Study title: Effects of Probiotics on Gut Microbiota, Endocannabinoid and Immune Activation and Symptoms of Fatigue in Dancers

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Study Protocol

1. Study Population Characteristics

A total of 51 female dancers initially expressed interest in participating in the study. From this group, participants aged 18 to 36 years, engaging in more than 8 hours of dance training per week and meeting the inclusion criteria were selected. Twenty-five individuals were excluded before randomization due to recent injuries, chronic diseases, the use of probiotics, prebiotics, or synbiotics, hospitalization, recent travel to tropical regions, or use of antibiotics, cannabinoid-based products, or corticosteroids within the past three months.

Ultimately, 26 dancers met the inclusion criteria. However, six withdrew early, citing scheduling conflicts and an inability to reconcile participation with training and personal commitments. Three participants did not attend scheduled blood/fecal collection appointments or collect the supplement/placebo. Thus, only 20 attended baseline sampling. Three of these later failed to complete the 12-week supplementation protocol. Although they participated in baseline sampling, they did not begin supplementation and provided no reason for withdrawal.

Seventeen dancers completed the full intervention without reporting adverse effects. Data from two were excluded during analysis: one due to an outlying BMI (overweight) and another for outlier values in biochemical markers (e.g., overdouble AEA levels). Ultimately, 15 participants (10 placebo, 5 probiotic) were included in the final analysis of serum markers (endocannabinoid and inflammatory markers), and 16 in fecal metabolomics (11 placebo, 5 probiotic). In the latter, only the participant with extreme BMI was excluded.

Maintaining participation proved difficult due to the demanding schedules of professional dancers. Long hours (often 8:00–20:00), performances, and irregular sleeping patterns made fasting morning appointments for blood collection challenging. Delivering stool samples on time and adhering to stable dietary habits over the 3-month intervention added further complexity. Only female students from the university's dance faculty met participation requirements. Recruitment attempts from external dance schools or theaters failed due to occupational demands or reluctance toward biological sample collection. As only two men applied, the final sample was restricted to women to avoid gender-related confounding effects in microbiome analysis.

2. Study objectives

The primary objective of this study was to evaluate the effect of a 12-week probiotic supplementation with *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 on gut microbiota composition, the gut metabolome, and endocannabinoid system markers including anandamide (AEA), cannabinoid receptor type 2 (CB2), and fatty acid amide hydrolase (FAAH) in professional dancers. Secondary objectives included the assessment of changes in serum inflammatory markers such as lipopolysaccharide (LPS), TNF-α, IL-1β, and IL-10, as well as the evaluation of gastrointestinal symptoms according to the ROME IV criteria, sleep quality measured by the Pittsburgh Sleep Quality Index, fatigue assessed with the Fatigue Assessment Scale, pain threshold determined by algometry, and coping strategies with stress assessed using the Mini-COPE questionnaire.

3. Study Design

This exploratory study was conducted as a randomized, double-blind, placebo-controlled trial. Participants were randomly assigned to either a probiotic or placebo group using block randomization (block size = 4), managed by the supplement manufacturer. Both participants and researchers were blinded to group allocation. The 12-week intervention consisted of daily supplementation with a probiotic containing *Lactobacillus helveticus* R0052 (Rosell-52, CNCM I-1722) and *Bifidobacterium longum* R0175 (Rosell-17, CNCM I-3470), commercially available as Sanprobi Stress (Sanprobi, Poland). Each capsule contained 3×10^9 CFU and was taken in the morning. Placebo capsules matched in shape and mass and contained maltodextrin and corn starch. Participants were instructed not to alter their diet or training habits and to report deviations such as illness, medication, or dietary changes. Adherence was monitored through daily intake diaries and return of unused capsules. All analyzed participants consumed all 84 doses.

4. Assessment Methods

Anthropometric and Functional Measures

Baseline assessments (1 week prior) included weight, height, BMI, and body composition (fat mass, lean mass, muscle mass) in resting conditions. Blood morphology (WBC, LYM) was measured via flow cytometry (Synergy 2 SIAFRT, BioTek) to screen for infections and assess

immunological and nutritional status. All participants were active dancers or students training ≥8 hours/week. Physical activity level was self-reported. Handgrip strength was assessed using a dynamometer (three trials per hand; average recorded). Pressure pain threshold was measured on the thumb flexor muscle using algometry (average of three values). Dietary intake was assessed through 3-day food diaries (2 weekdays, 1 weekend day), analyzed via Nuvero software to quantify energy, macronutrients, fiber, and cholesterol. Diet quality was assessed with the 14-item Mediterranean Diet Assessment Tool (MDAT) and verified through interview. No significant baseline dietary differences were found between groups.

Biochemical Analysis of Serum Markers (ECS, LPS, Cytokines)

Fasting venous blood samples (10 mL) were collected pre- and post-intervention. Serum was analyzed using ELISA kits (SunRed Biotechnology, China). CB2 and FAAH levels were assessed (sensitivity: CB2 \~0.285 ng/mL; FAAH \~0.116 ng/mL). AEA and LPS levels were measured using separate kits (AEA sensitivity \~7.125 ng/mL; LPS \~10.725 EU/L). Cytokines measured included TNF-α, IL-1β, and IL-10 (sensitivities \~2.827 ng/mL, \~15.013 pg/mL, \~9.012 pg/mL respectively). Intra- and inter-assay CVs were <10% and <12%, respectively.

Fecal Metabolomics

Stool samples (\~10–30 g) were self-collected and stored at –18°C at home, then transferred to –80°C. Untargeted metabolomic analysis was performed using LC–MS (UHPLC Sciex ExionLC with TripleTOF 6600+). Separation used a 45-minute gradient on a Phenomenex Luna Omega Polar C18 column. Mass spectra (m/z 50–850) were acquired using SWATH. Identification used Sciex OS v3.3.1, All-In-One library, NIST, and an internal lab database. Mass error tolerance was \~2 ppm.

Psychosocial and Subjective Measures

Validated questionnaires included: Pittsburgh Sleep Quality Index (PSQI; score 0–21), Fatigue Assessment Scale (FAS; 0–32), Mini-COPE (28 items; 14 strategies; grouped into active coping, avoidance, emotional support), and a Rome IV-based GI symptom questionnaire.

Statistical Analysis Plan (SAP)

1. Blood Biomarkers and Questionnaires

Analyses were performed in STATISTICA 13.3. Normality was assessed using the Shapiro–Wilk test. Between-group differences: Student’s t-test or Mann–Whitney U. Within-group pre-

post comparisons: paired t-test or Wilcoxon. Two-way repeated measures ANOVA was used to assess time, group, and interaction effects (η^2 effect sizes, Bonferroni post-hoc correction). Correlations between AEA, LPS, and other variables: Pearson or Spearman coefficients. $p < 0.05$ was considered statistically significant.

2. Metabolomic Data

Data were filtered to remove low stability signals (QC CV > 25%) and outliers (5th–95th percentile), log10-transformed, and analyzed using MetaboAnalystR 4.0. PCA visualized global variance; ASCA tested group and time effects (1000 permutations). Mixed models (Kenward–Roger correction) evaluated group × time interaction. Univariate t-tests (FDR-adjusted) identified differentially abundant metabolites. Measurement stability was confirmed using quality control (QC) samples. Results were interpreted as exploratory.

Ethics

The study was approved by the Bioethics Committee of Poznań University of Medical Sciences (Approval No. 412/22). Written informed consent was obtained from all participants. The study was registered at ClinicalTrials.gov (NCT05567653) and conducted in accordance with the Declaration of Helsinki and CONSORT 2010 guidelines. No protocol amendments were made.