

**STRESS & ANXIETY IMPROVEMENTS WITH ASHWAGANDHA AND  
B-VITAMINS**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of  
the requirements for the degree of Master of Science in Human Nutrition.

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## **ABSTRACT**

Therapies for improving chronic stress and anxiety is still an active area of research and development. The current available therapies and treatments are not reducing the number of individuals developing chronic stress, anxiety, or progressed conditions associated with adverse health effects. These adverse health effects include high blood pressure, obesity, and even metabolic disorders. A recently popular herbal supplement in the US, Ashwagandha, has gained attention in the reduction of stress and anxiety, and potentially possess other medicinal benefits. In fact, Ashwagandha has been used within the Ayurvedic branch of medicine for centuries. Separate from Ashwagandha, B-vitamins are used as another common alternative therapy to enhance mood and neuropsychological function. The present study examines the effects of the combination of B-vitamins and Ashwagandha supplementation on stress and anxiety, and other physiological responses. In this pre- post- study design, 40 women between the ages of 30 and 50 years old, with a body mass index between 25 and 35 kg/m<sup>2</sup>, were recruited. Qualified participants consumed a chocolate-flavored chew containing Ashwagandha extract and B-vitamins, twice a day, for four weeks. Stress and anxiety were measured using validated self-reported questionnaires. Additional measurements included heart rate, blood pressure, and salivary



stress biomarkers. Our data showed evidence that after four weeks of supplementation with B-vitamins and Ashwagandha, trait anxiety was reduced by 12.8% ( $p < 0.001$ ), state anxiety was reduced by 14.2% ( $p = 0.026$ ), negative affect was reduced by 15.1% ( $p = 0.012$ ), heart rate was increased by 4.9% ( $p = 0.001$ ), perceived stress was significantly reduced by 19.8% ( $p < 0.001$ ) evaluated through questionnaire, and momentary self-reported stress was reduced by 25.3% ( $p = 0.044$ ). Four weeks of supplementation did not significantly change blood pressure, BMI, or weight. A double-blind placebo-controlled study with a larger sample size is needed to ensure the validity of these findings.

## **Chapter 1**

### **INTRODUCTION**

Chronic psychological stress and anxiety are a global issue shown to possess detrimental health effects. They are a common contributor to weight gain<sup>1,2</sup>, high blood pressure<sup>3</sup>, depression<sup>4,5</sup>, and other prominent health issues<sup>6</sup>. These health conditions further increase the likelihood of developing harmful chronic health problems like obesity<sup>7</sup>, stroke, and cardiovascular disease<sup>3</sup> which are some of the leading causes of death yearly within the U.S<sup>8</sup>. Individuals who poorly cope with chronic stress and anxiety are also known to use drug stimulants (e.g. nicotine, alcohol, etc.)<sup>10,14,15</sup>. The severity of chronic stress and anxiety and their long-term effects on health is well understood; however, the availability of effective therapies and management is still needed to improve the overall quality of life.

It is recognized that psychological stress in America is a common issue. According to a 2015 survey by the American Psychological Association, over 70% of Americans indicated regularly experiencing psychological stress causing physical and emotional symptoms<sup>10</sup>. One in five individuals report that they do not manage their stress well<sup>11</sup>, and 45% of Americans have indicated lying awake at night due to stress-related issues<sup>12</sup>. Commonly surveyed stress related issues in America include money, work, family, and personal health concerns<sup>11</sup>. Interestingly, women are reported to have higher stress levels compared to men. Generation Xers (those born between 1965-1980; currently 40-54 years

old) and millennials (those born between 1980-1994; currently 25-39 years old) reported significantly higher psychological stress levels compared to other age groups<sup>28</sup> and reported to be significantly more likely to engage in unhealthy behaviors like increasing food intake, drinking alcohol, or smoking to manage their stress<sup>28</sup>.

Other stress and anxiety management therapies include seeking medical help or treatment from a physician. The common psychotropic medication prescribed for stress and anxiety are selective serotonin reuptake inhibitors (SSRI). SSRIs inhibit the neurochemical, serotonin, uptake process within the nerve cells that removes serotonin for excretion. Low serotonin levels are associated with poor mood including anxiety and depression<sup>24</sup>. SSRIs are often considered an anti-depressant medications although studies have shown that SSRIs are effective medications that treat anxiety<sup>25,26</sup>. A common problem for those taking SSRIs include withdrawal symptoms if one decides to stop taking the medication<sup>12,13</sup>. Withdrawal symptoms are caused by the sudden reduction in serotonin levels that can cause individuals to return to their original state. This may result in individuals seeking treatment in complementary alternative medicine (CAM) such as herbs, vitamins, or other supplements with limited potential side-effects. Finding a treatment without withdrawal side-effects for stress and anxiety is still necessary.

CAM therapy has shown to become prominently used in the American population to prevent or treat chronic conditions that include moods, stress and anxiety<sup>16</sup>. Commonly used essential vitamins, such as B-vitamins, have been proven to be beneficial for mood and cognition<sup>20,21</sup>. It has also been shown to be an effective therapy for the improvement of stress and anxiety<sup>30</sup>. Another recognized CAM therapy that has recently been shown to

improve stress and anxiety is an Ayurvedic herb that has been used for centuries, Ashwagandha. Ashwagandha is widely used in India for its various believed medicinal benefits that include inflammation, cognition, glucose control, stress, anxiety, and other conditions. Currently there are published studies that support Ashwagandha's potential to improve memory<sup>29</sup>, improve stress and anxiety<sup>17</sup>, type II diabetes prevention<sup>18</sup> and other ailments. A previous study assessed Ashwagandha's anxiolytic effects on 105 participants for 60 days. The results showed significance in decreased stress based on a 14-question Perceived Stress Scale Questionnaire (PSSQ) and serum cortisol<sup>17</sup>. This is one of the few available studies that assess the benefits of Ashwagandha on psychological stress. More clinical studies with other validated questionnaires, diverse ethnic subjects, and tools are needed to determine Ashwagandha's relationship to stress and anxiety reduction.

A potentially reliable tool, commonly used in the psychology field, to assess perceived stress is an ecological momentary assessment (EMA). EMAs are commonly performed using a technology-based application where assessments can be prompted by text-messages or email and performed on their cell-phone devices virtually anywhere with access to internet. EMAs are able to assess the current behaviors, moods, and individual experiences in real time which limits recall bias compared to other assessments. EMA is a validated method in studies for psychological and behavioral evaluations and found to be a valuable tool for stress and anxiety assessment<sup>22,23</sup>. This tool could be beneficial for an intervention study to better assess the momentary changes in stress and anxiety within individuals.

Therefore, given no study has assessed the effects of the combination of Ashwagandha and B-vitamins, a clinical intervention study is proposed to explore this as a potential treatment to help individuals manage their stress and anxiety and possibly reduce the likelihood of developing adverse chronic health conditions. The proposed study will evaluate stress and anxiety using validated self-reported questionnaires, EMAs, and salivary stress biomarkers. Other additional measurements taken include anthropometrics, blood pressure and heart rate to determine associated stress-related physiological responses. The hypothesis of this study is that a twice daily consumption of a chew with Ashwagandha (300mg) and B-vitamins, will reduce stress and anxiety and stress-related factors (i.e. weight, blood pressure) after 4-weeks in women between the ages 30 and 50.

## **Chapter 2**

### **LITERATURE REVIEW**

#### **2.1 Stress & Anxiety in America and Impact on Human Health**

Chronic psychological stress and anxiety are some of the most chronic mental health issues in the United States. Surveys conducted on stress in America found that in 2015, approximately 77% of the adult population indicated regularly experiencing stress causing physical and emotional stress symptoms<sup>10</sup>. Individuals between 25 and 54 years old were reported to have the highest reported stress compared to the other age groups combined<sup>48</sup>. These age gaps consist of the Generation X (born between 1965-1980; currently 40-54 years old) and millennials (born between 1980-1994; currently 25-39 years old). A majority of whom are within the working force and have children. Additionally, women compared to men have reported to indicate greater levels of stress<sup>10, 48</sup> and a need for mental health care<sup>46,47</sup>. In a 2007 survey, approximately 27 million adults (18 years old and older) being treated for anxiety or mood disorders; 67% of those adults were women<sup>47</sup>.

Chronic stress and anxiety have been associated with poor health outcomes that include weight gain<sup>1,2,7</sup>, high blood pressure<sup>3</sup>, and poor mental health (i.e. depression)<sup>4,5,9</sup>. These health issues can further result in obesity, cardiovascular disease, or even stroke. Appropriate management of stress and anxiety are essential for the reduction of chronic stress and better overall better health outcomes. Sadly, reported methods of stress and

anxiety management have frequently been associated with the engagement of unhealthy behaviors like binge eating<sup>31</sup>, drinking alcohol, or smoking<sup>28</sup>. These coping methods can lead to an even worse health outcome. For example, it is well understood that there is an association between binge eating and increased likelihood of obesity<sup>79</sup>, smoking and increases in lung cancer risk<sup>80</sup>, and alcohol abuse and increased risk in liver damage<sup>81</sup>. Thus, it is imperative to find effective stress and anxiety therapies to reduce the number of individuals engaging in unhealthy behaviors that risk their overall and future health.

## **2.2 Stress & Anxiety Symptoms and Assessment Methods**

Stress, anxiety, and fear are interrelated and thus have been used interchangeably within literature and research. Chronic stress is known to play a major role in the development of anxiety and other neuropsychiatric disorders<sup>34, 35</sup>. Underlying reasons for stress was found to be due to money, work, family and personal health concerns<sup>11</sup>, which could afflict anyone at any point in time. Individuals with anxiety “usually process fear-inducing information in excessive detail that overwhelms their ability to appraise it properly<sup>32</sup>.” Individuals are unable to control their worry that cause significant distress that include impairment in their everyday life, sleep disturbances, and irritability<sup>36</sup>. Symptoms vary from person-to-person and can be disabling for some. Anxiety screening assessments are necessary to prevent chronic stress and prevent the development of other neuropsychiatric disorders if left untreated.

Assessments for anxiety vary in the clinical field and research settings. Examples of some questionnaires in the clinical field that fit the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for evaluating and screening for generalized anxiety and

depression include the Patient Health Questionnaire 9-item scale, the Generalized Anxiety Disorder 7-item and Kessler Psychological Distress Scale. Two reliable and validated questionnaires for evaluating stress and anxiety commonly used in the research field include the State-Trait Anxiety Inventory (STAI)<sup>38</sup> and the Perceived Stress Scale (PSS) questionnaire<sup>39</sup>. Other questionnaires that have shown reliability in assessing stress, anxiety, and mood is the Positive and Negative Affect Schedule (PANAS)<sup>40-42</sup>. These tools have been well utilized and have shown to be reliable measures for the assessment of stress and anxiety.

### **2.3 Current Treatments for Stress & Anxiety and Limitations**

People with chronic stress and anxiety may seek management through medical treatment from a physician. Treatments for anxiety are antidepressants<sup>44</sup>, which are very costly. In a 2007 survey, the annual medical expenditures on anxiety and mood disorders was approximately \$37.8 billion and prescription treatments for anxiety or mood disorders was \$18.4 billion<sup>47</sup>.

The first line of treatment for stress/anxiety has been selective serotonin reuptake inhibitors (SSRIs) and sometimes selective norepinephrine reuptake inhibitors (SNRIs)<sup>43</sup>. SSRIs and SNRIs inhibit the neurochemicals, serotonin and norepinephrine, uptake process within the nerve cells that removes serotonin or norepinephrine for excretion. Individuals with low bioavailable serotonin and norepinephrine levels are associated with poor mood, anxiety, and depression<sup>24</sup>. These treatments increase the bioavailable serotonin and norepinephrine which has been shown to be an effective treatment for anxiety<sup>25,26</sup>. On the other hand, these currently available treatments have serious adverse effects, commonly a



buildup of tolerance and withdrawal symptoms when deciding to discontinue treatment<sup>12,13</sup>. Treatments targeting the mechanism for anxiety and symptoms of anxiety (i.e. fear) is still an active area of research and finding a cost-effective treatment without withdrawal side-effects for stress and anxiety is still necessary. This may result in individuals, especially those without health insurance, seeking treatment in complementary alternative medicine (CAM) such as herbs, vitamins, or other supplements.

## **2.4 B-vitamins**

CAM therapy is prominently used in America to prevent or treat chronic conditions<sup>16</sup>. CAM therapies include dietary supplements such as vitamins and minerals, herbal products, or even meditation. Those who use CAM therapy on a regular basis report stress relief and feelings of being more relaxed<sup>49</sup>. It is possible that for some, CAM therapy could provide a better treatment compared to pharmaceuticals to reduce chronic stress and anxiety.

In fact, studies have shown that a commonly used essential vitamin complex, B-vitamins, is beneficial for mood and cognition<sup>20,21</sup>. B-vitamins have also shown to be an effective treatment for the improvement of stress and anxiety<sup>30</sup>. B-vitamins consist of eight vitamins: B1 (thiamin), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B9 (folate), and B12 (cobalamin). These are essential vitamins that the body requires on a regular basis in order to function properly. B-vitamins are not made within our bodies meaning we rely on our diet from sufficient sources to maintain appropriate levels. B-vitamins are water soluble, meaning that the body excretes the vitamins very easily and little is stored within the body. Thus, daily sufficient B-vitamin

intake is highly recommended. A deficiency in any of the different B-vitamins can lead to the development of potential health disorders<sup>52,53</sup>. Foods that include B-vitamins are red meat, dark leafy vegetables, eggs, dairy products, and even whole grains. Vitamins B1, B6, and B12 are in fact used for the synthesis of neurochemicals like  $\gamma$ -aminobutyric acid (GABA) and serotonin<sup>50</sup>. Additionally, the bioavailability of B-vitamins can influence mood, and those with chronic depression and poor mental health have been found to have lower levels of serum B-vitamins<sup>51</sup>. Studies have shown that supplementation with B-vitamins does in fact improve mood, stress, and anxiety. The benefits of B-vitamins and other vitamins are continually being studied due to our essential needs and their impact on our overall health.

## **2.5 Ashwagandha**

Another recognized CAM therapy that has more recently been used by individuals to improve their stress and anxiety is an Ayurvedic herb. This herb is Ashwagandha, which has been used for centuries and more widely used in India for its various medicinal benefits that include inflammation, cognition, anti-diabetic properties, stress and anxiety, and other conditions<sup>27</sup>. The scientific plant name of Ashwagandha is "*Withania somnifera*," commonly used within literature. It has also been referred to as "Indian winter cherry," "Indian ginseng," or "winter cherry." The bioactive compounds of Ashwagandha are Withanolides<sup>54</sup>, which are the compounds within the plant that plays a beneficial role on our health. There is some collective evidence that has shown Ashwagandha has the potential to improve memory<sup>29</sup>, to prevent type II diabetes<sup>19</sup>, depression<sup>17</sup>, and other ailments<sup>55,56</sup>. The safety of Ashwagandha has been fairly evaluated and no toxicity has

been reported in different doses of Ashwagandha in in-vivo studies in rats<sup>58</sup> and clinical studies<sup>17,18,57</sup>.

Studies on Ashwagandha's anxiolytic benefits are limited but have been positive. In a randomized, double-blind placebo-controlled study that assessed the anxiolytic effects of 600mg Ashwagandha root extract daily on 64 subjects for 60 days showed a significant decrease in reported stress based on the 14-question Perceived Stress Scale Questionnaire (PSSQ) (reduction of 44%;  $P < 0.0001$ ), the General Health Questionnaire (reduction of 72.3%;  $P < 0.0001$ ), the Depression Anxiety Stress Scale Questionnaire (reduction of 71.6%;  $P < 0.0001$ ), and serum cortisol (reduction of 27.9%;  $P = 0.002$ )<sup>17</sup>. This is one of the few available studies that assess the benefits of Ashwagandha on psychological stress. More clinical studies with a diverse population and other validated questionnaires to evaluate stress and mood changes are needed to support its stress and anxiety benefits.

## **2.6 Salivary Biomarkers and Assessment of Stress**

Many published studies available on Ashwagandha have used serum cortisol to evaluate the changes in stress. Cortisol is a steroid hormone produced within our body, more specifically by the adrenal cortex, that is part of the hypothalamic-pituitary adrenocortical (HPA) axis. The HPA axis is a regulated feedback mechanism, neuroendocrine stress response, that serves to regulate responses to stress and other physiological functions (i.e. immune function) by releasing cortisol and other hormones. Cortisol has its own diurnal curve reaching its highest in the morning 30 minutes after waking up, and hitting its lowest at bedtime. There is extensive research that cortisol is a valid measure of chronic stress<sup>82,83</sup>. Cortisol can be measured through saliva, serum, and

hair. Although there is some disagreement between which measure of cortisol best represents chronic stress, salivary and hair cortisol appeared to be an appropriate and non-invasive measure<sup>70</sup>. Salivary also presents to be the most versatile in evaluating cortisol and HPA axis dysfunction correlated with depression<sup>60-62</sup>, and even anxiety<sup>62-64</sup>, by evaluating the cortisol awakening response (CAR). This is by calculating the area under the curve (AUC) from two obtained morning salivary samples: immediately upon waking up and 30 minutes after waking up (approximate time cortisol peak after wakening). Although CAR is able to evaluate HPA dysfunction, the most prominent method of evaluating changes in total cortisol levels at different timepoints in intervention studies is by calculating the AUC from samples obtained from morning to night within a day.

Another useful physiological stress biomarker emerging in research studies is salivary alpha-amylase (sAA). sAA is an enzyme produced within the salivary glands of acinar cells and is important for the digestion of carbohydrates. sAA has its own diurnal curve often declining 30 minutes after waking up, then slowly elevating within the day thereafter and declining towards the end of the day. sAA is a part of the autonomic nervous system and found to play a role in the biological process of stress. More recently it has shown to be a valid and reliable measurement for stress<sup>66-68</sup>. In comparison to cortisol, sAA may be more sensitive in response to stress<sup>69</sup>. Although few research studies are available on the use of sAA to evaluate chronic stress, future studies should consider using sAA as a supplemental biomarker for evaluating chronic stress over a period of time.

## 2.7 Ecological Momentary Assessment (EMA)

Within the neuroscience and psychology field of research on moods and emotions, self-reported data collected based on recall can be a limitation in research studies<sup>71-73</sup>. Recall bias has shown to influence the crucial evaluation of the fluctuation of moods and emotions<sup>74,75</sup>. A more recently developed method of assessment that's used in the psychology field, that may alleviate recall bias, are EMAs. EMAs assess momentary behaviors, moods and individual experiences in real time<sup>22,23</sup>, thus reducing recall or memory bias. EMAs differ from commonly used self-reported assessments by offering participants the benefit of reporting their moods and behaviors within their natural environment, at specific times, and away from research sites.

An EMA can be delivered through the internet by using programs created by external organizations (i.e. ReTAINE), that records self-reported momentary data multiple times throughout multiple days. EMA can be scheduled for specific days and prompt participants to complete a momentary assessment at a given time frame via text message. An EMA is a validated method in studies for psychological and behavioral evaluations and found to be a valuable tool for stress and anxiety assessment<sup>22,23</sup>. EMAs have been used in a large range of different studies that include cognitive therapy<sup>76</sup>, evaluation of stress, mood and illicit drug use craving<sup>77</sup>, and even drug relapse prevention<sup>78</sup>.

EMAs' wide range of use in evaluating behavior, changes in moods, and sensitivity to time-related changes<sup>22</sup> show benefits for intervention studies. In fact, studies have shown that EMAs could be a better measurement for intervention studies to better assess the momentary changes in stress and anxiety within each individual and may be more valuable

to evaluate treatments options for stress and anxiety<sup>74,75</sup>. Due to the lack of research in evaluating treatment outcomes with an EMA, this may be an important assessment to use to evaluate its efficacy.

### **Chapter 3**

#### **AIMS OF THE STUDY**

**Aim 1:** Determine the extent to which the consumption of chocolate chews, containing Ashwagandha and B-vitamins reduces stress and anxiety over a 4-week period based on self-reported questionnaires and EMA.

**Aim 2:** Determine the extent to which the consumption of chocolate chews reduce salivary stress biomarkers, cortisol, and alpha-amylase, over a 4-week period.

**Aim 3:** Determine if a 4-week intervention with chocolate chews reduce physiological responses related to chronic stress such as high blood pressure and heart rate.

## **Chapter 4**

### **METHODS**

#### **4.1 Study Design and Intervention**

In this pre-post-intervention study, a total of 40 participants were enrolled in the study. Participants were asked to come in for a total of four visits: initial screening, a baseline, a 2-week follow up, and a final visit (4-week follow up). Qualified participants were asked to consume a chocolate flavored chew twice a day for four weeks. Each chocolate chew contained 300mg of Ashwagandha extract and 25% of the recommended daily value of B-vitamins. So, each participant consumed 600mg of Ashwagandha extract and 50% of the recommended daily value of B-vitamins daily for 4-weeks. No placebo group intervention was used in the study. Researchers checked-in with all participants the first two days after starting the intervention to evaluate and record any adverse effects or side effects observed since starting the chocolate chew consumption. During each visit (baseline, 2-week follow up, 4-week follow up) a variety of questionnaires to evaluate stress and anxiety were taken by each participant as well as different anthropometric and physiological measurements. Participants were asked to collect salivary samples and complete EMA questionnaires outside of the research site for the purpose of the study.



## 4.2 Subject Recruitment, Inclusion & Exclusion Criteria

Flyer advertisements were posted and emailed around both the University of Delaware community for the recruitment of participants. Individuals interested in the study called or emailed the research lab to receive more information regarding the study. Those interested were scheduled for a phone pre-screening to determine if they met the inclusion or exclusion criteria. Information gathered during the phone screening included: name, email, phone number, sex, date of birth, if they owned a smartphone device with access to internet and Wi-Fi, ability to receive text messages, any known allergies to Ashwagandha, and the presence of any conditions that could inhibit their ability to participate in the study.

Inclusion criteria included body mass index (BMI) between 25-35kg/m<sup>2</sup>, ages between 30 to 50 years old, access to the internet and Wi-Fi on their smart phone devices and the completion of at least 80% of the EMAs prior to their scheduled baseline visit. Exclusion criteria included women who were pregnant, trying to conceive, had any chronic health issues that would limit their ability to participate, and those who developed an intolerance to the chocolate chew.

A total of 183 interested participants were phone-screened and 45 of those qualified for the study. Those that qualified over the phone were then invited to come in for an in-person screening visit at the University of Delaware Tower at STAR in Newark, DE. A total of 40 women, all of whom met all the inclusion criteria and provided written informed consent to participate, were enrolled in the study. A flow chart of the participants in the study is presented in Figure 1 (Appendix A.1).

## **4.3 Study Visits**

### **4.3.1 Screening Visit**

Participants were scheduled to come in for a total of four visits for the completion of the study. All visits were taken place at the University of Delaware Tower at STAR. The first visit was the screening visit to review and obtain consent from participants. Once consent was received, participants completed medical history (example shown in Appendix C.1), demographics questionnaires (example shown in Appendix C.2), anthropometrics (height, weight, waist and hip circumference), blood pressure, and heart rate measurements. EMA set-up on their cellphone devices were performed during this visit to ensure they received text-messages, that prompted them to complete an EMA. EMAs began starting the day after the screening visit. Additionally, each participant was instructed on how and when to complete the EMA (refer to Chapter 4.5 EMA), how and when to collect their saliva samples (example shown in Appendix C.9), and how to record a 3-day diet record (example shown in Appendix C.8) to log their food consumption. Participants were compensated \$20 for the completion of this visit. Participants were instructed to complete the first set of EMAs (Baseline EMA), a 3-day diet record, and collect three saliva samples the day before their scheduled visit.

### **4.3.2 Baseline Visit**

At the second visit, anthropometrics, blood pressure and heart rate were taken. Each 3-day diet record was reviewed by a researcher to obtain accurate measurements of recorded food items. Participants completed a Perceived Stress Scale Questionnaire (PSSQ)<sup>39</sup>, State-trait Anxiety Inventory (STAI) – form Y questionnaire<sup>38</sup>, Positive

Negative Affect Scale questionnaire (PANAS)<sup>41</sup>, and a Global Physical Activity Questionnaire (GPAQ)<sup>84</sup>. Participants were then instructed to start the intervention the following day for the next four weeks, totaling 28 days. The intervention given was a chocolate flavored taffy chew containing B-vitamins and Ashwagandha. A two-week supply of the chocolate chew (28 pieces) was provided to participants during their baseline visit. Participants were asked to record the times they consumed the chocolate chew each day and return any uneaten chews to evaluate compliance. In this study, EMAs were provided in seven-day increments. Participants were instructed to perform the second set of seven consecutive days of scheduled EMAs after 10 days of consuming the chews, midway into the intervention. Participants were compensated up to \$60 for this visit. Forty dollars of the \$60 were given for the completion of at least 80% of the EMAs (31 of 38 EMA), three saliva samples collected the day before the baseline visit, and completion of the 3-day diet record.

#### **4.3.3 Two-Week Follow Up**

The third visit, 2-weeks post-intervention, included the collection of all the same information and measurements as the baseline visit and questionnaires, except for the PSSQ. Each 3-day diet record was reviewed with a researcher to obtain accurate measurements of recorded food items. Uneaten chews were returned and recorded during the visit for compliance. Participants received an additional two weeks supply of the chocolate chews for the continuation of the intervention. Participants were instructed to complete the second set of EMAs and complete the scheduled EMAs for a final seven days. This third set of EMAs were completed after 20 days of starting the chocolate chew

intervention. Participants were compensated \$20 for this visit and for providing the three saliva samples performed the day before the visit.

#### **4.3.4 Four-Week Follow Up (Final Visit)**

The fourth visit, 4-weeks post-intervention, included the collection of all the same measurements, information, and questionnaires as the baseline visit. Each 3-day diet record was reviewed with a researcher to obtain accurate measurements of recorded food items. Uneaten chocolate chews were collected and recorded during the visit for compliance. Participants were compensated up to \$200 for this visit. Forty dollars of the \$200 were given for the completion of at least 80% of the second set of EMAs. Eighty dollars of the \$200 were given for the completion of at least 80% of the third set of EMA, three saliva sample collected the day before the final visit, and for the completion of the 3-day diet record. For participants who completed at least 90% of all scheduled EMAs (103 of 114 EMA) within this study, received \$80 of the \$200.

### **4.4 Demographics, Anthropometrics, and Physiological Measurements**

#### **4.4.1 Demographics**

Demographic information including age, education level, income, race, marital status, number of children, and employment status were collected during the screening visits.

#### **4.4.2 Anthropometrics**

##### Body Mass Index (BMI)

BMI was calculated from height (in cm) and weight (in kg) measurements taken during each visit. Height was taken on a stadiometer affixed to the wall, in centimeters. Participants removed their shoes, were asked to stand straight with their back and buttocks against the walls, with their heels touching the base of the measurer for proper measurement. Weight measurement was taken on a calibrated, leveled, digital scale in kilograms. Participants changed into scrubs during each visit to maintain consistency in each measurement. Participants removed their shoes and were asked to stand straight with their chins up. Centimeter conversions to meters were performed by dividing the measured centimeter by 100. BMI was calculated using the following equation<sup>59</sup>:

$$\text{BMI} = (\text{Weight (kg)}) / \text{Height (m}^2\text{)}$$

##### Waist and Hip Circumference

Waist and hip circumference measurements (in cm) were obtained during each visit using a flexible, non-stretchable, tape measure. Participants were asked to change into provided scrubs during each visit to maintain consistency. Measurements were taken anatomically on the right side of the body while standing. Waist measurement taken around the circumference of the abdomen at the umbilicus. Hip measurement taken around the largest circumference of the buttocks. Tape measure was checked to assure that it was parallel to the ground and untangled prior to recording the measurement.

### **4.4.3 Physiological Measurements**

#### Heart Rate & Blood Pressure Measurements

Two blood pressure and heart rate measurements were taken during each visit, three minutes apart. An appropriate blood pressure cuff was used based on the measurement around their upper arm using a flexible, non-stretchable, tape measure (in cm). Measurement was taken after five minutes of isolation within a quiet room. Participants were asked to keep both feet planted on the floor and remain silent during the period of measurements.

### **4.5 Questionnaires, 3-Day Diet Record, and EMA**

#### **4.5.1 Questionnaires**

##### PSSQ

A Perceived Stress Scale Questionnaire<sup>39</sup> (PSSQ) was completed only during the baseline and final visits. The validated self-reported questionnaire<sup>39</sup> consists of 10 questions based on a 5-point Likert scale to measure and assess individual perceived stress. Scoring was conducted based on the manual with scores ranging from 0 – 40. Scores within 0-13 were considered low stress. Scores ranging within 14 – 26 were considered moderate stress. Scores ranging within 27 – 40 were considered high perceived stress. The test re-test reliability has a Cronbach's alpha at  $>0.70$ <sup>65</sup>.

##### STAI Questionnaire

A State-Trait Anxiety Inventory (STAI) - Form Y<sup>38</sup> was completed during the baseline, the 2-week follow up, and the final visit. The validated self-reported questionnaire<sup>38</sup> consists of a two-part questionnaire: 20 questions to assess state or acute

anxiety characteristic and 20 questions to assess trait or chronic anxiety characteristics, based on a 4-point Likert scale. Scoring was calculated based on the provided directions ranging from 20 to 80 points for each questionnaire part. Scores ranging within 20 – 37 were considered no or low anxiety. Scores ranging within 38 – 44 were considered moderate anxiety. Scores ranging within 45 – 80 were considered high anxiety. The test re-test reliability of both trait and state anxiety range from 0.73–0.85<sup>107</sup>.

### PANAS-X

A Positive Affect Negative Affect Scale Questionnaire<sup>41</sup> (PANAS), version X, was completed during the baseline, the 2-week follow up, and the final visit. The validated self-reported mood questionnaire<sup>40,41</sup> consists of 60 total feelings or emotions rated on a 5-point Likert scale. Participants reported the extent to which they felt a certain feeling or emotion during the past week. The 60 different feelings and emotions in the questionnaire were then grouped and scored to evaluate the positive and negative mood affects. These terms can also be grouped into 11 other different types of emotional states as well. For the purpose of this study, only positive and negative affects were evaluated. Scoring was performed based on the grouped terms following the PANAS-X manual. The Cronbach's alpha for internal consistency range from 0.83-0.9 for positive affect, and 0.85-0.90 for negative affect<sup>41</sup>.

### GPAQ

A Global Physical Activity Questionnaire<sup>84</sup> (GPAQ) was completed during the baseline, the 2-week follow up, and during the final visit (4-week follow up). The validated self-reported questionnaire is used to assess weekly physical activity and physical activity

status. Participants were asked to maintain their habitual physical activity for the duration of the study. The validated questionnaire consists of 16 total questions to evaluate vigorous-intensity activity, moderate-intensity activity and sedentary behavior during the past week. Measurement was calculated based on the GPAQ guidelines and calculated as metabolic equivalents (MET) per week. Individuals with a total of  $\geq 150$  MET (or minutes) of moderate activity per week,  $\geq 75$  MET of vigorous activity per week, or a combination of  $\geq 600$  MET moderate and vigorous activity were considered physically active.

#### **4.5.2 3-Day Diet Record**

Participants were asked to complete a 3-day diet record prior to returning to each visit during the baseline, the 2-week follow up, and the final visit. Each participant was asked to maintain their habitual diet for the duration of the study and provide a detailed description of all consumed items on 3 self-chosen days (example of instructions shown in Appendix C.8). Participants were asked to choose 2 weekdays and 1 weekend day. Additionally, they were asked to choose days where they consumed similar foods regularly on either the corresponding weekday or weekend day. All 3-day diet records were reviewed with a researcher during the returning visit.

#### **4.5.3 EMA**

Ecological momentary assessments (EMA) were conducted at three different timeframes of the intervention (Baseline EMA, 2-week post-intervention EMA, and Final EMA) of the five total weeks of the study, in seven consecutive day increments. The EMA was delivered to smartphone devices via a prompted text-messages. All participants completed a total of 21 days of EMAs. On the days of their scheduled EMAs, participants



would randomly receive a text message, five times per day within their waking hours, prompting them to complete the EMA. Each EMA consisted of 60 questions from the validated daily stress inventory questionnaire<sup>85</sup>. The questionnaire consists of different stressors. Participants reported the following stressors that applied to them since the last completed EMA and rated each stressor and how much the stressor affected them.

Additionally, participants performed an EMA during the times they collected their saliva samples. These were performed the day prior to their baseline, 2-week follow up, and final visits. Participants collected three different saliva samples, for stress biomarker assessment, over the course of one day: the moment they wake up, 30 minutes after waking up, and at bedtime. Participants answered a total of six questions in each unprompted EMA. Calendars were provided to participants to keep track of all EMAs scheduled within the study (example shown in Appendix C.11).

## **4.6 Salivary Collection and Biomarker Assessments**

### **4.6.1 Saliva Collection**

Participants were asked to collect approximately 1,000 $\mu$ L (1mL) of their saliva at three different timepoints within a day. Salivary samples were collected at home and in cryovials provided. “SalivaBio Saliva Collection Aid” by Salimetrics cryovials were used for this study. Instructions were provided to all participants during each visit (example shown in Appendix C.9). Participants were asked to avoid the consumption of any foods or drinks and avoid brushing their teeth immediately prior to the saliva collection. Participants were asked to store their saliva samples within a refrigerator until their scheduled visit the following day.

A passive drool method was used to collect salivary samples. Salivary collection was performed only the day prior to their scheduled visits for the baseline, the 2-week follow up, and the final visit. Participants collected their saliva the moment they wake up, 30 minutes after waking up, and right before bedtime. After each saliva collection, an unprompted EMA was completed to use as collection timestamp. This is later used to calculate AUC of the total biomarker within the day.

#### **4.6.2 Salivary Biomarker Assessments**

All salivary samples were stored within  $-80^{\circ}\text{C}$  prior to assessment and quantification of biomarkers. All samples were thawed to room temperature prior to analysis. Cortisol and sAA enzyme-linked immunosorbent assay (ELISA) were performed using assay kits by Salimetrics. Cortisol samples were analyzed using the Synergy HTX Multi—Mode Plate Reader and sAA were analyzed using the TECAN Infinite 200 PRO. Area under the curve (AUC) was used to determine total concentration of biomarker within the same day of salivary collection. Equation to solve AUC followed Pruessner et al., 2003.

##### Cortisol Assay

The cortisol ELISA assay used was a competitive immunoassay. ELISA microtiter plates and reagents were stored at  $2-8^{\circ}\text{C}$  within the foil pouch. Both reagents and plates were brought to room temperature during the time of use. All reagents used were prepared within the same day of the ELISA assessment.

During the day of the assay, all samples were thawed, vortexed and centrifuged at  $1,500 \times g$  for 15 minutes. 25 microliters ( $\mu\text{L}$ ) of each salivary sample supernatant were used for the purpose of this experiment. All samples were run in duplicates, read at 450nm

and within 10 minutes of adding the stop solution. Samples greater than the cortisol range (3.0 µg/dL (82.77 nmol/L)) were diluted and rerun. Samples less than the cortisol range were rerun with concentrated samples. Cortisol concentrations were determined using a 4-parameter non-linear regression curve fit and using the average measured optical density (OD) values and percent bound calculations according to the Salimetrics kit manual.

#### sAA Assay

The sAA assay used was a kinetic enzyme assay. ELISA microtiter plates and reagents were stored at 2-8°C until time of use. Both reagents and plates were brought to room temperature during the time of use, except for the  $\alpha$ -Amylase substrate.  $\alpha$ -Amylase substrate was placed in a 37°C incubator shaker to avoid degradation. During the day of the assay, all samples were thawed, vortexed and centrifuged at 1,500 x g for 15 minutes. 10µL of each salivary sample supernatant were used for the purpose of this experiment. Three samples were run and read at a time: waking sample, 30 minutes after waking sample, and bedtime sample. Samples were immediately read in the plate reader after adding the  $\alpha$ -Amylase substrate using reverse pipetting method to avoid bubble formation. The enzyme binding activity was measured spectrophotometrically at 405 nm at exactly 1 minute prior to the addition of  $\alpha$ -Amylase substrate and at exactly 3 minutes after the addition of  $\alpha$ -Amylase substrate. All samples were run in duplicates.  $\alpha$ -Amylase activity was calculated using the following equation based on the difference in the two optical density (OD) measurements:  $\Delta\text{Abs.} \times 328 = \text{U/mL } \alpha\text{-Amylase activity}$ . Calculations followed the Salimetrics kit manual.

#### 4.7. Statistical Analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences (SPSS) version 26.0 and Prism 8 (Version 8.4.1) software program. Descriptive statistics were performed and summarized for all data. All measurements of stress and anxiety were compared to baseline to evaluate changes across time. Statistical significance was set at P value of  $<0.05$ . The statistical methods for the evaluation of statistical significance of each aims of the study were as follows:

Aim 1: Determine the extent to which the consumption of chocolate chews, containing Ashwagandha and B-vitamins reduce stress and anxiety over a 4-week period based on self-reported questionnaires and EMA assessments.

- A paired t-test was used to determine the changes in stress and anxiety from the baseline to the 2-week follow up visit and changes in baseline and the 4-week follow up visit for questionnaires and EMA results.

Aim 2: Determine the extent to which the consumption of chocolate chews reduce salivary stress biomarkers, cortisol and alpha amylase, over a 4-week period.

- A paired t-test was used to determine the biomarker changes from the baseline to the 4-week follow up.

Aim 3: Determine if a 4-week intervention with chocolate chews reduce physiological responses related to chronic stress such as high blood pressure and heart rate.

- A paired t-test was used to determine changes in physiological responses from the baseline versus the 2-week follow up visit, and the baseline versus the 4-week follow up visit.

## **Chapter 5**

### **RESULTS**

#### **5.1. Demographics, Participant Characteristics, & Compliance**

The demographic characteristics of all 40 women from diverse backgrounds are presented within Appendix A Table 1. The mean age within the study was  $43 \pm 6$  years old and the BMI of  $29.76 \pm 2.62$ . A majority of the participants were married (60%), had a 4-year college degree (32.5%) or master's degree (35%), had a household income above \$100,000 (47.5%), and were Caucasian/White (70%). The average compliance of the supplementation was 96.4% of the 39 participants who completed the study.

#### **5.2 Changes in Anthropometrics & Physiological Responses**

No significant changes in body weight, BMI, diastolic blood pressure, systolic blood pressure, and waist hip circumference ratio were found. Mean and standard deviations presented within Appendix A Table 2. Although a significant increase in heart rate by 4.9% ( $p = 0.001$ ) was found between intake of the chocolate chews and heart rate when comparing baseline and final visits.

#### **5.3 Changes in Self-reported Questionnaire Results and EMAs**

Significant reduction in perceived stress and anxiety based on questionnaire evaluation was found, correlated with chocolate chew consumption after two and four weeks. In the PSSQ, there was a reduction in stress score by approximately 19.8% ( $p$

<0.001) after the four-week consumption of chocolate chews. Mean and standard deviations are presented within Appendix A Figure 2 and Table 3. At baseline, the participant average PSSQ score was categorized as moderately stress. After the intervention, at 4-weeks, the participant average PSSQ score was categorized as low stress. Consistent findings were found in STAI trait anxiety scoring (Appendix A Table 3 and Figure 3). Individuals showed a reduction in trait anxiety by approximately 8.2% ( $p = 0.003$ ) at the 2-week follow up and 12.8% ( $p < 0.001$ ) at the 4-week follow up when compared to the baseline. Comparably STAI state anxiety showed reduction by 10.7% and 14.2% ( $p = 0.026$ ) 2-week follow up and 4-week follow up, respectively, compared to baseline. Additionally, there was a significant reduction in average reported perceived stress in EMA by 23.7% ( $p < 0.001$ ) after the 2-weeks follow up compared to the baseline. The 4-week follow up showed a significant 25.3% ( $P = 0.044$ ) reduction in perceived average stress compared to the baseline EMA. Mean and standard error presented in Appendix A Table 4 and Figure 5.

In addition to changes in stress and anxiety, negative mood changes (negative affect) evaluated from the PANAS-X questionnaire were significantly reduced by approximately 15.6% ( $p = 0.003$ ) at the 2-week follow up and remained constant until the remainder of the study. No significant changes in positive mood (positive affect) were found, although an increasing trend in positive mood scoring was found across the timepoints. The mean and standard error presented in Appendix A Table 3 and Figure 4.

No correlation was found between intake of the chocolate chews and changes in self-reported physical activity over 4-weeks.

#### **5.4 Changes in Salivary Biomarker Results**

There were no significant changes in either salivary cortisol or sAA biomarkers when comparing the baseline, the 2-week follow up, and the final visit (4-week follow up). Our data showed some slight increase in the salivary cortisol at the 2-week follow up of 1.88% (SE: 0.015;  $p = 0.841$ ) and 4-week follow up of 1.21% (SE: 0.012;  $p = 0.861$ ), compared to baseline. Additionally, sAA also showed slight increase of 8.52% (SE: 4.70;  $p = 0.367$ ) at the 2-week follow up but decrease of 0.64% (SE: 4.61;  $p = 0.945$ ) at the 4-week follow up, compared to baseline. These changes were both found to be statistically insignificant. The mean and standard deviation is presented in Appendix A Table 5.

## **Chapter 6**

### **DISCUSSION**

Improvements in mood, stress, and anxiety can be beneficial for our overall health and quality of life. Chronic stress and anxiety are common mental health concerns that affects millions and still requires effective therapies that will cause little to no potential side effects. The present study is one of the first to evaluate the benefits of the combination of B-vitamins and Ashwagandha on stress and anxiety. The present study enrolled 40 participants, although one participant withdrew from the study due to experiencing minor intolerance towards the chocolate chew. Within the study, we had a compliance rate of 96.4% with the supplementation amongst the 39 participants and no adverse effects were noted, indicating that supplementation with 50% of the recommended daily B-vitamins and up to 600mg of Ashwagandha per day is safe and well tolerated. This is similar to other reported studies with B-vitamins<sup>93</sup> and Ashwagandha supplementation<sup>87-89</sup>.

This present study found supportive evidence of improvements in stress and anxiolytic effects after supplementation with both Ashwagandha and B-vitamins from various validated questionnaires, similar to other studies<sup>87-89</sup>. Participants showed a significant reduction in perceived stress, momentary stress, state and trait anxiety, and reduction in negative moods. This study also uniquely used EMAs to evaluate the momentary stress and the fluctuation of changes in perceived stress during the intervention



period. The repeated measurements of momentary stress gathered from EMA strengthens the reported evidence of the stress-relieving benefits by assessing the changes in momentary stress throughout the day on a day-to-day basis. This approach has been suggested in some studies as a better method to evaluate treatments for anxiety disorders since mood, stress, and anxiety fluctuates throughout the day<sup>74,75</sup>. In this study, we averaged the reported stress from collected EMAs and compared it over the three timeframes of the study. Although stress-relieving and anxiolytic benefits have been reported, our present study did not determine any significant changes in salivary stress biomarkers.

Previous studies have suggested that stress and anxiety improvements from Ashwagandha supplementation may be due to its antioxidant properties or improvements of the HPA axis activity<sup>88</sup>. Ashwagandha has shown to contain numerous alkaloids, superoxide dismutase, and free-radical scavenging enzyme properties<sup>97</sup>. Alkaloids are naturally occurring compounds found to play anxiolytic and antidepressant benefits through the mediation of neurotransmitters<sup>98</sup>. A study evaluating the potential alkaloid mechanism of Ashwagandha in rats found monoamine oxidase (MAO) inhibition activity<sup>100</sup> which has been shown in other studies to be correlated with antidepressant-like effects<sup>99</sup> and has also been used in medications to treat depression. At this time, no subsequent study has been conducted to evaluate MAO inhibition activity with Ashwagandha supplementation. Other antioxidant properties have included superoxide dismutase and polyphenols that reduces oxidative stress and free radicals<sup>97</sup>. Studies have shown an association with cellular oxidative imbalance and anxiety disorders, which could

contribute to the anxiolytic effects of Ashwagandha supplementation<sup>101</sup>. B-vitamins may also enhance mood and provide anxiolytic benefits differently, by increasing the production of neurotransmitters<sup>53,94</sup>. Vitamins such as the B-6 vitamin is a rate-limiting cofactor for the production of neurotransmitters such as serotonin and  $\gamma$ -aminobutyric acid (GABA).

The present study failed to find significant changes in cortisol stress-induced biomarkers, which differs from published studies with Ashwagandha interventions. This could be due to the sample difference using salivary cortisol instead of serum cortisol, reportedly used by other studies. It is unclear why serum cortisol was the preferred method for evaluation in previous studies. Serum cortisol evaluates the total cortisol within the circulatory system that includes cortisol-binding globulin (CBG) (80% of the total), cortisol-binding albumin (10% of the total), and free cortisol. Salivary cortisol instead is a direct measure of the free cortisol and has been established to be an appropriate alternative measure of cortisol<sup>102-104</sup>. In fact, free cortisol is more sensitive to changes in response to stress, by reducing CBG and elevating the free cortisol significantly<sup>105</sup>. Thus, free cortisol may be a better measurement of stress. Although the results of this study did not find supportive evidence in changes in cortisol, this could potentially be explained by confounding factors that include use of mood or anxiety medications or use of hormonal contraceptives that needs to be considered<sup>91</sup>.

Our study also included the evaluation of another stress biomarker, sAA, which showed insignificant elevations. An explanation for the slight elevation is that sAA is a surrogate marker of norepinephrine, part of the sympathetic nervous system, and has been

established as a biomarker in response to stress<sup>106</sup>. Slight increase in sAA may be a result of the B-vitamin supplementation. B-vitamins are a cofactor for the synthesis of catecholamine neurotransmitters such as norepinephrine<sup>53</sup> which may result in slight increases from supplementation. Although, when comparing 2-week follow up to 4-week follow up sAA reduction was noted. A longer intervention study is needed to determine if further reduction in sAA would result from B-vitamin and Ashwagandha supplementation. Additionally, although participants received instructions on saliva collection method and specific times of sample collection, variations in method and time of collection can significantly cause errors<sup>91,95</sup>.

This present study did not find any improvements in weight, waist hip ratio, or blood pressure that are commonly influenced by chronic stress. The lack of change in anthropometrics and blood pressure align with a previous published 8-week long intervention studies<sup>87,96</sup>. Our study also evaluated physical activity across the three timepoints and found no significant changes, ensuring that physical activity was not a confounding factor in this study. Interestingly, our study reported a significant increase in heart rate which has not been reported in other studies. Heart rate increase can be associated with increase in norepinephrine, typically seen in elevated sAA. Our data does in fact show slight elevations in sAA after 2-week follow-up. Although heart rate increase remains, sAA returns to baseline levels after 4-week follow-up. Due to the short-term intervention, it is difficult to determine if there is a direct correlation.

Strengths of this study include the use of multiple different validated questionnaires to evaluate stress and anxiety, EMAs to record momentary and detailed assessments in the

variation of changes in stress throughout the intervention period, and the use of two biological stress markers. Limitations of this study include small sample size and short intervention period. Future research should evaluate the underlying mechanism of Ashwagandha, or withanolides, stress relieving properties. Additionally, a double-blinded placebo-controlled clinical trial study design, larger sample size, removal of confounding factors noted within this study and a longer intervention duration should be considered for future studies to support our findings.

## **Chapter 7**

### **CONCLUSION**

This study concludes that the consumption of B-vitamins and Ashwagandha daily for 4-weeks can potentially aid in stress and anxiety experienced by many. It could reduce perceived stress, anxiety, and decrease negative mood symptoms associated with stress and anxiety. Our results suggest that B-vitamins and Ashwagandha do not reduce physiological responses associated with stress such as blood pressure and may not influence salivary cortisol or sAA biomarkers of stress. Although this is contrary to the results of other studies conducted on Ashwagandha supplementation, this may be influenced by the length of study and method of assessment in comparison to these studies. Further studies are necessary to support these findings of anxiolytic benefits of B-vitamins and Ashwagandha supplementation on reduction of chronic stress. It is important to provide stress relieving therapies to reduce the rates of obesity, development of poor mental health, high blood pressure, comorbidities, and alcohol and tobacco use related to chronic stress and anxiety. A natural therapy could potentially be a more cost-effective treatment and reduce drug dependency associated with anxiety medication use. Future studies should conduct a longer, double blinded placebo-controlled study, with a larger sample size on stressed participants to validate these findings. Additionally, the mechanism to which Ashwagandha's anxiolytic benefits should be further investigated.

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## Appendix A

### TABLES AND FIGURES

**Table A.1.**  
**Baseline Characteristics**

<b>Variables</b>	<b>Mean ± Standard Deviation</b>
<b>Age, years</b>	43.10 ± 6.04
<b>Body height, cm</b>	162.90 ± 6.20
<b>Body mass, kg</b>	79.15 ± 9.84
<b>BMI (kg/m<sup>2</sup>)</b>	29.76 ± 2.62
<b>Education Level</b>	<b>n (%)</b>
Less than high school	0 (0)
High school or equivalent	1 (2.5)
Some college	8 (20.0)
2-year college degree	2 (5.0)
4-year college degree	13 (32.5)
Master's degree	14 (35.0)
Doctoral degree	1 (2.5)
Professional degree	1 (2.5)
<b>Household Income</b>	
Under \$25,000	1 (2.5)
\$25,000 - \$49,999	6 (15.0)
\$50,000 - \$74,999	7 (17.5)
\$75,000 - \$99,999	6 (15.0)
\$100,000 +	19 (47.5)
Prefer not to say	1 (2.5)
<b>Marital Status</b>	
Single never married	8 (20.0)
Separated / Divorced	7 (17.5)
Married	24 (60.0)
Widowed	0 (0)
Living with another	1 (2.5)
<b>Race</b>	
Hispanic / Latino	1 (2.5)
Not Hispanic / Latino	39 (97.5)
American Indian / Alaskan Native	0 (0)
Asian	2 (5.0)
Native Hawaiian / Islander	0 (0)
Black / African American	7 (17.5)
White	28 (70.0)
Mixed Race	2 (5.0)
Other	1 (2.5)

*Table 1: Baseline characteristics and frequencies of all 40 enrolled participants.*

**Table A.2. Anthropometric and Physiological Changes Assessment**

	<b>Baseline</b>	<b>2 weeks</b>	<b>4 weeks</b>
<b>Body weight, kg</b>	78.73 ± 9.611	79.22 ± 9.89	78.65 ± 9.79
<b>BMI, (kg/m<sup>2</sup>)</b>	29.62 ± 2.51	29.80 ± 2.54	29.59 ± 2.60
<b>SBP (mmHg)</b>	118.2 ± 12.17	118.4 ± 10.52	118.1 ± 9.578
<b>DBP (mmHg)</b>	79.28 ± 9.32	79.73 ± 9.32	79.08 ± 9.53
<b>HR (bpm)</b>	71.28 ± 9.36	73.17 ± 9.66	74.80 ± 9.35**
<b>WC/HC</b>	0.88 ± 0.04	0.89 ± 0.05	0.87 ± 0.14

Table 2: Values are presented as means ± standard deviation (SD). n=39. SBP – systolic blood pressure; DBP – diastolic blood pressure; HR – heart rate; WC/HC – ratio of waist to hip circumference. \*\* Significance of P < 0.05 compared with Baseline.

**Table A.3. Stress and Anxiety Measures**

	<b>Baseline</b>	<b>2 weeks</b>	<b>4 weeks</b>
<b>PSSQ</b>	17.26 ± 4.44	---	13.85 ± 4.15***
<b>STAIT</b>	37.15 ± 8.35	34.10 ± 5.99**	32.38 ± 6.05***
<b>STAIS</b>	30.90 ± 9.50	27.59 ± 8.96	26.51 ± 7.46*
<b>Positive Affect</b>	32.10 ± 6.42	33.46 ± 6.69	33.97 ± 6.15
<b>Negative Affect</b>	16.38 ± 5.63	13.82 ± 3.71*	13.90 ± 3.91*

Table 3: Values are presented as means ± standard deviation (SD). n=39. PSSQ – perceived stress scale questionnaire; STAIT – trait anxiety inventory scoring; STAIS – state anxiety inventory scoring; Positive Affect – positive affect mood; Negative Affect – negative affect mood. \*\*Significance of P<0.05 compared with Baseline. \*\*\* Significance of P<0.001 compared with Baseline.

**Table A.4. EMA Average Reported Stress**

	<b>Mean</b>	<b>Std. Error</b>
<b>Baseline</b>	0.1482	0.1640
<b>2 Weeks Post-Intervention</b>	0.1131*	0.1517
<b>Final</b>	0.1107*	0.0187

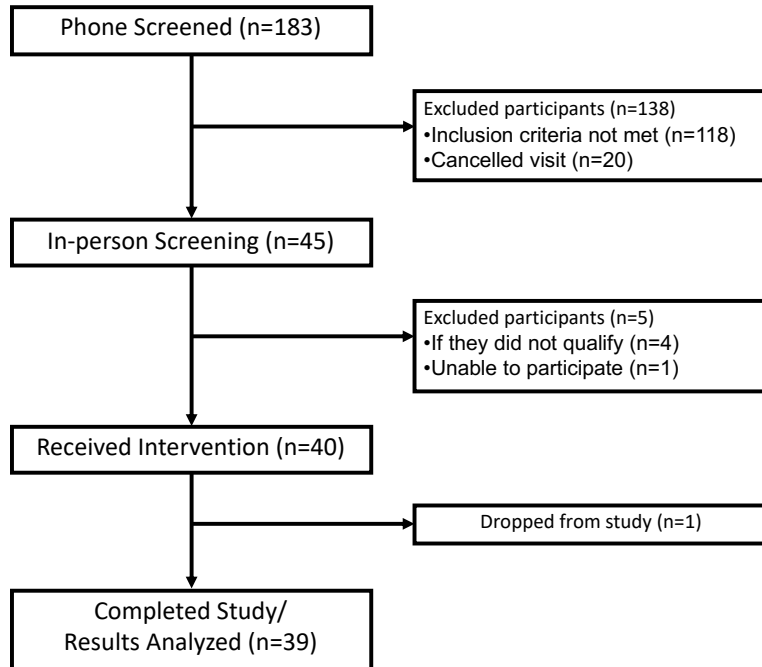
Table 4: Values are presented as means and standard error (Std. Error). n=39. Average reported stress from daily Ecological Momentary Assessment at three different timepoints of the study. \*Significance of  $P < 0.05$  compared with Baseline.

**Table A.5. Changes in Salivary Biomarkers**

	<b>Baseline</b>	<b>2 weeks</b>	<b>4 weeks</b>
<b>Cortisol (Total)</b>	<b>0.16 ± 0.10</b>	<b>0.16 ± 0.08</b>	<b>0.17 ± 0.07</b>
<b>α-amylase (Total)</b>	<b>50.37 ± 34.81</b>	<b>54.66 ± 38.43</b>	<b>50.05 ± 27.84</b>

Table 5: Values are presented as means ± standard deviation (SD). Total concentrations from area under the curve (AUC) using 3 salivary samples for each biomarker. No significant changes across timepoints found.

**Figure A.1. Flow of Study**





**Figure A.2. Changes in Perceived Stress**

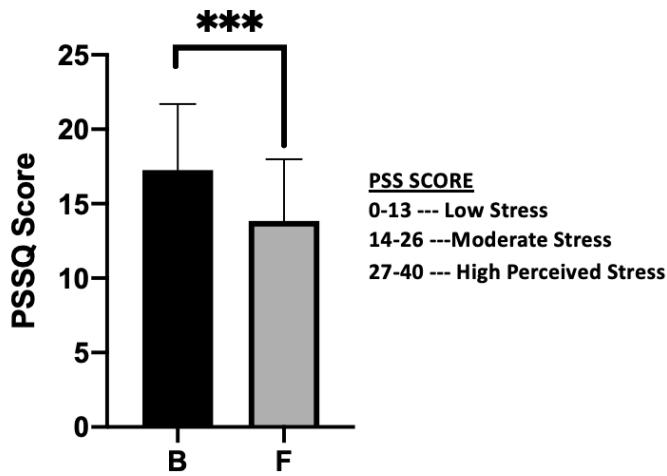


Figure 2: Values are presented as means  $\pm$  standard deviation (SD).  $n=39$ . PSSQ – Perceived Stress Scale Questionnaire.

**Figure A.3. Changes in Anxiety**

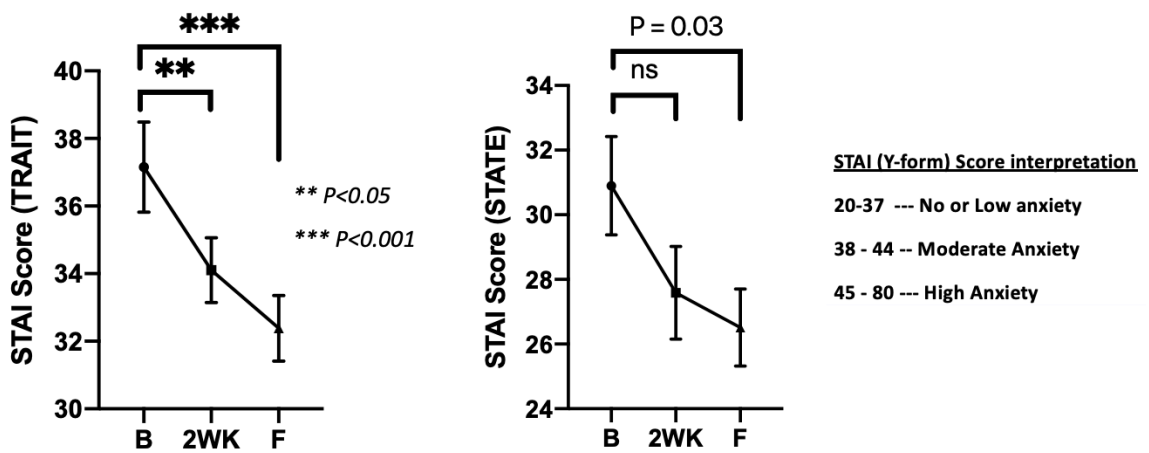


Figure 3: Values are presented as means  $\pm$  standard error (SE).  $n=39$ . STAI – State Trait Anxiety Inventory-Y form Questionnaire.

**Figure A.4. Changes in Mood**

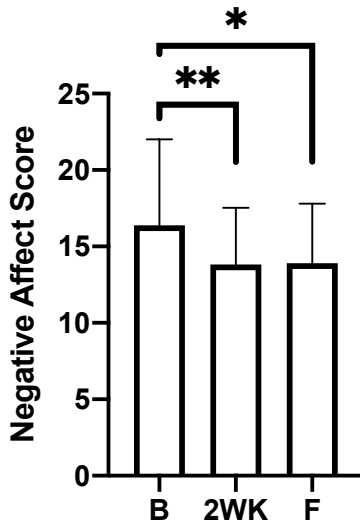


Figure 4: Values are presented as means  $\pm$  standard deviation (SD).  $n=39$ . Negative Affect – negative affect mood.

\*Significance of  $P<0.01$  compared with Baseline.

\*\* Significance of  $P<0.001$  compared with Baseline

**Figure A.5. Changes in Reported EMA Stress**

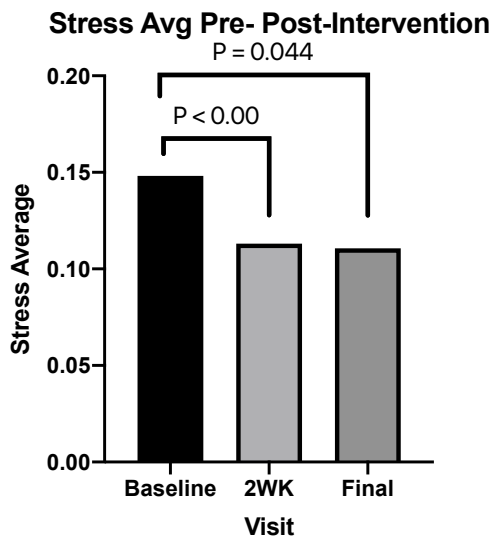


Figure 5: Stress average evaluated from reported EMA stress assessments. \*Significance of  $P<0.05$ .

## Appendix B

### IRB APPROVAL LETTER



RESEARCH OFFICE

210 Hallihen Hall  
University of Delaware  
Newark, Delaware 19716-1551  
Ph: 302/831-2136  
Fax: 302/831-2828

DATE: September 14, 2018

TO: Sheau Ching Chai, PhD, RDN  
FROM: University of Delaware IRB

STUDY TITLE: [1248818-2] Evaluating the efficacy of chocolate chew on stress relief

SUBMISSION TYPE: Revision

ACTION: APPROVED  
APPROVAL DATE: July 18, 2018  
EXPIRATION DATE: August 14, 2019  
REVIEW TYPE: Full Committee Review

Thank you for your submission of Revision materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All SERIOUS and UNEXPECTED adverse events must be reported to this office. Please use the appropriate adverse event forms for this procedure. All sponsor reporting requirements should also be followed.

Please report all NON-COMPLIANCE issues or COMPLAINTS regarding this study to this office.

Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Nicole Famese-McFarlane at (302) 831-1119 or nicolefm@udel.edu. Please include your study title and reference number in all correspondence with this office.

## Appendix C

### STUDY VISIT FORMS

#### C.1 Medical History Questionnaire

Have you ever had or have you now: (Please check at right of each item and if yes, indicate year of first occurrence)

	Yes	No	Year
High blood pressure			
Rheumatic fever			
Heart trouble			
Pain or pressure in chest			
Shortness of breath			
Asthma			
Pneumonia			
Chronic cough			
Head or neck radiation treatments			
Tumor or cancer (specify)			
Malaria			
Thyroid trouble			
Diabetes			
Serious skin disease			
Mononucleosis			

	Yes	No	Year
Hay fever			
Allergy injection therapy			
Arthritis			
Concussion			
Frequent or severe headache			
Dizziness or fainting spells			
Severe head injury			
Paralysis			
Disabling depression			
Excessive worry or anxiety			
Ulcer (duodenal or stomach)			
Intestinal trouble			
Pilonidal cyst			
Frequent vomiting			
Gallbladder trouble or gallstones			

## C.2 Demographics Questionnaire

### Demographic Questionnaire

**Gender:**  Male  Female

**Age:** \_\_\_\_\_

**Highest level of education:**

- Less than high school  High school or equivalent  
 Some college  2-year college degree (Associates)  
 4-year college degree (BS, BA)  Master's degree  
 Doctoral degree  Professional degree (MD, JD, etc)

**Annual household income:**

- Under \$25,000  \$25,000 - \$49,999  \$50,000 - \$74,999  
 \$75,000 - \$99,999  Over \$100,000  Prefer not to say

**Ethnicity and race:**

Choose one:

- Hispanic or Latino  Not Hispanic or Latino  Prefer not to say

Choose one or more:

- American Indian/Alaska Native  Asian  Native Hawaiian or Other Pacific Islander  
 Black or African American  White  Prefer not to say

**Current marital status:**

- Single never married  Married  
 Separated/Divorced  Widowed  Living with another

### C3. 3-Day Food Record Instruction

#### Diet Record Instructions

The week prior to your visit, choose **3 days** to record everything you eat and drink. Choose **two weekdays and one weekend day**. Please pick days that are typical for your current eating patterns.

On the first day, you have chosen, starting when you wake up write down everything you eat and drink that day. It may be best to carry around the small diary with you so you can record the food as soon as you have eaten it. It is important that you record each meal or snack immediately after it's eaten.

**1. Record all food and beverages consumed during a 24-hour period.**

Provide the following:

- **Date & time of day** the food or drink was consumed.
- **Name and Type of Food Eaten:** e.g. peanut butter, spaghetti
- **Brand or Restaurant Name:** e.g. McDonald's, Sargento, Quarter, etc.
- **Food or Beverage Characteristics:**
  - **Color:** e.g. green vs yellow beans; white vs. whole wheat bread
  - **Fat Content:** % fat (e.g. skim, 1%, 2%), leanness of meat (e.g. extra lean ground beef), fat claims (e.g. low-fat, fat-free)
  - **Freshness:** e.g. fresh, frozen, canned or dried
  - **Other Details:** e.g. sugar-free, reduced-sodium
- **Method of Preparation:** e.g. baked, fried

**2. Please measure and describe the amount of food eaten as best as possible. Refer to the next page for some helpful hints and examples.**

Common kitchen measurements include:

- Teaspoons and tablespoons
- Cups
- Ounces

**3. Record if anything was added when preparing food, such as oil (list specific kind), sauce, butter, or margarine, or other condiments, seasonings or toppings.**

- Example: adding Parmesan cheese to spaghetti, adding mayo to a sandwich

## C4. Saliva Collection Instruction

### Saliva Collection Instructions

PLEASE READ ALL INSTRUCTIONS BEFORE COLLECTION

- **When you will be collecting your saliva:**
  - **The day before visit 2**
    - You need one day's worth of saliva, which is 3 tubes of usable saliva with recorded times to continue to participate in the intervention trial.
  - **The day before visit 3**
  - **The day before visit 4**
- **When to collect:**
  - You will be collecting:
    - Immediately after waking up
    - 30 minutes after waking up
    - At bedtime

**\*IT IS VERY IMPORTANT THAT YOU FOLLOW THIS SCHEDULE\***

➤ **How to collect saliva:**

You will be performing what is called the "Passive drool method" to collect your saliva samples. You will uncap the collection tube and simply drool into the container. Please fill up the tube as much as you can. Once you have your collection, please get the collection tube to the refrigerator as soon as possible. If this is not immediately, that is okay, but please try to get it into a cool temperature within a few hours. Immediately after collecting your saliva sample and putting a cap on it, you must complete a Salivary Sample Assessment. Follow the instructions below "How to document saliva collection time in ReTAINE" to complete this assessment. This is necessary as it acts as a timestamp for your saliva. Failure to complete this immediately after collecting saliva could threaten your ability to continue this study and compensation.

Tips to follow:

- During the first two samples in each day, the one immediately after waking up and the one 30 minutes after waking up, please do not brush your teeth or eat or drink anything other than water.
- Avoid high acid, caffeine, or sugar foods immediately before saliva collection.
- Please document alcohol, caffeine, nicotine, and prescription/over the counter drugs used within 12 hours of saliva samples on the forms on the last three pages.
- Please also document physical activity and oral diseases if within 12 hours of a sample.
- Do not eat a major meal within 60 minutes of a saliva collection.
- If you have eaten, rinse mouth with water and wait 10 minutes to give sample.



## C5. Chocolate Chew Instruction

### Chocolate Chew Instructions:

The chocolate chew will be taken every day for the next 4 weeks. You will take it **TWICE** daily. Once in the morning and once in the afternoon.

**(Please take it 6 hours apart)**

When you return for the following visit, you should *return* any uneaten chocolate chews, if you happen to have forgotten to consume any of the chocolate chews.

You will be contacted by the researcher the first two days and asked if you had any symptoms or discomforts after taking the chocolate chew. Please make sure to confirm the phone number that will be best to contact you at.

Below, please complete the form to indicate the date and time you took the chocolate chew.

	<u>Morning</u>		<u>Afternoon</u>
<u>WEEK 1:</u>		<u>WEEK 1:</u>	
Day 1	_____	Day 1	_____
Day 2	_____	Day 2	_____
Day 3	_____	Day 3	_____
Day 4	_____	Day 4	_____
Day 5	_____	Day 5	_____
Day 6	_____	Day 6	_____
Day 7	_____	Day 7	_____
<hr/>		<hr/>	
<u>WEEK 2:</u>		<u>WEEK 2:</u>	
Day 1	_____	Day 1	_____
Day 2	_____	Day 2	_____
Day 3	_____	Day 3	_____
Day 4	_____	Day 4	_____
Day 5	_____	Day 5	_____
Day 6	_____	Day 6	_____
Day 7	_____	Day 7	_____

## C6. Participant Calendars

### March 2019

Sun	Mon	Tue	Wed	Thu	Fri	Sat
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20 SCREENING VISIT	21 EMA signal	22 EMA signal	23 EMA signal
24 EMA signal	25 EMA signal	26 EMA signal	27 EMA signal - Collect saliva sample - (3) Voluntary EMA	28 Baseline Visit	29 START CHOCOLATE CHEW <b>DAY 1</b>	30
31						

### April 2019

Sun	Mon	Tue	Wed	Thu	Fri	Sat
	1	2	3	4	5	6
7	8 EMA signal	9 EMA signal	10 EMA signal - Collect saliva sample - (3) Voluntary EMA	11 2-Week Visit EMA signal	12 EMA signal	13 EMA signal
14 EMA signal	15	16	17	18 EMA signal	19 EMA signal	20 EMA signal
21 EMA signal	22 EMA signal	23 EMA signal	24 EMA signal - Collect saliva sample - (3) Voluntary EMA	25 Final Visit	26	27
28	29	30				