Study Protocol

a) Research Design

This study employs a single-blind, parallel-group, two-arm randomized controlled trial (RCT) design, conducted in accordance with CONSORT 2010 guidelines. The trial compares daily passive listening to Mozart sonatas (intervention) against a silence control condition over a 4-week period. Assessments occur at baseline (Week 0) and immediately post-intervention (Week 4, within 48 hours of the final session) to evaluate pre–post changes. The design minimizes bias through computer-generated randomization and blinded outcome assessment while acknowledging that participant blinding is not feasible due to the nature of the auditory intervention.

b) Participants

The sample size was calculated using G*Power 3.1.9.7 with a F-test repeated-measures, within-between interaction, corresponding to a 2-group × 2-time-point mixed-design repeated-measures ANOVA. The parameters were set as follows: effect size Cohen's f = 0.25, $\alpha = 0.05$, power = 0.80, two groups, two measurements, correlation among repeated measures = 0.50, and nonsphericity correction $\varepsilon = 1$. Based on these assumptions, the minimum required sample size was 34 subjects in total (17 subjects per group). The study recruited 30 participants per group, resulting in a total sample size of 60 participants. A total of 60 healthy young adults (aged 18–30 years; 30 per arm) will be recruited from university campuses and community settings in Indonesia through flyers, social media, and word-of-mouth.

Inclusion criteria: Generally healthy young adults (18–30-year-old), have a job/ student undergoing college degree. Exclusion criteria: Antibiotic or probiotic/prebiotic use within the past 3 months; history of chronic gastrointestinal disorders (e.g., IBS, IBD); current smoking; diagnosed hearing impairment; professional musical training; pregnancy; use of corticosteroids or medications known to significantly affect cortisol levels; or any condition likely to confound stress or gastrointestinal symptom reporting. Eligibility screening used a structured questionnaire followed by written informed consent prior to the screening. The participants were allowed to drop/exit the study anytime and of their own accord, without any coercion.

c) Randomization and Blinding

Participants will be randomized in a 1:1 ratio to the Mozart music or silence group using computer-generated block randomization (block sizes of 4 or 6), stratified by sex and baseline perceived stress score (PSS-10 median split) to enhance group balance on key prognostic variables. Allocation concealment will be maintained through sequentially numbered opaque envelopes prepared by an independent researcher. Outcome assessors (laboratory technicians processing salivary cortisol samples and the statistician conducting final analyses) will be fully blinded to group assignment. Participants and the research coordinator cannot be blinded due to the obvious difference between music listening and silence, but they will be explicitly instructed not to disclose group allocation during assessments.

d) Grouping and Saliva Sampling

- Mozart Music Intervention Group (n=30): Participants will listen to a standardized 30-minute playlist featuring Mozart sonatas (e.g., Sonata for Two Pianos in D Major, K.448; Piano Sonata No. 16 in C Major, K.545; and selected movements chosen for their structured, predictable tempo ranging 60–120 bpm). The playlist will be delivered via a secure mobile app or pre-loaded device with noise-isolating headphones at a comfortable volume level (50–60 dB, verified with a sound-level meter). Sessions must be completed daily at a consistent time of day (preferably during a relaxed period, e.g., evening), in a quiet environment.

- Silence Control Group (n=30): Participants will sit quietly for 30 minutes daily with no auditory input (no music, podcasts, conversations, or other stimuli). They will use the same timing app for consistency but will be instructed to maintain complete silence.

To minimize expectancy and Hawthorne effects, both groups will receive identical instructions regarding posture, breathing, and avoiding additional stressors during the session. Adherence will be monitored using timestamped logs from the mobile app, supplemented by a daily paper diary and weekly researcher check-in calls or messages. A minimum compliance rate of 80% (≥23 out of 28 sessions) will be required for inclusion in per-protocol sensitivity analyses.

Approximately 3 ml of unstimulated saliva was collected in a 50 ml sterile tube (NEST, China) from each subject on the baseline prior to the study and 30-day post intervention/control. Prior to the downstream analysis, the saliva was centrifuged at 6000 rpm for 15 minutes to separate the debris from the saliva.

e) Data Collection via Survey

Several aspects of stress and gastrointestinal symptoms were collected via self-reporting validated survey. For stress aspect, we adapted and employed Perceived Stress Scale-10 survey (Harris et al., 2023) and Positive and Negative Affect Schedule (PANAS) survey (Andreu-Sánchez et al., 2023) to acquire whether Mozart Music Listening can affect both stress levels and mood of each participant. For gastrointestinal symptoms, we used Gastrointestinal Symptoms Ranking Scale (GSRS) (Alvarez et al., 2023) to assess the participants gastrointestinal conditions. The stool quality was also assessed via Bristol Stool Form Scale survey adapted from the survey created Shokouhi and colleague in 2022. The surveys were filled and collected on the baseline and 30 days after intervention/control.

f) Salivary Cortisol Quantification

The levels of salivary cortisol were quantified using Cortisol Saliva Competitive ELISA (Tecan, Männedorf, Switzerland, #Cat RE52611, #LOT ECO170). In brief, 50 µl of saliva samples, control, and standard were all pipetted into each respective wells in the given microtiter plate, followed by 100 µl enzyme conjugate in each well. The plate was then sealed and incubated while shaking at 18-25ºC on an orbital shaker (400-600 rpm) for two hours. The plate was washed four times with 250 µl 1x Wash Buffer for each well, and then dried by tapping the inverted plate unto a paper towel. 100 µl TMB Substrate solution was added to each well. The plate was then sealed and incubated while shaking at 18-25ºC on an orbital shaker (400-600 rpm) for 30 minutes. The reaction was stopped by adding 100 µl TMB Stop solution to each well. The color should change from blue to yellow. The mixture was then measured at the wavelength of 450 nm using a spectrophotometer (Tecan, Männedorf, Switzerland) within 15 minutes after pipetting the Stop Solution. The standard curve was generated by plotting the optical density of each cortisol standard wells and the concentration of each standard. The linear equation formula from the standard curve was then used to calculate the cortisol levels from each subject. The assay was done in duplicate.

g) Data Analysis

Data were analyzed using Statistical Packages for Social Sciences (SPSS) version 29 (IBM, Armonk, NY). Descriptive statistics were used to summarize participant characteristics and study variables. Categorical variables were presented as frequency and percentage, while continuous variables were presented as mean and standard deviation or median and interquartile range, depending on data distribution.

Before conducting inferential analyses, the normality of continuous variables was assessed using the Shapiro-Wilk test. For comparisons between the Mozart music intervention group and the silence control group, categorical variables were analyzed using the Pearson chi-square test. Continuous variables with normal distribution were analyzed using the independent t-test, whereas non-normally distributed variables were analyzed using the Mann-Whitney test.

Within-group comparisons between pre- and post-intervention measurements were conducted separately for each group. Normally distributed continuous variables, such as PANAS scores, were analyzed using the paired t-test. Ordinal or non-normally distributed paired variables, including PSS-10 categories, gastrointestinal symptom items, and Bristol Stool Form Scale categories, were analyzed using the Wilcoxon signed-rank test.

Changes in salivary cortisol levels from pre- to post-intervention were calculated for both groups. The difference in cortisol changes between the intervention and control groups was compared using the independent t-test. In addition, Bland-Altman analysis was performed to assess the agreement and mean difference between the intervention and control groups. All statistical tests were conducted using a significance level of $p < 0.05$.

h) Ethical Considerations

This study will be conducted in full compliance with the ethical principles outlined in the Declaration of Helsinki (World Medical Association, 2013) and according to the International Conference on Harmonization Good Clinical Practice (ICH-GCP) guidelines. The study has been approved by Institutional Review Board (IRB) Faculty of Dentistry, Universitas Trisakti with EC number: 071/S3/KEPK/FKG/04/2026.