THE EFFECT OF PROBIOTIC SUPPLEMENTATION ON SELF-REPORTED SLEEP QUALITY

By

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Abstract

Objectives: A growing body of evidence suggests that the microbiome plays an important role in mental health. Few studies have examined how probiotic supplementation affects sleep quality. The current study investigated the impact of probiotics on self-reported sleep quality and the role of anxiety in moderating this relationship. The study also assessed the role of vagal tone in mediating probiotic-induced improvements in self-reported sleep quality.

Methods: Forty-three undergraduates at the University of Kansas participated in a two-week study assessing the effect of probiotic supplementation on psychological and physiological processes. A sleep diary was collected daily. Measures of sleep, anxiety, and vagal tone were collected at baseline and post-intervention. Indices of sleep quality were analyzed using multilevel growth modeling. The moderating effects of treatment condition and initial anxiety were examined. The mediating effect of vagal tone was also assessed.

Results: Initial level of anxiety was found to moderate the effect of probiotic supplementation on sleep onset latency (SOL) and sleep efficiency. In participants without elevated anxiety, probiotic supplementation was associated with a significant decrease in SOL and a significant increase in sleep efficiency over the course of the two-week trial. The effectiveness of probiotic supplementation increased as anxiety decreased. Probiotic supplementation had no effect on SOL and sleep efficiency in participants with elevated levels of anxiety. No association was found between treatment condition and number of awakenings or minutes awake after sleep onset. Finally, change in vagal tone was not found to mediate change in sleep quality.
Conclusion: These findings suggest that the microbiome influences sleep quality. Manipulation of the microbiome via probiotic supplementation may be useful for treating individuals with disrupted sleep.

Keywords: sleep, microbiome, probiotic, anxiety, heart rate variability
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# Table of Contents

Abstract ........................................................................................................................................ iii

Acknowledgements ......................................................................................................................... v

Table of Contents ............................................................................................................................. vi

Table of Figures ............................................................................................................................... viii

Introduction ...................................................................................................................................... 1

Probiotics and Sleep Quality ............................................................................................................ 3

Anxiety as a Moderating Factor ....................................................................................................... 4

The Role of the Vagus Nerve ......................................................................................................... 5

Methods .......................................................................................................................................... 8

Participants ...................................................................................................................................... 8

Study Treatment .............................................................................................................................. 9

Instruments ...................................................................................................................................... 9

Procedure ....................................................................................................................................... 11

Statistical Analysis .......................................................................................................................... 11

Results ............................................................................................................................................ 13

Discussion ....................................................................................................................................... 17

Limitations ....................................................................................................................................... 23

Future Directions ............................................................................................................................ 24

Conclusion ....................................................................................................................................... 24
Table of Figures

Table 1 ........................................................................................................................................ 39
Table 2 ........................................................................................................................................ 40
Table 3 ........................................................................................................................................ 41
Table 4 ........................................................................................................................................ 41

Figure 1. Effect of probiotic supplementation on sleep onset latency in participants with low starting anxiety.................................................................................................................. 42

Figure 2. Effect of probiotic supplementation on sleep efficiency for participants with low starting anxiety.................................................................................................................. 43
The Effect of Probiotic Supplementation on Self-Reported Sleep Quality

The study of the microbiome and probiotics is a rapidly growing area in the fields of physical and mental health. The microbiome is the collection of approximately 100 trillion microorganisms found in the human gastrointestinal tract (Galland, 2014). This “microbial organ” contains a vast amount of genetic material and forms a symbiotic relationship with its host (Backhed, Ley, Sonnenburg, Peterson, & Gordon, 2005). The microbiome assists in harvesting otherwise inaccessible nutrients, such as cellulose and resistant starches (Backhed et al., 2005). It also supports the development of the immune system (Nikoopour & Singh, 2014). Formation of the microbiome begins prenatally and is strongly influenced by environmental factors (Li, Wang, & Donovan, 2014).

Unfortunately, modern lifestyles are detrimental to the microbiome. Due to the built environment, humans are no longer receiving exposure to the microorganisms necessary for a healthy microbiome (Hoisington, Brenner, Kinney, Postolache, & Lowry, 2015). Widespread antibiotic usage also plays a major role in promoting microbiome dysfunction (Blaser, 2016). Emerging evidence suggests this dysfunction can lead to a number of diseases (de Vos & de Vos, 2012). Alterations in the diversity of intestinal microbiota have been linked to health aberrations such as inflammatory bowel disease, obesity, and allergies (Kaser, Zeissig, & Blumberg, 2010; Ley, Turnbaugh, Klein, & Gordon, 2006; Stsepetova et al., 2007). Sudo et al. (2004) found that germfree mice—mice raised without exposure to microorganisms—displayed an exaggerated stress response. A disrupted circadian rhythm, in combination with a high-fat, high-sugar diet, has also been shown to alter the microbiome in mice (Voigt et al., 2014). This finding is particularly relevant as it represents modern sleeping and dieting habits.
One way of therapeutically altering the microbiome is through probiotics. Probiotics are microorganisms that improve the health of the host when administered (Williams, 2010). Products containing probiotics are abundantly available, and easily accessible by the general population. According to Williams (2010), the two most common species of bacteria used in probiotics are *Lactobacillus* (*L.* ) and *Bifidobacterium* (*B.* ): However, effects seem to depend on the specific strain. Rather than causing permanent change, probiotics tend to temporarily colonize the intestines (Alander et al., 1999). While primarily studied in patients with irritable bowel syndrome, emerging evidence suggests that probiotics may have psychotropic properties (Dinan, Stanton, & Cryan, 2013).

Probiotics can interface with the brain through the microbiome-gut-brain axis, a communication system involving both humoral and neural pathways (Bercik, Collins, & Verdu, 2012; Mayer, 2011). This exchange is bidirectional and allows reciprocal regulation between the gut and the brain (Cryan & O'Mahony, 2011). Microbiota can affect inflammatory and immune processes, as well as produce hormones and neurotransmitters (for review see Galland, 2014). By doing so, these organisms are capable of influencing the central nervous system. The microbiome is also closely associated with the enteric nervous system, which is the 200 to 600 million neurons of the gut considered to be the third branch of the autonomic nervous system (Mayer, 2011). The vagus nerve—a primary component of the parasympathetic nervous system—serves as a primary connection between the enteric and central nervous system, innervating foregut structures (Mayer, 2011). The vagus nerve will be discussed in greater detail later in this paper.

Recent studies have demonstrated the connection between probiotics and mental health. Treatment with *L. helveticus* and *B. longum* has been linked to reduction in stress, depression and
anxiety in both animals and humans (Ait-Belghaoui et al., 2014; Gilbert et al., 2013; Messaoudi et al., 2011). Benton, Williams, and Brown (2007) demonstrated that mood was significantly improved in low-mood individuals after administration of an L. casei milk drink. In animal models, Bifidobacterium infantis has been found to reduce pro-inflammatory responses and provide possible antidepressant effects through increased tryptophan release (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008). Ingestion of this strain has also been shown to reduce depressive behaviors, restore immune function and basal noradrenaline concentrations in rats following maternal separation (Desbonnet et al., 2010). In a study by Kantak, Bobrow, and Nyby (2014), treatment with L. rhamnosus GG was comparable to medication in attenuating OCD-like behavior in mice. These “psychobiotics” could represent a new form of treatment for psychological symptoms (Dinan et al., 2013).

Probiotics and Sleep Quality

Very little research has examined the effect of probiotics on sleep. Sleep is vital for normal physiological and psychological functioning. Unfortunately, 50-70 million Americans experience disordered sleep, inflicting substantial financial and societal cost (Institute of Medicine Committee on Sleep & Research, 2006). Sleep disturbance is a significant stressor that contributes to cognitive impairment, affective disruption and physical disease (McEwen, 2006). Mood or anxiety disorders are often comorbid in people with chronic insomnia (Kraus & Rabin, 2012). Modern lifestyles contribute to this growing health concern by shifting importance away from healthy sleep habits and towards other activities. Developing effective and safe treatments for disturbed sleep is a necessary public priority.

There is some preliminary, albeit inconclusive, evidence that probiotics can improve sleep. Mice treated with heat-killed L. brevis showed alterations in sleep patterns, which could
potentially treat disrupted sleep rhythms (Miyazaki et al., 2014). Yamamura et al. (2007) demonstrated that ingestion of an *L. helveticus* drink significantly improved sleep efficiency and number of wakening episodes in elderly patients when compared to baseline values. However, probiotics did not outperform placebo in this study. Furthermore, Diop, Guillou, and Durand (2008) demonstrated that treatment for three weeks with *L. acidophilus* and *B. longum* did not improve sleep symptoms, but did alleviate stress-related gastrointestinal symptoms. As it stands, the relationship between probiotics and sleep is still poorly understood. It should be noted that current studies have not looked at individuals with preexisting sleep difficulties. More severe sleep disturbance may make the therapeutic effects of probiotics more noticeable. For example, one study has found that probiotics improve mood, but only in individuals with poor mood at the start of the study (Benton et al., 2007). As such, given the findings that probiotics can positively affect psychological processes, it is expected that probiotic supplementation would have positive effects in people experiencing sleep disturbance.

*Hypothesis 1:* Probiotic supplementation will improve self-reported sleep quality in individuals with poor sleep.

**Anxiety as a Moderating Factor**

The relationship between the microbiome and disrupted sleep may involve other psychological processes. Anxiety, which is known to contribute to poor sleep, is of particular interest (Altun, Cinar, & Dede, 2012; Mirbagher, Gholamrezaei, Hosseini, & Sayed Bonakdar, 2014). Anxiety disorders tend to precede the development of insomnia, a relationship not seen in mood disorders (Johnson, Roth, & Breslau, 2006; Ohayon & Roth, 2003). Treatment of sleep disturbance is an important goal when treating generalized anxiety disorder, and medications managing anxiety symptoms can also improve sleep (Sheehan, Svedsater, Locklear, & Eriksson,
Concurrent improvements in anxiety and sleep can also be seen in other therapies (Bradt, Dileo, & Potvin, 2013; Cho, Min, Hur, & Lee, 2013; Clementi & Alfano, 2014). The close relationship between anxiety and sleep disturbance highlights the need to assess anxiety when investigating new sleep treatments.

As mentioned briefly in a previous section, certain strains of probiotics have been found to contain anxiolytic properties. Further evidence of this can be seen in recent animal model studies. *L. helveticus* interacts with genotype and diet to protect against anxiety in certain types of mice (Ohland et al., 2013). Heightened anxiety-like behavior in immunocompromised mice was normalized by administration of *L. rhamnosus* and *L. helveticus* (Smith et al., 2014). Administration of *L. helveticus* in rats also reduced anxiety-like behavior while reducing neuroinflammation and restoring serotonin transmission (Luo et al., 2014). Keeping this in mind, it is expected that probiotic supplementation would have a greater effect in improving disturbed sleep in individuals also presenting with heightened anxiety. This is because of the potential of probiotics to address disrupted sleep through multiple avenues of action. Individuals with high anxiety may present more opportunities for probiotics to have a therapeutic effect via anxiety-related and anxiety-unrelated pathways. In essence, probiotics would be capable of taking a multi-targeted approach to addressing disrupted sleep.

*Hypothesis 2*: Anxiety will moderate the beneficial effects of probiotics on sleep, and higher anxiety prior to treatment will relate to greater improvement in self-reported sleep quality.

**The Role of the Vagus Nerve**

Finally, it is important to understand how probiotics may influence disrupted sleep. As mentioned previously, the vagus nerve is a component of the microbiome-gut-brain axis.
Forsythe, Kunze, and Bienenstock (2012) have suggested that the vagus nerve is crucial in mediating the effects of microbiota on the brain of animals. Vagal restriction prevented probiotic-induced reduction of anxiety- and depression-related behavior in mice (Bravo et al., 2011). Bercik et al. (2011) also demonstrated the importance of vagal integrity in mediating probiotic-induced anxiolysis. These findings have marked vagal tone as a primary candidate of interest when assessing probiotic mechanisms of action.

One way to assess vagal tone is through measurement of heart rate variability (HRV), or the variation of the intervals between heart beats. HRV is highly dependent upon vagal input, particularly during respiratory sinus arrhythmia (Chess, Tam, & Calaresu, 1975; Porges, Doussard-Roosevelt, & Maiti, 1994). Respiratory sinus arrhythmia (RSA) is characterized by an increase in heart rate during inspiration and a decrease in heart rate during expiration (Grossman & Taylor, 2007). RSA can be assessed through spectral analysis of heart rate. Spectral analysis separates the variance of the signal into three discrete frequency bands: very low frequency (VLF), low frequency (LF), and high frequency (HF; European Society of Cardiology, 1996). High-frequency HRV (henceforth referred to as HF) is the frequency that corresponds with RSA, and therefore is widely accepted as an index of vagal tone (for review see European Society of Cardiology, 1996). High HF during resting conditions is thought to reflect a physiologically flexible state that facilitates adaptive response to stressors (Tonhajzerova, Mokra, & Visnovcova, 2013). This flexibility is also important for healthy psychological processes, such as emotional regulation and attention (Porges et al., 1994).

Reduced HF is associated with a multitude of mental health disorders. Hughes and Stoney (2000) found that depressed mood is related to a greater decrease in HF during stress. A recent meta-analysis also linked major depressive disorder with reduced HF, along with a shift
towards sympathetic dominance reflected by other measures of HRV (Kemp et al., 2010). Alcohol dependence is associated with reduced HF, and alcohol use negatively correlates with HF in college students (Quintana, McGregor, Guastella, Malhi, & Kemp, 2013; Udo et al., 2013). Post-traumatic stress disorder (PTSD), schizophrenia, bipolar disorder, and several types of anxiety disorder are all related to decreased HF as well (Minassian et al., 2014; Moon, Lee, Kim, & Hwang, 2013; Pittig, Arch, Lam, & Craske, 2013; Wahbeh & Oken, 2013).

While sleep is controlled through multiple mechanisms, the parasympathetic nervous system plays a prominent regulatory role. Toscani et al. (1996) and Baharav et al. (1995) demonstrated that HF increases continuously from sleep onset and reaches its highest value during slow-wave sleep. Parasympathetic control is strongest during these phases. During the REM phase, the sympathetic system gains dominance, and HF is greatly reduced (Cabiddu, Cerutti, Viardot, Werner, & Bianchi, 2012). This constantly shifting balance is indicative of a healthy autonomic nervous system and a normal sleep cycle.

A growing body of evidence exists connecting suppressed parasympathetic activity to sleep disturbance. Individuals with PTSD and disturbed sleep had significantly reduced HF compared to PTSD-resilient individuals (Kobayashi, Lavela, & Mellman, 2014). Self-reported sleep quality correlates with HF, and disrupted sleep in children is associated with decreased HF (El-Sheikh, Erath, & Bagley, 2013; Hovland et al., 2013; Michels et al., 2013). People with primary insomnia have been shown to have increased beta electroencephalography activity and lower HF HRV in slow-wave sleep, suggesting a state of hyperarousal (Maes et al., 2014). Overall, it appears that disrupted sleep is associated, at least in part, with disruption in autonomic balance: specifically, a shift towards increased sympathetic activity and decreased parasympathetic activity. Introduction of beneficial bacteria to GI tract would be expected to
increase vagal tone via the microbiome-gut-brain axis. As a result, this probiotic-induced vagal activity should restore autonomic balance and therefore improve sleep quality.

*Hypothesis 3:* The beneficial effect of probiotics will improve vagal tone assessed by increases in high-frequency heart rate variability, which will mediate the improvement in sleep.

**Methods**

**Participants**

Participants were undergraduate students recruited from the University of Kansas SONA research pool. Undergraduates were targeted both as a sample of convenience and because disrupted sleep is a widespread problem for this population (Hershner & Chervin, 2014). The SONA research pool consists of all students currently enrolled in Psychology 104 who have opted to participate in research. As this is part of a larger study, all participants were selected based on the dysphoric criteria of scoring greater than 10 on the Beck Depression Inventory (BDI; Beck, Steer, & Brown, 1996) during the SONA pre-screening survey. Participants were included in this study if they presented with a Pittsburgh Sleep Quality Index (PSQI) total value greater than 5—a score associated with poor sleep quality—during the initial study session (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). Fifty-two participants were eligible for this study. Data were available for 44 participants who completed the initial session and at least three sleep diaries during the supplementation period. One participant was an outlier (average value of sleep onset latency greater than 3 standard deviations from the overall mean) and was excluded from the data analysis. The final data analysis was conducted with 43 participants.

Because of the nature of participant selection, I was not able to fully control for equal group assignment beforehand. However, the main study used a randomized block design with a
block size of four to assign participants to a study condition. This meant that two out of every four participants were assigned to the same treatment condition. As such, participants were almost equally assigned to each condition in this study ($n = 20$ for placebo; $n = 23$ for probiotic).

**Study Treatment**

Probiotic supplementation consisted of one dose (two capsules) of regular PB8 (Nutrition Now, Vancouver, WA). Bacteria strains in this product included *L. acidophilus*, *L. plantarum*, *L. salivarius*, *L. rhamnosus*, *L. casei*, *B. lactis*, *B. bifidum*, and *B. longum*. Excipients were microcrystalline cellulose, inulin, magnesium stearate, and silica. PB8 contains 14 billion colony-forming units per dose. Placebo pills consisted of microcrystalline cellulose-filled capsules matched as closely as possible in size and shape to the PB8 capsules.

**Instruments**

**Beck Depression Inventory—II.** The BDI—II (Beck et al., 1996) is a 21-item self-report measure assessing depression severity. The intensity of each symptom is scored 0 to 3, with higher scores indicating greater severity of depression. The BDI demonstrates high reliability (Cronbach’s $\alpha = .81-.86$) and validity (Beck, Steer, & Carbin, 1988).

**Pittsburgh Sleep Quality Index.** Sleep quality was assessed through a modified PSQI (Buysse et al., 1989). The PSQI is a 19-item self-report measure assessing sleep quality across seven domains: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleep medication, and daytime dysfunction. The PSQI has acceptable reliability and validity (Buysse et al., 1989). The PSQI was modified to reflect sleep habits over the past two weeks, rather than the past month.

**Sleep diary.** The sleep diary was a 17-item self-report measure assessing different aspects of sleep during the previous night. The sleep diary used in this study was based off of the
Core Consensus Sleep Diary (CSD: Carney et al., 2012). The CSD was designed to standardize sleep self-monitoring.

**State portion of the State-Trait Anxiety Inventory (STAI).** The STAI consists of two 20-item self-report measures assessing state and trait anxiety severity (Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983). Items are rated on a 4-point scale with higher scores indicating greater anxiety. The STAI has high reliability and validity (Spielberger, 1989; Spielberger et al., 1983). The state portion of the STAI has been shown to be efficacious in detecting mental health disorders (Kvaal, Ulstein, Nordhus, & Engedal, 2005).

**Heart rate variability.** HRV was collected through a three-lead electrocardiogram (ECG; MindWare Technologies LTD., Gahanna, OH), using Ag/AgCl disposable electrodes and a sample rate of 1000 Hz. During the ECG data collection, participants were seated and performing a vanilla baseline task, which has been shown to improve between- and within-subject stability (Jennings, Kamarck, Stewart, Eddy, & Johnson, 1992; Udo et al., 2013). The vanilla baseline task required participants to count the number of times a rectangle of a certain color appeared on a screen, in order to create a standardized resting condition across participants. Task stimuli were created and presented using the E-prime software (Psychology Software Tools, Pittsburgh, PA). During the initial session, participants counted the number of blue rectangles; during the follow-up session, participants counted the number of purple rectangles. The 10-minute recording window was separated into two 5-minute intervals. The first interval was used to initiate a resting state and the second interval used to assess HRV.

HRV was collected and analyzed using HRV Analysis Software (MindWare Technologies LTD., Gahanna, OH). ECG data was visually inspected and manually corrected for
missing or skipped beats, artifacts, and misidentified R-peaks. HF power was selected as the measured variable.

**Procedure**

This study consisted of an initial session (Day 0), a 14-day supplementation period (Days 1-14), and a final session (Day 14 after final probiotic administration). The length of supplementation period was chosen based off of previous study methodology (Ait-Belgnaoui et al., 2014; Desbonnet et al., 2008). The initial and final session took place on the same day of the week, and as close to the same time as possible. During the initial session, participants were consented to the study. HRV was then collected, followed by questionnaire administration. Finally, participants were given their supply of study treatment, along with instructions concerning administration and performance of the adherence check. The entire process lasted approximately one hour. Experimenters and participants were blind to the assigned treatment condition.

The supplementation period began the day following the initial session. Participants took one dose of study treatment in the morning of each day. They then filled out the daily online sleep diary. An adherence check was included at the end of the sleep diary, which required the participant to upload a photo of him/herself taking the supplement. On Day 14, participants took their last dose in the morning prior to arriving at the final session. The final session consisted of HRV and questionnaire collection, identical to the first session. At the end of the session, participants were given a debriefing form, explaining the purpose of the study.

**Statistical Analysis**

Analyses were conducted using the lme4, glmmADMB and mediation packages in R 3.2.5 (Bates, Maechler, Bolker, & Walker, 2015; Fournier et al., 2012; R Core Team, 2016;
Tingley, Yamamoto, Hirose, Keele, & Imai, 2014). Sleep onset latency (SOL), number of awakenings, waking after sleep onset (WASO), and sleep efficiency were used as outcomes and were modeled as discrete Poisson variables. Sleep efficiency was modeled by using the total number of minutes asleep as the outcome variable and the log of total time in bed (SOL + minutes asleep + WASO + minutes in bed after awakening) as an offset variable. Examination of the distributions of the number of awakenings and WASO variables revealed a large quantity of responses valued at zero. To address this, models were corrected for zero inflation (Harrison, 2014). An alpha level of .05 was selected to test model estimate significance. Multilevel growth models (MGMs) were used to assess change in the outcome variables over the course of the study. Multilevel models are useful in analyzing grouped observations. A classic example of multilevel modeling involves assessing characteristics of students within classrooms. Students represent a level-1 variable, while the classroom to which a student belongs represents a level-2 variable. It is expected that students are more similar to their classroom-peers than to students in other classrooms. Multilevel modeling is a form of analysis that allows random effects to be estimated, which can account for variance unique to the grouping level. MGMs are an extension of this. In MGMs, repeated observations from an individual represent the level-1 variables, while the attributes of the individual represent level-2 variables. This type of analysis is useful in determining how between-individual variables (such as experimental condition) affect within-individual measurements over time.

For this study, four two-level MGMs were computed. Sleep onset latency (in minutes), number of awakenings, WASO (in minutes), and sleep efficiency were modeled separately as dependent variables. Observation-level random effects were utilized in the sleep onset latency model to correct for overdispersion (Harrison, 2014). For each model, predictor variables
included day of observation (Level 1), as well as study condition and initial STAI score (Level 2). Models were specified according to Bliese (2013) with values for day of observation representing the number of days since the start of treatment (i.e., 0, 1, 2, 3…). An interaction between time, treatment condition, and initial anxiety was estimated. A significant interaction estimate indicated that study condition and initial STAI score moderate change in the dependent variable over time. Appropriate lower order interactions were also estimated. Participant age and use of sleep medication were included as covariates to control for their effects. All models included a random intercept at the participant level.

Comparison between pre-treatment and post-treatment STAI, PSQI, and HF power values were each assessed using a two-factor repeated analysis of variance. The interaction between time and treatment condition was examined. Changes in PSQI subscales were assessed using Mann-Whitney U tests. One participant did not complete a follow-up session; therefore, data from 42 participants were available to compare pre- and post-treatment STAI and PSQI. Technical difficulties during HRV measurement meant that data from 37 participants were available to compare pre- and post-treatment HF power. Mediation analysis was performed in accordance with the procedures outline by Imai, Keele, and Tingley (2010). Treatment condition was selected as the independent variable, while change in PSQI scores was selected as the dependent variable. Change in HF power was selected as the mediator variable. A mediator model (mediator variable regressed on independent variable) and an outcome model (dependent variable regressed on independent and mediator variables) were estimated. Bootstrapping was performed using these two models to determine mediation and direct effects.

Results
Descriptive statistics are provided in Table 1. Participants were similar in starting characteristics across groups. The average initial sleep onset latency (SOL) for participants was 26.7 minutes (M placebo = 28.6 minutes, M probiotic = 25.0 minutes). These findings match previous studies of disturbed sleep in college students (Lund, Reider, Whiting, & Prichard, 2010). The average initial number of awakenings was 1.3 (M placebo = 1.2, M probiotic = 1.5), the average initial waking after sleep onset (WASO) was 12.5 minutes (M placebo = 10.2, M probiotic = 14.6), and the average initial sleep efficiency was 89.2% (M placebo = 88.7%, M probiotic = 89.6%). Participants had comparable scores on the BDI (M placebo = 19.7; M probiotic = 19.1) and the STAI (M placebo = 45.6; M probiotic = 47.5). A total of 11 participants (n placebo = 6; n probiotic = 5) used a sleep aid at least once throughout the study. For these participants, the average number of days a sleep aid was used was 7.09 (placebo = 4.67; probiotic = 10). The average number of adherence checks submitted, out of a possible 14, was 12.7 (M placebo = 12.6; M probiotic = 12.8).

First, a model for SOL was estimated. Results for the model can be found in Table 2. The interaction between study day, treatment condition, and initial STAI score was found to be statistically significant (β = 0.041±0.018, p = .023), indicating that the change in SOL over time is moderated by a combination of treatment condition and initial anxiety. In order to further explore the interaction between time, treatment condition, and initial anxiety, secondary analyses were performed for all models in which the interaction was significant. Each model was applied to two subsets of the data: one containing only participants with initial anxiety below the sample median of 47 (representing non-elevated anxiety), and one containing only participants with initial anxiety equal to or above the sample median (representing elevated anxiety). The purpose of each secondary analysis was to identify if the estimated relationship from the primary analysis
was true for both non-elevated and elevated anxiety participants. A significant interaction estimate in the secondary analysis indicated that the relationship applied to the corresponding sample subset.

Secondary analyses revealed that the three-way interaction for the SOL model was only statistically significant for non-elevated anxiety participants ($\beta = 0.146\pm0.041$, $p < .001$). There was no difference between treatment conditions at elevated levels of anxiety ($\beta = 0.019\pm0.050$, $p = .708$). Further detail regarding the interaction was obtained by estimating SOL reduction over the study given conditional values of initial anxiety. Conditional STAI scores were chosen based on the 1st quartile value (STAI score = ~39) and the average value (STAI score = ~47).

Estimated SOL recovery values are displayed in Table 3. Individuals with lower anxiety (1st quartile STAI score) receiving probiotic supplementation are expected to experience a 45.19% decrease in SOL over two weeks compared to an 8.05% decrease for individuals receiving a placebo. Individuals with average anxiety receiving probiotic supplementation are expected to experience a 36.05% decrease in SOL compared to a 31.61% decrease for individuals receiving a placebo. Probiotic supplementation is more effective than placebo across lower through average initial STAI values. This effectiveness is not present at elevated levels of initial anxiety. A hypothetical recovery trajectory for a lower-anxiety participant can be viewed in Figure 1.

Number of awakenings and WASO were also modeled using the same procedure as SOL. The interaction between study day, treatment condition, and initial STAI score was not found to be statistically significant for either the number of awakenings model ($\beta = 0.002\pm0.022$, $p = .930$) or WASO model ($\beta = 0.013\pm0.010$, $p = .208$). The interaction between treatment condition and time was also not found to be statistically significant for either the number of awakenings model ($\beta = 0.041\pm0.022$, $p = .62$) or WASO model ($\beta = 0.01\pm0.011$, $p = .376$).
Finally, a model for sleep efficiency was estimated. Results for this model can be seen in Table 2. The interaction between study day, treatment condition, and initial STAI score was found to be statistically significant ($\beta = -0.004 \pm 0.002, p = .042$), indicating that the change in sleep efficiency over time is moderated by a combination of treatment condition and initial anxiety. Secondary analyses revealed that this interaction was only statistically significant for non-elevated anxiety participants ($\beta = -0.011 \pm 0.005, p = .044$). There was no difference between treatment conditions at high initial levels of anxiety ($\beta = 0.000 \pm 0.005, p = .918$). Sleep efficiency increase over the course of the study was also estimated for the same conditional values of initial anxiety as used for the SOL model. Estimated sleep efficiency improvement values are displayed in Table 4. Individuals with lower anxiety receiving probiotic supplementation are expected to experience a 4.41% increase in sleep efficiency over two weeks compared to a 1.40% increase for individuals receiving a placebo. Individuals with average anxiety receiving probiotic supplementation are expected to experience a 2.67% increase in sleep efficiency compared to a 4.17% increase for individuals receiving a placebo. Similar to the SOL model, probiotic supplementation is more effective than placebo at improving sleep efficiency at lower levels of initial anxiety. This effectiveness decreases as initial anxiety decreases and becomes negligible at average levels of anxiety. There is no difference between treatment conditions at elevated levels of initial anxiety. A hypothetical recovery trajectory for lower-anxiety participant can be viewed in Figure 2.

The interaction between time and probiotic supplementation did not have a statistically significant effect on STAI-measured anxiety, $F(1,41) = 1.311, p = .259$, PSQI-measured sleep, $F(1,41) = .44, p = .511$, or HF power, $F(1,36) = 1.375, p = .248$. Post-treatment STAI (M placebo = 43.9, M probiotic = 48.0) and PSQI (M placebo = 9.3, M probiotic = 9.9) scores were
similar between groups. Probiotic supplementation did not have a significant effect on any PSQI subscale \((p > .05\) for all comparisons). Change in HF power did not mediate the relationship between treatment condition and sleep. The average mediation effect estimate is 0.02, 95% CI = -0.69-0.41, \(p = .91\). The average direct effect estimate is -0.36, 95% CI = -2.01-1.48, \(p = .79\). It should be noted that it was hypothesized that probiotic supplementation would improve PSQI-measured sleep quality and that HF power would act as a mediator on this effect. HF power would not be expected to act as a mediator without improvement on the PSQI.

**Discussion**

The primary goal of this placebo-controlled, double-blind investigation was to evaluate the degree to which supplementation with a commercial-grade probiotic could be effective in improving self-reported sleep quality in college undergraduates. Multilevel growth modeling was used to estimate change in four daily sleep diary metrics—sleep onset latency (SOL), the number of awakenings, waking after sleep onset (WASO), and sleep efficiency—over the course of the two-week study period.

Hypothesis 1 predicted that probiotic supplementation would improve self-reported sleep quality over placebo. Probiotic supplementation was not observed to have a significant main effect on any of the self-reported sleep parameters. This is most likely due to the interaction between treatment condition and initial anxiety. Hypothesis 2 predicted that probiotic supplementation would lead to a greater improvement of sleep quality in participants with higher initial anxiety. While initial anxiety did moderate the effect of treatment, the moderation was opposite of the hypothesized direction. Participants without elevated initial anxiety who received probiotic supplementation experienced a greater reduction in SOL over the course of the study compared to those who received placebo. This difference became greater as initial anxiety
decreased. The average lower-anxiety (1st quartile initial anxiety score) participant in the probiotic treatment condition experienced a 45.19% reduction in SOL (Table 3), equivalent to a 12.1-minute reduction. The average lower-anxiety participant in the placebo condition experienced only an 8.05% reduction in SOL, equivalent to 2.1-minute reduction. This means that there was a 37.14% (10-minute) difference in self-reported SOL change between treatment conditions for lower-anxiety participants. Secondary analyses revealed that there was no difference between treatment conditions in participants with elevated initial anxiety.

Similar results were seen regarding sleep efficiency. Probiotic supplementation was associated with significantly reduced sleep efficiency in lower-anxiety participants compared to placebo. The average lower-anxiety participant in the probiotic treatment condition experienced a 4.41% improvement in sleep efficiency, whereas the average lower-anxiety participant in the placebo treatment condition experienced a 1.4% increase in sleep efficiency (Table 4). Given a starting sleep efficiency of 89.66%, this equates to a 3.95% addition in sleep efficiency in the probiotic condition and only a 1.26% addition in sleep efficiency in the placebo condition. Again, probiotic supplementation had no effect on participants with elevated initial anxiety. These findings are broadly consistent with a previous report suggesting that the ingestion of beneficial food-based bacteria may help treat disrupted sleep (Yamamura et al., 2007).

Treatment condition was not found to be associated with any significant changes in the number of awakenings, or on the duration of wakefulness after sleep onset (WASO), over the course of this study. Notably, this is the first reported investigation of the effect of probiotic supplementation on either self-reported awakenings or WASO. Given the beneficial effect of probiotic supplementation on self-reported SOL and sleep efficiency, it is possible that self-report has insufficient sensitivity to detect probiotic-induced changes in number of awakenings
or WASO. Other measures of sleep disturbance, such as actigraphy or polysomnography may be of more use in detecting change in these indices.

The magnitude of the probiotic treatment effect in this study is comparable to some previously reported effects of active treatments for insomnia. For example, a meta-analysis by Huedo-Medina, Kirsch, Middlemass, Klonizakis, and Siriwardena (2012) found that, on average, non-benzodiazepine hypnotics decreased self-reported SOL by seven minutes over placebo—remarkably similar to the 9.8-minute reduction observed with supplementation in lower-anxiety participants in the present study. Likewise, cognitive-behavioral therapy for insomnia has been found to reduce self-reported SOL by 8.4 minutes, improve sleep efficiency by 17%, and to improve other indices of disrupted sleep by 25-50% in elderly patients with insomnia (Kay, Buysse, Germain, Hall, & Monk, 2015). Sleep restriction therapy has been found to reduce self-reported SOL by roughly 16 minutes over placebo, increase sleep efficiency by 17% over placebo, and reduce other measures of sleep disturbance by approximately 50% (Miller et al., 2014). The magnitude of the effect of probiotic supplementation on sleep efficiency was not as great in the present study as the other treatments. However, it should be noted that participants in the referenced studies had initial sleep efficiency values of 65-70% (Kay et al., 2015; Miller et al., 2014). That is because these comparative studies evaluated participants diagnosed with insomnia, in contrast to the present study which only targeted individuals with self-reported sleep disruptions. The use of a less severe sample could explain smaller treatment effect on sleep efficiency. Even with the reduced severity of sleep impairment among study participants, probiotic supplementation produced an effect similar to established treatments for sleep onset insomnia.
If the present study findings replicate, probiotics may have considerable clinical utility in helping address disrupted sleep. Probiotics are easy to administer and consume. There is a large body of evidence suggesting that probiotics are safe and low-risk (see Doron & Snydman, 2015). They are also easily accessible. The probiotic used in this study is available over-the-counter. The same cannot be said for existing sleep treatments. Pharmaceutical sleep aids, like most pharmaceutical treatments, can have harmful side effects (Mallon, Broman, & Hetta, 2009). Psychological treatments are generally side-effect free but require substantial time and effort. Probiotics promise to avoid those consequences while maintaining a clinically-relevant therapeutic benefit.

The potential utility of probiotics in treating sleep disturbance is consistent with the broader premise of a connection between the microbiome and mental health (Dinan et al., 2013). Probiotic microbes tend to temporarily colonize the gut after their ingestion (Alander et al., 1999). This colonization can help restore a healthier, more adaptive microbial balance to the gut (Williams, 2010). In the case of this study, improvements in SOL and sleep efficiency can presumably be attributed to restored microbial balance. Even if the introduced microorganisms were not colonizing the gut, the results of this study suggest that probiotics are fulfilling a function not currently addressed by the microbiome. Either way, it appears that sleep disturbance can be alleviated through manipulation of the microbiome.

Most importantly, this study emphasizes the role that lifestyle plays in mental health. If an imbalanced microbiome is involved in sleep disruption, then it is necessary to determine how this imbalance arises. A person’s microbiome develops early in life and is molded by lifestyle (Falony et al., 2016; Li et al., 2014). Further investigation into the microbiome may reveal methods to both address microbial imbalance and to prevent imbalance from arising in the first
place. It may turn out that the best way to treat sleep disturbance and other forms of mental illness in the future is to implement lifestyle changes targeting the microbiome.

As mentioned previously, probiotic supplementation was only better than placebo in participants without elevated initial anxiety. Furthermore, the treatment effect increased as initial anxiety decreased. It should be noted that probiotic supplementation did not have a significant effect on anxiety in this study. This could account for the lack of effectiveness of probiotics in improving disrupted sleep in people with elevated initial anxiety. It was hypothesized that the sleep-related therapeutic effect of probiotics would be enhanced in individuals with high anxiety because of the purported anxiolytic properties of probiotics (Bercik et al., 2011; Luo et al., 2014). By concomitantly addressing anxiety, probiotics would presumably have the potential to positively impact sleep through multiple mechanisms. In the absence of probiotic-induced anxiolysis, however, there is no hypothesized reason to suspect that initial anxiety would moderate the effect of probiotic supplementation.

Given the findings from the present study, it is possible that anxiety interferes with probiotic-induced improvement in sleep. For example, stress is actually capable of altering the structure of the microbiome, and it can reduce microbial diversity and richness (Bailey et al., 2011; Gur, Worly, & Bailey, 2015). Anxiety might reduce probiotic effectiveness in treating sleep disturbance by inducing more severe microbiome dysfunction. Accordingly, the use of adjuvant treatment approaches, such as an increased probiotic dosage or the use of different strains, may be needed to compensate for the inhibiting effect of anxiety. However, there is at present insufficient evidence to arrive at a definite conclusion. The relationship between sleep, anxiety, and probiotics is an area that has received little attention. Further research is needed to elucidate the role anxiety plays in regulating probiotic-induced sleep improvement.
Despite the anxiety-related reduced effectiveness, it is important to emphasize that probiotics typically outperformed placebo at improving SOL and sleep efficiency among study participants across non-elevated initial anxiety levels. Past research has shown that only 37% of individuals with sleep problems also have a comorbid anxiety disorder (Roth et al., 2006). Furthermore, Ohayon, Caulet, and Lemoine (1998) reported that 54.3% of people with a diagnosis of primary insomnia do not experience symptoms of anxiety. It appears that a significant portion of people with sleep disturbance are not simultaneously suffering from anxiety. As such, probiotic supplementation has the potential to be a powerful effective therapeutic agent for this population.

The final goal of this study was to help determine if a relationship exists between probiotic supplementation, sleep, and vagal tone. The vagus nerve is a major connection between the enteric and central nervous systems (Mayer, 2011). Hypothesis 3 predicted that probiotic-related vagal tone improvement, measured through HF, would mediate improvement in sleep. Probiotic supplementation did not have an effect on HF, or PSQI-measured sleep. These findings fail to support a beneficial effect of probiotics on vagal tone. They also do not support vagal tone as a mechanism of action for improved sleep. Currently, no studies have examined the effect of probiotics on HRV. Because probiotics did not influence PSQI-measured sleep, it is difficult to rule out HF as a potential mediating variable. Further experimentation would be needed to thoroughly assess the role of the vagus nerve in probiotic-induced sleep improvement.

The lack of vagal tone improvement could also help explain why the expected interaction between initial anxiety and treatment condition was not seen. There is evidence that probiotics can reduce anxiety through immune-based pathways (Smith et al., 2014). However, previous studies have also found that the anxiolytic properties of probiotics could be eliminated by
severing the vagus nerve (Bercik et al., 2011; Bravo et al., 2011). It is possible that the anxiolytic properties of probiotics rely primarily upon vagal pathways, rather than other branches of the microbiome-gut-brain axis. If so, addressing anxiety-related sleep disturbance would require the use of probiotics that can affect vagal tone.

While vagal tone should remain a potential mechanism of interest, the results of this study suggest that it may not be related to the sleep-related therapeutic effects of probiotics. Other pathways within the microbiome-gut-brain axis should be examined for their role in regulating sleep disturbance. The microbiome prominently regulates both innate and adaptive immunologic responses within the body (Galland, 2014). There is evidence that inflammation and immune activation alters sleep architecture (for review see Ali, Choe, Awab, Wagener, & Orr, 2013). Microbiota are also capable of producing metabolites, hormones, and neurotransmitters that can influence the central nervous system (Galland, 2014). Any of these pathways could have been responsible for the improvement in SOL seen in this study.

**Limitations**

There are potential limitations to the current findings. First, this study utilized a relatively short supplementation period. Previous studies investigating the psychotropic properties of probiotics have used a supplementation period of two weeks or longer (eg/ Benton et al., 2007; Yamamura et al., 2007). The two-week supplementation period used in this study falls on the short end of required probiotic administration and may have been insufficient to detect changes in HRV or psychological questionnaires.

Other limitations can be attributed to the methodological design of the study. Due to the small sample size, this study may not have been adequately powered to detect all of the meaningful effects of probiotic supplementation. The use of a non-clinical sample may have
contributed to this reduced power. This study could have also been improved through the use of objective sleep measures, such as actigraphy or polysomnography, as well as stronger supplementation adherence checks.

A final limitation is the generalizability of the findings. As this study was part of a larger experiment, recruitment targeted participants with elevated symptoms of depression. It would be important to replicate these findings with a more heterogeneous sample.

**Future Directions**

The next step is to follow up on the results of this study and determine if probiotics can influence objective measures of sleep disturbance. Examples of objective sleep measures include actigraphy. One benefit to using actigraphy is that it minimizes the influence of the placebo effect (Neukirch & Colagiuri, 2015). This would make it easier to identify unique effects of probiotics.

It is also necessary to determine if probiotics are inducing reliable change within the microbiome. It is assumed that probiotics provide benefits by restoring the microbiome to balance. However, without assessing the composition of the microbiome, this cannot be known for sure. This study provides rationale for assessing change in the composition of the microbiome following probiotic treatment for sleep disturbance.

Finally, it is important to determine how long probiotic-induced reductions in sleep disturbance last. Probiotics typically have transient effects, meaning that improvements in disrupted sleep may have a limited window of benefit (Alander et al., 1999). Establishing a duration of effect for probiotics would be necessary for the development of appropriate treatment strategies.

**Conclusion**
This study provides evidence of the potentially important role that the microbiome plays in regulating psychological and physiological processes. It suggests that probiotic supplementation could be used to help reduce sleep disruption. Support for probiotics as a treatment for mental illness and related disorders is growing rapidly. Exploration of the microbiome’s role in mental health promises to illuminate the connection between lifestyle and psychopathology.
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<table>
<thead>
<tr>
<th><strong>Participant characteristics</strong></th>
<th>Overall ( (N = 43) )</th>
<th>Placebo ( (n = 20) )</th>
<th>Probiotic ( (n = 23) )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>Gender ( n \ (%)^</em> )</em>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>25 (58.1)</td>
<td>12 (60.0)</td>
<td>13 (56.5)</td>
</tr>
<tr>
<td>Male</td>
<td>17 (39.5)</td>
<td>7 (35.0)</td>
<td>10 (43.5)</td>
</tr>
<tr>
<td><strong>Sleep Medication Usage ( n \ (%) )</strong></td>
<td>11 (25.6)</td>
<td>6 (30.0)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td><strong>Sleep Efficiency Initial ( % ) (SD)</strong></td>
<td>89.2 (10.0)</td>
<td>88.7 (12.2)</td>
<td>89.6 (7.9)</td>
</tr>
<tr>
<td><strong>Mean (SD)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age in years</td>
<td>19.6 (3.2)</td>
<td>19.0 (1.5)</td>
<td>20.1 (4.2)</td>
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<tr>
<td>Adherence (max = 14)</td>
<td>12.7 (2.7)</td>
<td>12.6 (3.3)</td>
<td>12.8 (2.1)</td>
</tr>
<tr>
<td>SOL Initial Min</td>
<td>26.7 (26.0)</td>
<td>28.6 (28.3)</td>
<td>25.0 (24.2)</td>
</tr>
<tr>
<td>Awakenings Initial</td>
<td>1.3 (1.4)</td>
<td>1.2 (1.2)</td>
<td>1.5 (1.6)</td>
</tr>
<tr>
<td>WASO Initial Min</td>
<td>12.5 (20.1)</td>
<td>10.2 (12.2)</td>
<td>14.6 (25.2)</td>
</tr>
<tr>
<td>PSQI Initial</td>
<td>8.6 (2.6)</td>
<td>8.5 (2.3)</td>
<td>8.7 (2.9)</td>
</tr>
<tr>
<td>PSQI Final**</td>
<td>9.6 (2.2)</td>
<td>9.3 (2.0)</td>
<td>9.9 (2.3)</td>
</tr>
<tr>
<td>STAI Initial</td>
<td>46.6 (10.3)</td>
<td>45.6 (10.6)</td>
<td>47.5 (10.1)</td>
</tr>
<tr>
<td>STAI Final**</td>
<td>46.0 (10.2)</td>
<td>43.9 (9.5)</td>
<td>48.0 (10.6)</td>
</tr>
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<td>BDI Initial</td>
<td>19.3 (7.0)</td>
<td>19.7 (7.8)</td>
<td>19.1 (6.4)</td>
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<tr>
<td>BDI Final**</td>
<td>15.3 (6.07)</td>
<td>15.6 (6.7)</td>
<td>15.0 (5.6)</td>
</tr>
<tr>
<td>HF Initial***</td>
<td>457.1 (372.0)</td>
<td>514.9 (382.7)</td>
<td>402.2 (363.2)</td>
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<tr>
<td>HF Final***</td>
<td>500.4 (357.9)</td>
<td>547.9 (441.3)</td>
<td>455.3 (260.4)</td>
</tr>
</tbody>
</table>

*Note. SOL – Sleep onset latency; WASO – Wake after sleep onset; PSQI – Pittsburgh Sleep Quality Index; BDI – Beck Depression Inventory; STAI – State-trait Anxiety Inventory (state version)*

* One participant declined to answer; ** \( N = 42 \); *** \( N = 37 \)
### Table 2

*Sleep Onset Latency (SOL) and Sleep Efficiency Model Results*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SOL Model</th>
<th>Sleep Efficiency Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intercept</strong></td>
<td>2.771 (0.162) ***</td>
<td>-0.109 (0.014) ***</td>
</tr>
<tr>
<td><strong>Level 1 (observation specific)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>-0.027 (0.014) *</td>
<td>0.003 (0.001) *</td>
</tr>
<tr>
<td>Sleep Medication</td>
<td>0.406 (0.188) *</td>
<td>-0.026 (0.018)</td>
</tr>
<tr>
<td><strong>Level 2 (participant specific)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>0.264 (0.222)</td>
<td>-0.021 (0.019)</td>
</tr>
<tr>
<td>STAI initial (centered)</td>
<td>0.015 (0.154)</td>
<td>-0.007 (0.013)</td>
</tr>
<tr>
<td>Age (centered)</td>
<td>-0.127 (0.100)</td>
<td>0.014 (0.008)</td>
</tr>
<tr>
<td>Condition*STAI</td>
<td>-0.188 (0.218)</td>
<td>0.022 (0.019)</td>
</tr>
<tr>
<td><strong>Cross-level interaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day*Condition</td>
<td>-0.005 (0.019)</td>
<td>-0.001 (0.002)</td>
</tr>
<tr>
<td>Day*STAI</td>
<td>-0.027 (0.013) *</td>
<td>0.002 (0.001)</td>
</tr>
<tr>
<td>Day<em>Condition</em>STAI</td>
<td>0.041 (0.018) *</td>
<td>-0.004 (0.002) *</td>
</tr>
</tbody>
</table>

*Note.* Estimates and standard errors (in parentheses). The fixed effects must first be exponentiated in order to yield interpretable likelihoods.

***p<.001; *p<.05
### Table 3

**Sleep Onset Latency (SOL) reduction (%)**

<table>
<thead>
<tr>
<th></th>
<th>Low anxiety</th>
<th></th>
<th>Average Anxiety</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>2 weeks</td>
<td>1 day</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.60</td>
<td>8.05</td>
<td>2.68</td>
<td>31.61</td>
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<tr>
<td>Probiotic</td>
<td>4.20</td>
<td>45.19</td>
<td>3.14</td>
<td>36.05</td>
</tr>
</tbody>
</table>

### Table 4

**Sleep Efficiency increase (%)**

<table>
<thead>
<tr>
<th></th>
<th>Low anxiety</th>
<th></th>
<th>Average Anxiety</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>2 weeks</td>
<td>1 day</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.10</td>
<td>1.40</td>
<td>0.29</td>
<td>4.17</td>
</tr>
<tr>
<td>Probiotic</td>
<td>0.31</td>
<td>4.41</td>
<td>0.19</td>
<td>2.67</td>
</tr>
</tbody>
</table>
Figure 1. Effect of probiotic supplementation on sleep onset latency in participants with low starting anxiety.
Figure 2. Effect of probiotic supplementation on sleep efficiency for participants with low starting anxiety.