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Does Lactobacillus reuteri Probiotic Treatment Improve Sleep Quality in Rhesus Macaques (Macaca mulatta) Displaying the Self-injurious Phenotype?

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Does *Lactobacillus reuteri* Probiotic Treatment Improve Sleep Quality in Rhesus Macaques (*Macaca mulatta*) Displaying the Self-injurious Phenotype?

A Thesis Presented

By

PETER NIKOLAI MCGINN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Does *Lactobacillus reuteri* Probiotic Treatment Improve Sleep Quality in Rhesus Macaques (*Macaca mulatta*) Displaying the Self Injurious Phenotype?

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To Samantha (Sam) Suchovic.
ACKNOWLEDGEMENTS

To Melinda, I cannot thank you enough for your endless patience and guidance. When I came to you in the summer of 2015 I was failing my classes, I couldn’t wake up on time for anything, and I had no confidence in my ability to be a scientist let alone make it through school. You decided to take a chance on me and my entire life changed. Through my many mentors in the lab, through countless presentations and lab projects, and most importantly from your mentorship, I slowly came to believe in myself. I wish I could put into words just how much of a role model you have been to me. Thank you.

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To my family and friends. There isn’t enough room to say all that needs to be said so I will just keep it simple, thank you.
ABSTRACT

DOES LACTOBACILLUS REUTERI PROBIOTIC TREATMENT IMPROVE SLEEP QUALITY IN Rhesus Macaques (MACACA MULATTA) DISPLAYING THE SELF INJURIOUS PHENOTYPE?

FEBRUARY 2019

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Directed by: Professor Melinda A. Novak

Self-injurious behavior (SIB) is a complex phenotype that occurs with an increasing prevalence of about 7-34% in humans and 10-12% in non-human primates (NHPs). This study evaluated the efficacy of probiotic Lactobacillus reuteri as a treatment for self-injurious behavior (SIB) and sleep disruption in rhesus macaques. The treatment was proposed to alleviate mild self-biting, sleep disruption, and reduce chronically elevated hypothalamic-pituitary-adrenocortical (HPA) axis activity, all hallmark features of monkeys with this condition. The probiotic preparation included two strains of L. reuteri (L. reuteri ATCC PTA 6475 & L. reuteri DSM 17938) containing on average 200 million colony forming units per chewable tablet. The study was conducted on 14 rhesus macaque monkeys (9 males) housed at the University of Massachusetts Amherst. To our knowledge this is the first time that a Lactobacillus strain has been used as a treatment for SIB in rhesus macaques. This study utilizes motion-activated infrared camera technology, modified enzyme-immunosorbent-assays (EIAs) techniques to measure hair cortisol concentrations, and daily behavioral observations to provide an overall assessment of the behavioral, physiological, and sleep associated implications of probiotic treatment on SIB and control non-human primates (NHPs). Administration of L
*reuteri* modestly decreased biting behavior in monkeys with SIB ($F_{n,m} = 5.64$, $p=0.02$) and showed overall decrease in nighttime activity across all subjects but did not normalize SIB to nonSIB values. Hair cortisol values are pending. These findings and the findings of previous work further strengthen the argument for probiotics as an efficacious treatment for SIB behavior.
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CHAPTER 1

INTRODUCTION

This study is a continuation of previous work evaluating the efficacy of probiotics to treat self-injurious behavior (SIB) and sleep disruption in rhesus macaques. It explores the efficacy of *Lactobacillus reuteri*, in alleviating the dominant SIB behaviors such as mild self-biting and hair-pulling as well as restoring balance to a chronically elevated hypothalamic-pituitary-adrenocortical (HPA) axis activity. The probiotic preparation includes two strains of *L. reuteri* (*L. reuteri* ATCC PTA 6475 & *L. reuteri* DSM 17938) containing on average 200 million colony forming units per chewable tablet. These strains have been shown in human and rodent models to influence the neurological, immune, and gastro-intestinal systems. To our knowledge this is the first time that a *Lactobacillus* strain has been used as a treatment for SIB in rhesus macaques and may begin to fill in the many gaps left by the focus on rodent models.

This study utilizes motion-activated infrared camera technology, modified enzyme-immunosorbent-assays (EIAs) techniques to measure hair cortisol concentrations, and daily behavioral observations to provide an overall assessment of the behavioral, physiological, and sleep associated implications of probiotic treatment on SIB and control non-human primates (NHPs).

1.1 Self-injurious Behavior (SIB)

SIB is idiopathic, meaning symptoms are not detected until onset, thus establishing cause and effect relationships is challenging (Novak 2003). However, one can retrospectively identify relevant factors in the pathology. The current working model of SIB in NHPs, as identified by our lab, is a coupling of early life stress in the form of
nursery rearing (Lutz et al. 2003, 2007) in concert with allelic variation in certain candidate genes related to serotonin expression and opioid receptor activity (Novak 2003, Chen et al. 2010). The presence of genetic factors and early life trauma does not guarantee SIB pathology, but retrospectively is typical of the majority of subjects displaying SIB behavior (Chen et al. 2010).

Interestingly, SIB can vary in intensity. It can present in mild forms: such as hair pulling or infrequent and mild self-biting. However, a more severe form includes both a higher incidence of SIB along with bites leading to wounds requiring veterinary care (Novak 2003). Mild cases are typically monitored but not treated. Deep wounding behaviors are more variable and can increase in frequency due to increased exposure to chronic stress such as cage relocation (Davenport et al. 2008).

Despite the variation described above, our lab has identified a number of key correlates of SIB such as chronically dysregulated hypothalamic-pituitary-adrenocortical axis activity (Davenport et al. 2006), increased anxiety (Tiefenbacher et al. 2005, Major et al. 2009), and increased sleep disruption that are documented in both mild and severe manifestations (Davenport et al. 2008, Stanwicks et al. 2017). It is important to also acknowledge that the prevalence of SIB in captive NHPs is not necessarily a result of captivity as the condition has also been documented in free-ranging Japanese macaques (Grewal 1981).

SIB is an increasingly significant problem in the human population and shares some commonalities with this pathology in monkeys. In humans, SIB is referred to as non-suicidal self-injurious behavior (NSSI) which is under consideration as a discrete disorder in DSM-5. NSSI can take several forms including but not limited to: burning, cutting, or even repeated picking at skin or hair (Simeon & Favazza 2001, Butler &
NSSI can be a symptom of both genetic disorders such as Cornelia de Lange syndrome (Oliver et al. 2009) and psychiatric disorders like borderline personality disorder (Andover et al. 2011) but may also be triggered by early-life trauma (i.e. sexual abuse) (Briere & Gil 1998). It is suggested that NSSI may in fact fall into two subtypes; a reactive form in response to stressors and a compulsive form without a discernable stimulus (Simeon & Favazza 2001). Similar to rhesus macaques, humans with NSSI have sleep disruption (Brylewski & Wiggs 1999, Symons et al. 2000) and increased anxiety (Klonsky et al. 2003, Liu et al. 2017).

Rodent research aligns with the correlates and predisposing factors of SIB found in human and macaque literature. The caveat being that rodent models of SIB are almost entirely chemically or surgically induced. Behaviors can range from mild self-biting and deep wounding (Guo et al. 2018, Kasim & Jinnah 2002) to excessive and repetitive grooming behaviors termed “barbering” (Greer & Capecchi 2002). Rodent SIB can be induced in a variety of ways including but not limited to environmental manipulation or through physiological stressors such as prolonged restraint (Guo et al. 2018), genetic mutations such as alterations or deletions in the Hoxb8, Shank3, and Sapap3 genes (Greer & Capecchi 2002, Peca et al 2011, Welch et al. 2007), and through intracerebral injections of pharmacological agents like muscimol (Baumeister & Frye 1984).

Despite this artificial nature of induced rodent SIB, there remains important and key overlaps. As previously noted, rodent SIB behavior can be exaggerated with stressors and changing environmental demands such as isolation (Kasim & Jinnah 2002). Similarly, increased self-injury is thought to be paired with increased corticosterone levels implicating a role of the HPA axis in rodent SIB behavior (Guo et al. 2018).
Finally the role of genetic predisposing factors as previously mentioned, suggest that rodent SIB is a matter of not only nurture but also nature.

To our knowledge there is lacking evidence for spontaneously occurring rodent SIB that closely mimics either human NSSI or macaque SIB. The similar but different nature of induced rodent SIB reinforces the need for rhesus macaques as a bi-translational model bridging the gap between rodent and human SIB research.

1.2 Sleep Disruption

One of the features of SIB in monkeys is some form of sleep disruption (Davenport et al. 2008). The role of sleep in rhesus macaques with SIB has been well documented in our lab over three separate studies noting a robust SIB effect in the form of delayed sleep onset and increased nighttime activity (Davenport et al. 2008, Stanwicks et al. 2017, and Guresh unpublished data 2017). The three studies looked at two separate groups of rhesus macaques with varying intensity of SIB (i.e. wounding or mild biting) and measured nighttime activity during different years and at different times of the year. Regardless of these experimental manipulations the SIB effect has held firm. One primary aim of this study is to determine whether improvement in sleep quality will result in decreased SIB pathology.

Nighttime activity has proven difficult to study in macaque species due to their curious nature and dexterity. Sleep activity recording technologies requiring direct contact with the subject must be strongly attached and carefully protected. Most sleep studies require macaque restraint or surgical implantation (Balzamo et al. 1998, Darbin et al. 2009). While these techniques are appropriate for traditional sleep studies, the restraints and external equipment may increase SIB behavior potentially confounding
results. Some studies have used less invasive techniques utilizing collars and actigraphy technology to measure movement (Barrett et al. 2009, Golub & Hogrefe 2016). While the devices can be securely and safely attached, they record all movements and do not give indication of type, eliciting stimulus, or context and often need to be paired with other measures.

The study of macaque sleep disruption has improved in recent years. The initial Davenport study utilized a 1-minute point sampling method to scan nighttime activity taken once during and after cage relocation. Each 1-minute segment was scored on a scale of 0-3, with “0” indicating no nighttime activity (Davenport et al. 2008). Self-biters responded to the major life stress of relocation with exacerbated self-biting and increased nighttime activity (Davenport 2008). The scoring system proved to be tedious and subjective. The methodology was improved in a study utilizing infrared motion activated cameras, infrared light, and surveillance software set to record videos throughout the night any time the monkey moved (Stanwicks et al. 2017). The new technology allowed for subjects to be studied each week and eliminated the need for point sampling as the software only recorded upon detected movement. The SIB subjects once again showed increased sleep disruption and delayed sleep onset (Stanwicks et al. 2017). With the improved recording methodology, a secondary and smaller relocation study was conducted in response to an administratively mandated room change. The unpublished data reinforced the previous work, again demonstrating increased sleep disruption and delayed sleep onset in SIB monkeys, but did not show any effect of the relocation (Guresh Unpublished 2017). This current study will use a slightly more refined technology in which the cameras have built in infrared lights to observe sleep effects of *Lactobacillus reuteri* in both control and SIB subjects.
Similar to our non-human primate models, NSSI in humans is correlated with sleep disruption (Symons et al. 2000). A benefit to human sleep studies is that they are often paired with subjective survey tools and indices that can provide valuable insight into perceived quality of sleep, daily anxiety scores, depression, and overall stress (Liu et al. 2017, Hysing et al. 2015). These in-depth questionnaires can be coupled with physiological indicators such as salivary melatonin concentrations, cortisol in blood plasma, and nighttime sleep measures (i.e. EEG, EMG, EOG).

Non-suicidal self-injurious behavior (NSSI) has been increasingly studied in humans revealing important connections between sleep and this disorder. Poor sleep and nightmares are associated with non-suicidal self-injury in adolescents (Liu X et al. 2017). Additional research is now beginning to suggest that HPA axis activity and sleep may have a bi-directional relationship. In cases of insomnia, increased HPA axis activity may be a causative factor preventing sleep and increasing instances of wakefulness (Buckley & Schatazberg, 2005 & Backhaus et al. 2003). In contrast, HPA axis activity dysregulation is a symptom of obstructive sleep apnea (Buckley & Schatazberg, 2005). Further research is focused on determining the ability of sleep disruption to predict daytime behavior and physiological correlates. In one study of 205 adults living in community housing for people with intellectual disabilities, sleep disruption was correlated with increased anger and challenging daytime behaviors (Brylewski & Wiggs 2001).

The majority of SIB rodent research focuses on creating and refining robust models of SIB and potential treatment alternatives. Little emphasis has been placed on directly connecting the effects of nighttime activity or sleep disruption to the rodent SIB phenotype. One study attempted to connect sleep deprivation and the SIB correlates of
increased anxiety or environmental stimulation. The study demonstrated that rats with moderate d-amphetamine-induced SIB showed altered dopaminergic receptors leading to increased self-mutilating behaviors after experiencing 48hrs of REM sleep deprivation (Lara-Lemus et al. 1997). Outside the realm of SIB, sleep deprivation has shown both psychotropic and physiological effects suggesting an additional role in SIB subjects. A study of rodents treated with clomipramine, a treatment known to induce clinical depression in rodents, demonstrated increased REM sleep fragmentation and longer periods of nonREM (Savelyev et al. 2012). These studies show that even in rodent models sleep and depression may be closely related to SIB as sleep deprivation increases a subject’s vulnerability to stress. This concept is supported in an additional study exploring the effects of sleep deprivation on rat neuroendocrine function showing that sleep deprivation increased stress reactivity and predisposed subjects to a hypersensitivity to stress (Meerlo et al. 2008). While there is lacking rodent research in this specific field, we can make a strong case for a relationship between sleep disruption and SIB based on the overlaps in human, macaque, and nonSIB rodent sleep literature.

1.3 Chronic HPA Axis Dysregulation

One important factor that appears to be related to SIB is dysregulation of the hypothalamic-pituitary-adrenal axis (HPA). This axis is one arm of the stress response system. Cortisol is an important biomarker for stress and has several downstream affects such as preparing the body for the “fight or flight” response via glucose break down (Kyrou et al. 2008). More importantly, a negative feedback loop is initiated post-cortisol release inhibiting further HPA activity (Kyrou et al. 2008). Complications can arise when this negative feedback system fails to initiate properly, and excess cortisol is produced
(hypercortisolemia) (Nieman et al. 2008) or when repeated exposure to chronic stressors leads to reduced stress response (Kyrou et al. 2008).

Cortisol can be measured in several ways including: fecal and urine samples, in saliva, and in blood samples. These methods are problematic because they are point samples and influenced by the natural circadian rhythm of cortisol. In the early morning cortisol levels naturally reach peak concentrations and decrease over time (Meyer & Novak 2012). However, these sampling matrices do not provide an estimate of chronic stress exposure. More recently, a chronic assessment has been developed by measuring cortisol in hair (Davenport et al. 2006). This sampling matrix provides an integrated measure of cortisol concentrations over 3 months in macaques.

Dysregulation of the HPA axis has been consistently observed both in plasma and hair samples in monkeys. Monkeys with SIB show chronically lower levels of plasma cortisol in response to the stress of sedation and venipuncture (Tiefenbacher et al. 2000, 2004) and chronically elevated levels of hair cortisol. It is also interesting to note that one of the major hormones in the HPA axis is corticotrophin-releasing hormone (CRH) which has been reportedly implicated in episodes of insomnia (Kyrou et al. 2008) further strengthening the argument for a relationship between sleep disruption in NHPs and HPA axis activity. Chronic elevations of the HPA axis, as determined by measuring cortisol in hair, have been observed in monkeys with SIB (Davenport et al. 2008).

However, the literature surrounding HPA axis activity in humans with NSSI is lacking. One recent human study of 26 NSSI and 26 healthy control subjects reported higher salivary cortisol levels 30 minutes post-awakening in subjects with NSSI as compared to controls, but did not observe change in hair cortisol levels (Reichl et al. 2016). A previous study of 14 females with NSSI and 14 controls, from many of the same
authors, found hyporesponsive HPA axis activity suggesting salivary cortisol levels were reduced in human with NSSI (Kaess et al. 2012). Both studies report altered HPA axis activity, but the nature of the change is unclear. The etiologies of both human and nonhuman SIB are varied and represent an important area for further research. Hair cortisol values will shed light on pre and post probiotic treatment and give valuable context to deciphering sleep related effects.

HPA axis activity appears to be slightly more researched in rodent models of depression and environmental stressors, and less so in rodent SIB research. Examples of this relationship can be seen in early maternal separation models involving CD1 mouse strains. These mice demonstrated stunted development of HPA axis which in turn resulted in a blunted ACTH response leading to weakened ability to adapt to stressors (Schmidt et al. 2002). Further research in clomipramine treated rat pups has suggested that the hippocampus is able to communicate directly with the hypothalamus via brain derived neurotrophic factor (BDNF). This ability to modulate CNS stress pathways provides yet another connecting point potentially explaining the correlation between HPA axis dysregulation and SIB behavior (Savelyev et al. 2012). The literature still remains divided on the exact relationship of HPA axis activity and rodent SIB. In contrast a study in 2012 using pemoline induced rat SIB models suggested that exposure to high environmental stress (floor shock) had little significant influence on SIB (Bloom et al. 2012). These result can be explained in a number of ways such as the degree of SIB severity and the nature of the shock stimulus, but still point toward a spectrum of SIB pathologies. An additional study involving pemoline induced rats and pretesting anxiety scores on the elevated plus maze test, in agreeance with the Bloom study, explains that pre-anxiety screening does not significantly correlate with susceptibility to SIB. The
paper does however show the use of anxiogenic drugs with induced SIB seemingly exaggerates the pathology (Yuan & Devine 2016). This paper elucidates the difficulty in deciphering an exact cause and effect relationship in the SIB pathology. The contending literature leaves much room for further research and warrants more comparison between induced rodent SIB with spontaneously occurring SIB or NSSI.

1.4 Psychoactive Treatment

An effective treatment for SIB must demonstrate influence over at least two of three correlates; sleep disruption, cortisol concentrations, and anxious behaviors. SIB itself has been reduced through pharmacotherapy but none of these studies examined sleep disruption, cortisol concentrations, or anxious behaviors. Various drugs have shown some efficacy in reducing SIB including naltrexone (an opiate antagonist (Kempf et al. 2012), fluoxetine (a selective serotonin reuptake inhibitor SSRI) (Fontenot et al. 2009), and guanfacine (an alpha 2 adrenergic receptor agonist) (Freeman et al. 2015). Administration of an anxiolytic drug in the form of benzodiazepine (Tiefenbacher et al. 2005) was effective in some animals but others were made worse.

Whether efficacy persists after drug removal remains largely unknown. Additionally, it is unclear if further treatment is necessary or if drug dependency would require continued administration to maintain improved SIB conditions. This continued treatment represents a large financial burden and disqualifies many research animals from further study.
1.5 Probiotic Treatment as an Alternative

Probiotics are organisms that exist in a mutualistic manner within the gut microbiome in animals and convey some benefit to their host. Having first been discovered in the 1900s they have since become an important and promising treatment alternative in both humans and animals. Probiotics may prove to be a long-term treatment with the ability to colonize the gut microbiome in just 2 weeks with little to no severe or psychotropic side-effects as reported for pharmacotherapy. The microbial density will, however, decrease in the following weeks following removal of probiotic supplementation suggesting the need for continued administration (Bouhnik et al. 1992).

Probiotics can be ingested orally as both live and heat-treated strains, transferred between healthy and unhealthy humans via an uncommon procedure termed fecal microbiota transplant (FMT) (Van Nood et al. 2013), and are also found naturally within healthy human and animal microbiomes (Morita et al. 2008).

One genus of gram negative bacteria, *Lactobacillus*, has been closely studied in recent years. Included in this genus is one of the more common strains, *Lactobacillus reuteri* (*L. reuteri*), naturally found in the gut flora of mammals and birds (Sarra et al. 1985, Naito et al. 1995). Commonly it can be isolated from meat and milk products and more importantly it can be passed maternally to offspring via breast milk (Sinkewicz & Nordstrom 2005). An important distinction is that *L. reuteri* is found in the gut of animals in species specific strains (Naito et al. 1995).

Outside the realm of sleep, *Lactobacillus* has already begun to show promise as a treatment for some disease and immunological threats. For example, daily dietary supplementation of *L. reuteri* has been shown to confer resistance to potent gram-negative bacteria such as *Helicobacter pylori* (Imase et al. 2007, Francavilla et al. 2008),
Salmonella typhimurium (Casas & Dobrogosz 2000), and Escherichia coli (Edens et al. 1997). Further research has determined a potential mechanism in which L. reuteri releases a broad-spectrum antibiotic, reuterin that inhibits the growth and development of certain gram-positive and negative bacteria in the gut (Talarico et al. 1988). Lactobacillus strains have seen increased usage in irritable bowel syndrome (IBS) clinical trials (Szajewska et al. 2005) and in adolescent abdominal pain research. L. reuteri has even been demonstrated to improve oral health by out-competing bacterial Streptococcus strains thought to attack enamel (Nikawa et al. 2004) and reducing incidences of gingivitis (Krase et al. 2006).

Behavioral stress related impacts of Lactobacillus strains have been recently shown in rodent models. One strain, Lactobacillus rhamnosus, was demonstrated to modulate emotional behavior (Bravo et al. 2011) and in a separate mouse study, administration of L. reuteri showed reduced stress and anxiety (Marin et al. 2017). Another recent study involving Lactobacillus helveticus saw beneficial effects on emotional learning in rats exposed to early-life stress (Cowan et al. 2016).

Most human clinical trials exploring probiotic treatments revolve around gastrointestinal disorders, but some studies have begun to look at behavioral and anxiety reducing benefits of certain strains. Aptly named “Psychobiotics” these specific strains of probiotics work through the novel pathway known as the brain-gut axis (Dinan et al. 2013). These probiotics include a range of strains including Lactobacillus, Escherichia, Candida, Streptococcus, and Bifidobacterium to name a few (Dinan et al. 2013). Examples can be found in the emerging literature as administration of Lactobacillus helveticus taken in combination with Bifidobacterium longum resulted in anxiolytic effects in a study of humans and rodents (Messaoudi et al. 2011). A separate study
observed reduced anxiety after thrice daily administration of *Lactobacillus casei* to human patients suffering from chronic fatigue syndrome (Rao et al. 2009). A second study of young adults preparing for national medical exams demonstrated a reduction in salivary cortisol levels and response to stress during administration of *Lactobacillus casei* (Takada et al. 2017). These aforementioned studies already begin to highlight the efficacy for *Lactobacillus* probiotics as a potential treatment for stress, mood, and anxious behaviors in humans.

In addition to its many potential uses in combating pathogens, *L. reuteri* is already being used in a commercial context. Currently it is used in avian livestock operations as a growth supplement to encourage body weight increases and reduce response to stress of overcrowding (Casas et al. 1998). It has also seen some use as a treatment for children suffering from colonic and intestinal complications (Urbanska et al. 2016, Shronikova et al. 1997). It is currently marketed for human consumption as an immune-strengthening, stress reducing, and gut health bolstering daily supplement.

One area lacking in literature is the relationship of *L. reuteri* and sleep. While *L. reuteri* effects on sleep have not been directly studied, other *Lactobacillus* strains such as *Lactobacillus brevis* (Miyazaki et al. 2014), *Lactobacillus casei* (Takada et al. 2017), and *Lactobacillus plantarum* (Dhaliwal et al. 2017) have shown promise in improving sleep quality and efficacy. Despite growing evidence of the ability for *Lactobacillus* probiotics to regulate sleep via the brain-gut axis pathway, clear consensus has not been reached. It is, however, conceivable that different strains within the genus may have overlapping functions providing further motivation for use of the *L. reuteri* strain.

The decision to use *Lactobacillus reuteri* was made with the knowledge that 12 of our 14 subjects were surrogate peer-reared (SPR), meaning they received an alternative
milk formula in absence of breast milk which arguably may be the primary source of *L. reuteri* in macaques. The remaining 2 subjects were maternal peer-reared (MPR) by mothers that were surrogate peer-reared. Therefore we can, with reasonable confidence, claim that our 14 subjects are likely to be deficient in *L. reuteri*. Indeed, recent findings demonstrate the presence of *L. reuteri* in the gut of maternally reared rhesus monkey but not in the gut of nursery reared monkeys (Dettmer, 2018) This lack of previous exposure coupled with important aforementioned research on probiotic effects, and the important factor that our subjects both tolerate and ingest the *L. reuteri* probiotic make it an ideal treatment candidate for this study.

1.6 Objectives of this study.

The goal of this study was to evaluate the efficacy of *L. reuteri* administration on biting behavior, sleep disruption, and HPA axis activity in rhesus macaques. Our hypothesis was that monkeys with SIB showed heightened reactions to stressors. Because lactobacillus probiotics appear to reduce stress reactivity, we predicted that administration of *L. reuteri* would decrease sleep disruption, sleep onset time, and nighttime activity. In addition we predicted a decrease in hair cortisol levels and mild self-biting during administration.

It is important to remember that while we hypothesize that probiotic treatment can reduce the factors related to stress, it might not represent a complete cure. Sleep disruption is a symptom of SIB, and therefore it is more accurate to suggest we are treating the symptoms that may worsen SIB. The results of this study could lead to more clinical trials of probiotic treatment in both human and animal models. By testing
multiple strains of probiotic we can begin to unravel specific strain effects and potentially create individualized probiotic treatments.

The study involved an ABA design in which subjects received 3 months of placebo (pretest), followed by three months of *L. reuteri* (test), and followed by 3 months of placebo (posttest). Thus, each subject served as its own control. The phases were separated by a 2 week window to control for probiotic washout. Within subject designs are preferred when environmental conditions are highly stable because they minimize between subject variability.
CHAPTER 2

METHODOLOGY

2.1 Subjects

This probiotic nighttime behavior study was conducted on 14 rhesus macaque monkeys (9 males) housed at the University of Massachusetts Amherst. The monkeys ranged in age from 13-25 years with an average age of 17 years. Eight monkeys (3 females and 5 males) had a mild biting pathology (SIB) which did not require medical or veterinary intervention with the remaining 6 subjects (2 females and 4 males) serving as controls. SIB status was determined from twice daily 5-minute behavioral samples collected both in the AM and the PM. For our experiment, 12 monkeys had been nursery reared at the NIH prior to transfer to UMass and 2 were born at UMass and reared by their nursery reared mothers in social groups.

Eight of the subjects (SIB = 3) were housed in pens with dimensions of roughly 4x6x8 ft. while the remaining six (SIB = 5) were housed in Allentown cages with standard dimensions 5x6x2 ft. Pen housed subjects were maintained on bedding of wood shavings rotated weekly, while Allentown subjects could forage through wood shavings between the cross section of the metal flooring. All housing environments were spot cleaned daily and sanitized biweekly. All subjects were housed and cared for in accordance with the Animal Welfare Act and the NIH Guide to the Care and Use of Laboratory Animals. The subjects and their backgrounds are listed below in Table 1.
Twice daily, the primates were fed monkey chow (Lab Diet) which was supplemented with fruit or vegetable snacks in the morning. A vigorous program of enrichment included spacious cage housing, exceeding the standards set by the Animal Welfare Act that included multiple perches and portable or stationary manipulable objects. Additionally, the primates received daily enrichment consisting of TV, forage stimulation, and edible puzzles on a weekly rotating schedule. The subjects were carefully maintained on 13hr/11hr light dark cycle with light onset at 7 am. Four monkeys were pair housed and, for this study, each pairing was treated as one subject as the nighttime behavior could not be accurately assigned to specific individuals. One pair consisted of two SIB subjects whereas the other pair consisted of two control subjects. Three additional pairs were housed in grooming contact only. The remaining subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>DOB</th>
<th>Age</th>
<th>Sex</th>
<th>SIB</th>
<th>Maternal Model*</th>
<th>Pen Type</th>
</tr>
</thead>
<tbody>
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<td>19</td>
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<td>Pen</td>
</tr>
<tr>
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<td>Pen</td>
</tr>
<tr>
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<td>Pen</td>
</tr>
<tr>
<td>V42</td>
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<td>No</td>
<td>SPR</td>
<td>Pen</td>
</tr>
<tr>
<td>ZA65 &amp; ZA56</td>
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<td>Allentown</td>
</tr>
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<td>ZA54</td>
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<td>Pen</td>
</tr>
<tr>
<td>I18</td>
<td>04.23.93</td>
<td>25</td>
<td>M</td>
<td>No</td>
<td>SPR</td>
<td>Allentown</td>
</tr>
<tr>
<td>N01</td>
<td>04.18.03</td>
<td>15</td>
<td>M</td>
<td>Yes</td>
<td>MPR</td>
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</tr>
<tr>
<td>ZA31 &amp; ZA01</td>
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<td>16</td>
<td>M</td>
<td>No</td>
<td>SPR</td>
<td>Pen</td>
</tr>
<tr>
<td>ZA02</td>
<td>01.29.02</td>
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<td>M</td>
<td>Yes</td>
<td>SPR</td>
<td>Allentown</td>
</tr>
<tr>
<td>ZA63</td>
<td>05.22.02</td>
<td>16</td>
<td>M</td>
<td>Yes</td>
<td>SPR</td>
<td>Pen</td>
</tr>
<tr>
<td>N02</td>
<td>02.18.05</td>
<td>13</td>
<td>F</td>
<td>Yes</td>
<td>MPR</td>
<td>Allentown</td>
</tr>
</tbody>
</table>
were housed individually or in grooming contact. Extensive attempts to pair house these subjects had resulted in severe aggression.

### 2.2 Probiotic Administration

During the pretest and posttest phases the monkeys received a small chewable orange flavored tablet placebo from Bioserv. During the probiotic phase subject received a mandarin orange flavored chewable table with two strains of *L. reuteri* (*L. reuteri* ATCC PTA 6475 & *L. reuteri* DSM 17938) containing on average 200 million colony forming units per chewable tablet. The probiotic is commercially available as Gastrus by the company BioGaia. The author was blind to probiotic phase start throughout the study.

### 2.3 Equipment

This current study utilized the following equipment: two Dell Inspiron 3000 laptops connected to three 1.0 Megapixel USB Cameras (Power DC5V) with built in IR light. The infrared cameras were connected via USB to the iSPYCONNECT 64-bit motion detection recording software for windows. The built in IR light allowed the cameras to seamlessly shift between light and dark cycles within colony rooms. iSPYCONNECT 64-bit software is an open source tool most commonly used in home security and motion detection. The software relies on direct USB connection to the cameras and pre-programmed recording requirements making it an ideal software for this study.
2.4 Procedure

Nighttime video surveillance was conducted on all subjects during designated weeks with 3-4 monkeys scored per night for 4 nights per week, resulting in all subjects scored in a given week. The subjects were randomized across rooms using “The Hat” application for Windows operating system. The 12 subject IDs were listed and then randomized for 10 seconds before ordering the results. The subjects were randomized such that no subject could be recorded on the same day of the week during sequential weeks for more than two weeks. For example, if after three randomization intervals V27 was selected for Monday video collection three weeks in a row the entire group was again randomized for another 10 seconds.

For this study, a recording schedule was established to begin at 6:00PM and to end at 7:00AM the next day. Each camera was set up prior to 6:00PM and oriented so that it faced across from the subject’s cage with substantial room to include the entire cage frame within the camera lens (Screenshot 1). Laptops were positioned outside of the room and connected to cameras via a long USB cord under the colony room door. The laptops were on throughout the night and their placement outside of the room reduced light disruption during recording.

**Screenshot 1**: Example of nighttime recording with (left) subject V27 laying in lateral recumbency on perch and (right) subject V43 laying prone in hammock.
Movement detection zones allowed the experimenter to focus the camera onto the subject and eliminate interference from the surrounding environment (Screenshot 2). Pre-programmed recording time allowed the experimenter to set up the cameras prior to the recording period further reducing disruption prior to lights off. The iSPY program was configured to record at the highest sensitivity of 0.001 pixel changes and set to stop recording 5 seconds after the movement ceased. Threshold values correspond to the total amount of pixel change detected across an image. 0.001 is the lowest threshold setting available and corresponds to percentage of total pixel color change. Recording was initiated if 0.1% of total pixels in the detection zone changed gray scale color.

![Screenshot 2: Example Detection zone (left side) on subject ZA63. Movement is only detected within the light gray box, therefore subject ZA54’s (right) movements will not be detected.](image)

### 2.5 Nighttime Activity Measures

Nighttime activity data were extracted the following day by manually merging all video clips taken the previous night and using the file explorer program to obtain our measures of interest (see Table 2 below for measures and their definitions). The iSPY program records and saves the generated videos for each camera automatically and
separately files them. The experimenter reviewed camera footage recorded during the time frame, rated video quality, and merged the files for later analysis. Videos were visually evaluated to ensure that movement was properly recorded, recording was not interrupted, and subjects were clearly visible during the night. If the recording was insufficient or disrupted in any way the trial was considered a mistrial and replaced by an additional recording.

The measures below (Table 2) were recorded and converted into seconds rounding up to the nearest hundredths place. The files were manually sorted so that only data collected between 8:00PM and 7:00AM were processed. By highlighting multiple clips, the experimenter could determine the total amount of time in seconds across the TMT, TMT1HR, and TMT11HR intervals (Screenshot 3). To calculate the VideoSum, Num10s, Num30s, and LV measures, the experimenter highlighted all the videos and sorted by video length. Once the files were ordered by magnitude they could be easily sorted for the aforementioned categories by highlighting the files of interest.
Table 2: Nighttime Activity Measures and Their Definitions

<table>
<thead>
<tr>
<th>Measure</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Movement Time</td>
<td>TMT</td>
<td>The sum of all movements recorded from 8:00PM – 7:00AM.</td>
</tr>
<tr>
<td>Total Movement Time in First Hour</td>
<td>TMT1HR</td>
<td>Total movement time recorded in first hour of lights off from 8:00PM – 9:00PM.</td>
</tr>
<tr>
<td>Total Movement Time in Last Hour</td>
<td>TMT11HR</td>
<td>Total movement time recorded in last hour from 6:00AM – 7:00AM.</td>
</tr>
<tr>
<td>Longest Video</td>
<td>LV</td>
<td>The longest single video recorded in each trial.</td>
</tr>
<tr>
<td>Number of Videos Per Session</td>
<td>VideoSum</td>
<td>The total number of video clips generated during 11 hours of recording.</td>
</tr>
<tr>
<td>Number of Greater than 10 second videos</td>
<td>Num10s</td>
<td>The sum of all motion detection clips longer than 10s during recording from 8:00PM – 7:00AM.</td>
</tr>
<tr>
<td>Number of Greater than 30 second videos</td>
<td>Num30s</td>
<td>The sum of all motion detection clips longer than 30s during recording from 8:00PM – 7:00AM.</td>
</tr>
<tr>
<td>Total Movement Time 2 Hours Prior to Lights off</td>
<td>TMTDAY</td>
<td>Total movement time recorded in the two hours before lights off from 6:00PM-8:00PM.</td>
</tr>
</tbody>
</table>

Screenshot 3: Manual scoring of TMT between 8:00PM – 7:00AM. Note that not all videos generated are shown, but they are all selected and the TMT can be seen in the right-hand details column.
2.6 Exclusion Criteria for the Nighttime Trials

Videos were considered mistrials if: the session was interrupted or stopped recording, cameras had shifted angle, or a subject in another cage came into frame. Often times an automatic PC update could result in a failed trial or unknowingly alter the presets. The total number of videos scored and videos that were excluded can be found in table 3 below. We had an overall rate of 16% of trials were excluded due to exclusion criterion or software malfunctions.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total Videos</th>
<th>Scored Videos</th>
<th>Mistrials</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27</td>
<td>33.00</td>
<td>28.00</td>
<td>5.00</td>
</tr>
<tr>
<td>V38</td>
<td>32.00</td>
<td>29.00</td>
<td>3.00</td>
</tr>
<tr>
<td>V43</td>
<td>30.00</td>
<td>27.00</td>
<td>3.00</td>
</tr>
<tr>
<td>V42</td>
<td>27.00</td>
<td>26.00</td>
<td>1.00</td>
</tr>
<tr>
<td>ZA65 &amp; ZA56</td>
<td>30.00</td>
<td>28.00</td>
<td>2.00</td>
</tr>
<tr>
<td>ZA54</td>
<td>32.00</td>
<td>27.00</td>
<td>5.00</td>
</tr>
<tr>
<td>I18</td>
<td>34.00</td>
<td>27.00</td>
<td>7.00</td>
</tr>
<tr>
<td>N01</td>
<td>32.00</td>
<td>27.00</td>
<td>5.00</td>
</tr>
<tr>
<td>ZA31 &amp; ZA01</td>
<td>32.00</td>
<td>28.00</td>
<td>4.00</td>
</tr>
<tr>
<td>ZA02</td>
<td>38.00</td>
<td>25.00</td>
<td>13.00</td>
</tr>
<tr>
<td>ZA63</td>
<td>36.00</td>
<td>26.00</td>
<td>10.00</td>
</tr>
<tr>
<td>N02</td>
<td>33.00</td>
<td>29.00</td>
<td>4.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>389.00</strong></td>
<td><strong>327.00</strong></td>
<td><strong>62.00</strong></td>
</tr>
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</table>
2.7 Videos Per Phase

Table 4 below describes the number of videos per subject per phase after exclusion criteria was applied.

<table>
<thead>
<tr>
<th></th>
<th>Subject</th>
<th>Subject PRE</th>
<th>Subject TEST</th>
<th>Subject POST</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>V27</td>
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<td>11.00</td>
<td>9.00</td>
<td>28.00</td>
<td></td>
</tr>
<tr>
<td>V38</td>
<td>11.00</td>
<td>9.00</td>
<td>9.00</td>
<td>29.00</td>
<td></td>
</tr>
<tr>
<td>V43</td>
<td>8.00</td>
<td>10.00</td>
<td>9.00</td>
<td>27.00</td>
<td></td>
</tr>
<tr>
<td>V42</td>
<td>9.00</td>
<td>8.00</td>
<td>9.00</td>
<td>26.00</td>
<td></td>
</tr>
<tr>
<td>V28</td>
<td>9.00</td>
<td>10.00</td>
<td>9.00</td>
<td>28.00</td>
<td></td>
</tr>
<tr>
<td>ZA54</td>
<td>7.00</td>
<td>11.00</td>
<td>9.00</td>
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<tr>
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<td>10.00</td>
<td>9.00</td>
<td>27.00</td>
<td></td>
</tr>
<tr>
<td>N01</td>
<td>7.00</td>
<td>11.00</td>
<td>9.00</td>
<td>27.00</td>
<td></td>
</tr>
<tr>
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<td>ZA63</td>
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<td>10.00</td>
<td>9.00</td>
<td>26.00</td>
<td></td>
</tr>
<tr>
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<td>10.00</td>
<td>10.00</td>
<td>9.00</td>
<td>29.00</td>
<td></td>
</tr>
</tbody>
</table>

2.8 Estimates of Self-Biting Behavior

Modified frequency behavior was collected twice daily in 1-hour intervals at 9:00-10:00 AM and 4:00-5:00 PM. Observers would position themselves in colony rooms opposite the subject of interest and record behavior in 15 second intervals for a total of 5 minutes (20 intervals). Subject order was randomized every day, and the observers had to meet a 90% reliability criterion before being allowed to take behavioral data. Self-biting rates were derived from this measuring system.

2.9 Evening Activity Measures

Evening total movement time was considered in the final analysis. Evening total movement was scored during 6:00PM to 8:00PM period prior to lights off. 6:00PM is the
chosen start time to avoid any human interference as standard husbandry and research practices end around 5:00PM daily. It is a unique period of uninterrupted activity with lights on.

2.10 Daytime Proxy Measures

In order to explore daytime behavior when video surveillance was not feasible due to husbandry and research projects, we created a daytime proxy measure for our modified frequency 5-min samples. The proxy measure was created by analyzing AM and PM modified frequency data during each phase for each subject. The intervals of modified frequency where the subject was recorded in visual explore or 5 intervals of social contact were counted as periods of inactivity. The number of intervals of inactivity were then subtracted from 20 to create an activity score.

2.11 Hair Cortisol Extraction

In addition to behavioral and activity measures, hair cortisol was used as a biological marker for overall stress in our subjects. Hair cortisol is extracted using a novel methodology pioneered in the Meyer’s lab (Meyer et al. 2014). The methodology follows a number of steps to extract the cortisol. First the sample was washed twice to remove surface cortisol contaminants using 5mL of high performance liquid chromatography (HPLC) grade isopropyl alcohol on a 3min inversion cycle using a rotator. After two cycles of washing, the isopropyl was removed and the sample dried for 2-3 days. The sample was then ground to a fine powder using a bead beater. In the next step, methanol was added and the sample incubated at room temperature for 18-24hrs on constant inversion. The sample was then centrifuged at 10,000 rpm for 5 minutes. And
the supernatant extracted and placed in a separate and sterile microcentrifuge tube for methanol evaporation in a vacuum evaporator. The hair cortisol was then reconstituted in an enzyme immunosorbent assay (EIA) diluent, which was then assayed using a standard commercial EIA kit. All samples for this study were run in Dr. Meyer’s lab.

2.12 Data Analysis

The data were analyzed using Analysis of variances (ANOVA) with SIB status as the between subject variable and phases as the repeated measure. The analysis specifically looked at: TMT, ≥Num10s, ≥Num30s, TMT1HR, TMT11HR, TMTDAY, ProxyTMT, and LV. Findings were also confirmed with the Wilcoxon signed-rank non-parametric test. PRISM graphing software was used to create the graphs below. The results of the ANOVA were further analyzed using post hoc comparisons of the phase means with a Bonferroni correction for multiple comparisons. There are three comparisons. 1) Pre vs Test, 2) Test vs Post, and 3) Pre vs. Post.
CHAPTER 3
RESUL T S

3.1 Self-Biting Behavior

Administration of L reuteri modestly decreased biting behavior in monkeys with SIB (see Figure 1 for the group means; F(2,12) = 5.64, p= 0.02). A comparison of individual subjects revealed that 6 out of 8 SIB monkeys showed a decrease in biting behavior. The remaining 2 subjects showed little change.

![Figure 1: Average of SIB self-biting rates (PRE), probiotic (PRO), and placebo (POST) phases.](image)

3.2 Nighttime Activity

Administration of L reuteri decreased nighttime activity in all monkeys irrespective of SIB status as revealed by significant effects of phase. However, despite the decrease in nighttime activity (most notably in SIB monkeys), monkeys with SIB continued to show higher levels of nighttime activity compared to nonSIB monkeys. Specific findings are described below and a table of summary statistics is located at the end of this section.
3.3 Total Movement Time

There was a phase effect as TMT was significantly reduced by probiotics in both groups (See Figure 2; \(F_{(2,20)}=6.03\) p=0.01). SIB subjects had significantly increased total movement time compared to controls and irrespective of phase (See Figure 3; \(F_{(1,10)}=10.46\) p=0.01). There was no significant effect of SIB by phase interaction \(F_{(2,20)}=0.20\) p=0.82).

![Figure 2: Average combined SIB and control nighttime total movement time across placebo (PRE), probiotic (PRO), and placebo (POST) phases](image)

![Figure 3: Average of SIB compared to control nighttime total movement time across placebo (PRE), probiotic (PRO), and placebo (POST) phases.](image)

3.4 Total Movement in the First Hour

Similarly, movement time in the first hour was reduced by probiotic treatment in both groups (See Figure 4; \(F_{(2,20)}=6.17\) p=0.01). However, SIB monkeys continued to show more activity in the first hour after light offset compared to nonSIB monkeys (See Figure 5; \(F_{(1,10)}=12.04\) p=0.01). There was no significant SIB by phase interaction \(F_{(2,20)}=1.79\) p=0.19).
3.5 Total Movement in the Last Hour

There was an overall phase effect demonstrating reduced activity after probiotic administration in both groups (See Figure 6; $F_{(2,20)}=4.29 \ p=0.03$). There was a marginal main effect of SIB showing that SIB monkeys continued to show more activity during the hour before light onset. (See Figure 7; $F_{(1,10)}=4.52 \ p=0.06$). There was no significant effect of SIB by phase interaction $F_{(2,20)}=1.88 \ p=0.18$.

Figure 4: Average combined SIB and control SIB nighttime total movement time in the first hour across placebo (PRE), probiotic (PRO), and placebo (POST) phases

Figure 5: Average of SIB compared to control nighttime total movement time in the first hour across placebo (PRE), probiotic (PRO), and placebo (POST) phases.

Figure 6: Average combined SIB and control nighttime total movement time in the last hour across placebo (PRE), probiotic (PRO), and placebo (POST) phases

Figure 7: Average of SIB compared to control nighttime total movement time in the last hour across placebo (PRE), probiotic (PRO), and placebo (POST) phases.
3.6 Longest Video

Once again, *L. reuteri* treatment reduced the length of the longest video time in both groups. (See Figure 8; F(2,20)=7.00 p=0.01. There was also a SIB effect as the SIB group generated on average significantly longer videos (See Figure 9; F(1,10)=5.54 p=0.04) There was no significant effect of SIB by phase interaction F(2,20)=1.09 p=0.35).

![Figure 8: Average combined SIB and control longest nighttime video generated across placebo (PRE), probiotic (PRO), and placebo (POST) phases.](image1)

![Figure 9: Average of SIB compared to control longest nighttime video generated across placebo (PRE), probiotic (PRO), and placebo (POST) phases.](image2)

3.7 Total Number of Videos Generated

Probiotic treatment was associated with a reduction of total videos generated across phase. (See Figure 10; F(2,20)=4.42 p=0.03). There was also a SIB effect as the SIB group continued to generate on average significantly more videos (See Figure 11; F(1,10)=5.89 p=0.04.) There was no significant effect of SIB by phase interaction F(2,20)=0.35 p=0.71.)
3.8 Total number of Videos Generated ≥10s

As in all previous measures, there was a phase effect demonstrating reduction of ≥10second videos generated in both groups. (See Figure 12; $F_{(2,20)}=3.39$ $p=0.05$. There was also a SIB effect as the SIB group continued to generate on average significantly more ≥10s videos (See Figure 13; $F_{(1,10)}=6.59$ $p=0.03$.) There was no significant effect of SIB by phase interaction $F_{(2,20)}=0.93$ $p=0.41$.)
### 3.9 Total number of Videos Generated ≥30s

There was no overall phase effect as the number of ≥30second videos generated in both groups remained the same across treatment conditions. (See Figure 14; \( F_{(2,20)}=1.88 \) \( p=0.18 \)). There was however a significant SIB effect as the SIB group generated on average significantly more ≥30s videos (See Figure 15; \( F_{(1,10)}=5.05 \) \( p=0.05 \)). There was no significant effect of SIB by phase interaction \( F_{(2,20)}=1.85 \) \( p=0.18 \).

![Figure 14](image1) ![Figure 15](image2)

**Figure 14:** Average combined SIB and control ≥30s nighttime videos across placebo (PRE), probiotic (PRO), and placebo (POST) phases.  
**Figure 15:** Average of SIB compared to control ≥30s nighttime videos across placebo (PRE), probiotic (PRO), and placebo (POST) phase.

### Table 5: Table of Significant Nighttime Measures

<table>
<thead>
<tr>
<th>Behavior</th>
<th>SIB Effect</th>
<th>Phase Effect</th>
<th>SIB x Phase Interaction</th>
<th>Pre vs. Test</th>
<th>Test vs. Post</th>
<th>Pre vs. Post</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIME VALUES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMT</td>
<td>10.46, ( p=0.01 )</td>
<td>6.03, ( p=0.01 )</td>
<td>0.20, ( p=0.82 )</td>
<td>( p=0.08 )</td>
<td>( p=0.93 )</td>
<td>( p=0.02 )</td>
</tr>
<tr>
<td>TMTHR1</td>
<td>12.04, ( p=0.01 )</td>
<td>6.17, ( p=0.01 )</td>
<td>1.79, ( p=0.19 )</td>
<td>( p=0.01 )</td>
<td>( p=1.00 )</td>
<td>( p=0.08 )</td>
</tr>
<tr>
<td>TMTHR11</td>
<td>4.52, ( p=0.06 )</td>
<td>4.29, ( p=0.03 )</td>
<td>1.88, ( p=0.18 )</td>
<td>( p=0.01 )</td>
<td>( p=1.00 )</td>
<td>( p=0.01 )</td>
</tr>
<tr>
<td>Longest VID</td>
<td>5.54, ( p=0.04 )</td>
<td>7.00, ( p&lt;0.01 )</td>
<td>1.09, ( p=0.35 )</td>
<td>( p&lt;0.01 )</td>
<td>( p=1.00 )</td>
<td>( p&lt;0.01 )</td>
</tr>
<tr>
<td><strong>FREQ. VALUES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total VIDS</td>
<td>5.89, ( p=0.04 )</td>
<td>4.42, ( p=0.03 )</td>
<td>0.35, ( p=0.71 )</td>
<td>( p=0.16 )</td>
<td>( p=0.96 )</td>
<td>( p=0.06 )</td>
</tr>
<tr>
<td>10&gt;sec VIDS</td>
<td>6.59, ( p=0.03 )</td>
<td>3.39, ( p=0.05 )</td>
<td>0.93, ( p=0.41 )</td>
<td>( p=0.08 )</td>
<td>( p=0.01 )</td>
<td>( p&lt;0.01 )</td>
</tr>
<tr>
<td>30&gt;sec VIDS</td>
<td>5.05, ( p=0.05 )</td>
<td>1.88, ( p=0.18 )</td>
<td>1.85, ( p=0.18 )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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3.10 Daytime and Early Evening Behavior

Two measures of daytime activity were examined, and a summary of the statistical results are reported in table 6. In the first, the surveillance software was used to measure activity in the two hours before sleep onset. There was no overall phase effect as the number of seconds of early evening activity remained unchanged in both groups as a function of treatment condition. (See Figure 16; \(F_{(2,20)}=0.33\) p=0.72). However, there was a significant SIB effect as the SIB group showed elevated activity compared to controls (See Figure 17; \(F_{(1,10)}=13.47\) p=0.01). There was no significant SIB by phase interaction \(F_{(2,20)}=0.07\) p=0.93).

![Figure 16](image)

**Figure 16:** Average of SIB compared to control total movement time 2-hours before light offset across placebo (PRE), probiotic (PRO), and placebo (POST) phases.

To determine whether all of our nighttime effects were caused by basic differences in activity level, we examined our twice daily 5-minute modified frequency samples. We created a proxy activity measure from the AM and PM modified frequency samples by counting intervals in which no motor activity was present. There was no effect of SIB, Phase, or SIB by Phase interaction.
Figure 17: Average of SIB compared to controls evening modified frequency scores across placebo (PRE), probiotic (PRO), and placebo (POST) phases.

Table 6: Table of Significant Daytime Measures

<table>
<thead>
<tr>
<th>Behavior</th>
<th>SIB Effect</th>
<th>Phase Effect</th>
<th>SIB x Phase Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-8 pm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMT</td>
<td>13.47, p&lt;0.01</td>
<td>0.33, p=0.72</td>
<td>0.07, p=0.93</td>
</tr>
<tr>
<td>MF Samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ProxyTMT</td>
<td>0.28, p=0.76</td>
<td>0.13, p=0.88</td>
<td>0.14, p=0.97</td>
</tr>
</tbody>
</table>

3.11 Hair Cortisol Concentrations

Hair cortisol values were available only for the Pre and Probiotic Phases. Monkeys with SIB showed elevated hair cortisol compared to controls ($F_{(1,12)}=7.28$, p=0.02, see Figure 18). Even though hair cortisol values appear to go down with probiotic treatment, there was no significant phase ($F_{(1,12)}=0.87$, p=0.37) or SIB by phase interaction ($F_{(1,12)}=0.91$, p=0.36). At an individual level, 5/8 SIB subjects show a reduction in hair cortisol values. Two subjects showed no change and these were the same subjects that showed no decrease in biting behavior. One SIB subject that showed a reduction in biting with treatment and an increase in hair cortisol.
Figure 18: Average of SIB compared to controls hair cortisol values across placebo (PRE), probiotic (PRO), and placebo (POST) phases.
CHAPTER 4

DISCUSSION

We predicted that administration of *L reuteri* would decrease self-biting behavior, decrease sleep disruption, and reduce HPA axis activity in SIB monkeys. As expected, SIB monkeys showed a reduction in biting behavior and sleep disruption with probiotic treatment. These findings are consistent with the results of another study in which we showed that treatment with *Bifidobacterium infantis* reduced biting behavior in this same group of monkeys (manuscript in preparation). However, it should be noted that neither of these probiotic strains eliminated biting behavior entirely.

The decrease in nighttime behavior for SIB was only partially confirmed. Unexpectedly, both the SIB and nonSIB monkeys showed reduced nighttime activity with probiotic administration. This suggests that the effect is not selective to SIB monkeys and in fact benefitted both groups. This finding was unexpected because the nonSIB monkeys showed minor sleep disruption in the pretest phases. While nighttime activity appeared to decrease in SIB monkeys, it still did not normalize to the level of the controls, and there were no significant SIB by phase interactions.

We subsequently analyzed the SIB monkey and nonSIB monkey data separately for significant effects of treatment phases. In the SIB monkeys, significant effects of phase were detected for 3 measures (total movement time in the first hour, total movement time in the last hour, and length of the longest video). Marginally significant effects (*p* = < 0.09) were detected for 2 measures (total movement time and number of videos ≥ 10 seconds). In examining the nonSIB group, there was only one phase effect (total movement time in the last hour). Thus, we can suggest that SIB monkey nighttime
activity was reduced by the administration of *L. reuteri* and that probiotic treatment had a greater effect in SIB subjects.

The usual expectation in this ABA design was that elevated nighttime activity would be reinstated in the second A phase as compared to the probiotic treatment phase. However, reductions in nighttime activity continued through the second A phase. There are two possible explanations for this finding: the first being that probiotic efficacy continued into the posttest and the second is due to another unidentified variable. To tackle the question of probiotic efficacy persisting, more posttest data need to be collected to see if nighttime activity increases. Depending on the results, we can determine whether the efficacy continues to remain or eventually returns to baseline, suggesting that the probiotic takes longer to subside than the 3 months we initially chose.

The second possible explanation is that there may be an unidentified variable such as seasonality or an environmental change that caused continued suppression of nighttime activity only during the posttest. The posttest to this study occurred during late spring to early summer. We can rule out late spring to summer effects because we have evaluated and never seen changes in behavior that are unique to summer. It should also be pointed out that these monkeys live in an indoor environment under a constant day/night cycle. Another more likely explanation is individual variability. No obvious changes either in husbandry practices, enrichment experiences, or care staff occurred during any phase of this study. Thus, it seems unlikely that an unknown environmental factor was responsible for the continued improvement of the monkeys during the posttest period.

SIB monkeys also showed a reduction in cortisol concentrations during treatment. This effect was also reported when the same group of monkeys was treated with another psychoactive probiotic, *Bifidobacterium infantis*. These findings are consistent with both
human and rodent studies in which treatment with these compounds either reduced HPA axis activity directly (Janik et al. 2015, Marin et al. 2017, Liang et al. 2015, Sudo & Chida et al. 2004) or when preloaded, modulated the reaction to a novel stressor (Kato-Kataoka et al. 2016)

The effects of psychoactive probiotics on these characteristics can be partially explained by an exploration of the brain-gut axis mechanisms. The brain gut axis is a unique pathway involving communication of the microflora to the brain through modulation of the immune system, neuroendocrine, and the neural pathways to exert its influence. However, it should be noted that many of these pathways are best explored and represented in rodent research and may not reflect the diversity of human and NHP conditions.

One of the more notable hypotheses surrounding the mechanism of psychobiotics is that transcription of GABA (inhibitory neurotransmitter) and glutamate (excitatory neurotransmitter) are somehow upregulated in varying cortical regions and down regulated in others by probiotic gut flora. More specifically this was shown in clinically healthy rodent models given oral doses of L. rhamnosus probiotic (Janik et al. 2015). The hypothesized mechanism still remains unclear but can be explained through increased regulation of GABA and glutamate related genes, metabolic products of altered gut flora, or by direct synthesis of GABA via bacteria after administration of probiotics. The effects of increased GABA can be seen in an older study that replicated the SIB phenotype in rodents by giving intranigral injections of GABA agonist muscimol. In the experiment the rodent subjects were given direct injections of muscimol into regions of the substantia nigra which resulted in dose dependent SIB behavior (Baumeister & Frye 1984). These
studies make a strong case for GABA as a major component in the brain gut axis and possible explanation for probiotic effect on SIB.

Another proposed mechanism is through the production and release of neuropeptides and or metabolites from microflora in the gastrointestinal tract. These products are able to cross the blood brain barrier to act directly on the hypothalamus and other important brain regions. Many of these pathways influence locations that regulate stress, anxiety, and depression. Examples of this can be seen in the research by Marin and collaborators who showed that the addition of Lactobacillus probiotic had the ability to outcompete other commensal microflora resulting in a decreased production of kynurenine and a return to homeostasis reversing the effect of previous stressors (Marin et al. 2017).

Rodent research further supports the idea of a more closely related HPA axis and SIB pathology. One such paper has touted the ability of Lactobacillus helveticus NS8 to improve rodent stress in a chronic restraint depression model. The authors argue that administration of the probiotic lead to reduced plasma cortisol levels, anxiety, and depression scores on elevated maze, sucrose preference tests, open field test, object recognition, and object placement testing (Liang et al. 2015). The paper also tested biochemical markers noting increase BDNF and restored hippocampal serotonin (Liang et al. 2015). This study strengthens what the emerging literature has repeatedly shown, that the microbiome can actively alter neural pathways and have reversing effects on stress and depression, two proposed hypotheses that repeatedly intersect our working model of SIB.

Additionally, research has looked at the post-natal period in mouse models where the microbiome is still developing and colonization has yet to be completed. This
developmental period represents a time of greater susceptibility to disease but also to stress. One particular experiment observed germ-free mouse strains in direct comparison to specific pathogen free mice and mouse models inoculated with B.\textit{infantis} and showed that germ free mice had increased corticosterone and ACTH levels, reduced BDNF expression, and most notably showed that elevated HPA axis response could be partially resolved with fecal microbiota transplant of feces from the SPF mice treated with probiotic (Sudo & Chida et al. 2004).

All of these proposed mechanisms have merit and prove that the explanation behind our unique findings may not be so one-dimensional. Additional research is warranted as determining the exact relationship may be the key to creating an effective treatment and eventual cure for SIB in humans and non-human primates.

The finding of increased nighttime activity raises the question as to whether the differences in activity are specific to the night. When we examined the 2 hours before light offset, SIB monkeys were more active than nonSIB monkeys although this difference in activity was unaffected by treatment, remaining consistent across all phases. There are potential explanations for this finding. The first is that perhaps during those two quiet hours before sleep the subjects’ total movement time increases in anticipation of sleep. Alternatively, a second hypothesis is that SIB is associated with trait-like hyperactivity. Thus, we measured activity during our 5-modified frequency samples. It was not possible to use the surveillance software during the workday because of the numerous activities of animal care and research staff. We created the proxy measure outlined in the methods section. As reported in the results section the proxy measure did not show any activity differences between SIB and control subjects and like the evening measure, the daytime activity was unaffected by the treatment. Thus, it seems unlikely
that the SIB monkeys in this population differed from controls by being hyperactive. However, because the evening measure identified significant differences, it remains possible that the presence of observers equalized the activity, or conversely, the proxy measure was not an accurate assessment tool.

Future directions might involve exploring a multi-strain probiotic or perhaps probiotics used in conjunction with other exogenous substances such as melatonin. The number of probiotics becoming commercially available are on the rise and powerful combinations of probiotics may hold the key to creating individualized treatment plans. Experimental designs that compare the efficacy of multi-strain probiotics with and without *L. reuteri* could give indication of what specific role *L. reuteri* plays in reduction of SIB and nighttime behavior. In addition to testing multi-strain probiotics, the use of fecal analysis to determine the exact density and growth of probiotics in subjects could also explain some of our findings. Pre and post fecal analysis and microbiome genotyping could also explain the specific mechanism used by *L. reuteri* as demonstrated in the literature. A valid future direction is to answer the question of whether certain bacteria are being out competed by the addition of probiotics or if an entirely different mechanism is at play.

Another important consideration is whether activity is really indicative of a specific sleep disruption or whether it is predicated by hyperactivity across the day and night activity. We must then consider whether probiotics reduce both nighttime and daytime activity. It would also be valuable to attempt to measure subjects more than two times per week or to measure all animals in the same room at the same time to determine synchrony of behaviors. Synchrony could answer the question of whether subjects become habituated to the movements of the other macaques within the colony room or if
their behavior is affected by the other subjects. It would be useful to also combine
techniques as suggested in the articles by Barrett 2009 and Golub & Hogrefe 2016 where
they studied actigraphy data from implanted sensors and collars. This would be beneficial
in creating a better sense of sleep disruption. It would be valuable to graph the time
stamps for the 30 second videos to determine the exact spread of activity. This could
answer the question of whether SIB subjects have more sleep disruption on one end or
another of the night. Coupling this data with actigraphy information could clarify the type
of activity and help further refine the novel VRNA system. Another important future
direction is to increase the number of subjects and include different populations if
possible. One unanswered question is whether the severity of SIB correlates with the
severity of sleep disruption, as such it would be important to test whether *L.reuteri* would
have a greater or equal effect on subjects considered intense self-biters. A follow up
study could incorporate the use of neural imaging to look at changing neural plasticity in
subjects undergoing probiotic treatment.

An important next step is determining the exact relationship of SIB and sleep.
Further exploration is needed to determine whether sleep is driven by SIB pathology or if
SIB pathology is increased by sleep disruption. The use of sleep aids, especially
nonpharmacological aids like melatonin or herbs like chamomile, will be invaluable in
understanding the complex SIB phenotype.
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