

***Lactobacillus helveticus* and *Bifidobacterium longum* taken in combination reduce the apoptosis propensity in the limbic system after myocardial infarction in a rat model**

Stéphanie-Anne Girard^{1,2}, Thierno Madjou Bah¹, Sévan Kaloustian^{1,2}, Laura Lada-Moldovan^{1,2}, Isabelle Rondeau^{1,2}, Thomas A. Tompkins³, Roger Godbout^{1,4} and Guy Rousseau^{1,2*}

¹Centre de Biomédecine, Hôpital du Sacre-Coeur de Montréal, 5400 Boulevard Gouin Ouest, Montréal, Que., Canada H4J 1C5

²Département de Pharmacologie, Université de Montréal, Montréal, Que., Canada H3C 3J7

³Institut Rosell-Lallemand, 8480 Boulevard Saint-Laurent, Montréal, Que., Canada H2P 2M6

⁴Département de Psychiatrie, Université de Montréal, Montréal, Que., Canada H3C 3J7

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Myocardial infarction (MI) stimulates the release of pro-inflammatory substances that induce apoptosis in the limbic system. Pro-inflammatory cytokines are considered as the root cause of apoptosis, although the mechanism is not fully explained and/or understood at this time. In addition, depression may induce gastrointestinal perturbations that maintain the elevated levels of pro-inflammatory cytokines. It has been shown that some specific probiotic formulations may reduce gastrointestinal problems induced by stress and the pro/anti-inflammatory cytokine ratio. Therefore, we hypothesised that probiotics, when given prophylactically, may diminish the apoptosis propensity in the limbic system following a MI. Male adult Sprague–Dawley rats were given probiotics (*Lactobacillus helveticus* and *Bifidobacterium longum* in combination) or placebo in their drinking-water for four consecutive weeks. A MI was then induced in the rats by occluding the left anterior coronary artery for 40 min. Rats were killed following a 72 h reperfusion period. Infarct size was not different in the two groups. Bax/Bcl-2 (pro-apoptotic/anti-apoptotic) ratio and caspase-3 (pro-apoptotic) activity were reduced in the amygdala (lateral and medial), as well as in the dentate gyrus in the probiotics group when compared with the placebo. Akt activity (anti-apoptotic) was increased in these same three regions. No significant difference was observed in Ca1 and Ca3 for the different markers measured. In conclusion, the probiotics *L. helveticus* and *B. longum*, given in combination as preventive therapy, reduced the predisposition of apoptosis found in different cerebral regions following a MI.

Probiotics: Myocardial infarction: Apoptosis: Limbic system

Myocardial infarction (MI) induces the release of pro-inflammatory substances that may affect the function of other tissues^(1,2). For example, we have observed that 3 d after MI, different structures of the limbic system such as the amygdala, the hippocampus or the hypothalamus present an increase in apoptosis^(3,4). Although the link is not clearly established, this cell death may account for post-MI depression that we have documented in the present experimental model^(3,5). Reduction in this early apoptosis by pharmacological interventions results in an attenuation of the depressive behaviour⁽³⁾, and thus seems to be beneficial since post-MI depression patients present a three to four times increase in mortality as compared with non-depressive patients^(6,7).

Pro-inflammatory cytokines are among the different substances that may explain the presence of apoptosis in the limbic system after MI⁽⁴⁾. Inhibition of the synthesis of pro-inflammatory cytokines by pentoxifylline is sufficient to prevent apoptosis in the limbic system⁽⁴⁾. This observation leads us to predict that interventions that induce a shift in

the anti-/pro-inflammatory cytokine ratio must reduce the apoptosis tendency in the limbic system after MI.

Stress conditions, such as depression, may affect other organs that could perpetuate this condition. For example, it has been reported that stress predisposes individuals to develop functional bowel disorders or exacerbate symptoms of irritable bowel syndrome by decreasing mucosal barrier function^(8,9) and thus increasing translocation of bacterial lipopolysaccharide from gram negative bacteria^(10,11). Increased bacterial lipopolysaccharide translocation may result in maintenance of the activation of the inflammatory response system and elevated pro-inflammatory cytokines.

To prevent this gastrointestinal problems, probiotics defined as live micro-organisms, which, when consumed in adequate amounts confer a health benefit on the host, have been applied as an alternative approach of prevention and therapy. Probiotics may exert beneficial antibacterial effect on pathogens through the production of antibacterial substances, decrease adhesion of both pathogens and their toxins, increase barrier functions

Abbreviations: AR, area at risk; MI, myocardial infarction; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

* **Corresponding author:** Guy Rousseau, fax +1 514 338 2694, email guy.rousseau@umontreal.ca

and inhibit pro-inflammatory cytokine production^(12,13). It has been reported that a probiotic formulation beneficially affects the human stress response and its impact may be mediated through the gut–brain axis. In healthy volunteers suffering from stress-induced gastrointestinal symptoms in which the combination of probiotics *Lactobacillus helveticus* and *Bifidobacterium longum* was given showed a significant reduction in gastrointestinal symptoms⁽¹⁴⁾. Other studies with *L. helveticus* have shown that it can reduce *Escherichia coli*-induced lesions⁽¹⁵⁾ and modulate motility (in stress studies⁽¹⁶⁾), whereas *B. longum* has been shown to downregulate TNF- α ⁽¹⁷⁾ and maintain remission in ulcerative colitis patients⁽¹⁸⁾ indicating a potential anti-inflammatory action. Therefore, we hypothesise that the regular intake of two biotherapeutic microbes, *L. helveticus* and *B. longum*, in combination in a probiotic formulation as a prophylactic agent, may diminish the apoptosis propensity induced by the inflammatory condition observed after MI in different brain regions.

Materials and methods

Experimental groups (animals and housing)

A total of thirty-five rats were used in the present experiment. They were 10-weeks-old adult male Sprague–Dawley rats (Charles River Canada, St-Constant, Que., Canada) weighing between 325 and 350 g (at the beginning of the experiment). The rats were housed individually under constant conditions (temperature of 21–22°C and humidity of 40–50%). The animals were maintained on a 12-h dark–light cycle which began at 08.00 hours. Chow pellets (5075-US Charles River Rodent) and tap water were available *ad libitum* throughout the study. An acclimatisation period of 5 d after delivery by the supplier was allowed before the rats were randomly distributed to one of two groups, probiotics (n 18) or placebo (n 17). Both of the groups underwent a 40 min occlusion of the left anterior descending coronary artery. The animals were fed over a 4-week period and were killed following 3 d of reperfusion.

Probiotic treatment

The commercial probiotic given was a combination of two genus; *L. helveticus* R0052 and *B. longum* R0175 (Probio' Stick™ provided by Institut Rosell, Inc., Montreal, Que., Canada). The probiotics were administered by dissolving the freeze-dried culture or the vehicle only (maltodextrin) in 200 ml of drinking tap water. Each rat in the probiotic group received a daily dose of 10^9 colony forming units/ml. The drinking solution was newly prepared every second or third day for four consecutive weeks. Water intake was monitored throughout the entire investigation to ensure enough bacteria were administered. The body weight of each rat was also monitored every second or third day.

In vivo surgical procedure

Animals underwent anaesthesia following a ketamine/xylazine (50 and 5 mg/kg, respectively) intramuscular injection. Subsequently, the rats were intubated and anaesthesia was maintained using isoflurane (1.5%) ventilation. Electrocardiogram

and heart rate were monitored throughout the procedure using electrodes placed on their paws. A left thoracotomy was carried out at the fifth intercostal space permitting the occlusion of the left anterior descending coronary artery using a 4–0 silk suture (Syneture; Covidien, Mansfield, MA, USA). Ischaemia was confirmed by alterations of the ST segment and myocardial surface cyanosis. After 40 min of ischaemia, the thread was removed permitting reperfusion of the myocardial tissue. After the thorax was sutured by means of a 2–0 silk suture (Syneture; Covidien), the animals were given an antibiotic injection (15 000 IU penicillin G; Duplocillin LA, Intervet Canada Ltd, Whitby, Ont., Canada) as well as an analgesic injection (2 mg/kg butorphanol) before being returned to their respective cages.

Decapitation measurements, the area at risk of the heart and myocardial infarction size and tissue dissection

After 3 d of reperfusion, the rats were restrained in a cone bag and rapidly decapitated. Decapitation was preferred as the killing method to avoid any alteration of biochemical pathways that could arise ensuing anaesthesia or CO₂ exposure. The heart was taken and the brain was placed on a dish positioned on ice. Brain regions were identified according to the atlas of Paxinos & Watson⁽¹⁹⁾; frontal cortex, prefrontal cortex, hippocampus (Ca1, Ca3 and dentate gyrus), amygdala (medial and lateral parts) and hypothalamus (anterior and posterior parts). Tissues were frozen in liquid nitrogen and kept at –80°C until needed.

The heart was removed and the left anterior descending coronary artery was occluded at the same site to establish the area at risk (AR) with infusion of Evans Blue (0.5%) by retrograde perfusion into aorta. The heart was then placed at –80°C for 5 min and sliced in four to five transverse sections of 2 mm. Each section was incubated 5 min at 37°C in a triphenyltetrazolium chloride solution (1%, pH 7.4) to better distinguish the area of necrosis (I) from AR. MI was expressed as a percentage of necrosis (I) of the AR ($(I/AR) \times 100$). Additionally, AR was expressed as a percentage of left ventricle area.

Caspase-3 activity

Cytosolic proteins were extracted in lysis buffer (1% Triton X-100, 0.32 M sucrose, 10 mol/ml 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris; pH 8.0), 5 mmol/ml ethylenediamine tetra-acetate, 2 mmol/ml DL-1,4-dithiothreitol, 1 mmol/ml phenylmethanesulphonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml pepstatin A and 10 g/ml aprotinin). Enzymatic reactions were carried out in a reaction buffer (50 mmol/ml Tris (pH 7.5), 5 mmol/ml MgCl₂, 1 mmol/ml ethylene glycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.1% 3-[cholamidopropyl dimethylammonio]-1-propanesulphonate and 1 mmol/ml dithiothreitol), with 25 mg of proteins and a fluorogenic substrate, *N*-acetyl-asp-glu-val-asp-7-amido-4 methylcoumarin (40 μ mol/ml). Reactions were incubated at 37°C for 3 h and stopped with the addition of 0.4 M NaOH and 0.4 M glycine buffer. Fluorescence was quantified using a spectrofluorometer (Photon Technology International, Lawrenceville, NJ, USA) at an excitation wavelength of 365 nm and an emission wavelength of 465 nm.

Western blot

Brain tissue samples were lysed in a buffer containing protease and phosphatase inhibitors (leupeptin, microcystine and benzamide). After solubilisation, equal amounts of proteins (60 µg) in each line were loaded on a 10% SDS-PAGE, and after migration, proteins were transferred onto a nitrocellulose membrane. Primary antibody directed against Akt (1:2000), phospho-Akt (1:1000; NEB Biolabs, Knowl Piece, Hitchin, UK), Bax or Bcl-2 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was incubated overnight at 4°C. After three washes, a secondary peroxidase-coupled antibody (1:5000) was added for 1 h at room temperature (anti-rabbit Ig (IgG)–horseradish peroxidase from Santa Cruz Biotechnology). A Renaissance Chemiluminescence kit (Perkin Elmer, Mississauga, Ont., Canada) was used to visualise the bands, and the quantitative analysis was conducted with a Kodak Image Station. After quantification, membranes were placed in stripping buffer (0.1 M glycine, 1% SDS, pH 2.0, 1 h at room temperature). The same procedures were repeated with the other antibody (Akt or phospho-Akt) to obtain the phospho-Akt:Akt ratio.

Statistics

Results are expressed as means with their standard errors. Differences between groups were evaluated using Student's *t* test. $P < 0.05$ was considered significant. All the variances were equal and the data normally distributed.

Results

Infarct size

AR, expressed as a percentage of left ventricle area, was similar for both groups (placebo 53.0 (SEM 4.3)%; probiotics 62.1 (SEM 8.3) % of left ventricle; $P = 0.36$). Following 40 min of ischaemia and 72 h of reperfusion, the myocardial infarct size (I/AR) represented 44.1 (SEM 2.5) % of the AR for the placebo group when compared with 45.2 (SEM 3.3) % for the probiotics group ($P = 0.80$). There were no significant differences between the probiotic and the placebo groups.

Bax/Bcl-2 content

Bax/Bcl-2 ratio was measured in five different regions (Fig. 1). A significant decrease in Bax/Bcl-2 ratio was observed in the dentate gyrus, medial and lateral amygdala in the probiotic group when compared with the placebo group. In contrast, Ca1 and Ca3 regions indicate no significant difference in the ratio between groups.

Caspase-3 activation

After 72 h of reperfusion, caspase-3 activity was significantly reduced in the probiotic group in the lateral amygdala, medial amygdala and dentate gyrus when compared with the placebo group (Fig. 2). No significant difference was observed between the groups in the Ca1 and Ca3 regions.

Phospho-Akt:Akt content

Phospho-Akt:Akt ratios were significantly different between the placebo and probiotic groups in the three different brain regions, lateral amygdala, medial amygdala and dentate gyrus (Fig. 3). No significant difference was observed between groups in the Ca1 and Ca3 regions.

Discussion

The data obtained in the present study are the first reported observation that the gut–brain axis can modulate the apoptosis propensity observed in the limbic system after MI. However, there are numerous observations in the literature suggesting a link between depression and gastrointestinal diseases⁽¹¹⁾. It has been observed that intestinal mucosal dysfunction, characterised by an increased translocation of gram-negative bacteria, plays a role in the inflammatory pathophysiology of depression inducing the sickness behaviour⁽¹¹⁾. In a mouse model, depression increases the sensitivity to experimental colitis, which can be reversed by antidepressants⁽²⁰⁾. It is also reported that patients with irritable bowel syndrome have a high prevalence of psychiatric disorders suggesting a link between brain and gut. The mechanisms are not clearly established, but it has been suggested that prolonged exposure to stress can induce low-grade inflammation, causing ultrastructural epithelial abnormalities, alter bacterial–host interactions allowing an increase in bacteria translocation⁽¹⁰⁾, which in turn affects the brain. For example, treatment of mice with lipopolysaccharide increases insulin transport across the blood brain barrier by about threefold. The brain endothelial cells, which comprise the blood brain barrier, secrete many substances including cytokines and such secretion can be stimulated from one side of the blood brain barrier with release into the other side⁽²¹⁾. However, in contradiction to this hypothesis, Verdu *et al.*⁽²²⁾ showed

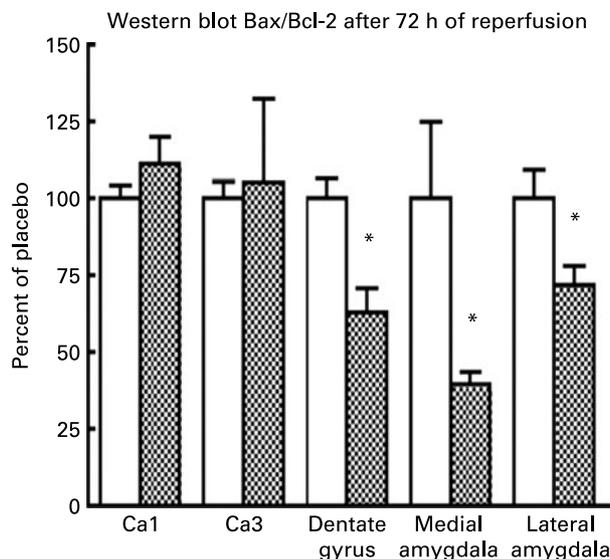


Fig. 1. Bax/Bcl-2 ratio in the different regions assessed by western blot ($n = 6-8$ /group; * $P < 0.05$ indicating a significant difference between the placebo (□) group and the probiotic (▨) group: Ca1, $P = 0.26$; Ca3, $P = 0.86$; dentate gyrus, $P = 0.007$; medial amygdala, $P = 0.034$; and lateral amygdala, $P = 0.01$).

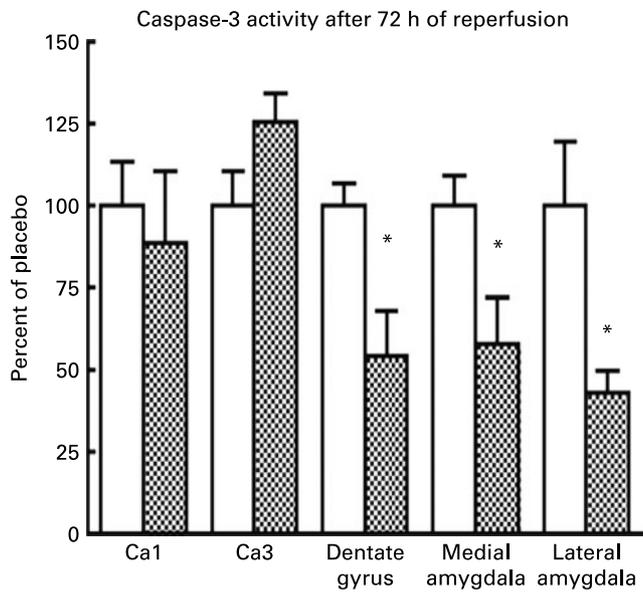


Fig. 2. Caspase-3 activity in the different regions assessed by *in vitro* spectrofluorescence (n 6–8/group; * P <0.05 indicating a significant difference between the placebo (□) group and the probiotic (▨) group: Ca1, P =0.67; Ca3, P =0.09, dentate gyrus, P =0.017; medial amygdala, P =0.026; lateral amygdala, P =0.015).

that the bacterial content of the gut influenced the rate of recovery of host pathophysiology induced by chronic *Helicobacter pylori* infection, including behavioural changes, and that these changes were not associated with modulation of intestinal permeability.

Probiotics have multiple and diverse influences on the host, which may include antimicrobial activity, enhancement of barrier function and immunomodulation. Recent investigations have suggested specific probiotics and gut microbiota can impact the immune system at both a systemic and a mucosal level⁽²³⁾. Studies show that certain probiotic bacteria

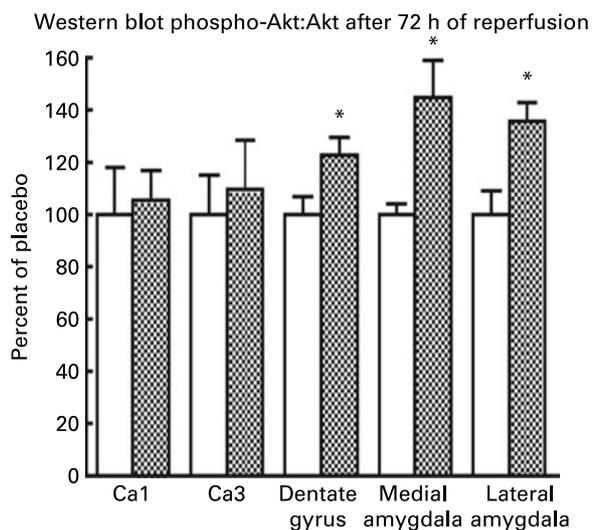


Fig. 3. Akt activity in the different regions assessed by the ratio of phospho-Akt on total Akt evaluated by western blot (n 6–8/group; * P <0.05 indicating a significant difference between the placebo (□) group and the probiotic (▨) group: Ca1, P =0.80; Ca3, P =0.69; dentate gyrus, P =0.032, medial amygdala, P =0.004; lateral amygdala, P =0.01).

may increase the production of anti-inflammatory cytokines including IL-10⁽¹²⁾. Alternately, probiotics could inhibit pro-inflammatory cytokine production such as TNF- α and IL-8, two pro-inflammatory cytokines⁽²⁴⁾. The alteration of the pro/anti-inflammatory cytokine balance may explain the reduction in apoptosis that we observed in the different limbic regions. While the mechanisms have not been elucidated, it has been suggested that pro-inflammatory cytokines participate in the limbic cell death after MI⁽⁴⁾.

The effect of probiotics on the susceptibility of apoptosis observed in the amygdala and in the dentate gyrus is similar to drugs with anti-inflammatory properties tested in our MI model such as cytokine inhibitor⁽⁴⁾, anti-depressant⁽³⁾ or A_{2A} adenosine receptor agonist⁽²⁵⁾.

In the present study, we observed that probiotics are unable to reduce the apoptosis observed in the Ca1, suggesting that different mechanisms are involved in this region. Apoptosis can be induced by at least two different pathways, extrinsic and intrinsic, although there are some proteins that are common to both such as caspase-3⁽²⁶⁾. Our data from amygdala and dentate gyrus suggest that the extrinsic signalling pathway is involved in the apoptosis in these regions as shown by the change in the Bax/Bcl-2 ratio. In contrast, intrinsic signalling pathway may be involved in the apoptosis observed in Ca1. It has been shown that the Ca1 region of the brain is the most sensitive to hypoxia⁽²⁷⁾. It is well known that hypoxia can activate the intrinsic signalling pathway⁽²⁸⁾, and thus can explain why probiotics are unable to reduce the apoptosis tendency in Ca1. However, previous data indicate that Ca1 apoptosis can be attenuated in the presence of pentoxifylline. This observation leads us to formulate the hypothesis that in addition to hypoxia, cytokines must also be involved in the apoptosis observed in Ca1. This can be verified by the effect of pentoxifylline⁽⁴⁾ on the Bax/Bcl-2 ratio. The effect of *L. helveticus* and *B. longum* in combination on the extrinsic apoptotic pathway is probably not sufficient to attenuate apoptosis susceptibility in this region.

We also observe a higher level of activation of Akt in presence of the combination of *L. helveticus* and *B. longum* when compared to the placebo group. Akt plays a critical role in the proliferation, differentiation and apoptosis; and inhibition of the PI3K/Akt invariably leads to cell cycle arrest and/or apoptosis^(29–32). One possibility that explains the present result is a low molecular weight soluble factor released from the bacteria that stimulates Akt activation⁽¹³⁾. Alternatively, it has been shown that some proteins isolated from bacteria may activate Akt⁽³³⁾. However, this is less probable since this protein needs to be present in the limbic system to activate Akt.

Myocardial reperfusion is associated with an important inflammatory response that can modulate the infarct size^(34,35). Since specific probiotic strains are capable of acting on the balance between pro/anti-inflammatory cytokines, the probiotics used in our model had the potential of attenuating the inflammatory process and the infarct size. However, the absence of the effect of probiotics on MI size is in accordance with our previous results indicating that pentoxifylline, a cytokine synthesis inhibitor, did not affect the infarct size⁽⁴⁾. Although many studies seem to indicate that the reduction in pro-inflammatory cytokines has a beneficial effect on infarct size^(36,37), other studies show that diminution of pro-inflammatory cytokines such as TNF- α has no beneficial effect^(38–40). Overall, in our experimental model,

the administration of *L. helveticus* and *B. longum* in combination as a prophylactic agent seems to have only a minor effect on myocardial infarct size. However, this lack of effect eliminates the infarct size as a possible explanation for the reduction of apoptosis susceptibility in the limbic system.

In conclusion, the probiotic preparation containing both *L. helveticus* and *B. longum* in combination reduced the apoptosis susceptibility observed in the limbic system after MI, but did not have any significant effect on myocardial infarct size.

Ethics statement

The investigation conformed to the *Animal Care guidelines* published by the Canadian Council and the procedures were approved by the Local Animal Care Committee.

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