

Use of a non-invasive biomarker salivary alpha-amylase to assess the role of probiotics in sleep regulation and stress attenuation in surgical patients: A randomised double-blind clinical trial

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ABSTRACT

Background and Aims: The influence of gut microbiota on human behaviour, stress and sleep is currently a novel topic of research. A prospective double-blind randomised trial was planned to find out whether probiotics by alteration of the gut microbiome can allay surgery-related stress and improve sleep. **Methods:** A total of 160 elective surgical patients were randomised to receive either probiotic or placebo capsule twice daily for four and a half consecutive preoperative days. They were subjected to the Perceived Stress Scale (PSS) questionnaire, a psychomotor vigilance task (PVT) and estimation of salivary alpha-amylase (SAA), a well-known biomarker of stress on the evening of admission and on the evening just before the day of scheduled surgery. Data were analysed using Chi-square test, Student's *t*-test and Mann-Whitney test. **Results:** On comparing between the two groups, there was a significant difference in PSS scores, SAA levels and PVT scores after probiotic/placebo therapy. There was a decrease in the PSS scores by 11.38% ($P < 0.05$) and SAA levels by 11.38% ($P < 0.05$) and an increase in the PVT scores (12.13%, $P < 0.05$) following probiotic treatment. There was a mean difference of 52.85 in SAA levels in probiotic group and - 69.32 in placebo group with a definite fall in SAA levels in probiotic group, which showed that these patients had reduced stress levels and improved psychomotor vigilance implying improved sleep. **Conclusion:** Gut microbiome alteration with probiotics results in lowering of psychological stress and sleep improvement in the preoperative period in surgical patients.

Keywords: Gastrointestinal microbiome, probiotics, salivary alpha-amylase, sleep, stress

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INTRODUCTION

The concept of a brain-gut microbiota (BGM) axis has recently emerged.^[1] There is evidence in human volunteers and small mammals that gut microbes communicate with the central nervous system through parallel and interacting channels including neural, endocrine, immune and metabolic pathways to shape the architecture of sleep and also the stress reactivity of the hypothalamo-pituitary axis.^[2-5] The mechanisms of signal transmission are complex and not fully elucidated.^[4]

A prospective double-blind randomised trial was planned wherein gut microbes of surgical patients were

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manipulated with the help of probiotics. Probiotics are live non-pathogenic microorganisms that, when administered in adequate amounts, confer a health benefit to the host.^[6] Our primary objective was to find out whether probiotics allay surgery-related stress and anxiety and improve sleep. The secondary objective was to find out if probiotic intake is associated with any side-effects.

METHODS

A prospective double-blind randomised clinical trial was planned in a tertiary medical college hospital from 1 February 2018 to 31 January 2020. Institutional Ethics Committee approval was obtained. The trial was registered with the Clinical Trials Registry of India (CTRI/208/03/12276). Written informed consent was obtained from the patients before recruitment for the trial. A total of 160 patients of either sex posted for elective major surgeries under general/regional anaesthesia aged between 18 and 60 years and having the ability to read, hear, write and speak our local language were included. Patients on chemotherapy/radiotherapy/oral antacids, those with gut/psychiatric/neurological symptoms, oral lesions, patients with history of gastric bypass surgery/intestinal resection, those with moderate/severe pain, pregnant/immune-compromised/diabetic patients and patients with autoimmune diseases/milk allergy/history of colour blindness were excluded.

The sample size was determined based on a previous study by Minowa *et al.*^[7] A sample of 73 was taken to provide a power of 80% with a confidence interval of 95% based on the assumption that administration of probiotics would demonstrate at least a 25% difference in sleep and stress levels of the patient. In each group, 80 patients were recruited considering dropouts.

The study participants were randomised according to a computer-generated randomisation number table into two groups– Group *P* (placebo) and Group *PB* (probiotic) [Figure 1]. Group concealment was done with the help of sequential, opaque, numbered, similar-looking envelopes containing either *P* or *PB* capsule. Group allocation was done and envelope with drug was administered to patient on admission to surgery wards by a junior resident, blinded to the study drug. Basic patient information [Table 1] and information about antibiotic intake in study period was obtained.

On the evening of admission, a sample of the recruited patient's saliva was drawn in a plain plastic container by spitting method. The sample was refrigerated in our hospital laboratory. The participants were instructed to refrain from eating and brushing their teeth 1 h prior to taking the salivary sample. The Perceived Stress Scale (PSS) Questionnaire [Annexure 1] in the local dialect version and the psychomotor vigilance task (PVT) were administered to the patients and the responses were noted.^[8] The probiotic/placebo capsule depending on the group of the patient was then given with sips of water that evening after dinner and twice daily after lunch and dinner for the next four preoperative days. The probiotic formulation ('Providac' capsules) contained *Lactobacillus acidophilus* (LA) and *Bifidobacterium* (BB). The placebo was a multivitamin capsule. Every day, during ward rounds for pre-anaesthetic examination, a junior resident who is one of the researchers would ask the patients for occurrence of side effects of probiotics like abdominal bloating, constipation and thirst. On the evening before the day of scheduled surgery, between 7 and 8 p.m., another sample of the patient's saliva was drawn and refrigerated. The PSS Questionnaire and PVT were given to the patient and the responses were noted by a researcher blinded to the study drug. Once a week, all collected refrigerated samples were subjected to assay for the non-invasive biomarker, salivary alpha-amylase (SAA).

The present study was double-blinded because neither the patient nor the doctor administering the study drugs knew the identity of the drug being administered (probiotic/placebo).

The AA kinetic reaction kit (Salimetrics) used by us in our study is specifically designed and validated for the kinetic measurement of SAA activity. It is intended only for research use in humans and some animals. It utilises a chromogenic substrate, 2-chloro-*p*-nitrophenol, which can be spectrophotometrically measured at 405nm.

The PVT is a simple task administered on the mobile phone wherein the subject touches an icon as soon as the light appears. The light will turn on randomly every few seconds for 5–10 min. The main measurement of this task is to see how many times the icon is not touched when the light is on.

Data were statistically described in terms of percentages, frequencies (counts, number of cases),

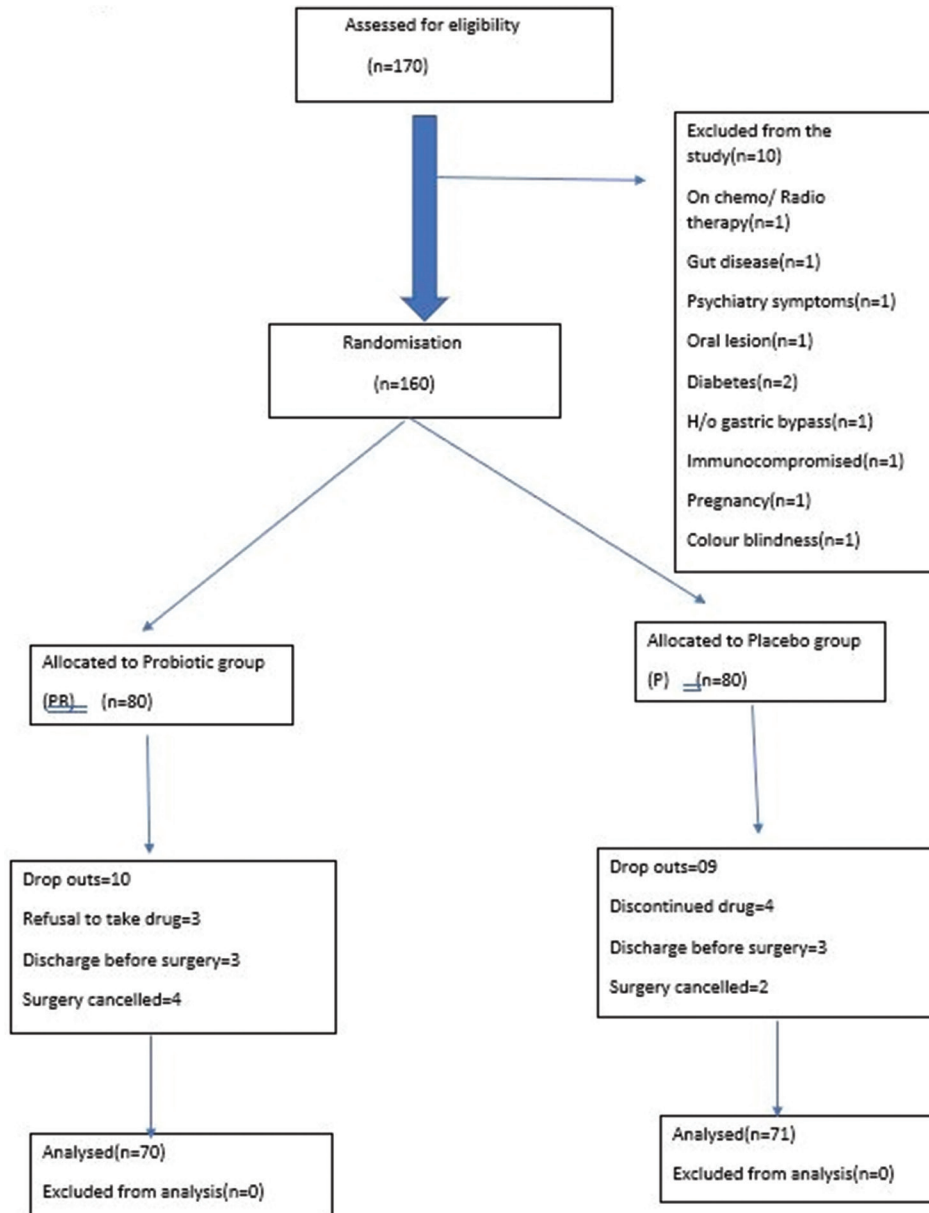


Figure 1: Diagram for the flow of participants through each stage of the study

mean ± standard deviation (SD), mean difference and SD of difference.

Chi-square test was applied for the categorical data (patient demographics)[Table 1]. Student’s *t*-test was applied for parametric data. Mann–Whitney test was applied to compare the non-parametric data between the groups. A *P*-value less than 0.05 was considered statistically significant. *P* < 0.01 was considered highly significant. All statistical analyses were performed using Statistical Package for Social Sciences version 23(SPSS23) (International Business Machine, New York).

RESULTS

On statistical analysis of demographic data with respect to patient age, gender and type of diet, it was found by Chi-square test that both groups were comparable with no significant difference between the groups [Table 1]. Three cases in *P* group and two in *PB* group were on antibiotics and this difference was not significant.

The primary outcomes of the study were changes in the SAA levels, PSS and PVT scores. For comparison of the PSS scores between the two groups, Student’s *t*-test was applied. It was found that PSS scores were

Table 1: Comparison of demographic data in both groups

	Group				Total		P
	Probiotic		Placebo		Count	%	
	Count	%	Count	%			
Age (years)							
30 and below	15	21.4	11	15.5%	26	18.4	0.393 NS
31-40	20	28.6	17	23.9	37	26.2	
41-50	18	25.7	17	23.9	35	24.8	
51-60	11	15.7	21	29.6	32	22.7	
>60	6	8.6	5	7.0	11	7.8	
Total	70	100.0	71	100.0	141	100.0	
Sex							
F	18	25.7	24	33.8	42	29.8	0.294 NS
M	52	74.3	47	66.2	99	70.2	
Total	70	100.0	71	100.0	141	100.0	
Diet							
Veg	34	48.6	39	54.9	73	51.8	0.450 NS
Non-veg	36	51.4	32	45.1	68	48.2	
Total	70	100.0	71	100.0	141	100.0	

Chi-square test was applied; NS: non-significant; veg-vegetarian

14.94 ± 5.77 and 13.24 ± 5.55 before and after the probiotic, respectively, with ($P = 0.00$) [Figure 2]. The PSS levels were non-significant in both the groups for before and after the probiotic/placebo treatment.

Student's *t*-test was applied to compare the PVT scores (in percentage) between the two groups. It was found that there was no significant statistical difference in the PVT scores ($P = 0.17$) before the start of probiotic/placebo treatment and there was a significant difference ($P = 0.03$) in PVT scores, 77.26 ± 24.64 before and 86.63 ± 17.88 after the drug PB [Figure 3].

On application of Student's *t*-test to compare the SAA levels between the two groups, it was found that there was a highly significant difference ($P = 0.00$) in the SAA levels 464.28 ± 128.78 (group PB) and 411.3 ± 128.55 (group P) that were recorded after the completion of probiotic/placebo therapy ($P = 0.00$) but the difference in SAA levels before the starting of probiotic/placebo therapy was not significant ($P = 0.49$) [Table 2].

t-Test was applied separately to compare each parameter (PSS score/PVT score/SAA levels) within each group before and after study drug/placebo therapy period. It was found that there was a highly significant difference in both groups for each of the parameters ($P = 0.00$) [Table 3]. This means that both placebo and probiotic formulations produce a significant change in PSS scores, PVT scores and

salivary amylase levels after administration for four and a half days, with a decrease in PSS scores, increase in PVT scores and a fall in SAA levels in probiotic group and the reverse with placebo group.

Mann-Whitney test was applied to compare the changes in various parameters between the two groups; again a highly significant difference was found in both groups for each parameter (PSS scores, PVT scores and SAA levels [Table 3]). This shows that both placebo and probiotic formulations produce a significant change in PSS scores, PVT scores and SAA levels after administration for four and a half days; nevertheless, it was observed that with respect to SAA levels, there was a mean difference of 52.85 in the probiotic group and - 69.32 in the placebo group. This shows that there is a definite decrease in the SAA levels after probiotic administration. No probiotic-related side effects were observed in any of the patients.

DISCUSSION

Anxiety and stress are said to start appearing in the patient as soon as the surgical procedure is planned. Anxiety related to surgery or anaesthesia cannot be separated. Various interventions, both pharmacological and non-pharmacological, have been tried to reduce preoperative anxiety.^[9,10] Pre-surgical patients were therefore selected as our study population.

The autonomic nervous system (ANS) controls the activity of the salivary glands. ANS activation due to stress results in the secretion of SAA.^[11,12] Several

Table 2: Comparison of salivary amylase levels between the groups

Parameter: salivary, amylase (units/ml)	Group	n	Mean	Std. deviation	95% Confidence interval for mean		P
					Lower bound	Upper bound	
Before drug P	Probiotic	70	464.28	128.78	433.57	494.99	0.49
	Placebo	71	446.34	175.93	404.70	487.98	
After drug P	Probiotic	70	411.43	128.55	380.78	442.08	0.00
	Placebo	71	515.66	185.88	471.66	559.65	

t-Test was applied; $P < 0.01$ highly significant

Table 3: Comparison of PSS, PVT scores and salivary amylase levels

Parameter	Group	Observation point	n	Mean	SD	Mean difference	SD of difference	Change (%)	P	Mann-Whitney P
PSS	Probiotic	Before drug P	70	14.94	5.77	1.70	2.02	11.38	0.00	0.000
		After drug P	70	13.24	5.55					
	Placebo	Before drug P	71	16.55	5.05					
		After drug P	71	17.44	5.49					
PVT (%)	Probiotic	Before drug P	70	77.26	24.64	-9.37	21.12	12.13	0.00	0.000
		After drug P	70	86.63	17.88					
	Placebo	Before drug P	71	82.56	20.98					
		After drug P	71	78.42	25.02					
Salivary, amylase (units/ml)	Probiotic	Before drug P	70	464.28	128.78	52.85	64.35	11.38	0.00	0.000
		After drug P	70	411.43	128.55					
	Placebo	Before drug P	71	446.34	175.93					
		After drug P	71	515.66	185.88					

t-Test was applied for within-group comparison, $P < 0.01$, SD: standard deviation. Mann-Whitney test was applied for between-group comparison, $P < 0.05$ - significant, PSS: Perceived Stress Scale, PVT: Psychomotor vigilance test

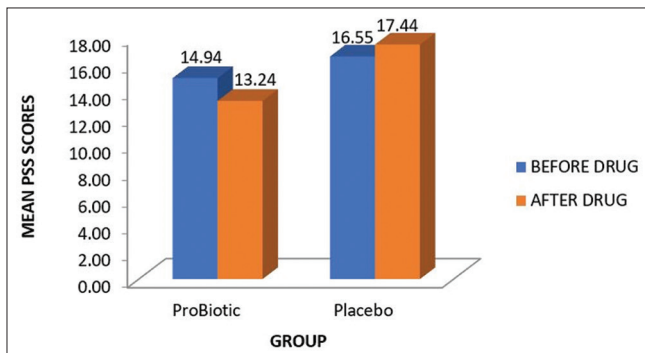


Figure 2: Comparison of PSS scores between the groups

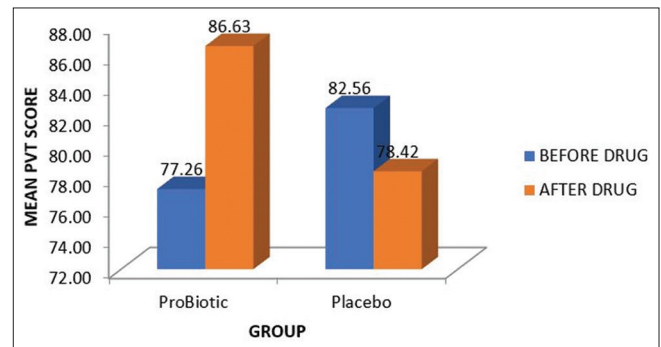


Figure 3: Comparison of PVT scores (percentages) between the groups

studies have indicated that SAA increases significantly during psychological stress and decreases by relaxation intervention and hence SAA is a good surrogate marker of sympathetic nervous system activation under stressful conditions.^[13,14] SAA has been also found to correlate with the amount of sleep a person receives; hence, we chose it as an objective indicator in our study.

The patients in our study had variable PSS scores, PVT scores and SAA levels before the start of either placebo/probiotic therapy. This is expected, because patients awaiting surgery may experience different levels of anxiety and stress depending on factors like patient age, gender, cultural and physiological status, personality development, level of education, diseases,

drug treatment, type and extent of planned surgery and previous surgical experience;^[14,15] however, both groups were comparable in terms of age, gender and type of diet and the difference in the basal PSS, PVT scores and SAA levels was not statistically significant in the patients. Environmental factors like dietary habits, drug treatment, intestinal motility, stool frequency and consistency can influence the gut microbiota composition.^[16] An equal number of vegetarians and non-vegetarians formed the population in our study and all of them received our hospital diet which includes vegetarian food without curds and buttermilk.

Antibiotics can alter gut flora significantly; nevertheless, not many of our study cases were receiving antibiotics.

In our study population, both probiotic and placebo produced a change in PSS, PVT scores and SAA levels after preoperative administration for 4 days; that means, both probiotic and placebo produced a change in stress and sleep levels; nevertheless, probiotic administration produced a significant decrease in the PSS scores, an increase in PVT scores and a decrease in SAA levels, whereas placebo administration produced an increase in the PSS scores, a decrease in the PVT scores and an increase in the SAA levels. All this goes to show that with placebo, the stress level increased and psychomotor vigilance decreased, maybe because of increased stress and reduced sleep; however with probiotic administration, the preoperative stress levels decreased and psychomotor vigilance improved, again possibly because of reduced stress and improved sleep. The significant fall in SAA levels after probiotic administration in our study population again shows the reduction in preoperative stress levels in the patients. This shows that the strategy of altering the gut microbiome with probiotics did produce a reduction in stress. Tension and stress are major predictors of sleep quality.^[17,18] So, if the stress was less as per SAA levels, it is assumed that sleep quality too would have been better.

SAA can be measured at the patient's bedside using a portable instrument;^[19] but we did not possess this tool. We got SAA estimated in the laboratory by enzymatic principle. This method has a sensitivity of 0.4 U/ml and an assay range of 2–400 U/ml.

Various authors have measured psychological stress and anxiety by various kinds of scales. We chose to use the PSS questionnaire for stress assessment and a mobile app for the PVT. The PSS is the most widely used psychological instrument for measuring the perception of stress, especially in stress perception research. It measures the degree to which situations in one's life are appraised as stressful.^[6] The PVT measures sustained attention and gives a numerical measure of sleepiness by counting the number of lapses in attention of the tested subject.

In the last few decades, an increasing number of studies on humans and small mammals have indicated a role of probiotics in regulating mood, cognition and response to stress via the bi-directional BGM axis.^[1,5]

A study in medical students showed that daily consumption of LB casei strain Shirota helped to maintain sleep quality during periods of stressful periods like during examinations.^[18]

A recent systematic review by Yang, Ju and Cheen of 21 studies on humans found that regulation of intestinal bacteria with probiotic and non-probiotic interventions was effective in treating anxiety.^[20] The probiotic formulation used in our study contained LA and BB; nevertheless, the most frequently used genera of probiotics are LB and BB.^[21] *Enterococcus*, *Streptococcus* and the yeast *Saccharomyces* are other commonly used probiotic preparations.

Several systematic reviews with probiotics have concluded that the overall safety record of probiotics is good;^[20,22-24] nevertheless, no side effects of probiotics were reported in our study cases.

The typical dosages of probiotics vary based on the product, but the common dosages for LB and BB range from 10 to 20 billion colony forming units (CFU) per day for adults. The probiotic formulation used in the current study population contained 5 billion CFU of LA and 12 billion CFU of BB per capsule. One capsule was given 12 hourly; so the dosage was well within the recommended range.

Our study presents several limitations. The exact mechanism by which the probiotics decreased stress and improved sleep is not known. Gut bacteria are said to directly stimulate intrinsic primary afferent neurons of the enteric nervous system to send messages to the brain via the vagus nerve which is said to be a key communicator between gut microbes and the CNS.^[4,5] Short-chain fatty acids (SCFAs) produced by fermentation of host dietary carbohydrate by LB and BB have been implicated as major signalling molecules mediating host-microbe communication via the enteroendocrine cells and enterochromaffin cells (ECCs). SCFAs regulate 5-HT release from the ECCs.

A break in the cold chain/a person with a particularly acidic stomach/slow digestion might kill the probiotic microbes before they reach the large intestines alive. All these factors could have affected our actual dose of probiotics; we, however, tried to maintain uniformity by avoiding patients on antacids and also keeping the probiotic refrigerated. Most study intervention times with probiotics for anxiety symptoms are in the range of 4–8 weeks. The intervention time in our study was only four and a half days. This might have been too short to significantly increase the population of newly introduced gut microbiota.

There are no studies to date by Indian researchers on the topic of the link between the gut microbes and the brain.^[25] Our study appears to be the only study to analyse biomarkers like SAA in relation to gut microbes. We feel that more trials with a careful complex analysis of causality and association between gut microbes and the brain are necessary.

CONCLUSION

Our study concludes that probiotics reduce stress and improve sleep in the surgical patient and this suggests a high possibility of a link between the gut microbes and the brain.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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ANNEXURE 1: PERCEIVED STRESS SCALE

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

01	In the last few days, how often have you been upset because of something that happened unexpectedly?	0	1	2	3	4
02	In the last few days, how often have you felt that you were unable to control the important things in your life?	0	1	2	3	4
03	In the last few days, how often have you felt nervous and 'stressed'?	0	1	2	3	4
04	In the last month, how often have you felt confident about your ability to handle your personal problem?	0	1	2	3	4
05	In the last few days, how often have you felt that things were going your way?	0	1	2	3	4
06	In the last few days, how often have you felt that you could not cope with all the things that you had to do?	0	1	2	3	4
07	In the last few days, how often have you been able to control irritation in your life?	0	1	2	3	4
08	In the last few days, how often have you felt that you were on top of things?	0	1	2	3	4
09	In the last few days, how often have you been angered because of things that were outside your control?	0	1	2	3	4
10	In the last few days, how often have you felt difficulties were piling up so high that you could not overcome them?	0	1	2	3	4

0=Never; 1=almost never; 2=sometime; 3=fairly often; 4=very often

Scoring ranging from 0 to 13 would be considered low stress

Scoring ranging from 14 to 26 would be considered moderate stress

Scoring ranging from 27 to 40 would be considered high perceived stress