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Investigating the role of perceived stress on bacterial flora activity and salivary cortisol secretion: a possible mechanism underlying susceptibility to illness

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**Abstract**

This study examined the impact of academic stress on salivary cortisol concentrations and lactic acid bacteria activity. Whole, unstimulated saliva samples and faecal samples were collected from 23 healthy undergraduate students ( $23.0 \pm 6.8$  years; range 18-44) over two one-week periods: during the beginning of semester (low stress baseline condition) and during the first week of exams (high stress condition). Students also completed a series of questionnaires measuring perceived levels of stress, gastrointestinal symptoms, and nutritional intake. Significant findings indicated that faecal lactic acid bacterial levels were lower during the high stress condition. Paralleling this, students rated perceived levels of stress as being greater during the exam period compared to the baseline condition. The findings from this study have provided further insight into the link between stress and gastrointestinal flora activity in humans.

## **Introduction**

Growing interest in psychoneuroimmunology (PNI) research has provided evidence that stressors can have a pervasive impact upon an individual's psychological and physiological well-being. A meta-analysis studying the relationship between psychological stress and the immune system found acute stressors (for example, public speaking) were associated with the up-regulation of natural immunity and the down regulation of specific immunity, producing an adaptive change in the immune system allowing the stressor to be dealt with quickly (Segerstrom & Miller, 2004). Chronic stressors (for example, unemployment) were associated with suppression of both natural immunity and specific immunity, therefore resulting in potentially detrimental changes in immune functioning (Segerstrom & Miller). Several studies have also linked stressors to increased disease states, for example, hypertension, diabetes and gastrointestinal disorders (Drossman, 1998; Gabry *et al.*, 2002; Milde *et al.*, 2003), and increased susceptibility to common viral and bacterial infections (Cohen *et al.*, 1991).

Despite extensive research exploring the influence of stress on immune functioning and well-being, little is known about the impact of stress on the gastrointestinal microflora. The digestive tract contains the largest component of the immune system in the human body (Jones *et al.*, 2006). Several studies have provided evidence that stress is related to digestive problems and gastrointestinal disorders (Drossman, 1998; Walker *et al.*, 2001), however there has been limited research investigating the underlying mechanisms. To date, several animal studies have found that stress reduces the concentration of lactobacilli, beneficial lactic acid bacteria in normal gut flora, resulting in an environment more favourable to pathogens (Bailey & Coe, 1999;

Bailey *et al.*, 2004). The only human study in this area has been limited to neuroemotional stress induced by extreme conditions during spaceflight (Lizko *et al.*, 1987), and no studies have investigated the impact of psychological stress on gastrointestinal flora under everyday stressful conditions. However, a growing body of evidence suggests that disruption of the indigenous GI microflora may also contribute to the onset and maintenance of irritable bowel syndrome (Madden & Hunter, 2002; Malinen *et al.*, 2005).

Research in this area is of significance as the gastrointestinal microflora is fundamental to maintaining host health and normal gut function (Bailey & Coe, 1999). The number of bacteria in the human gastrointestinal tract is estimated to be as high as  $10^{12}$  cells/g of contents, which exceeds the number of eukaryotic cells forming the human body by a factor of 10 (Tlaskalova-Hogenova *et al.*, 2004; Volker, 2004; Zoetendal *et al.*, 2004). These indigenous microflora have several beneficial effects on the host, the most important of which includes the prevention of colonization by potential pathogens (Berg, 1996).

PNI research has shown that one of the main mechanisms by which stress affects immune functioning is through activation of the hypothalamus-pituitary-adrenal (HPA) axis (Cohen & Williamson, 1991; Smyth *et al.*, 1998). The primary consequence of HPA-axis activation is the secretion of cortisol, a type of glucocorticoid (steroid) hormone. Short-term secretion of cortisol enhances immunological functioning by providing energy through metabolism of carbohydrates, proteins and fats (Mader, 2000; Talbott, 2002). In contrast, hyper-secretion of cortisol leads immunosuppression (Ritchey *et al.*, 2002; Heuser & Lammers, 2003; Lozovaya & Miller, 2003; Segerstrom & Miller,

2004). Several studies have investigated the relationship between daily stressors and cortisol level, with findings providing evidence that higher cortisol secretion is related to higher levels of stress (Ockenfels *et al.*, 1995; Smyth *et al.*, 1998; Vedhara *et al.*, 1999). Several studies have also demonstrated that secretory immunoglobulin A (sIgA, the main component of the mucosal immune system) is sensitive to psychological stress, with chronic stress associated with lowered sIgA levels (Bosch *et al.*, 2004).

The aim of the present study was to identify the effect of stress on physiological well-being among humans, including gastrointestinal flora activity (specifically lactic acid bacteria) and salivary cortisol levels. Academic stress was used as a model of everyday stress amongst students, as this has been found in several studies to have a pervasive impact on their psychological and physiological well-being (Humphrey & McCarthy, 1998; Ross *et al.*, 1999; Shaikh, 2004; Hong, 2005). It was hypothesized that there would be a reduction in the concentration of beneficial lactic acid bacteria in the stress condition, and that this reduction would be associated with an increase in cortisol concentration.

## **Materials and method**

### **Participants**

Twenty three science students, 16 women and 7 men ( $23.0 \pm 6.8$  years), of Swinburne University of Technology volunteered to participate in this study. Students were informed about the study verbally during a lecture and through the university online teaching resource. To be eligible for the study, participants had to be free of significant psychological or physiological conditions. Subjects were excluded if they were taking

any medication that may have interfered with the biological measures (i.e. taking corticosteroids, antibiotics, probiotics). The protocol was approved by the Human Research Ethics Committee of Swinburne University of Technology, and an informed consent form was signed by all the subjects who agreed to participate in the protocol.

### **Protocol design**

Testing took place over two one-week periods. The first week of testing was during week 6 of the first teaching semester providing a low stress condition (low stress week). The second week of testing (approximately 7 weeks later) was during the first week of examinations providing a high stress condition (high stress week). Participants were instructed to complete the Initial Questionnaire at the onset of the study in the low stress week. A Daily Diary Questionnaire was provided for each of day of testing, containing a section to be completed in the morning and in the evening. Two Weekly Questionnaires were provided, to be completed at the end of the low stress week and the high stress week.

### **Questionnaire based measures**

Data was collected using three questionnaires; the Initial Questionnaire, the Daily Diary Questionnaire, and the Weekly Questionnaire. The Initial Questionnaire consisted of a number of demographic items assessing age, gender, and student status. The Daily Diary Questionnaire included an item used to assess a person's perceived level of daily stress and an item used to assess a person's perceived level of daily gastrointestinal symptoms, measured on a 10cm visual analog scale. A daily nutritional questionnaire

(Knowles, 2004) measured intake of the five food groups, liquid consumption of tea, coffee, and alcohol, and number of cigarettes smoked. The Weekly Questionnaire consisted of the K10 Scale (Kessler *et al.*, 2003), which measures non-specific psychological distress. The digestive symptom subscale from the Physical Health Questionnaire (PHQ, Barton *et al.*, 1995) was used to measure weekly digestive problems. The stress subscale for the Depression, Anxiety and Stress Scale (DASS<sub>21</sub>, Lovibond & Lovibond, 1995) was used to measure perceived weekly stress.

### **Saliva sampling and analysis**

For each day of testing, saliva samples were to be provided upon waking, as recommended by Pruessner *et al.* (1997). During the low stress week, two consecutive days of sampling were required. During the high stress week, five days of sampling were required: day before first exam, day of first exam, day after first exam, third day after first exam, and five days after first exam. For each sample participants were instructed to provide approximately 1 mL of saliva into sterile 2 mL polypropylene tubes, and to immediately refrigerate samples. Upon receipt at the laboratory, saliva samples were stored at -20°C until assayed.

Salivary cortisol was assayed with a Cortisol Enzyme Immunoassay kit (EIA, Assay Designs, Inc., Biocore Pty. Ltd.). Saliva samples were thawed, and a 1:4 dilution was made with Assay Buffer. A micro-plate reader capable of reading at 405nm, with correction between 570 and 590nm, was used to measure the optical density of samples. The concentration of cortisol in samples was calculated using an immunoassay software package (SoftMAX), utilizing a 4 parameter logistic curve fitting program. All samples

from a participant were analyzed in one assay. Inter- and intra-assay coefficient of variation was less than 10.5% and 13.4%, respectively. The specificity was 100% for cortisol, 3.64% for progesterone and less than 0.10% for estradiol, cortisone and androstenedione (related steroid compounds).

### **Faecal sampling and analysis**

Faecal samples were collected using sterile faecal containers with a scoop attached to the lid. For the low stress week and high stress week participants were asked to provide at least three samples each week, and at any time of the day. Participants were instructed to collect enough faeces to at least half fill the container (approximately 1 gram) by either (1) placing a separate clean container with a wide opening or plastic wrap or newspaper in the toilet bowl, and to pass faeces directly into the container or onto the plastic wrap or newspaper, or (2) scoop faeces from used toilet paper. Participants were instructed to screw the lid on firmly, place the container in a zip-lock plastic bag, and refrigerate. Upon receipt at the laboratory, faecal samples were stored at 4°C, until assayed.

All microbiological media were supplied by Oxoid Ltd. (Basingstoke, UK) and prepared according to the manufacturers instructions with de-ionised water, and autoclaved at 121°C for 20 minutes before use. One gram of faeces was diluted into 1mL of Quarter-Strength Ringer Solution and homogenized by vortex mixing. Further serial 10-fold dilutions were made in Mann-Rogosa-Sharp (MRS) Broth. A serial 10-fold dilution of samples up to  $10^{-5}$  was appropriate. Afterwards, 0.1 mL samples of each dilution were spread on MRS agar for quantification of total lactic acid bacteria. MRS



plates were incubated for 3 days at 30 °C in a candle jar with 10% carbon dioxide and reduced oxygen. Total lactic acid bacteria were quantified by counting colony-forming units (CFU) on the MRS plates corresponding to the last readable dilution.

## **Results**

### **Statistical methods**

Data was analyzed using the Statistical Package for Social Sciences (SPSS) for Windows Statistical Package Version 14.0. Because of the small sample size paired *t*-tests were used to analyze the data, and not repeated measures ANOVA. Initial screening of the data indicated there were no significant differences for gender across the variables, therefore group results were analyzed.

*t*-tests among the measures are given below in Table 1. The daily measure of perceived stress and the weekly measure of stress, digestive symptoms, and psychological distress significantly increased from baseline week to exam week. The physiological measure of cortisol and the daily measure of gastrointestinal upset increased from baseline week to exam week, however this was not statistically significant. The physiological measure of bacterial count significantly decreased from baseline week to exam week.

Figure 1 shows the general trend for the effect of stress on normal gut flora over time. A continuous reduction of bacterial levels during the period of academic stress was observed. During the examination period, bacterial levels the day before the first exam were relatively normal when compared to the bacterial levels observed in the baseline

(low-stress) week. For the day of the first exam bacterial levels dropped, however this decrease was not significantly below levels observed in the baseline period. This drop in bacterial level continued for first and second post exam days, with this decrease being significant. There was a slight rise in bacterial levels on the third post exam day, however bacterial levels remained significantly lower than baseline levels on the fourth and fifth post exam days.

### **Controlling for potential confounding variables**

Changes in dietary habits, caffeine and alcohol consumption, and smoking from baseline week to exam week were examined as these variables may potentially confound the effect of stress on the results (Smyth *et al.*, 1998). The results given in Table 2 show that the number of servings of vegetables, dairy products, and consumption of alcohol and tea decreased from baseline week to exam week. *t*-test analysis indicated this decrease was only significant for servings of vegetables. The number of servings of meat, cereal and fruit, coffee consumption, and number of cigarettes increased from baseline week to exam week. *t*-test analysis indicated that the only significant increase was for coffee consumption.

### **Discussion**

It is well known that perceived stress has a detrimental effect on immunological functioning. Numerous studies have provided evidence that one of the main mechanisms by which this occurs is through the hyper-secretion of cortisol. However little is known about the impact of stress on the gastrointestinal microflora of humans.

The findings that bacterial count significantly decreased in the high-stress week compared to the baseline week among human participants is unique to this study. However, these results are consistent with several animal studies providing evidence that stress decreased the number of beneficial gut flora (Bailey & Coe, 1999; Bailey *et al.*, 2004). This may be due to bacteria responding to stress-induced alterations in gut physiology, such as increased bicarbonate production or an inhibition of gastric acid and gut mucous secretion, resulting in an environment less conducive to beneficial bacteria (Bailey & Coe). While there was a continuous reduction in bacterial count during the period of academic stress, additional investigation measuring bacterial levels beyond the stress period is needed to examine the time of recovery of bacterial flora.

The observation that cortisol concentration did not significantly increase in the stress week compared to the baseline low stress week is inconsistent with several studies showing that during time of stress cortisol is produced at higher levels (Ockenfels *et al.*, 1995; Smyth *et al.*, 1998; Vedhara *et al.*, 1999). Cortisol levels usually display a diurnal variation, with highest concentrations after waking, then declining throughout the day. The current study collected a single saliva sample in the morning. This is when cortisol is usually maximal, which may have led to a ceiling effect. Aggregation of repeated cortisol samples taken over a day could provide a more sensitive measure of the cortisol-stress response by overcoming the possible masking effects of diurnal variation of cortisol. Future research would benefit by employing collection procedures, where participants are asked to provide timed saliva samples throughout the day.

It is possible that the observed effects of stress on the gastrointestinal microflora may be mediated by cortisol. Previous research has shown that maternal separation in

infant monkeys resulted in large cortisol responses as well as disruptions in gastrointestinal microflora (Bailey & Coe, 1999). Research has also found that injecting pregnant rats with cortisone (cortisol) is associated with reduced concentrations of beneficial bacteria in the small intestine of pups after birth (Schiffrin *et al.*, 1993). Cortisol may mediate the effect of stress on gastrointestinal microflora by altering the acidity of gut secretions and gut motility (Bailey & Coe). Additional investigation is needed to further explore the potential of cortisol to mediate the effects of stress on gastrointestinal microflora.

It is also possible that the observed decrease in bacterial count during the high stress condition may be related to other physiological mechanisms associated with the stress response. It has been previously demonstrated that chronic stress is associated with a lowered sIgA response (Bosch *et al.*, 2004). Secretory IgA interferes with the ability of bacteria to adhere to and penetrate mucosal surfaces, and consequently limits the colonization of pathogens (Miletic *et al.*, 1996). A decline in levels of sIgA decreases resistance to microbial pathogens, however less is known about the effect sIgA has on the commensal microflora of the gut. Another factor that may influence the commensal microflora is bowel transit time, the time taken for food to travel through the digestive tract. Several studies have demonstrated that bowel transit time is affected by stress, with psychological stress accelerating colonic transit (Ditto *et al.*, 1998; Plourde 1999). Changes in transit time have also been shown to alter microbial growth in the colon (Wang *et al.*, 1996; Erbil *et al.*, 1998). Several studies have provided evidence that the HPA-axis (which stimulates the release of cortisol) is involved in the regulation of the sIgA response and bowel transit time (Monnikes *et al.*, 1993; Amsterdam *et al.*, 2000).

The assessment of these physiological parameters were beyond the scope of this study, however future research would benefit by including these measures in the investigation of the effects of stress on bacterial flora activity.

Despite the exploratory nature of this study, there are several other limitations that need to be acknowledged. Firstly, previous research has indicated that several possible confounding factors can influence cortisol concentration and/or bacterial flora activity, including smoking, eating, alcohol and caffeine consumption (Smyth *et al.*, 1998). In this study, the number of servings of vegetables significantly decreased, while the consumption of coffee significantly increased from the baseline condition to the stress condition. Therefore these variables may have influenced the effect of stress on the biological measures and need to be considered in future studies. Secondly, not all participants provided faecal samples for each day of testing, therefore, only grouped averages were reported. Finally, the continued reduction in flora levels may have been due to the additional stressors of other exams undertaken by participants during the study period.

These findings have implications for chronic intestinal disorders (such as irritable bowel syndrome), as there has been an observed increase in their incidence, and an increase in stressors commonly confronted by individuals (Russel, 2000). This study may provide evidence of the importance of perceived stress in the development and perpetuation of these disorders, lending to the importance of stress management in prevention and treatment. These results may also provide the impetus for further research into the use of probiotics to prevent the development of gastrointestinal problems during times of stress.

In summary, the present study is the first to demonstrate that non-extreme 'every day' stress events affect the integrity of the indigenous gastrointestinal microflora of humans. Bacterial numbers decreased from the baseline week to the stress week. Future research needs to further explore the interrelationship between stress-induced changes in cortisol concentration and bacterial flora activity, and susceptibility to gastrointestinal disorders. Additional biological parameters associated with the stress response (e.g. bowel transit time, sIgA response) should be examined in association with alterations in bacterial flora activity.

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Table 1

*Mean, Standard deviation and t-test for Baseline week compared to Exam week.*

| Measure               | Low-stress week                       | High-stress week                      |                             |
|-----------------------|---------------------------------------|---------------------------------------|-----------------------------|
|                       | <i>M(SD)</i>                          | <i>M(SD)</i>                          |                             |
| Daily stress          | 2.82(1.28)                            | 4.34(1.24)                            | $t(22)=-7.23, p<0.05^{***}$ |
| Weekly stress         | 4.65(4.69)                            | 6.57(3.80)                            | $t(22)=-3.30, p<0.05^{**}$  |
| K10                   | 6.00(3.93)                            | 7.52(4.36)                            | $t(22)=-2.69, p<0.05^*$     |
| Phq digest            | 10.91(2.84)                           | 12.82(3.71)                           | $t(22)=-4.07, p<0.05^{**}$  |
| Gastro upset          | 1.09(1.00)                            | 1.38(1.23)                            | $t(22)=-1.48, p>0.05$       |
| Cortisol <sup>1</sup> | 5.17(2.59)                            | 5.60(3.33)                            | $t(22)=-0.73, p>0.05$       |
| CFU/mL                | $6.38 \times 10^7 (9.16 \times 10^7)$ | $2.95 \times 10^7 (6.04 \times 10^7)$ | $t(22)=2.35, p<0.05^*$      |

Note. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . CFU = Colony forming units.

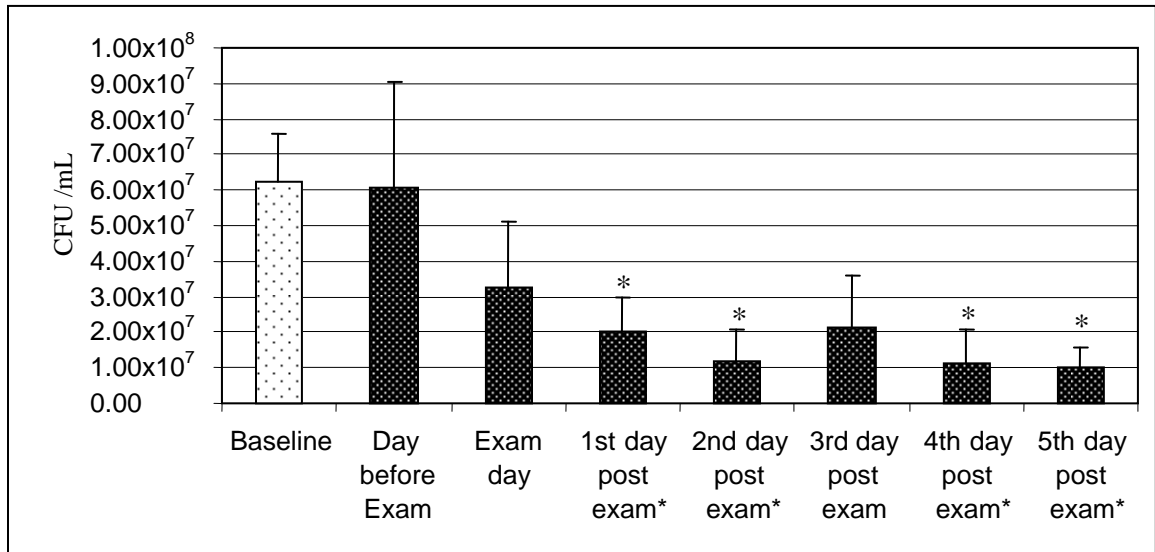
Table 2

*Changes in Dietary Habits, Caffeine and Alcohol Consumption and Smoking from*

*Baseline week to Exam week.*

| Measure    | Low-stress week | High-stress week |                         |
|------------|-----------------|------------------|-------------------------|
|            | <i>M(SD)</i>    | <i>M(SD)</i>     |                         |
| Meat       | 1.25(0.53)      | 1.33(0.51)       | $t(22)=-0.85, p>0.05$   |
| Cereal     | 1.95(0.70)      | 2.09(0.79)       | $t(22)=-1.08, p>0.05$   |
| Vegetables | 1.86(0.60)      | 1.66(0.63)       | $t(22)=2.17, p<0.05^*$  |
| Fruit      | 1.29(0.63)      | 1.35(0.68)       | $t(22)=-.51, p>0.05$    |
| Dairy      | 1.52(0.50)      | 1.52(0.60)       | $t(22)=0.05, p>0.05$    |
| Alcohol    | 1.22(1.61)      | 1.07(1.34)       | $t(22)=0.89, p>0.05$    |
| Coffee     | 0.67(0.87)      | 0.84(1.00)       | $t(22)=-2.14, p<0.05^*$ |
| Tea        | 0.87(0.92)      | 0.81(1.00)       | $t(22)=0.41, p>0.05$    |
| Smoking    | 1.77(4.68)      | 2.32(5.39)       | $t(22)=-1.21, p>0.05$   |

Note.  $*p<0.05$



**Figure 1 Knowles *et al***

**Legend to Figure 1.**

Impact of stress on normal flora of gastrointestinal tract. Note. \* $p < 0.05$  when compared to baseline. CFU= Colony forming units/mL.