Inhibitory Effects of *Lactobacillus reuteri* on Visceral Pain Induced by Colorectal Distension in Sprague Dawley rats

Running Title:

*Lactobacillus reuteri* inhibits visceral pain

Takeshi Kamiya, Lu Wang, Paul Forsythe, Gudrun Goettsche, Yukang Mao, Yufang Wang, Gervais Tougas, John Bienenstock,

Intestinal Disease Research Program, McMaster University

and The Brain-Body Institute, St. Joseph’s Healthcare Hamilton

Abbreviations:

Inflammatory bowel disease (IBD), Lactobacillus reuteri (LR), Irritable bowel syndrome (IBS), Colorectal distension (CRD)

Correspondence:

Dr. John Bienenstock

The Brain-Body Institute, St. Joseph’s Healthcare Hamilton

Department of Pathology & Molecular Medicine, McMaster University

50 Charlton Ave. E. Martha Wing, Room H304, Hamilton, Ontario, Canada L8N 4A6

Email: bienens@mcmaster.ca
ABSTRACT

**Background and Aims:** Probiotic bacteria are being investigated as possible treatments for many intestinal disorders. The present study aimed to explore the effects of live, heat-killed or gamma irradiated *Lactobacillus reuteri* on cardio-autonomic response and single fiber unit discharge in dorsal root ganglia to colorectal distension in healthy *Sprague Dawley* rats housed under conventional conditions. The effects of this treatment on somatic pain were also examined.

**Methods:** 1 X 10^9 bacteria were given by gavage for 9 days. Colorectal distension occurred under anesthesia. Heart rate was measured through continuous electrocardiography. Single fiber unit discharge was recorded from the 6th left lumbar dorsal root ganglion. Somatic pain was evaluated by the tail flick and paw pressure tests. **Results:** Colorectal distension caused a pressure-dependent bradycardia in the control (native medium) group. Treatment with live, heat-killed or gamma irradiated bacteria as well as their products (conditioned medium) prevented the pain response even during the maximum distension pressure (80 mmHg). Both viable and non-viable bacteria significantly decreased dorsal root ganglion single unit activity to distension. No effects on somatic pain were seen with any treatment. **Conclusions:** Oral administration of either live, or killed probiotic bacteria or conditioned medium inhibited the constitutive cardio-autonomic response to colorectal distension in rats through effects on enteric nerves. These data may provide a novel explanation for beneficial probiotic effects on visceral pain.
INTRODUCTION

Probiotics are defined as living microorganisms which, upon ingestion in adequate numbers, exert health benefits beyond inherent general nutrition.[1] Commensal bacteria associated popularly with probiotic activity are *Lactobacilli*, *Bifidobacteria* and *Streptococci* and non-pathogenic *Escherichia coli*. They are also important and normal constituents of the human gastrointestinal microflora. Probiotics have been used in the treatment of bacterial or viral induced acute intestinal infections such as antibiotic associated diarrhea,[2][3] *Clostridium difficile* infection,[4] traveller’s diarrhea and rotavirus diarrhea.[5][6] In recent years, some clinical studies have shown the therapeutic effects of different strains of probiotics in the treatment of chronic inflammatory bowel disease (IBD) including ulcerative colitis,[7][8] Crohn’s disease [9] and pouchitis,[10][11] or prevention of allergic disease [12] such as atopic eczema and asthma. In addition, some investigators have reported that probiotic treatment has been effective for the attenuation of experimental colitis seen in interleukin-10 deficient mice,[13][14] trinitrobenzene sulfonic acid (TNBS) or dextran sulfate sodium (DSS) chemically induced colitis in mice [15][16] and transgenic HLA-B27 rats.[17]

Irritable bowel syndrome (IBS) is one of the most common chronic disorders in which abnormal discomfort or pain is associated with defecation or a change in bowel habit, in the absence of other disease that could explain the symptoms.[18] It is likely that various factors contribute to the causation of symptoms, but no final mechanisms have yet been agreed upon. However there is general agreement that increased sensitivity to visceral pain and/or disordered autonomic function is one of the mechanisms involved in symptom generation in patients with IBS.[19][20][21] The autonomic pathways are also a major mediator of the visceral pain response and altered autonomic balance may alter visceral perception.[22] The magnitude of the autonomic response to visceral stimulation such as distension can be used to quantify the intensity of visceral perception and of
visceral autonomic function in animal models. Colorectal distension (CRD) is an established technique \cite{23}\cite{24} for delivering noxious visceral stimuli to rats. There have been a few clinical trials \cite{25}\cite{26} which have suggested that treatment with various probiotic bacteria can improve IBS symptoms. In a recent study, Kim et al reported \cite{27} that a probiotic formulation containing 8 different probiotic species, VSL\#3, appeared to be promising in the relief of abdominal bloating in patients with diarrhea-predominant IBS. O’Mahony et al have recently also shown \cite{28} that oral administration of \textit{Bifidobacterium infantis} significantly improved the symptoms including the abdominal pain/discomfort score in IBS patients in a randomized controlled trial. However the exact mechanism of action of probiotics in IBS is poorly understood. The functional effects of probiotics on the autonomic nervous system and visceral perception have not yet been examined in otherwise unstimulated healthy animals housed under conventional conditions.

The reason we chose to study \textit{Lactobacillus reuteri} (LR) was that it has been described as having a positive effect on the incidence of diarrhea in children \cite{29} and also inhibited the onset of colitis in transgenic IL-10 deficient mice. \cite{14}

In the present study, we investigated the effects of live LR, a probiotic bacterium, on cardio-autonomic responses and single fiber unit discharge in dorsal root ganglia to CRD in healthy male \textit{Sprague-Dawley} rats. In addition, to explore the mechanisms of action of probiotic therapy, we tested whether nonviable (gamma irradiated or heat killed) LR or some structural components or secreted products of this microorganism (conditioned media) were also effective. The effects of live or heat killed LR on somatic pain were also examined.
MATERIALS AND METHODS

a) Animals:

All experiments were performed using male *Sprague-Dawley* rats (Charles River Breeding Laboratories, Saint Constant, QC, Canada) weighing 360-514g. Rats were housed in the Central Animal Facilities in micro-isolator cages equipped with filter hoods, under controlled temperature (20°C), with a 12:12 hr light-dark cycle, and free access to food and water. Animals were fasted for 18 hr prior to experiments, but had unrestricted access to water. All experiments were approved by the Animal Care Committee of McMaster University, and all procedures were conducted in accordance with the Guidelines of the Canadian Council on Animal Care.

b) Bacterial preparations:

*LR* were purchased originally from the America Tissue Type Culture Collection (ATCC #23272). From frozen stocks (-80°C), *LR* were inoculated in fresh Man-Rogosa-Sharpe liquid medium (MRS broth; Difco Laboratories, USA) and grown at 37°C under anaerobic conditions for 48 hr. Bacteria were put into 50 ml sterile tubes in 40 ml MRS broth. After 2 days, bacteria had grown so much that they settled on the bottom of the tubes. Tubes were centrifuged at 2000 rpm for 15 min at room temp, washed twice with sterile PBS until the color was white and measured 32 (yellow) exactly in a Vitek colorimeter when appropriately diluted. This reading gives a concentration of $6 \times 10^8$ bacteria/ml. 8.5 ml of bacterial suspension were centrifuged in 15 ml tubes at 2000 rpm for 15 min at room temp; supernatants were discarded and bacteria resuspended in 1 ml of MRS broth to give a concentration of $5 \times 10^9$ bacteria per milliliter. Heat killed *LR* were prepared by heating aliquots of viable bacterial suspensions for 20 min at 80°C. Resuspended viable *LR* cells were killed by gamma irradiation with Cobalt 60 for 20 hr at 8.05 Gy/min. The resulting viability was determined by plating on MRS agar plates under anaerobic conditions for 72 hr at 37°C. No
bacterial growth was detected in either the heat killed or irradiated LR preparations.

c) Treatment protocol:
Rats were divided into five groups in the experiments. After handling for 1 week, they were fed by gavaging $1 \times 10^9$ organisms in 0.2 ml daily for 9 days (live $n=30$, heat killed $n=9$, gamma irradiated $n=6$ or conditioned medium $n=6$). Medium preparations consisted of either native or centrifuged (cell free) medium after 48 hr of bacterial culture (conditioned medium). Rats treated with native medium served as controls. Gavage was performed every morning using an 18 gauge blunt needle in unanesthetized animals.

d) Cardio-autonomic response to colorectal distension:

a) Experimental protocol
Rats were anesthetized with a Ketamine hydrochloride (75 mg/kg) and Xylazine (10 mg/kg) mixture, given intraperitoneally. Supplemental anesthesia was given throughout the study as required. A 5cm length plastic balloon, affixed to a Teflon catheter with a length of approximately 20 cm, was inserted intrarectally into the distal colon so that the tip was 6 cm from the anus. The catheter was connected to a barostat system composed of a flow meter and pressure control program (Distender, G&J Electronic Inc. Toronto, Canada). The cardiac response to CRD was measured while inflating the balloon with air to pressures of 50, 70 and 80 mmHg for 60 sec. Only one set of distensions per animal per day was performed. In order to let the animal recover from the previous distension, 10 min rest was allowed between each distension.

b) Data acquisition
The methodology has been published previously elsewhere.[30] In brief, continuous recordings of heart rate were performed through a surface electrocardiogram (ECG), obtained through 3 needle
electrodes applied to the left and right shoulders and the right hind leg. The signal was amplified and recorded on a personal computer using a commercial data acquisition program (Experimenter’s Workbench, DataWave Technologies, Longmont, CO, USA). Heart rate was measured for 60 sec before, during and after the CRD, for a total of 180 sec. Heart rate data were presented as the average heart rate for every 10 sec period during distension. Changes in heart rate were expressed as percent change compared to the resting heart rate prior to the distension in each animal.

e) Single fiber-unit discharge (Dorsal root ganglion) responding to CRD:

Four groups of rats treated with native medium (control, n=8), viable (n=6), heat killed (n=6) and gamma irradiated (n=7) bacteria were tested for effects of probiotic treatment on the visceral sensory afferent pathway response to colorectal stimuli by examining single unit discharge induced by CRD.

a) Experimental protocol

The methodology has been previously published.[31] Briefly, after anesthesia with Ketamine hydrochloride (90 mg/kg) and Xylazine (20 mg/kg), a laminectomy was performed to expose the 6th left lumbar dorsal root ganglion (LL6 DRG) and its root (LL6 DR). The skin and connective tissue were used to create a pool in which nerve tissue was immersed in mineral oil at 36-37°C to protect the spinal cord and exposed LL6 DRG. Bipolar tungsten electrodes were used to record single unit discharge. The LL6 DR between LL6 DRG and spinal cord was hooked on the negative electrode. A fine filament of nerve peeled off with forceps from LL6 DR and the proximal end of the filament was wrapped around the tip of the positive electrode. The electrodes were connected to the headstage (AI-402, Axon Instruments, CA, USA).

b) Data acquisition
The signals were amplified and processed through the programmable signal conditioner (CyberAmp 380, Axon Instruments, Foster City, CA, USA). The amplified signals were digitalized at 2 KHz by an A/D converter and stored on a personal computer using the same data acquisition program as for ECG data.

6) **Somatic pain measurement:**

Three groups of rats treated with native medium (control, n=5), viable (n=6) or heat killed (n=5) bacteria were examined for effects of probiotic treatment on somatic pain. Assessment of somatic pain was evaluated by measuring the threshold to thermal (Tail flick test) or mechanical stimuli (paw pressure test).

a) **Thermal stimuli; Tail flick test**

The tail of the rat was immersed, up to 5 cm from the tip, in a water bath (Poly Science Div. of Preston Ind. Inc., Niles, IL, USA) at a noxious temperature of 50±1°C. The latency from onset of stimulation to tail withdrawal was recorded.[32]

b) **Mechanical stimuli; Paw pressure test**

Nociceptive thresholds, expressed in grams, were measured with the Randall-Selitto analgesimeter (probe tip diameter 1 mm) (Ugo Basile, Biological research apparatus, Milan, Italy) by applying increasing pressure at a constant rate on the rat paw until the paw was withdrawn.[32]

7) **Microbiologic analysis:**

Stool samples were collected before and after probiotic treatments. Each sample was weighed, homogenized and diluted in 1 ml of 40 % glycerol/PBS solution. The dilutions were spread onto *Lactobacilli* selective MRS agar and anaerobically inoculated in triplicate at 37°C and incubated for 72 hr. *Lactobacilli* colonies were counted on the MRS plate. The data were expressed as
colonies/mg of feces.

8) Statistical analysis:
All data except somatic pain parameters were presented as mean± SEM. Student’s t test (paired), ANOVA (repeated or unrepeated) and the Newman-Keuls multiple comparison test were used as appropriate. Somatic pain parameters were expressed as median (range) and evaluated by the Mann-Whitney test. A p value < 0.05 was considered statistically significant.

RESULTS

Base line heart rate:
The base line resting heart rate was measured for each rat, for 60 sec prior to CRD. The resting heart rates were expressed as mean average beats per min. In control anesthetized animals this was between 190 and 404 /min, with a mean rate of 246.7± 6.2 /min. No significant differences in the resting heart rate were seen among groups (data not shown).

Effect of LR on heart rate response to CRD:
CRD caused a decrease in heart rate in the control group (n=10) that peaked at various times post distension, depending on the distension pressure (Figure 1). The response began at the moment of balloon inflation and persisted throughout distension. This response to the CRD was not observed in the group treated with live LR, even during the maximum distension pressure (80mmHg) (Figure 2). The inhibition of heart rate response to CRD by LR remained significant compared to the control in a subgroup analysis based on animals’ body weight.

Similar inhibition of cardio-autonomic responses was seen in the heat killed, gamma irradiated and conditioned media groups (Figure 3).
**Effect of LR on single fiber-unit discharge in response to CRD:**

LL6 DR basal single unit discharge at rest in control group was significantly higher than that of any other groups. The stimulus-response curve is shown in Figure 4. A pressure-dependent colonic afferent fiber response to CRD is evident in all groups. Responses to 60 mmHg were significantly reduced to < 60% of the control group by the feeding of viable or non-viable (heat killed and gamma irradiated) bacteria. The administration of viable and both types of non-viable bacteria significantly decreased single unit activity to CRD compared with the medium group at all pressures.

**Effect of LR on somatic pain:**

Tail flick latencies and paw withdrawal thresholds due to pressure were used as the thermal pain and mechanical pain parameters, respectively. Table 1 shows the results of these tests in each group (Table 1). There were no significant differences between the control, viable and heat killed groups in either the tail flick or paw pressure tests.

**TABLE 1: Effect of Probiotic Treatment on Somatic Pain**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Viable (n = 11)</th>
<th>Heat Killed (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail Flick (seconds)</td>
<td>5.2 (3.7-6.3)</td>
<td>5.8 (4.3-8.7)</td>
<td>5.0 (3.3-7.5)</td>
</tr>
<tr>
<td>Paw Pressure (grams/mm²)</td>
<td>287 (203-508)</td>
<td>259 (196-437)</td>
<td>433 (293-599)</td>
</tr>
</tbody>
</table>

Data are expressed as median (range). Control: native medium group, Viable: viable group, Heat Killed: heat killed group. NS: Statistically not significant when compared to control.
Microbiologic evaluation:

After administration of live bacteria, the fecal concentration (67.4 ± 23.6 x10^5 colonies/mg feces) of *Lactobacilli* increased significantly when compared with the basal level (1.5 ± 1.4 x10^5 colonies/mg feces). Numbers did not change in any of the other groups (n=6-15).

DISCUSSION

We examined the effects of oral treatment of a probiotic bacteria, *Lactobacillus reuteri*, on the cardio-autonomic response to CRD in *Sprague-Dawley* rats. The findings of this study were as follows: (a) Noxious CRD caused a decrease in heart rate in anesthetized healthy control rats. (b) Oral administration of both live and nonviable (heat killed and gamma irradiated) LR attenuated the cardiac and colonic afferent fiber responses to CRD. This inhibitory effect on pain was also reproduced with conditioned medium. (c) There was no effects of these treatments on somatic pain.

It is widely accepted that the intestinal microflora play an important role in the health of the host and also possesses immunomodulatory capacity.[33][34][35] A disturbance in the balance of normal intestinal microflora, or the host response to this, has been demonstrated to play a critical role in the pathogenesis of IBD.[33][36] Non-pathogenic bacteria may modify immune responses of the intestinal mucosa through interaction and signaling at mucosal surfaces. Probiotic bacteria offer a means of modifying the enteric microflora, and their therapeutic effects may include competitive interaction with commensal and pathogenic flora, production of antimicrobial metabolites, or influence on the immune response by modulating mucosal and systemic immunity,[33][34] epithelial function [33][37] and improving nutritional and microbial balance in the intestinal tract. However, the exact mechanisms of probiotic action are not well understood.
The most interesting finding in this study is the elimination of any cardio-autonomic response to the highest CRD pressures in normal rats after treatment with live or dead probiotic bacteria and even after treatment with conditioned medium. The existence of brain-gut relationships suggests that autonomic pathways through sensory afferent nerves are involved in informing the brain of the physiological and pathological events that occur in visceral organs. Several models of visceral perception have been demonstrated [23][24][30] in animals based on the study of visceromotor or cardiovascular responses to gastrointestinal and colonic distension. Cardiovascular responses to CRD reflect the intensity of sensory perception or the integrated autonomic function. In IBS patients, some investigators suggest that visceral hypersensitivity is involved in the etiology of the disorder, while others emphasize the importance of disturbed autonomic function.[38] Our results suggest that a particular probiotic, live or dead, or even products found in culture medium, can reduce the normal visceral sensitivity to CRD.

There is little evidence from published research that similar effects of probiotic organisms have been seen before on the autonomic nervous system. Kamm et al. [39] have recently shown in pigs that a probiotic yeast, *Saccharomyces boulardii* seemed to decrease the number of calbindin positive myenteric neurons. This was the only marker of many tested which showed any change following probiotic treatment: these included calcitonin gene-related peptide, nitric oxide synthase, vasoactive intestinal polypeptide and substance P. *L. farciminis* treatment inhibited a delayed hypersensitivity model of colitis in rats, probably through NO release.[40] Monocontamination of germ-free rats with *L. acidophilus* or *Bifidobacterium bifidum* has been shown to reduce the migrating myoelectric complex period.[41] In the latter study neuropeptide Y was decreased in the blood following conventionalization with full intestinal microflora, suggesting reduced inhibitory control of intestinal propulsion.

In our study, administration of conditioned media and nonviable bacteria (both heat killed and
gamma irradiated) inhibited the autonomic response to CRD. Previous studies indicated [42] that heat killed *Lactobacillus acidophilus* retained adhesion capacity to the epithelial receptor sites. Furthermore, bacterial peptidoglycan, an essential component of Gram-positive bacterial cell walls, is known to activate Toll-like receptors (TLR2). Indeed, the composition of lipoteichoic acid in the cell wall of a probiotic bacterium, *Lactobacillus plantarum*, has been recently shown to modulate both pro- or anti-inflammatory immune responses.[43] Bacterial lipopolysaccharide also interacts with TLR2 and TLR4.[44][45] Many commensal organisms can influence innate immune mechanisms through Toll receptors. On the other hand, nonviable gamma irradiated bacteria, but not heat killed bacteria had an attenuating effect on experimental colitis in mice, and this immunoregulation was mediated by TLR9–probiotic DNA motif interaction.[15][46] However, since heat killed organisms were equally effective as gamma irradiated in our studies, we infer that there are differences between the mechanisms of action of probiotics on inhibition of mucosal inflammation through Toll receptors and the elimination of autonomic responses to distension.

We found that oral administration of *Lactobacilli* had an inhibitory effect on constitutive discharge in colonic afferent fibers in the dorsal root ganglia. Furthermore, the measurements of single fiber discharge frequency in response to CRD paralleled the cardioautonomic effects observed on CRD. This clearly showed that the effect was localized to the intestine or its neuronal connections to the spinal cord. We previously demonstrated [47] that colonic distension produced a volume dependent bradycardia mediated through sympathetic afferent and cholinergic vagal efferent pathways in *Sprague-Dawley* rats. Under anesthesia, neither the thalamus nor the cerebral cortex are necessary for the evocation of cardio-autonomic response to CRD. The brainstem and hypothalamus integrate autonomic responses. We could find no effect of probiotic treatment on somatic pain in either of the standard tests employed. Taking these results on somatic pain into
consideration, our results obtained with the DRG single-unit discharge suggest that the observed effects were peripheral and not central (brain).

An interesting recent study has shown that another strain of *Lactobacilli (paracasei)* attenuated smooth muscle hypercontractility post *Trichinella* infection.[48] This effect was heat labile and a component in the conditioned medium also had the same effect. The effect of gamma irradiated organisms was not tested. The authors concluded that the effect was likely mediated by the immune response to infection and direct effects on smooth muscle. Again Verdu et al. very recently showed that administration of *L. paracasei* or the spent culture medium prevented the antibiotic-induced increase in visceromotor response and inflammatory activity, and effectively decreased immuno-staining for substance P.[49] In their model the direct effect of probiotic treatment on modulation of visceral perception by the autonomic nervous system in healthy animals was not tested. These results clearly show that another *Lactobacillus* strain can modulate inflammation-associated visceral hypersensitivity responses in a murine model. In view of our findings in healthy conventionally housed rats, an additional important mechanism to explain their observations might be through a more direct effect of the probiotic on a component of the nervous system. Alternatively, the involvement of visceral pain perception resulting from inflammation may have a different functional mechanism.

In conclusion, this is the first study to examine the effect of probiotic bacteria on autonomic function and visceral perception *in vivo* in normal animals. Oral administration of live or dead LR and even conditioned medium showed marked inhibitory effects on the cardio-autonomic response to CRD in *Sprague-Dawley* rats. These data provide supportive evidence for further explanation of the effect of probiotics on visceral pain and provide a novel mechanism of effect of probiotics in the treatment of patients with functional bowel disorders such as IBS.
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Conflict of interest: This is a statement to declare all authors have no competing interests
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FIGURES AND FIGURE LEGENDS

Figure 1: Time course of cardiac response to CRD of 50, 70 and 80 mmHg in control (native medium) group rats. Post 1 and 2 represented an average of heart rate response during first and last 30 seconds after distension. Data presented as mean±SEM of % change in heart rate compared with resting heart rate (n=10). * p<0.05 vs. resting.

Figure 2: Effect of oral administration of live Lactobacillus reuteri (LR) on cardiac response to CRD of 50, 70 and 80 mmHg. Data presented as mean±SEM of maximum % change in heart rate during distension compared with resting heart rate. Treatment: Control (n=10), Viable LR (n=30), * p<0.01 vs. control.

Figure 3: Effect of various probiotic treatments on cardiac responses to CRD. Data presented as mean±SEM of maximum % change in heart rate during distension compared with resting heart rate. Treatment: Control (n=10), Viable LR (n=30), Heat killed LR (n=9), Gamma irradiated LR (n=6), Conditioned media group (n=6), * p<0.01 vs. control.

Figure 4: Effect of various probiotic treatments on LL6 DR single-unit discharge to CRD. Data presented as mean±SEM. Treatment: Control (n=8), Viable LR (n=6), Heat killed LR (n=6), Gamma irradiated LR (n=7), * p<0.05 vs. various treatment groups.
Figure 1

The figure shows a graph plotting heart rate as a percentage of resting heart rate over time for different CRD conditions and blood pressures. The x-axis represents time in seconds, ranging from Rest to Post 2, with intervals at 10, 20, 30, 40, 50, 60, Post 1, and Post 2. The y-axis represents the percentage of resting heart rate, ranging from 85% to 110%. Different symbols and lines indicate data points for 50 mmHg, 70 mmHg, and 80 mmHg blood pressures. Asterisks denote statistically significant differences.
Figure 2

![Graph showing the relationship between intracolonic pressure (mm Hg) and % resting heart rate for Control (n=10) and Viable (n=30) samples.](image-url)

- **Control, n=10**: The % resting heart rate remains relatively constant across different intracolonic pressures.
- **Viable, n=30**: There is a noticeable decrease in % resting heart rate as intracolonic pressure increases, indicated by a star (*) on the graph.
Figure 3

![Graph showing % Resting heart rate against Intracolonic pressure (mm Hg) for different conditions: Control, Viable, Heat killed, Gamma irradiated, and Conditioned media.](image)

- **Control, n=10**
- **Viable, n=30**
- **Heat killed, n=9**
- **Gamma irradiated, n=6**
- **Conditioned media, n=6**
Figure 4

- **Control, n=8**
- **Viable, n=6**
- **Heat killed, n=6**
- **Gamma irradiated, n=7**

**Intracolonic pressure (mm Hg)**

**Single unit discharge (freq./sec)**

* denotes statistical significance.
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