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## Publication Date
2021

## DOI
10.1177/17448069211022952

Peer reviewed

*Short Report*

MOLECULAR PAIN

Molecular Pain
Volume 17: 1–7

DOI: 10.1177/17448069211022952
journals.sagepub.com/home/mpx
SAGE

# A role for gut microbiota in early-life stress-induced widespread muscle pain in the adult rat

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**Abstract**

Adult rats that experienced neonatal limited bedding (NLB), a form of early-life stress, experience persistent muscle mechanical hyperalgesia. Since there is a growing recognition that the gut microbiome regulates pain and nociception, and that early-life stress produces a long-lasting impact on the gut microbiome, we tested the hypothesis that persistent muscle hyperalgesia seen in adult NLB rats could be ameliorated by interventions that modify the gut microbiome. Adult NLB rats received probiotics, either *Lactobacillus rhamnosus* GG (10 billion CFU/150 ml) or De Simone Formulation (DSF) (112.5 billion CFU/150 ml mixture of 8 bacterial species), in their drinking water, or non-absorbable antibiotics, rifaximin or neomycin, admixed with cookie dough, to provide 50 mg/kg. Mechanical nociceptive threshold in the gastrocnemius muscle was evaluated before and at several time points after administration of probiotics or antibiotics. Adult NLB rats fed probiotics *L. Rhamnosus* or DSF, antibiotics, as well as rats fed non-absorbable antibiotics rifaximin or neomycin, had markedly attenuated muscle mechanical hyperalgesia. We hypothesize that persistent skeletal muscle hyperalgesia produced by NLB stress may be, at least in part, due to a contribution of the gut microbiome, and that modulation of gut microbiome using probiotics or non-absorbable antibiotics, may be novel therapeutic approaches for the treatment of chronic musculoskeletal pain.

**Keywords**

Neonatal limited bedding, myalgia, nociceptors, probiotics, antibiotics, microbiome

Date Received: 22 April 2021; Revised 13 May 2021; accepted: 18 May 2021

## Introduction

There is an increasing appreciation that the gastrointestinal (gut) microbiome affects the course and severity of many biological processes and diseases, including pain.[1–6] Most studies evaluating the role of the gut microbiome in pain have focused on modulation of visceral pain and hypersensitivity (e.g. preclinical models, as well as in inflammatory bowel disease and colitis),[7–10] and others have shown that the gut microbiome may affect chemotherapy-induced neuropathic pain.[11,12] And, while some clinical evidence shows that there may be a relationship between gut dysbiosis and fibromyalgia symptoms,[13,14] no studies have directly investigated whether manipulation of the gut microbiome affect musculoskeletal pain. In this study we tested the hypothesis that persistent muscle pain in adult rats (produced by early-life stress) is affected by modulating gut microbiome. Using a model of early life stress-induced adult muscle pain, based on the disruption of maternal care by limiting bedding/nesting material (neonatal limited bedding, NLB).[15,16]

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## Methods

### Animals

Primiparous timed-pregnant female Sprague Dawley rats were obtained from Charles River (Hollister, CA). Dams were housed with their litter in standard cages on postnatal days 0 – 1. On postnatal day 2, litters were assigned to limited bedding (NLB) or standard care (control) conditions, or received corticosterone. Behavioral experiments were performed on 220–280 g adult female rats.

Animals were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12 h light/dark cycle (lights on 7 am–7 pm) and environmentally controlled conditions; ambient room temperature (21–23°C), with food and water available *ad libitum*. Their care and use in experiments conformed to National Institutes of Health guidelines and measures were taken to minimize pain and discomfort. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

### NLB stress

We used the NLB protocol, a well-established model of early-life stress.[17] Dams and their pups were housed in standard cages on postnatal days 0 and 1, and beginning on postnatal day 2, dams and their pups were placed in cages fitted with a custom stainless steel mesh grid bottom (Ancare, Bellmore, NY), raised ~2.5 cm from the floor of the home cage, to provide space for collection of urine and feces.[18] The nesting/bedding material provided consisted of one sheet of paper towel (~112 × 22 cm), and no environmental enrichment. Litters were left undisturbed during postnatal days 2 – 9. From postnatal day 10 until weaning, dams and pups were again housed in standard cages with normal bedding (Paperchip® animal bedding, Shepherd Specialty Papers, Watertown, TN), and standard enrichment. On postnatal day 21 pups were weaned and same sex rats housed 3 per cage, in standard housing conditions.

### Probiotic and antibiotic feeding

*Probiotics.* Rats receiving probiotics were divided into 3 groups: the control group received only tap water for drinking, in another group the drinking water contained *Lactobacillus rhamnosus* GG (10 billion CFU/150 ml *L. rhamnosus*, Culturelle®, Amerifit, Inc, Cromwell, CT), and in the third group the drinking water contained De Simone Formulation (DSF) (112.5 billion CFU/150 ml mixture of *L. acidophilus, L. plantarum, L. casei, L. delbrueckii* subspecies *bulgaricus, Bifidobacterium breve, B. longum, B. infantis,* and *Streptococcus salivarius* subspecies *thermophilus*, VSL Pharmaceuticals, Inc, Towson, MD). Drinking water containing probiotics was made fresh each day. Water or probiotic-containing water was provided to rats *ad libitum* for 8 days.

*Non-absorbable antibiotics.* Typically, administration of non-absorbable antibiotics to rats is accomplished by gavage feeding. However, in order to eliminate stress associated with gavage feeding (stress would affect nociceptive threshold), we employed an established method of voluntary oral administration,[19] in which rifaximin or neomycin was admixed with 4 g of cookie dough mix (Pillsbury™ Sugar Cookie dough); this method of feeding has been shown to have an ingestion reliability of 99.9–100% in Sprague Dawley rats. To acclimate rats, they were fed cookie dough for 3 days prior to being fed the cookie dough-antibiotic mix. Rats receiving antibiotics were divided into 3 groups: the control group fed cookie dough, another group, cookie dough contained rifaximin (to provide 50 mg/kg; Xifaxan, Salix Pharmaceuticals, Inc.), and a third group, cookie dough containing neomycin sulfate (to provide 50 mg/kg; MilliporeSigma, Burlington, MA). Rats were fed cookie dough (plain, or with non-absorbable antibiotics) daily for 10 days.

### Mechanical nociceptive threshold in skeletal muscle

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a Chatillon digital force transducer (model DFI2, Amtek Inc., Largo, FL).[16] Rats were placed in cylindrical acrylic restrainers designed to minimize restraint stress and allow extension of their hind legs from lateral ports. To acclimatize rats to the testing procedure, they were placed in restrainers and exposed to the testing procedure, daily for 3 days, prior to starting experiments. On the day of the experiment, rats were placed in a restrainer for 30 minutes before experimental manipulations. To determine nociceptive threshold, a 7-mm diameter probe attached to the force transducer was applied to the gastrocnemius muscle to deliver an increasing compression force. The nociceptive threshold was defined as the force, in Newtons, at which the rat withdrew its hind leg. Mechanical nociceptive thresholds was determined by measuring the mean of 3 withdrawal thresholds taken at 5-min intervals; one hind limb of each rat was used. All behavioral testing was done between 10 am and 4 pm (no differences in baseline nociceptive threshold was observed over this time period), and was performed blind to treatment condition.

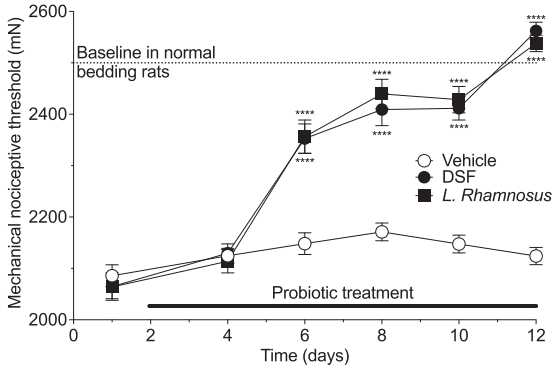


**Figure 1.** Probiotics DSF and L. Rhamnosus reverse NLB-induced muscle mechanical hyperalgesia. Adult NLB rats received tap water (vehicle), or tap water containing DSF (112.5 billion CFU/150 ml) or L. Rhamnosus (10 billion CFU/150 ml). NLB rats were hyperalgesic (dashed lines indicate muscle mechanical nociceptive threshold in rats raised on standard bedding) prior to probiotics. While rats receiving tap water showed no change in nociceptive threshold, both probiotics significantly increased nociceptive threshold in males (2- way ANOVA, interaction $F_{8,132} = 21.67$, $P < 0.0001$, Tukey's multiple comparison test showed significant differences for both probiotics from vehicle control rats on days 6 – 12 ****$P < 0.0001$); all groups n = 12.

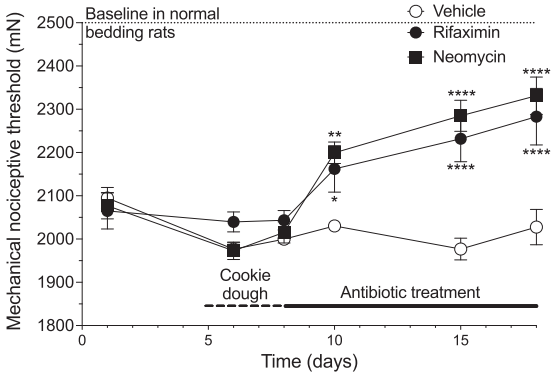


**Figure 2.** Antibiotics, rifaximin and neomycin, attenuate NLB-induced muscle mechanical hyperalgesia. Adult NLB rats were fed 4 g cookie dough (vehicle), or cookie dough containing rifaximin (50 mg/kg in 4 g cookie dough) or neomycin sulfate (50 mg/kg in 4 g cookie dough). The muscle mechanical nociceptive threshold of NLB rats was lower (i.e., they were hyperalgesic) compared to rats raised on standard bedding (threshold indicated by dashed lines) prior to antibiotic feeding. While rats receiving vehicle (cookie dough) showed no change in nociceptive threshold, both antibiotics significantly increased nociceptive threshold (2-way ANOVA, Time x Antibiotic treatment interaction $F_{16,171} = 5.78$, $P < 0.0001$). Dunnett's multiple comparison test showed significant differences for both antibiotics from vehicle control rats on days 10 – 22, *P,0.05, **$P < - 0.005$, ****$P < 0.0001$); Control n = 6, both rifaximin and neomycin n = 10.

### *Statistical analyses*

Group data are expressed as mean ± SEM of n independent observations. Statistical analysis of experimental data, conducted using Prism 9 (GraphPad Software, San Diego, CA), employed two-way analysis of variance (ANOVA) for groups with equal numbers (Figure 1), or a mixed-effect analysis (restricted maximum likelihood) for groups with unequal numbers (Figure 2). Where there was a significant main difference between treatment groups, Dunnett's post-hoc test was used. The accepted level for significance was $P < 0.05$.

## Results

### *Administration of probiotics, DSF and L. Rhamnosus, in adult rats attenuates NLB-induced muscle hyperalgesia*

Adult NLB rats treated with a probiotic, either DSF or *L. Rhamnosus*, showed markedly higher mechanical nociceptive threshold in the gastrocnemius muscle compared to control (vehicle-fed) rats (2-way repeated measures ANOVA, time × antibiotic treatment, $F_{8,132} = 21.67$, $P < 0.0001$, Figure 1). And, by day 10 of probiotic feeding, nociceptive threshold was similar to that seen in rats raised on standard bedding during postnatal days 2–9.

### *Administration of rifaximin and neomycin, in adult rats, attenuates NLB-induced muscle hyperalgesia*

Adult rats that had been exposed to the limited bedding protocol during postnatal days 2–9 (NLB), have a lower mechanical nociceptive threshold compared to rats that had standard bedding during the same postnatal period. When rats were fed non-absorbable antibiotics, rifaximin or neomycin, muscle mechanical hyperalgesia in NLB rats was markedly attenuated compared to control (vehicle-fed) rats (2-way repeated measures ANOVA, time × antibiotic treatment, $F_{8,79} = 7.211$, $P < 0.0001$; Figure 2). Antibiotic feeding was stopped after 10 days, and by the seventh day of normal diet, nociceptive threshold decreased to the level seen in vehicle-fed rats.

## Discussion

In this study, we found that persistent skeletal muscle hyperalgesia seen in adult rats that had been exposed to early-life stress was markedly attenuated by administration of non-absorbable antibiotics (rifaximin, neomycin), or of probiotics (DSF, *L. Rhamnosus*). It is likely that the marked attenuation of the persistent muscle hyperalgesia by these treatments is due to the effect of changes in the gut microbiome, since both the antibiotics[20–23] and probiotics have been shown to affect gut microbiome diversity.

It is well-established that early-life stress affects gut microbiome,[24–29] a dysbiosis that persists into adulthood.[24,25,29] Importantly, in addition to local effects, such as visceral pain[30,31] and gut permeability,[26,27] gut dysbiosis has been implicated in several systemic pathologies, including altered behavior (e.g. depression and anxiety),[24,32,33] Parkinson's disease,[34] increased hypothalamic pituitary adrenal axis (HPA) activity,[35] systemic lupus erythematosus[36] and systemic inflammation.[37,38] And, there is a growing appreciation that the gut microbiome may contribute to chronic extra-abdominal pain states,[1,3–6] for example in cutaneous inflammatory hyperalgesia[39] and paclitaxel-induced cutaneous thermal and mechanical nociception.[11] However, to the best of our knowledge evaluation of the role of the gut microbiome on skeletal muscle mechanical hyperalgesia has not previously been evaluated.

The mechanism by which early-life stress produces muscle hyperalgesia the persists in to adulthood has yet to be determined. However, it is known that early-life stress (maternal separation), produces a change in beta diversity (i.e. a change in microbial composition, or dysbiosis), for example a deficiency in *Lactobacillus*,[26,40] *Staphylococcus* and *Mucispirillum*,[29] *Clostridium*, *Bilophia*[30] and increases in *Bacteroides*,[29] *Alloprevotella* and *Acetivibrio*.[30] Other studies have observed additional changes in gut microbiome following early-life stress and with 5,000 – 10,000 bacterial species it is going to be challenging to determine which bacterial species contribute to local or systemic hyperalgesia. However, altering the gut microbiome population by administration of locally-acting antibiotics or by probiotics has been shown in preclinical and clinical studies to attenuate stress-induced visceral hyperalgesia,[22,23,30,41] as well as attenuate neuropathic cutaneous mechanical allodynia and thermal hyperalgesia.[42,43] How probiotic and antibiotic administration can ameliorate hyperalgesia, which is currently unknown, may depend on more than one mechanism. One mechanism may depend on modifying stress-induced gut dysbiosis. For example, early-life stress-induced gut dysbiosis and gut permeability, is ameliorated by probiotics,[18,26,30,44] as well as by the antibiotic, rifaximin.[22,45] Stress-induced increase in gut permeability results in increased levels of lipopolysaccharide (LPS) and inflammatory cytokines, an effect that is reversed by probiotics.[46] Importantly, in addition to affect the gut microbiome, early-life stress produces a decreased expression of tight junction expression in the gut,[47,48] leading to persistent increase gut permeability,[26,49] which allows for systemic leakage of bacteria products, such as LPS,[45] proinflammatory cytokines,[50,51] and bacterial translocation from the gut.[52] Since LPS, inflammatory cytokines and bacteria act directly on nociceptors to decrease nociceptive threshold and increase neuronal excitability,[53–58] since early-life stress-induced increased gut permeability persists in to adulthood,[59] leakage of LPS, cytokines and/or bacteria could contribute to hyperalgesia seen in adult NLB rats. While NLB-induced stress increase in gut permeability could affect the pharmacokinetics of the poorly absorbable antibiotics used in this study, rifaximin reverses stress-induced gut permeability to normal, non-stressed levels,[22,45] and neomycin also reduces gut permeability.[60] Given these effects of the antibiotics, it is unlikely that the increased gut permeability seen in NLB adult rats would significantly affect antibiotic pharmacokinetics. In addition to leakage of mediators or bacteria, the gut microbiome may affect nociception via direct action on the vagus nerve. We[61,62] and others[63,64] have shown that activity of vagal visceral afferents have a marked effect on somatic nociceptor sensitivity. And, since afferent vagus neurons innervating the gut sense resident bacteria and their mediators and metabolites[2,65,66] this could be another mechanism whereby the gut microbiome influences nociceptive threshold. While our data indicate that the probiotics and antibiotics we used produce an antihyperalgesic effect on NLB-induced hyperalgesia, it is possible that these agents could have independent analgesic effects. However, to the best of our knowledge, none of these agents has been reported to be analgesic following oral administration.

In summary, persistent mechanical hyperalgesia in adult rats exposed to early-life stress (NLB) is attenuated by interventions that modify the gut microbiome, either through administration of probiotics or non-absorbable antibiotics. We hypothesize that the gut microbiome and/or gut permeability is altered by early-life stress, which results in enhanced nociceptive excitability, either by action of gut microbiome-derived pronociceptive mediators and/or via modulation of vagal afferent activity. Our results suggest novel therapeutic approaches for the treatment of chronic musculoskeletal pain involving modulation of gut microbiome using probiotics or non-absorbable antibiotics.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the NIH grant AR063312.

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