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Department of Psychology – Faculty of Health Sciences

Assessment of bacteria gene expression changes in host oral microbiome during stress

A pilot study

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Assessment of bacteria gene expression changes in host oral microbiome during stress: a pilot study

Undersøkelse av bakterie genuttrykk i oral bakterieflora under stress: en pilot studie

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Preface

The human body and all the intricate processes in which it functions have always fascinated me. Health psychology became a natural field of interest as it integrates the whole human body, both psychology and physiology. In recent years the field of research on the gut microbiota has exploded, providing evidence for a strong connection between the gut microbiota and the brain. This research can serve as an important key in understanding the interaction between the body and the mind. As I wanted to gain a better understanding of the connection between the brain and the gut microbiome, there was no question in my mind about the topic for my thesis. When I contacted Professor Sven Pettersson he told me about his idea of an explorative pilot study. The aim was to investigate if this connection could be measured in saliva, by detecting bacteria gene expression changes in the oral microbiome in relation to stress.

I want to thank both of my supervisors Professor Sven Pettersson and associate professor Gerit Pfhul. Proffessor Pettersson for taking me on board as a part of his research group (Microbiome-Host interaction), and associate professor Pfhul for helping me plan the experiment and reading correction. Further I want to thank research assistant Alicia Kang for helping me in the lab, as well as giving me feedback on my report and protocols, and last but not least I want to thank all of the participants in my experiment.

Summary

The key objective of this study was to assess changes in the oral microbiome in relation to acute stress. Saliva samples were collected to analyze stress hormones and bacterial transcriptomic profiles. The participants underwent a socially evaluative cold pressor task (SECPT), reliably activating the HPA axis and eliciting pain and physiological responses in the participants, respectively. The level of salivary cortisol is related to the activation of the HPA axis, and serves as a reliable biomarker for stress assessment . The transcriptome is constantly responding to environmental conditions, and stress can be a major influence. By profiling the bacterial transcriptome in relation to the stressor, we could gain information about which genes are being actively expressed. If we are able to develop a protocol for capturing correlations between changes in oral bacterial transcriptome and stress, in search of new biomarkers, it will provide the field with a novel tool in measuring stress using saliva. Furthermore, it may be used to assess individuals at risk of developing chronic stress and depression.

Keywords: Gut-microbiota-brain axis, oral microbiome, transcriptomics, HPA-axis

Hippocrates stated more than 2000 years ago that, "all disease begins in the gut" (Hippocrates quotes quotable quote, n.d.). The link, between the brain and the gut in health and disease has been recognized by scientists for centuries (Mayer, 2011). Previously it was believed that only certain pathogenic organisms could be impacting the brain such as Toxoplasma gondii, HIV, Rabies and Syphilis to name a few (Mortensen et al., 2007; Price et al., 1988; Prosniak, Hooper & Dietzschold, Koprowski, 2000; Little, 2005). However, in the last decade research has discovered that the impact of microorganisms on the brain is not only limited to these few pathogens. Research is now discovering the tremendous impact of the gut microbiota on the brain and its impact on behavior, e.g. stress reactivity, anxiety and depression (Foster & Neufeld, 2013). This connection is bidirectional, and has been termed the gut-brain axis (GBA) (Montiel-Castro, González-Cervantes, Bravo-Ruiseco, & Pacheco-López, G. 2013; O'Mahony et. al., 2009). For example, stress affects the gut microbiome and the gut microbiome affects the level of stress, hence digestive problems are often stressful to the individual (Grenham, Clarke, Cryan & Dinan, 2011; Gur, Worly & Bailey, 2014). Further, chronic stress has tremendous consequences on both physical and mental health and is associated with depression. The main signalling system controlling endocrine responses during stress and acting as an important connection between the brain and the gut immune system is the Hypothalamic–pituitary–adrenal (HPA) axis (Dinan et al., 2006). To better understand the connection between the microbiome and stress reactivity I have done an explorative pilot study.

Firstly, I will review the concept of stress, and then provide some anatomical and physiological overview. Finally, I will explain the background to the method used in this study, i.e. transcriptome profiling.

Stress

Stress and its impact on health and disease is a complex relationship. There is little doubt that stress influences both physical and mental health. Stress can be defined as a negative emotional experience followed by biochemical, physiological, cognitive and behavioral alterations directed towards changing the stressor or reducing the impact of the negative feeling. Stress arises when a person experiences insufficient coping resources. In our modern society the physiological changes in relation to stress are still present, however the increased bodily activation does not serve the purpose it was originally meant to serve: fight or flight. Instead, people experience long-term physiological activation accompanied by excretion of stress hormones (Taylor, 2011). This chronic excretion of stress hormones can have damaging effects on the body: suppressing cellular immunity, neurodegeneration in the

hippocampus and production of hemodynamic changes such as high blood pressure, increased heart rate and neurochemical imbalances. The latter can provoke the development of psychiatric diseases (Lovallo, 2005; Taylor, 2011).

History. Walter Canon was the first person to research how optimal bodily functions were kept in homeostasis. To maintain homeostasis Cannon proposed that a negative feedback system regulated the autonomic system. He also defined the term "fight or flight" response, which can be described as an acute onset of behavioral and physiological changes, in response to a threatening stimulus, where the organism has to evaluate whether to fight or flight in order to survive (Taylor, 2011). Though Cannon was the first to define the term stress, another pioneering researcher, Hans Selye, made the concept of stress widely known. He became the first to understand the physiological mechanisms when an organism was exposed to physiological challenges. Working with animals, he noticed a consistent pattern in response to different types of challenges. Selye argued that this constituted the universal core of all stress responses, because all application of severe stressors always produced this set of physiological changes, and termed it the "general adaption syndrome". Based on his research he drew the conclusion that all stressors irrespective of being physical or psychological would elicit the same physiological reactions. This interpretation of stress served as a basic understanding of the stress response for many studies (Dickerson & Kemeny, 2004; Lovallo, 2005). However, this definition has been challenged. Not all stressors elicit the same physiological activations in the body. Experimental studies have shown that different types of stressors elicit different HPA activation, and thus not all stressors produce this universal pattern of response (Dickerson & Kemeny, 2004). Still, built upon Selye's observations of physiological responses to stress, researchers developed the concept of allostatic load. This concept refers to the changing physiology in response to the stressor. Allostatic load builds up over time, and is the physiological consequence of chronic or repeated stress exposure (Taylor, 2011). *Many* of the negative health effects associated with allostatic load can be explained by a dysfunctional HPA axis. HPA axis dysfunction has been related to a variety of negative health conditions such as anxiety and depression (Chen et al., 2015; Dedovic, & Ngiam, 2015; med stud).

Good and bad stressors. The physiological stress reaction is highly dependent upon the emotional response. Positive emotions in relation to stress often lead to increases in cardiovascular and catecholamine responses without activating cortisol. A physical demanding strain such as exercise is stressful for the body, but does not accompany powerful negative emotions such as a true fight or flight situation would. The individual has complete

control over the situation, the physical activity is time limited and positive emotions follows. Therefore, exercise will not induce high levels of cortisol.

Events that threaten a person's beliefs and commitments will most likely cause negative emotions and generate the fight or flight response even though the event is not physically harmful. Such stressors are often referred to as psychological stressors. An essential characteristic of this type of stress is the lack of a clear onset or offset, which may elicit a feeling of little or no control (Lovallo, 2005). Life events such as divorce, death of a loved one or problems at work are examples of psychological stressors, and are often viewed as bad stressors. Negative emotions in relation to stress, are often associated with increased cortisol in order to accompany the cardiovascular and catecholamine activation (Lovallo, 2005). However, it is worth noticing that the physical response to psychological stressors are highly variable, and recent reviews are finding inconsistent cortisol activity regarding psychological stressors. In other words, not all types of negative situations may trigger cortisol elevation, and the specific situational elements related to cortisol activity are not clearly identified (Dickerson & Kemeny, 2004).

To reduce the negative feelings associated with the stressful event, adjustments by some form of coping behavior is required. However if the coping strategies are insufficient or lacking, the negative feelings will persist, thus leading to chronic elevation of cortisol. Chronic cortisol elevations is a form of long-term stress, and is often what may be termed as psychological stress. The stressor is not present in itself, however the individual still feels threatened by it, and thus it is present in the mind as a psychological stressor (Lovallo, 2005; Taylor, 2011). Psychological stressors are what laypeople mean by stress, however the research literature has a broader perspective of what stress is (explained in the following paragraphs).

Physiology of the stress response

Autonomic nervous system. The autonomic nervous system consists of three branches: the sympathetic, parasympathetic, and enteric. Nuclei situated in the brainstem modulate the three branches antagonistically. Sympathetic activation stimulates the adrenal medulla to secrete the stress hormones adrenaline and noradrenalin. This leads to increased blood pressure, heart rate, sweating and constriction of the peripheral arteries. Parasympathetic activity reduces heart rate, whereas sympathetic activity increases heart rate. Activation of the enteric nervous system is under the control of the aforementioned branches, however, the action is reversed. Parasympathetic activity increases the activity of the digestive organs, whereas the sympathetic activity tends to inhibit this activity (Lovallo,

2005). The enteric nervous system (ENS) is the most specialized system of the three branches and differs both anatomically and functionally. It acts mostly independent from the brain and spinal cord, and consists of 100 million neurons controlling the gastro-intestinal system. The ENS has been referred to as the second brain due to its complexity and similarity in signaling molecules with the brain (Gershon, 1998; Mayer, 2011). This supports the growing knowledge about the importance of the gastro-intestinal system regarding behavior and emotional responses.

HPA axis. The HPA axis is the main endocrine signaling system involved in the stress response and controlled by a negative feedback system, similar to what Canon first proposed. The negative feedback loop can best be described by using an analogy of a thermostat, in which the thermostat shuts off a furnace when the room temperature has increased above the level of set point. In this case the hypothalamus can be referred to as the thermostat, and it receives input about the state of the room temperature (organs) and then sends signals back to the same organs through autonomic nervous system and endocrine messengers which act as the furnace. HPA activation is stimulated by corticotropin releasing (CRH) hormone excreted from the hypothalamic paraventricular nucleus (Karlén, Ludvigsson, Frostell, Theodorsson & Faresjö, 2011; Lovallo, 2005). CRH then acts on the anterior pituitary gland, which releases adrenocorticotropin hormone (ACTH) and beta-endorphin. ACTH then stimulates the adrenal cortex causing it to release cortisol into the bloodstream (Dickerson & Kemeny, 2004; Lovallo, 2005). Cortisol will then reach the hypothalamus through the bloodstream and its contact with cerebrospinal fluid in the ventricles binding to target cells, which inhibits secretion of CRH (Lovallo, 2005). Less CRH leads to less ACTH, which in turn leads to less stimulation of the adrenal cortex leading to less secretion of cortisol (Dickerson & Kemeny, 2004; Lovallo, 2005). Figure 1 illustrates this.
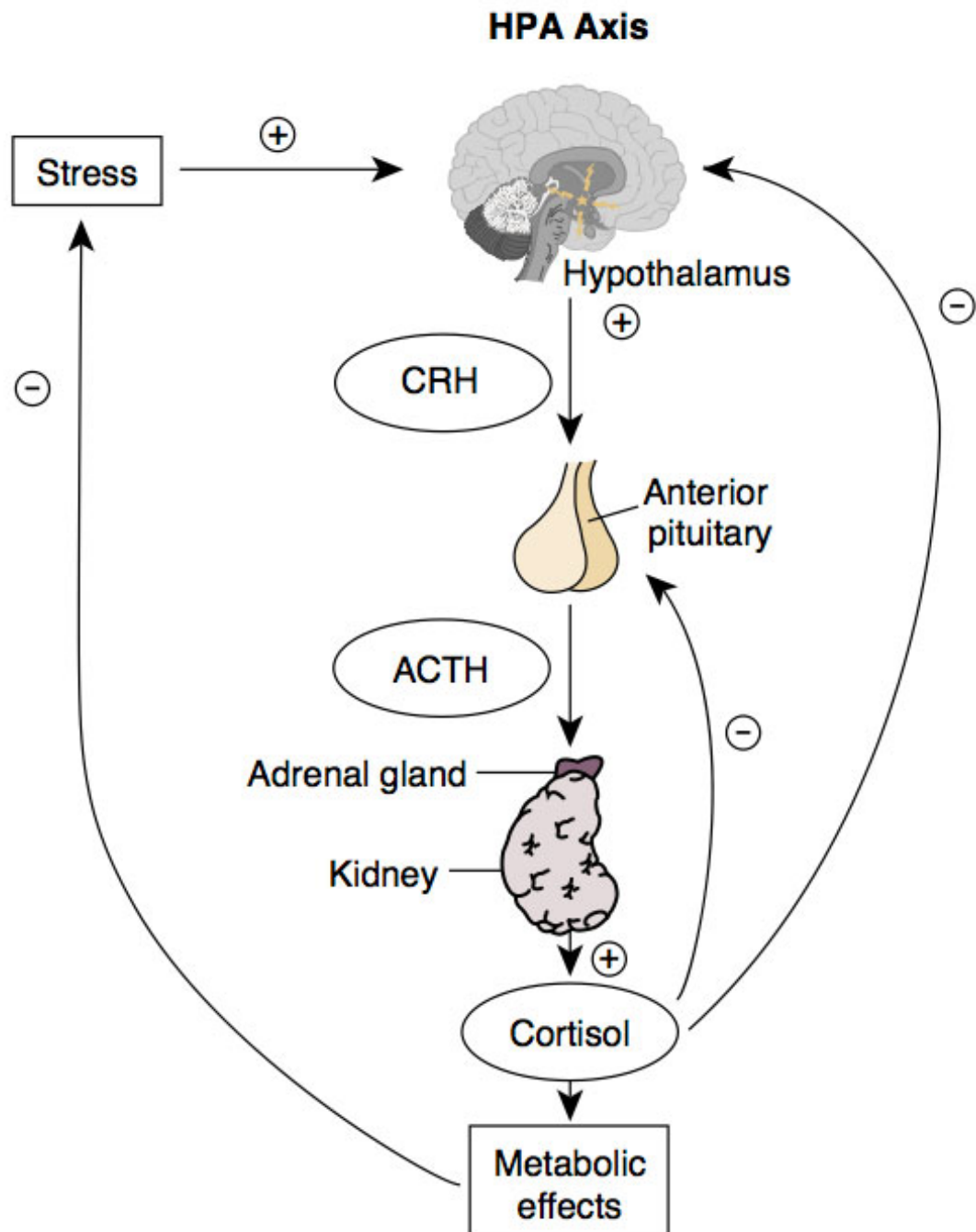
HPA Axis

Figure 1. The HPA axis is controlled by a negative feedback loop. Stress is perceived by the brain and the hypothalamus will excrete CRH to the anterior pituitary which then excretes ACTH to the adrenal gland, which will send cortisol into the blood stream. The brain will then detect high levels of cortisol in the blood stream, which in turn will lead to less excretion of CRH, ACTH and cortisol, and thus demonstrating the negative feedback loop ("Negative Feedback", n.d.).

The HPA axis can be assessed through different approaches, by measuring diurnal cortisol secretion in response to a stress task, a pharmacological challenge (drug / food

intake), and the cortisol awakening response (Dedovic, & Ngiam, 2015). Two interrelated systems are involved in the stress response: the sympathetic adrenomedullary system (SAM), which is the sympathetic activation of the autonomic nervous system, and the HPA axis, the main endocrine signaling system. The stress response is triggered by the cerebral cortex, which evaluates the situation as either threatening or harmful. The information is thereafter sent to the hypothalamus, which activates the sympathetic nervous system, and this response is seen as the classical fight or flight response (Lovallo, 2005; Taylor, 2011).

Cortisol. Cortisol (in humans) and corticosterone (in rodents) is one of the major stress hormones, which also plays an important role in normal autonomic regulation. Certain levels are required to exert effects on the cardiovascular system. When stress levels increase cortisol boosts this activation. This leads to the release of stored glucose and fats into the bloodstream, giving fuel to a potential fight-or-flight situation. Cortisol acts as an anti-inflammatory substance but may also inhibit the functioning of the immune system. When levels are high, cortisol may alter immune system tissues and suppress immune system responses (Lovallo, 2005).

The cortisol circadian rhythm is regulated through neurons in the paraventricular nucleus in the hypothalamus (Lovallo, 2005). This rhythm is important for maintaining normal organ regulation, as poor long-term health, chronic stress and depression has been associated with a dysfunctional rhythm (Dinan, 1994; Lovallo, 2005). The peak level of cortisol is in the early morning 45-60 minutes after waking up, declining throughout the day with a slight rise during midday meal, and varying levels in the afternoon reaching lowest concentration in the late evening and during sleep ("how to measure stress in humans," 2007; Ivkovic et al., 2015). However not every individual has this normal cortisol pattern. Research has reported both inter-individual differences and intra-individual differences.

In regards to laboratory induced stressors Dickerson & Kemeny (2004) conducted an extensive meta-analysis of 208 laboratory studies of acute psychological stressors in relation to cortisol excretion. They found that cortisol peaks around 0-20 min after stress exposure and returns to baseline 21-41 min post stressor. Different stress events elicited different recovery time of cortisol to baseline. Uncontrollable, social-evaluative stressors were associated with both greater peak responses and persistent cortisol elevations, up to 60 min post stressor. In this study, a socially evaluative cold pressor task (SECPT) was being used, which is a common laboratory stressor task that has been shown to activate the HPA axis leading to cortisol release (Skoluda et al., 2015).

Based on animal work, corticosterone (and most likely also cortisol) production is

regulated by the gut. Studies on mice have shown that the intestinal epithelial cells (IEC) which are the cells that form the surface of the large and small intestine, play a role in regulating corticosterone secretion, and that the microbiota altered this production (Mukherji, Ye, Kobiita & Chambon, 2013). This may explain one of the links in which microbes may influence stress reactivity, anxiety and depression through the gut-brain axis, and to better understand this connection the following paragraphs will further explain this axis (Hussain, 2013).

The gut-brain axis

The gut-brain communication system enables the brain to modulate the functionality of the gut, involving motility, secretion and mucin production by cells in the mucosal system (Collins, Surette & Berick, 2012). Stress responses and overall behavior have been shown to modulate this axis and thus impact all of the functions mentioned above, promoting changes in the bacterial composition (Collins et al., 2012; Dinan & Cryan, 2013; Heijtz et al., 2011). Gastrointestinal (GI) disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disorder (IBD) are all influenced by the gut-brain axis. In patients with IBS, increased ACTH, cortisol and proinflammatory cytokine response was found. This indicated an overactivation of the HPA axis in this disorder (Dinan, et al., 2006). Further, this may explain the high comorbidity among GI disorders and mood disorders such as depression and anxiety (Dantzer, O'Connor, Freund, Johnson & Kelley, 2007).

Gut-microbiota-brain axis. The human microbiome is so extensively integrated in bodily functions and metabolic processes that many researchers now refer to it as our second genome or the forgotten organ (Collins et al., 2012; Cryan, et al., 2012; O'Hara & Shanahan, 2006). The population of bacteria, viruses, and other microorganisms in or on the human body can be defined as the human microbiome or microbiota (Nelson et al., 2010). Bacteria in the gastrointestinal (GI) tract can influence brain chemicals and thus impact function and behavior. Clinical studies of germ free (GF) mice compared to non-GF mice show that the gut microbiota influences the regulation of stress, anxiety, mood, cognition and pain (Cryan & Dinan, 2012). This indicates that the gut microbiota has a crucial role in the bidirectional gut-brain axis, and that it might be more appropriate calling it the gut-microbiota-brain axis. Accordingly, it is more appropriate to include both, a top-down signaling system and a bottom-up pathway of communication (Figure 2). Hence, microscopic organisms in the gut play a crucial role in regulating mood and behavior (Grenham, et al., 2011; Gur, et al., 2014).
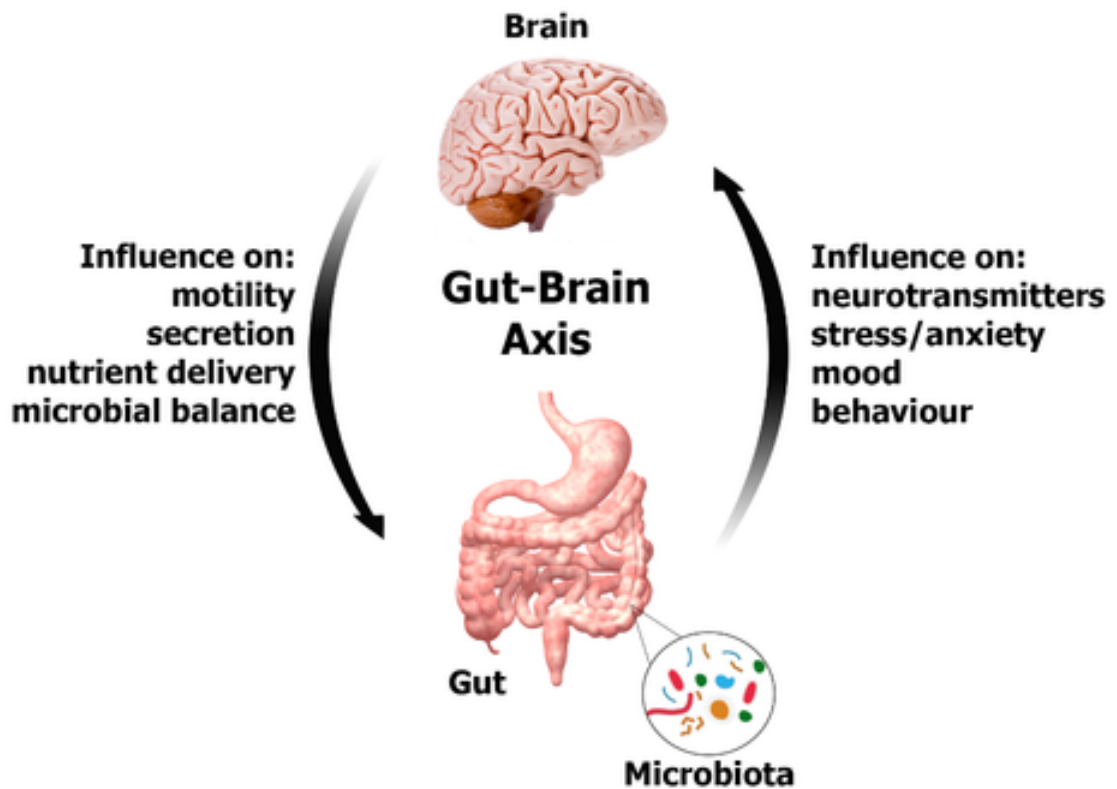
Figure 2. The gut-brain axis. The brain influences the gut by regulating motility, secretion, nutrient delivery and microbial balance. Whereas the gut and the gut microbiota influences the brain through neurotransmitters, which will affect behavior and feelings of stress and anxiety ("Beyond addiction," 2016).

Microbiota, stress and depression. Corticolimbic structures in the brain are involved in the bi-directional communication pathway and regulate the gastrointestinal function by sending signals to the gut. Important components involved in this communication are the HPA axis and the immune system. As mentioned above, the microscopic organisms in the gut play a crucial role in regulating mood and behavior; and the main pathway regulating stress reactivity is the HPA axis. The gut microbiota plays a crucial role in both programming of the HPA axis early in life and stress reactivity over the lifespan (Clarke et al., 2013). Major depression is influenced by the same stress regulation system, the HPA axis, and alterations such as increased cortisol and CRF coupled with decreased ability to suppress cortisol is evident in depressed patients, including higher levels of cytokines. Thus, one of the links between stress, depression and the gut microbiota might be the microbes' ability to influence the HPA axis and the immune system.

Germ free animal models have shown increased stress responses and reduced anxiety-like behavior. The GF mice in comparison to specific pathogen free mice (SPF) showed

exaggerated corticosterone (CORT) and adrenocorticotrophin (ACTH) in response to restraint stress, indicating a direct link between the microbiota and HPA axis reactivity (Dinan & Cryan, 2013; Foster & Neufeld, 2013). The hyperactivation of the HPA axis showed to be reversible by using a specific strain (bifidobacterium infantis) of probiotic bacteria (Dinan & Cryan, 2013). Probiotic bacteria can be defined as beneficial live organisms. They may exert positive health effects when consumed in adequate amounts, such as reduction of inflammation, infections and allergic disorders (Bravo et al., 2012). Reduction in anxiety behavior in animals and reduction of cortisol levels in humans have also been reported by the use of probiotics (Al-Asmakh, Anuar, Zadjali, Rafter & Pettersson, 2012).

Through bacterial gene expressions a study showed that the microbiota altered the production of corticosterone. The antibiotic induced microbiota depleted mice had defects in the intestinal epithelial cell gene expressions involved in production of corticosterone (mouse cortisol). This resulted in overproduction of corticosterone in the intestinal epithelial cell (Mukherji et al., 2013). This indicates a strong connection between microbial gene expression and overproduction of the stress hormone corticosterone, linking microbes to the regulation of stress reactivity (Figure 3).

Stress has also an impact on specific bacterial strains by promoting and inhibiting the growth of different bacterial types. One of the mechanisms behind this is through the neuroendocrine stress related hormones. Norepinephrine has been shown to increase the growth of both commensal and pathogenic bacteria. Human and animal studies have both shown that a specific strain of probiotic bacteria (lactobacilli) was reduced by stress, and the reduction correlated with stress-indicative behaviors (Gur et al., 2014). Recently in humans, a link between specific bacteria and depression has been discovered. Increased gut permeability and related bacterial translocations may be a factor resulting in increased inflammation and thus contributing to depression (Naseribafrouei et al., 2014). Disruption of the intestinal barrier can be reversed by probiotic supplements (Cryan & Dinan, 2012). These studies provide evidences for the link between the gut-microbiota, behavior, and stress reactivity (Luna & Foster, 2015).
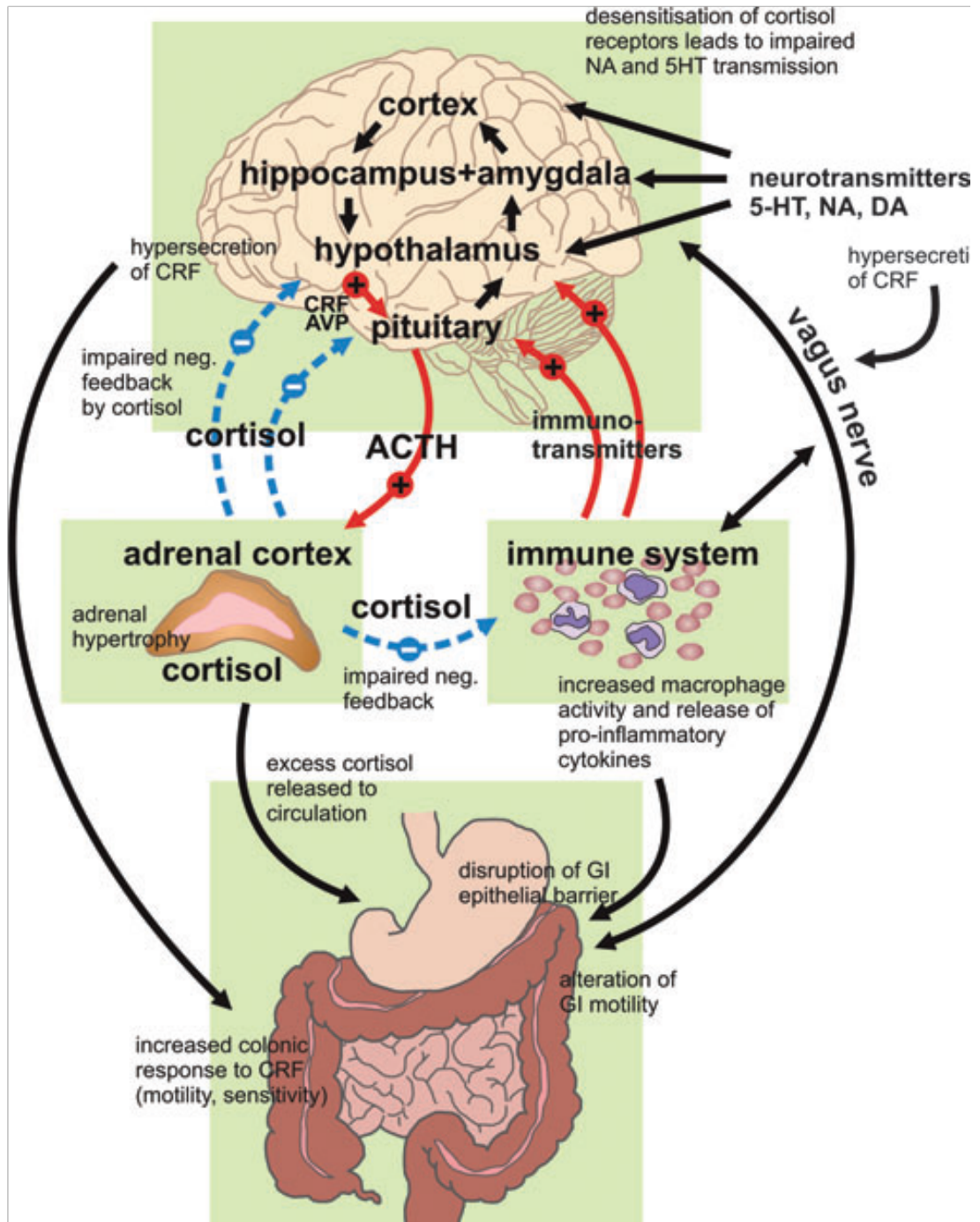
Figure 3. The gut-brain axis connection in chronic stress and depression. Corticolimbic structures in the brain alter gastrointestinal function demonstrating the bi-directional communication system. The immune system and the HPA axis play important roles in the regulation of this axis (Dinan & Cryan, 2013).

Oral microbiome

The digestive system begins in the mouth and has a direct connection to the gastrointestinal tract through the pharynx[1], esophagus[2] and the larynx[3]. The pharynx, also known as the throat, is hosting a rich microbial diversity containing up to 700 species of aerobic and anaerobic organisms organized in complex biofilms, only outnumbered by the colon. From the mouth to the colon, an estimate of over 1000 bacterial cells per day move downstream. The 45% overlap between the oropharyngeal and colonic microbiota, strongly supports the hypothesis that the oral microbiome influences the composition of the gut microbiome (Segata et al., 2012). The oral microbiome is mostly known to be involved in dental caries and gingivitis. However, the oral microbiome can be linked to an extensively amount of general health conditions and disease, and thus reflecting the individuals overall health (Refulio et al., 2013). Diseases that have been linked to the composition of the oral microbiome involve alveolar osteitis, tonsillitis, bacteremia, endocarditis, stomach ulcers, brain and liver abscesses, stroke, diabetes, pneumonia and premature birth (He, Li, Cao, Xue & Zhou, 2014).

The biofilms in the mouth may host pathogenic microbes, which may provide the host with a continual source of pathogenic microbes to the gut, causing chronic or recurrent stomach, small intestine, or colon dysbiosis (Refulio et al. 2013; Segata et al., 2012). High prevalence of Helicobacter pylori in dental plaque has been reported in patients with gastric Helicobacter pylori infection, suggesting that pathogenic dental biofilms can reduce or prevent antibiotic treatment success. Plaque-induced chronic periodontitis (CP), which is mainly caused by microbial dental biofilms, has a high comorbidity rate with inflammatory bowel disease (IBD), as well as with chronic stress and depression. Refulio et al. (2013) found a strong positive correlation between salivary cortisol levels and the presence of periodontitis, which agrees with previous findings. Increased HPA responses can be linked to inhibition of immune cells, causing the periodontal tissue to be vulnerable to periodontal pathogens. This indicates that subjects with high salivary cortisol may be at an increased risk

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[1] Pharynx is Greek and means throat. It is the cone-shaped passageway leading from the oral and nasal cavities in to the esophagus and larynx ("Pharynx," n.d.).
[2] Esophagus, a relatively straight muscular tube through which food passes from the pharynx to the stomach ("Esophagus," n.d.)
[3] Larynx, also called voice box, a hollow, tubular structure connected to the top of the windpipe (trachea); air passes through the larynx on its way to the lungs. The larynx also produces vocal sounds and prevents the passage of food and other foreign particles into the lower respiratory tract ("Larynx," n.d.).

for developing chronic periodontitis. The mechanisms involved in the connection between chronic periodontitis and salivary cortisol levels still remain uncertain (Refulio et al., 2013).

Chronic stress (as mentioned previously) is a high risk factor for developing diverse systemic conditions and diseases, presumably through activation of the HPA axis. HPA activation leads to increased salivary cortisol levels (Lovallo, 2005; Taylor, 2011). Salivary cortisol has therefore been used as a biomarker for assessing the association between chronic stress and periodontitis (Refulio et al., 2013).

Measuring stress

Saliva as a scientific biomarker: Saliva serves as an appropriate biomarker in health and behavior-related research due to its many advantages. Cortisol, the main stress hormone can be measured in saliva, which has a long history in the stress research field. The microbiota inhabiting the oral cavity is specific to each individual, enabling saliva to be used as a diagnostic, epidemiological and forensic tool.

Cortisol is the most studied biomarker for stress, and the levels in saliva reflects the biologically active free form of cortisol, which provides a more reliable measure than serum cortisol measured in blood. Therefore, salivary cortisol is a reliable marker for the activation of the HPA axis (Dickerson & Kemeny, 2004; Ivkovic et al., 2015; Saiyudthonga, Suwannaratb, Trongwongsab & Srisurapanonb, 2010).

The social evaluative cold pressor task (SECPT): The cold pressor task (CPT) is a commonly used experimental stressor task, however, only minor cortisol response has been found, and thus resulting in a limited HPA activation (Skoluda et al. 2015; Schwabe, Haddad & Schachinger, 2008). Notably, an increased cortisol excretion was found when adding a socially evaluative component to the regular CPT (Giles et al., 2014; Schwabe et al., 2008). Therefore the participants in this study were given deceptive information about video recordings (Appendix E).

Transcriptome profiling

Expression profiling

In the human body, most cells contain a nucleus with DNA. Specific to each cell, only a subset of all genes are actively expressed. The genes that are "turned on" in that particular cell is what decides the unique property of that cell type. The "turning on" or "off" is the transcriptome profile of a cell. By using microarray techniques researchers are able to reveal the expression levels of hundreds or thousands of genes within a cell. That is, RNA-

sequencing has recently been developed in order to look at transcriptome profiles by the use of deep-sequencing technologies (Wang, Gerstein & Snyder 2009). These techniques are based on DNA microarray technology (Tyrell & Schoolnik, 2003). This method provides a more precise measurement of transcriptomes than other methods (Wang, et al., 2009). Thus, it allows assessing the changes in transcriptomes due to immediate environmental changes. RNA sequencing facilitates the ability to look at changes in gene expression, and it reveals the presence and quantity of RNA in a biological sample at a given moment in time. Results from the expression profile in a microarray experiment when conducted properly, can be used for various research properties such as identification of regulated genes, metabolic pathways, and signaling systems among others. When conducting transcriptomic research, assessment of RNA quantity and quality is an important part, and should be done before any transcriptomic profiling. RNA is highly prone to degradation, and interference from contaminants that may be present in the sample. Assessing the quantity and quality of the RNA can be done through various methods: Optical density (OD) measurement is a common technique used in research on bacterial cultures, via Nano-Drop spectrophotometer, other ways to measure the quality and quantity is through agarose gel-electrophoresis, classical gel OD measurement, or with high innovative technologies like Bioanalyzer 2100 (Agilent Technologies, USA) and Experion (Bio-Rad Laboratories, USA). In this experiment the NanoDrop ND-2000 spectrophotometer (Thermo Scientific), and then Bioanalyzer were applied (Fleige & Pfaffl, 2006; Matlock, Beringer, Ash, Allen & Page, n.d.). Predominantly the A260/280 ratio was used for the NanoDrop ND-2000 spectrophotometer (Thermo Scientific), which shows the quality and level of protein contamination. A ratio greater than 1.8 is usually considered an acceptable indicator of good RNA quality (Fleige & Pfaffl, 2006).

The following project is an explorative pilot study. The main aim is to explore whether environmental-induced stress can change the oral transcriptomic profile. That is, will RNA expression change in response to the stressor induced?

Methods

Participants

Ten laboratory staff members from the Lee Kong Chian School of Medicine, Host Microbiota Interactions, Nutrigenomics and Metabolism group (4 male; mean age 29 years, and 6 females; mean age 29 years) were recruited during a weekly combined laboratory meeting, by asking if they were interested in participating in a study that seeks to investigate

possible gene expression changes in oral microbiome in relation to acute stress. The study was approved by the Singhealth Research Ethics Committee (CIRB Ref: 2016/2555). Written consents were obtained from all subjects prior to participation. No exclusion criteria were applied, due to low number of staff members to recruit participants from.

Design

A within-subject design was conducted where each participant underwent three sessions (two baseline non-stress condition and one stress condition) in a fixed order (baseline, stress condition and baseline), on three separate days. The participants gave three saliva samples each day. This controlled for cortisol diurnal variation and bacterial transcriptomic changes. The exposure to the stressor was conducted in the afternoon between 3:00 p.m. and 4:00 p.m., to control for cortisol circadian rhythms. The cortisol levels at these time points are fairly low, and thus preferable in order to get a better comparison between the baseline and the stress condition ("How to Measure stress in humans," 2007*).*

Baseline. The baseline saliva samples were collected the day before the stress condition and the day after the stress condition, at three specific time points: 3:00 p.m., 3:30 p.m. and 4:00 p.m.

SECPT. The Socially evaluative cold pressor task is a common laboratory stress test (Giles et al., 2014; Schwabe et al., 2008; Skoluda et al., 2015). The participants were instructed to immerse their non-dominant hand in ice-cold water (4-5 °C) while being watched and videotaped by the experimenter. The maximum time for keeping the hand in the ice-water was set to three minutes (Mitchell, MacDonald & Brodie, 2004). However this was not disclosed to the participants, as they were being instructed to keep it there as long as they could manage. Saliva was collected before the stressor task at 3:00 p.m., then 20 minutes after the SECPT (approximately 3:30 p.m.) and at 4:00 p.m. (for more details se Appendix B and D) (Giles, et al., 2014; Schwabe et al., 2008; Skoluda et al., 2015).

Stress-o-meter scale. Right after taking their hand out of the ice-water the participants were asked to rate how stressed they felt on a stress-o-meter scale (Appendix F) ranging from 0-10 (0= no stress, 10= extreme stress).

Perceived Stress Scale. On the two baseline days the participants were asked to rate their feelings and thoughts of stress in their life during the last month. The scale (Appendix C) is designed to measure the degree to which situations in one's life are appraised as stressful. The items are easy to understand, and the response alternatives are simple to grasp. This was applied in order to measure any potential long-term stress.

Debriefing. After the SECPT condition the participants were given a short debriefing (Appendix G).

Procedure

The day before the saliva collection started, participants received a saliva sample collection instruction sheet (Appendix B). On the day of the first and second baseline the participants were given a self-reported questionnaire (Perceived Stress Scale) about their feelings and thoughts the last month (Appendix C).

Next, the participants were asked to imagine chewing their most favorite food, while they gently moved their jaws in chewing movements. This process will generate saliva, and the saliva is then allowed to pool briefly under the tongue before being gently "drooled" into a sterile collection vial. For each sampling the participants were asked to donate 5 ml of oral drool sample, i.e. a total of 45 ml for the three time points and three measurement days.

For baseline, the participants were given collection vials before the collection times, and drooled the sample at their own convenient location. After the drool had been collected in the vial, it was immediately put on ice while transported to the lab. In the lab the saliva samples were pipetted into smaller aliquots; 400 µl of saliva were distributed into 4 aliquots for measuring cortisol; 1 ml of saliva were distributed into 3 aliquots containing 5 ml RNAlater in 50 ml falcon tubes for RNA protection and stabilization.

At the experimental session (SECPT), the participants entered the experimenter room one at a time and were asked to read a brief information about the study (Appendix E). They were then instructed to immerse their non-dominant hand up to and including their wrist in a box filled with ice-cold water (4-5 °C). They were instructed to look into the camera and to keep their hand in the water for as long as possible. The experimenter watched the participants during the test. The tolerance time was recorded, and they were instructed to rate their stress levels on a stress-o-meter (Appendix F) ranging from 1-10 (0 = no stress, 10 = extreme stress) right after taking their hand out of the ice-cold water. Saliva was collected before the experiment at 3:00 p.m., 20 minutes after the SECPT and at 4:00 p.m.

Debriefing. In the written consent form (Appendix A) and in the brief information sheet (Appendix E) the participants were being informed about video recordings to analyze their facial expression. After the SECPT the participants were given a short debrief where they were being told that: 1) The information about video recordings were false. 2) They received this information to elicit a higher stress response. 3) Research shows that a regular CPT does not elicit high levels of cortisol, but by adding a socially evaluative component (video recordings and being watched by the experimenter) increases cortisol excretion. 4)

This study is using cortisol as a measurement of the stress response, and it was therefore important to try and obtain a marked increase of cortisol excretion.

Cortisol extraction

      Saliva kit. The Abcam Cortisol ELISA Kit (ab154996) was selected as it is designed to give an accurate quantitative measurement of Cortisol in saliva (for more details about the procedures see Appendix A). The acronym ELISA stands for enzyme-linked immunosorbent assay. It is quick and easy and designed to handle a large amount of samples rapidly at the same time, and is often the preferred method in research and diagnostics. The end product of the assay will be colored, and this color correlates with the amount of analyte present in the original sample ("An Introduction to ELISA", n.d.).

RNA extraction

      RNA was extracted using standard beat beating protocol using QIAzol reagent, followed by column purification using the RNeasy mini kit (See Appendix H for a more detailed explanation of the procedures).

      RNA precipitation. RNA precipitation was conducted on samples below 40 ng/µl (participant 2,3 and 8) using the standard 3M sodium acetate protocol, in order to increase the RNA concentration and quality (See Appendix H for a more detailed explanation of the procedures).

Data analysis

      The time they kept their hand in ice-cold water was recorded in seconds. All participants' immediate subjective stress feeling was noted on a stress-o-meter ranging from 0 – not stressful at all to 10 – extremely stressful.

      Cortisol changes were expressed as: SECPT_Cortisol – baseline cortisol for the three time points (where baseline cortisol is the average from the two baselines, i.e. before and after SECPT).

      Salivary transcriptomic data: Three of the five participants (2, 3 and 8) that showed the desired cortisol results were picked for metatranscriptomic sequencing. A total of 27 samples from three participants (three tubes of samples for each condition), were sent to a sequencing facility Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University for metatranscriptomic preparation and analysis. In order to generate metatranscriptomic data, RNA concentration had to be above 40 (ng/µl), and the quality had to be between OD 1.9 and 2.1 (A 260/280).

Statistical analysis

The salivary cortisol data were analysed by Repeated Measures ANOVA with both baseline conditions averaged, the stress condition, and time (3:00 p.m., 3:30 p.m. and 4:00 p.m.) as within participant factor. The increase in cortisol was calculated as the difference between the time point 2 at the stress and baseline condition over the baseline condition, i.e. the relative increase in cortisol.

Hypotheses:

  1. The SECPT leads to an increased cortisol excretion

  2. Increased cortisol levels is related to changes in the salivary transcriptome

Results

The Perceived Stress Scale showed that 8 out of ten participants fell under or within the average stress level range, while 2 participants reported above 1 standard deviation of average stress ranges (reported ranges fell within 6 to 25 on a questionnaire with 10 questions, average score 13). The 10 participants kept their hand 23 to 123 seconds in the ice-cold water. They rated the stress as 3 up to 9 on a stress-o-meter scale from 0 to 10. Cortisol increased in 5 participants post SECPT. The time in ice water and the stress-o-meter correlated negatively, Spearman's rho = -.529, p = .116. That is, the more time spent in the cold water the lower the score on the stress-o-meter was.

Salivary cortisol

Analysis comparing the stress task to the baseline conditions showed no statistically significant increased cortisol response to the SECPT 20-minutes post stress, $F(1, 9) = 3.578$, $p = .091$, eta2 = .284 . However, there was a small to medium effect size and overall an increase from 9.51 (SD 3.7) ng/ml in baseline to 13.72 (SD 8.7) ng/ml in the test condition at 3:30 p.m., see figure 4. The time spent in ice water was positively correlated to the increase in cortisol levels, Pearson's r = .444, p = .198.
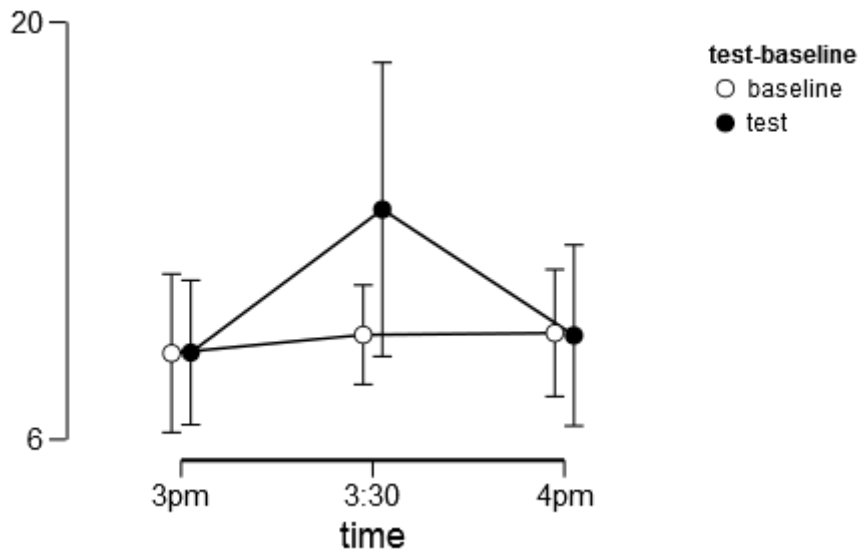
Figure 4. Salivary cortisol at three time points for three conditions (baseline 1, test and baseline 2). Cortisol responses increased 20 minutes after the SECPT. Error bars represent 95% confidence intervals.

RNA percipitation

The concentration of the RNA samples ranged from 53.3-280.8 ng/µl, the RNA quality ranged from 1.53-2.13, this was measured using NanoDrop ND-2000 spectrophotometer (Thermo Scientific).

Table 1

Concentration (ng/µl) and quality (A 260/280) of the RNA samples of participant 2, 3 and 8, for all three conditions (baseline 1, test and baseline 2)

Samples	Participants	ng/µl	A 260/280	A 260/230
1 BL 1.1	2	280.8	2.12	0.6
2 BL 1.2	2	157.7	2.09	0.6
3 BL 1.3	2	163.8	1.94	1.11
4 T 1	2	102.9	2.09	0.3
5 T 2	2	92.5	1.96	1.41
6 T 3	2	53.3	1.94	1.42
7 BL 2.1	2	236.6	2.13	2.17
8 BL 2.2	2	129.8	2.13	0.23
9 BL 2.3	2	146.4	2.07	2.03
10 BL 1.1	3	93.9	1.96	1.48
11 BL 1.2	3	66.1	1.53	0.58
12 BL 1.3	3	77.3	1.93	1.09

13 T 1	3	76.6	1.93	1.27
14 T 2	3	68.6	1.98	1.28
15 T3	3	79.5	1.95	1.28
16 BL 2.1	3	137.3	1.98	1.42
17 BL 2.2	3	103.4	1.91	1.2
18 BL 2.3	3	253.9	1.95	1.41
19 BL 1.1	8	232.9	1.93	1.51
20 BL 1.2	8	97.6	1.98	1.49
21 BL 1.3	8	97.3	1.95	1.29
22 T 1	8	235	1.91	1.37
23 T 2	8	117.8	1.94	1.5
24 T 3	8	139.6	1.95	1.43
25 BL 2.1	8	209.1	1.95	1.54
26 BL 2.2	8	91.1	2.02	1.81
27 BL 2.3	8	138.5	1.93	1.34

Results from the sequencing company (SCELCE)

The three samples (participant 2, 3 and 8) sent for metatranscriptomic sequencing did not achieve the minimum quality and standards measured by a bioanalyzer. Quality control reports that RNA samples were found to be heavily degraded, hence, no transcriptomic profile was generated.

Discussion

Salivary cortisol is a frequently used biomarker in stress research (Kemeny & Dickerson, 2004; Shirtcliff, Granger, Schwartz & Curran 2000). In this present pilot study, I investigated whether the oral microbial transcriptome would respond to stress or not. Five out of ten participants showed increased cortisol levels 20 minutes after the SECPT. The remaining five participants showed random cortisol values, with no specific relation to the SECPT. The effects were not statistically significant though, which might be explained by the small sample size (N=10). Despite the small sample size the SECPT showed to work in half of the participants, indicating that keeping the hand in ice-cold water while being socially evaluated (videotaped and watched by the experimenter) enabled a stress response in five participants. The cortisol clearly rose from baseline 1 to baseline 2 and declined in baseline 3, but did not decline to the level of baseline 1. Hence, indicating that the cortisol response lasted beyond 20 minute and up to 60 minute post stressor. Previous research on different forms of stressors shows that cortisol peaks around 0-20 minute and returns to baseline 21-41 minute post stressor. However different stressors elicit different cortisol recovery time, and research clearly shows that experiments which includes a form of social-evaluation creates

greater peak responses and persistent cortisol elevations, up to 60 minute post stressor (Dickerson & Kemeny, 2004). The results from this experiment demonstrates this long lasting cortisol response, and thus further indicating the effect of the experimental design.

The correlations showed that the more time spent in ice-cold water, the greater the cortisol increase and the lower the perceived stress. Intuitively this seems contradictive, however a possible explanation might be that the participants who were able to keep their hand in the ice-cold water for a long time (60< seconds) felt less pain, and therefore reported feeling less stressed, even though they kept their hand in the ice-cold water for a longer time. Salivary cortisol levels showed to be highly variable, and the participants showed both inter-individual differences and intra-individual differences (in the cortisol values). This might have various different explanations. Not all stressors produce the same physiological changes in the body depending on various factors, such as perception of the stressor, gender, age and context (Dickerson & Kemeny, 2004; Lovallo, Faragb, Vincentc, Thomasb, & Wilson, 2006). Uncontrollable factors such as: concurrent stress, illness, food and drink intake, and minor daily life activities may have interfered. For some of the participants the SECPT might not have been sufficient enough in eliciting a stress response, or the stress response were too small and declined too rapidly in order to be detected in the saliva sample drawn after the SECPT. Other factors such as varying cortisol circadian rhythms might explain the varying cortisol values between their own baseline values as well as in between the participants. Some participants may be prone to wake up early in the morning, while others are more prone to wake up later, which may cause different cortisol values when the samples were drawn and the experiment conducted (Roenneberga et al., 2007).

Based on the perceived stress scale the two participants that fell above the average stress range could presumably be chronically stressed. However more throughout assessment is needed to determine that, and their cortisol responses were not distinct from the other participants.

In order to obtain a better control of confounding variables a relaxation period of 20-30 minutes before and after the SECPT could have been included in the study design. However this was difficult in the current experiment as all of the participants were occupied in different research projects and duties that were time constricted. Therefore extending the time period of the experiment was not convenient.

Holding their hand in ice-cold water while being watched and videotaped was perceived as very stressful to most of the participants (see Appendix I), and thus it could be perceived as causing some negative emotions. Research has previously shown that cortisol is

often released in response to negative emotions (Lovallo, 2005). However, it is being shown by more recent research that the physical response to psychological stressors is immensely variable, and not as directly correlated as first believed (Dickerson & Kemeny, 2004). The specific situational element required to elicit a cortisol response has yet to be clearly identified. This may contribute to the explanation of why half of the participants did not release a cortisol response. Future research could focus on identifying more specific situational elements, which are correlated to a cortisol response. However, by determining other biomarkers related to stress, one might not need to do further research on cortisol related situational elements.

The main focus in this study was on identifying changes in gene transcription. However, these data could not be gathered. The RNA extraction protocol adapted in this study was made based on a published paper (Pandit, Cooper-White & Punyadeera, 2013), a standard laboratory RNA extraction protocol and also according to manufacturers' instructions (*RNeasy Protect Saliva Mini Handbook,* 2010), before it was later optimized into one protocol that we followed (see Appendix H). The optimization process also included various methods of collection and preservation of samples, which are as follows: fresh oral drool, oral drool snapfreezed in liquid nitrogen, oral drool preserved in RNAlater and, the use of whole saliva, pelleted cells[4] and cell supernatant[5], to achieve maximum recovery and quality of RNA. Results from the optimization process showed that oral drool snapfreezed in liquid nitrogen and cell pellets provided the highest yield and quality according to the NanoDrop ND-2000 spectrophotometer (Thermo Scientific), however RNA was still found to be heavily degraded when measured using a bioanalyzer, thus resulting in the inability to produce any metatranscriptomic data. A likely explanation is due to high amounts of salivary ribonucleases from various sources that are naturally occurring in saliva, thus resulting in rapid degradation. This fact may be the reason for the lack of reports on human salivary RNA (Park, Li, Yu, Brinkman & Wong, 2006). Whole saliva may not provide accurate results as it may contain foreign substances such as food debris (Li, Zhou1, St. John & Wong, 2004). Therefore other phases of saliva is preferable, such as cell pellets, and snapfreezing, which gave the highest quality in this study. The quality and quantity of the RNA is critical in order to have any success in any gene expression RNA-based analysis. Low quality RNA may

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[4] Pelleted cell: "the sediment portion that accumulates during centrifugation" ("Pellet," n.d.).

[5] Supernatant: "the usually clear liquid overlying material deposited by settling, precipitation, or centrifugation" ("Supernatant," n.d.).

compromise the results greatly, and contribute to highly expensive, time consuming and labour sensitive applications, which may come out as useless. It is therefore highly recommended and necessary to work with high-quality intact RNA from the start in research and diagnostics (Fleige & Pfaffl, 2006). For future research I would recommend to use a more specific quantification machine than the NanoDrop ND-2000 spectrophotometer (Thermo Scientific). By using more specific measurement tools such as the Agilent 2100 Bioanalyzer system or a qubit fluorometer one could more accurately quantify the concentration and quality (Fleige & Pfaffl, 2006; Sundberg et al., 2013).

In order to better monitor human health and diseases, more informative and specific biomarkers in saliva is greatly needed. As for now it is not known how RNA and ribonucleases[6] can coexist in saliva (Park et al., 2006). In order to optimize the RNA quality, identification of the specific RNA ribonuclease, which is not inactivated by RNAlater, could facilitate the creation of new reagents or other methods to completely conquer and avoid RNA degradation. Therefore it is important to know more about the constituents in saliva in order to develop such biomarkers more accurately (Li et al., 2004). Thus more investigation of procedures on how to extract and store saliva in order to maximize the quality will be an important contribution for future research. If we were able to establish the oral microbial transcriptome, it could serve the field with more knowledge about the salivary constituents. It is still completely novel, as there are no published papers as of yet on this specific topic. There are however, metagenomic data derived from salivary DNA published in recent years. The motivation in this study to use RNA instead of DNA is to enable the ability to detect the functionality of the genes without simply quantifying them (Tringe & Rubin, 2005; Tyrell & Schoolink, 2003).

Simply being alive is the only requirement for experiencing stress in some way. Our modern world is filled with all kinds of stressors that affect our mental and physical health in different ways (Taylor, 2011). The effects of chronic stress may lead to a dysfunctional HPA axis and persistent high levels of cortisol, which has shown a strong connection to major depressive disorder and maladaptation to adverse events (Dedovic & Ngiam, 2019; Chen et al., 2015). Studies on medical students suggest they are experiencing chronic stress and serious psychological problems such as anxiety and suicidal thinking (Rosiek, Rosiek-Kryszewska, Leksowski, & Leksowski, 2016). This may further affect the microbiome, as stress related hormones have the ability to influences the microbiota by increasing the growth

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[6] Ribonuclease: "is an enzyme that catalyzes the hydrolysis of RNA" (Ribonuclease, n.d.).

of both commensal and pathogenic bacteria. Specific strains of good bacteria (lactobacilli) can be reduced by stress, in both animals and humans and the reduction correlates with the stress-indicative behaviors (Gur et al., 2014). Chronic stress is directly linked to mental health problems, and suicidal thinking, which can lead to serious consequences, and now research show that the microbiota is strongly involved in these processes (Gur et al., 2014; Lovallo 2005; Rosiek, et al., 2016). More research on this connection could serve tremendous benefits, and saliva is considered a convenient biomarker in research.

Saliva serves many important factors in the body, and it is needed for digestion of food, protection from microorganisms and even speaking. The content of microorganisms is distinct compared to other bodily fluids such as blood (Li et al., 2004). Using saliva provides several advantages compared to other traditionally used biomarkers. It can simplify further investigation of the connection between stress response and the microbiome as saliva is more convenient to collect, does not need medically trained personnel, easier to be used for large scale surveys, inexpensive, non-invasive, safer and gives a relatively accurate reflection of many unbound, biologically active serological markers in the circulation (Shirtcliff, Granger, Schwartz & Curran 2000; Whembolua, Granger, Singer, Kivlighan, & Marguin, 2006). Blood, urine and stool as biomarkers may limit enrollments in scientific studies, as they are more invasive and hazardous than saliva. Other disadvantages are that blood has a shorter half-life than saliva, a phlebotomist is required, and is more expensive (Li et al., 2004; Pandit, Cooper-White, & Punyadeera, 2013; Shirtcliff, Granger, Schwartz & Curran 2000). Urine and stool can be more challenging to collect and may be viewed as stigmatizing (Feigelson et al., 2014; Lecky, Hawking & McNulty 2014; Whembolua, et al., 2006).

The research on the gut-microbiota brain axis is ground breaking as it changes the current understanding of how the body works (Aziz, Dore, Emmanuel, Guarner & Quigley, 2013). It has been known for centuries that microbes can impact the brain, and the impact was thought to be dominantly damaging. The damage was mostly due to direct microbial invasion of the brain, however we are starting to learn that microbes are communicating through various pathways without direct invasion of the brain, which may also turn out to be beneficial (Aziz, et al., 2013; Mortensen et al., 2007; Price et al., 1988; Prosniak, Hooper & Dietzschold, Koprowski, 2000; Little, 2005). The gut-microbiota brain axis is in the phase of discovery and thus the full functionality is not yet understood, nor is the biological importance of stressor-induced alterations of the microbiota (Aziz, et al., 2013; Galley & Bailey, 2014). However, studies demonstrate that some aspects of stressor-induced increases in immune system reactivity are dependent upon the microbiota (Galley & Bailey, 2014;

Kelly, et al., 2015). If we can discover ways to measure the stress related changes in saliva, it will provide a solid base for further investigation, which may investigate in depth what these changes specifically mean, if there are any. Further this may link the gap between the body and the mind, as the gut-microbiota-brain axis directly connects the brain to important bodily functions and microorganisms involved in these functions (Aziz, et al., 2013; Mayer, 2011). There is no longer doubt that physical illness is followed by psychological stress and psychological stress can lead to physical illness demonstrating the link between the body and the mind (Taylor, 2011).

If one is able to measure salivary transcriptomic changes in relation to stress, it will provide the literature with a novel tool for measuring impacts of stress on the human microbiota, as well as a tool for investigating the functionality of the microbiome. Future research could develop the technique further and investigate if it is liable in discovering and predicting individuals in the risk of becoming chronically stressed and depressed. Further it would be interesting to see if the participants not showing cortisol responses would show any transcriptomic changes. If they did, this method could serve as a more stable biomarker than cortisol for measuring stress. New treatments for stress and depression could be another benefit by doing further research on this topic, as reports shows reduction of cortisol by the use of probiotics (Al-Asmakh et al., 2012).

Further it would be interesting to look into which genes that are actively expressed and if there are specific patterns that can be decoded. If so, could these patterns give an explanation for the variations in the stress response inter-individual and intra-individual. The research so far has mostly focused on rodents, and more research on humans is needed in order to better understand the impact of the microbiome in stress and health in our day to day lives.

Conclusion

Salivary cortisol is sensitive and may be influenced by uncontrollable factors. RNA degrades quickly making it difficult to produce any metatranscriptomic data. However, if this can be overcome, it would provide novel ways of investigating stress related microbial changes in humans, as well as opening up doors for future discoveries.

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Appendix A

Consent to Participate in a Research Study
Lee Kong Chian School of Medicine   Nanyang Technological University, Singapore

Title of Study:	**Assessment of bacteria gene expression changes in host oral microbiome during stress**				

Investigators:

Name:	Prof. Sven Pettersson	**Dept:**	Metabolism Group	**Phone:**	6576 7335
Name:	Kang Yi Hui, Alicia	**Dept:**	Metabolism Group	**Phone:**	6576 7340
Name:	Kaurin Sunniva (Research Attachment Student)	**Dept:**	Metabolism Group	**Phone:**	82482491

Introduction
- You are being asked to be in a pilot research study which seeks to assess bacteria gene expression changes in host oral microbiome in relation to stress.
- You were selected as a possible participant because you are a healthy individual.
- We ask that you read this form and ask any questions that you may have before agreeing to be in the study.

Purpose of Study
- The purpose of the study is to identify possible new biomarkers in saliva as a result of stress.
- Ultimately, this research may be presented as an abstract/talk in conferences or in a paper or used as preliminary data for the application of a grant to monitor stress in medical students over a long period of time.

Description of the Study Procedures
- If you agree to be in this study, you will be asked to do the following things:

  1. Donate a total of 45 ml of Oral Drool sample, 5 ml per time point (over 3 time points: 3:00 p.m., 3:30 p.m., 4:00 p.m.) per day, for 3 days across a span of 2 week.
  (This is inclusive of 6 baseline collections and 3 collections on the experimental day).

  Video recordings of your face will be collected only to be used for scientific purpose in this study. The video recording will provide information about facial expression during the stressor task, in order to obtain a better evaluation of the individual responses to the stressor.

Risks/Discomforts of Being in this Study
- There are no significant risks being in this study apart from mild discomfort when the stressor is being administered and slight inconveniences during sample collection days.

Benefits of Being in the Study
- The benefit of your participation enables us to evaluate if there are any possible new biomarkers of stress in saliva.

Confidentiality
- This study is anonymous. We will not be collecting or retaining any information about your identity.
- The records of this study will be kept strictly confidential. Research records will be kept in a locked file in Ms Alicia Kang's office, and all electronic information will be coded and secured using a password protected file. We will not include any information in any report we may publish that would make it possible to identify you.

Right to Refuse or Withdraw
- The decision to participate in this study is entirely up to you. You may refuse to take part in the study *at any time* without affecting your relationship with the investigators of this study. Your decision will not result in any loss or benefits to which you are otherwise entitled. You have the right not to answer any single question, as well as to withdraw completely from the study at any point during the process; additionally, you have the right to request that the researcher does not use any of your material.

Right to Ask Questions and Report Concerns
- You have the right to ask questions about this research study and to have those questions answered by me before, during or after the research. If you have any further questions about the study, at any time feel free to contact me, **Alicia Kang at aliciayh@ntu.edu.sg** or by telephone at **6576 7340**.
- If you have any problems or concerns that occur as a result of your participation, you can report them to the **Professor Sven Pettersson** at the number above.
- Alternatively, you may also contact Nanyang Technological University, IRB department at irb@ntu.edu.sg for any concerns in regards to the study or your rights as a participant.

Consent
- Your signature below indicates that you have decided to volunteer as a research participant   for this study, and that you have read and understood the information provided above. You will be given a signed and dated copy of this form to keep, along with any other printed materials deemed necessary by the study investigators.

Please tick the appropriate box(s) below:

- Antibiotic treatment
- Smoker

Subject's Name (print): _____

Subject's Signature: _____   Date: _____

Investigator's
Signature:

Date:

Appendix B

Saliva collection instruction sheet

Study Title: Assessment of bacteria gene expression changes in host oral microbiome during stress

- 30 minutes before sample collection rinse your mouth with water and avoid eating and drinking to ensure that your mouth is free of food or other foreign substances.
- No brushing of teeth within 45 minutes prior to sample collection.
- Dental work should not be performed within 24 hours prior to sample collection.
- Saliva samples visibly contaminated with blood should be discarded and recollected.
- No strenuous exercise should be performed 1 hour before sample collection.

Procedure for collection:

1. Imagine chewing your most favorite food, while you gently move your jaws in chewing movements.
2. Allow saliva to pool in the mouth.
3. Tilt your head forward and drool into the collection vial/tube.
4. Repeat until sufficient sample is collected (min 5 ml).
5. Place sample on ice immediately.

Appendix C

Perceived Stress Scale

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, you will be asked to indicate by circling how often you felt or thought a certain way.

Name _____ Date

_____

Age _____ Gender (Circle): M F Other _____

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

1. In the last month, how often have you been upset

because of something that happened unexpectedly?................................. 0 1 2 3 4

2. In the last month, how often have you felt that you were unable

to control the important things in your life? 0 1 2 3 4

3. In the last month, how often have you felt nervous and "stressed"? 0 1 2 3 4

4. In the last month, how often have you felt confident about your ability

to handle your personal problems? 0 1 2 3 4

5. In the last month, how often have you felt that things

were going your way?.. 0 1 2 3 4

6. In the last month, how often have you found that you could not cope

with all the things that you had to do? 0 1 2 3 4

7. In the last month, how often have you been able

to control irritations in your life?.. 0 1 2 3 4

8. In the last month, how often have you felt that you were on top of things?.. 0 1 2 3 4

9. In the last month, how often have you been angered

because of things that were outside of your control?.................................. 0 1 2 3 4

10. In the last month, how often have you felt difficulties

were piling up so high that you could not overcome them? 0 1 2 3 4

Please feel free to use the Perceived Stress Scale for your research.

Appendix D

Study Procedure

One day before the participation day the participants received a saliva sample collection instruction sheet (Appendix B). On the day of the first baseline the participants were given a self-reported questionnaire (Perceived Stress Scale) about their feelings and thoughts the last month (Appendix C). This was to see if the participants underwent long-term psychological stress.

Saliva collection. The participants were instructed in "Passive drool" a common salivary collection method in research (Granger et al., 2007). This method is advantageous as it prevents the interference from materials to stimulate or absorb the sample, and enables the measurement of multiple salivary biomarkers including withdrawal of large sample volume (Douglas et al., 2006; Granger et al., 2007).

The participants were asked to imagine chewing their most favourite food, while they gently moved their jaws in chewing movements. This process will generate saliva, and the saliva is then allowed to pool briefly under the tongue before being gently "drooled" into a sterile collection vial. The participants were asked to donate a total of 45 ml of oral drool sample divided on three days. For each of the three time points (15.00- 15.30 and 16.00) during the day, 5 ml of saliva were collected.

Baseline. The baseline saliva samples were collected the day before the stress condition and the day after the stress condition, at the specific time points (15.00-15.30 and 16.00).

SECPT. In the written consent form the participants were being informed about video recordings to analyze their facial expression. This was a deception as no recordings were taken. The participants entered the experimenter room one at a time and were asked to read a brief information about the study (Appendix E). They were then instructed to immerse their non-dominant hand up to and including their wrist in a box filled with ice-cold water (3-4 °C). They were being instructed to look into the camera and to keep their hand in the water for as long as possible. The experimenter watched the participants during the test. The tolerance time was recorded. After the test they were given a rating scale (1 = no stress,10 = very stressed), and asked to rate how stressed they felt during the experiment. Saliva were collected before the experiment at 15.00, right after the stressor, and 20 minutes after the SCPT and at 16.00 (Giles, et al., 2014; Skoluda et al., 2015; Schwabe, Haddad & Schachinger, 2008)
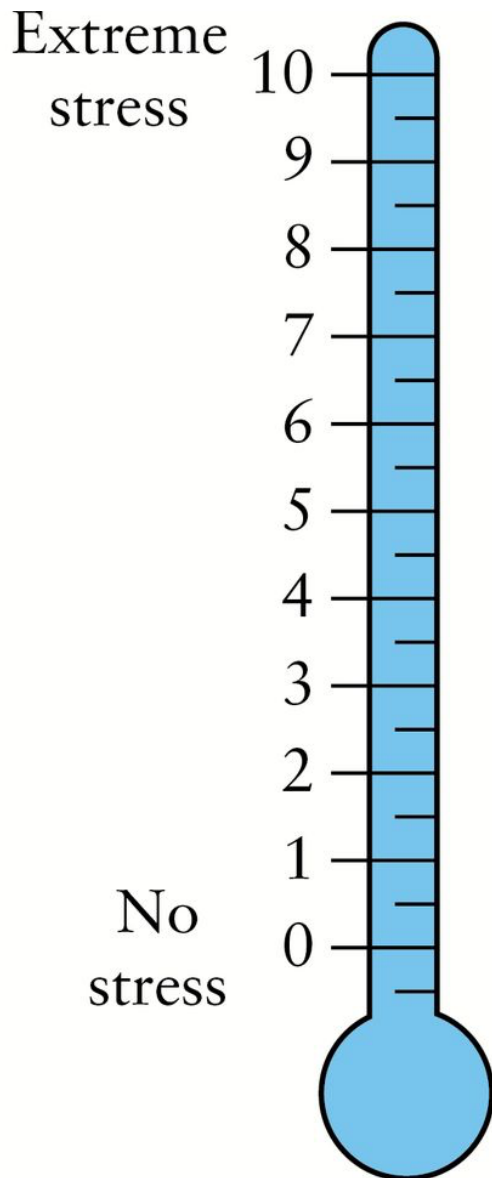
Appendix E

Information about the study "Assessment of bacteria gene expression changes in host oral microbiome during stress"

The key objective of this study is to assess for any bacteria gene expression changes in the oral microbiome in relation to acute stress. Unlike the genome, which is fixed for a given cell, the transcriptome can vary in response to environmental conditions. The participants (you) will therefore be exposed to a cold pressor task (CPT). The test is short and mild, but is still thought to elicit some pain and physiological responses associated with stress. You shall place your non-dominant hand up to and including the wrist in ice-cold water, and keep it there for as long as you manage, while looking into the video camera. Video recordings of your face while doing the CPT, will be used to analyse facial expression in order to obtain a better evaluation of the individual (your) responses to this cold experience.

Appendix F

Stress-o-meter

Please circle the number (0-10) on the "stress-o-meter" that best describes how much stress you experienced during the test

Appendix G

Debriefing after the SECPT

After the SECPT the participant were given a short debriefing: You were given false information about video recordings of your facial expression during the SECPT. You received this information to elicit a higher stress response. The research shows that a regular cold pressor task (CPT) does not elicit high levels of cortisol, but by adding a socially evaluative component (video recordings and being watched by the experimenter) will increase cortisol excretion. In our study we are using cortisol as a measurement of the stress response. It was therefore important to try and obtain a marked increase of cortisol excretion.

Appendix H

Saliva sample collection and RNA extraction

Saliva Cortisol measurements

Cortisol levels were measured using Abcam cortisol ELISA kit (ab154996) according to manufacturer's instructions. Prior to use, all reagents, samples and controls were equilibrated to room temperature. 1 X washing solution was made by diluting 50ml of 10X Washing solution in 450 ml of Deionized water, and then mixed thoroughly and gently. Respective amounts of 1X Cortisol-HRP Conjugate were prepared prior to use, following a standard protocol of 10 µl Cortisol-HRP Conjugate (concentrated) solution to 1 ml incubation buffer. The HRP-Conjugate was mix thoroughly and gently for 5 minutes on a rotating mixer, and kept stable at room temperature for 3 minutes.

Assay procedure. 1) The required number of ELISA strips were removed. 25 µl of Standards, Quality Control and Samples were added into their respective wells. 2) 200 µl of 1X Cortisol-HRP conjugate were added into each well, except for one well, which was left blank for substrate blank. 3) The wells were incubated at 37°C, for one hour in the dark. 4) After an hour, the contents in the well were aspirated. Each well was soaked for a minimum of 5 seconds and washed three times with 300 µl of 1X washing. The remaining fluids were being carefully removed by tapping the strips on tissue paper prior to the next step. 5) 100 µl of TMB substrate solution were added into the wells. 6) The wells were then incubated for 15 minutes at room temperature, in the dark. 7) 100 µl of stop solution were added into all wells in the same order and rate as in step 5, and shaked gently. 8) The absorbance of the samples were measured at 450 nm immediately after adding the stop solution.

Saliva RNA extraction preparation/Method of saliva collection and preservation

Procedure. 1) From the collection tube that was placed on ice, 1 ml of saliva was pipetted into a smaller collection tube (10ml) containing 5ml of RNAlater (Ambion, AM7021). Samples were mixed by inversion and left at room temperature overnight before storage at -80ºC. This procedure was performed on saliva samples of the first baseline condition and test condition. For the second baseline condition, saliva was pipetted into smaller aliquots (400ul) without RNAlater in microcentrifuge tubes (due to lack of RNAlater) and snap-freeze in liquid nitrogen, before storage at -20.

RNA-Extraction method for saliva

2) Saliva samples were thawed on ice and pelleted at 11 000*g* for 20 min at 4° The cell pellet was homogenized using 0.4g of zirconium silica beads (0.1mm and 1mm) at highest speed (30Hz) for 3 minutes using the QIAgen TissueLyzer II system (Hilden Germany) after addition of 800 µl of QIAzol reagent (Maryland USA). 5) 200 µl of chloroform (Sigma, 25693) was added to each tube and vortexed before incubation at room temperature for 5 minute. 7) The samples were then centrifuged at 10 000*g* for 10 min at 4°C. The upper aqueous layer was transferred to a fresh, labelled microcentrifuge tube and an equal volume of isopropyl alcohol (Sigma, 109827) was added. Samples were incubated at -20 for a minimum of 30 min before centrifugation at 10 000*g* for 15 mins at 4°C. 9) The pellet was washed with 1 ml of 70% molecular-grade ethanol and air dried in the biological safety cabinate for 30 mins before resuspension in 20 µl RNase-free water. RNA samples were cleaned up using the RNeasy Mini Kit (QIAgen, 74106) according to manufacturer's instructions. The quality and quantity of the isolated RNA were measured using the NanoDrop ND-2000 spectrophotometer (Thermo Scientific).

Samples were sent to Singapore Centre on Environmental Life Sciences Engineering (SCELSE), Nanyang Technological University for library preparation and transcriptomic analysis.

RNA precipitation. 2 µl of carrier (Glycoblue Coprecipitant, Thermofisher Scientific) was first added to each RNA sample to aid in visibility and recovery of nucleic acids. In short, 1:10 volume of 3 M Sodium acetate and 150 µl of 100% ethanol were used to precipitate each RNA sample overnight at -20°C. Samples were pelleted at top speed (13200 rpm), 4°C, for 30 minutes (Eppendorf 2454 R, Germany) on the subsequent day and washed with 1 ml of 80% ice-cold ethanol. The RNA samples were pelleted and left to air-dry for approximately 30 minutes in the Biological Safety Cabinet (Esco Class II Type A2). 10 µl of nuclease-free water was used to dissolve the RNA pellet before quantification using NanoDrop ND-2000 spectrophotometer (Thermo Scientific)

Appendix I

Summary table

Table 2

The participants' score on the Percieved stress scale, time hand held in ice-water and stress-o-meter scale ranges

Participants	Perceived stress score (mean)	Time in ice-water (sec)	Stress-o-meter range
1	16, 5	23	9
2	8,5	80	5
3	9,5	50	7
4	11,5	35	3
5	24	47	9
6	17,5	123	8
7	17	25	9
8	10,5	26	9
9	20	24	8
10	25	120	5