the intestinal microbiota, this will remain hypothetical.

In conclusion, the present study provided the first evidence of a positive outcome of probiotics combination (*L. helveticus R0052* and *B. longum R0175*) on post-MI depression. The beneficial impact of probiotics combination also includes the maintenance of intestinal barrier integrity, which may contribute to the inflammatory state observed after MI.

Limitations

One concern is the possible physical consequences of thoracic surgery and MI itself in the capacity of rats to perform physically demanding behavioural tasks. We have previously shown, however, that sham and MI rats, having undergone the same thoracic surgery as reported here, performed similarly in the Morris water maze task, a test of motor performance and spatial memory requiring an intact hippocampus[11,44], whether they were treated with the anti-depressant, desipramine, or saline, and improved with desipramine on the forced swimming test. The present results also indicated that, for similar myocardial infarct size, probiotics-treated MI rats were as efficient as sham controls on motor output measures such as swimming and escape time in the forced swimming test, which suggests that the level of injury was not sufficient to interfere with the results. Another concern is that more inflammatory cytokines should be analysed in order to support the notion according to which anti-inflammatory mechanisms underly the behavioural effects of probiotics reported here

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