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Intake of *Lactiplantibacillus plantarum* HEAL9 reduces the inflammatory markers soluble fractalkine and CD163 during acute stress: A randomized, double blind, placebo-controlled study

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ABSTRACT

The intestine and the brain are connected via the brain-gut axis and the intestinal microbiota influences the immune activation and signaling molecules that are involved in the stress response. The aim of the study was to investigate if intake of the probiotic strain *Lactiplantibacillus plantarum* HEAL9 (LPHEAL9) for four weeks could counteract elevated cortisol and inflammation levels in subjects with chronic stress that are exposed to an acute stress test (Trier Social Stress Test, TSST). Seventy participants were included, and 63 participants completed the study (LPHEAL9, $n = 32$; placebo, $n = 31$). Cardiovascular reactivity and cortisol levels were affected by the TSST, but no differences between the groups were observed. Intake of LPHEAL9 did, however, result in significantly decreased plasma levels of two inflammatory markers (soluble fractalkine and CD163) compared to placebo. In conclusion, intake of LPHEAL9 for four weeks may reduce inflammatory markers coupled to acute stress in chronically stressed individuals.

1. Introduction

The intestine and the brain are connected via the brain-gut axis. The intestinal microbiota influences psychological reactions such as stress by bidirectional communication via neural, endocrine and immune pathways [17,37,38]. This results in activation of the immune system and signaling molecules that are involved in the stress response. Stress can also bring about changes in the microbiota and a reduction of the genus *Lactobacillus* has been observed in stressed individuals compared to non-stressed individuals [27]. Thus, modification of the microbiota by consumption of probiotic bacteria may modify stress responses. Intake of probiotics has in animal models shown to reduce anxiety-like behavior and influence the brain activity. In humans, intake of probiotics have shown to reduce stress related gastrointestinal symptoms, circulating proinflammatory cytokines, and the stress-related hormone cortisol [27].

Psychological stress during an extended period (chronic stress) is a source of concern in many postindustrial countries as it results in stress-related health problems affecting the healthcare system and contributing to sickness absence. WHO has stated mental health as one of the fundamental components of health and wellbeing. The global economic impact of mental disorders has been estimated to reach more than US$ 16 million million between 2011 and 2030 [49]. During stress, there is an increase in the sympathetic nervous- and immune system crosstalk leading to release of a pro-inflammatory response, which in the short run is beneficial for the body's capacity to fight the trigger. However, in the long run as in a chronic stress state this may instead suppress the effects of the acute response and lead to negative health effects caused by low-grade inflammation [39]. Low-grade inflammation is linked to several common diseases, such as cardiovascular diseases, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), type 1 diabetes, depression and Alzheimer's disease [10].

To gain further knowledge about stress-related health problems, and the acute reactions caused by a stress trigger, the Trier Social Stress Test

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(TSST) is a widely used tool to induce psychosocial stress in a laboratory setting [28]. The TSST consists of two parts; one job-interview setting and one arithmetic task. It can be performed both in a real setting, as well as in a virtual reality world [25].

Results from studies are mixed and still much are unknown about the relation between acute and chronic stress [44]. We have earlier used a virtual TSST to investigate the acute stress response in men with chronic stress [32]. The study showed that subjects with chronic stress got a higher level of cortisol and IL-1β after the TSST in comparison to subjects that did not have chronic stress. The aim of the present study was to investigate if intake of the probiotic strain *Lactiplantibacillus plantarum* HEAL9 (LPHEAL9) for four weeks before the TSST could counteract the elevated cortisol and inflammation levels seen in subjects with chronic stress. LPHEAL9 has earlier been used in studies in combination with one other strain (*Lacticaseibacillus paracasei* 8700:2) and shown positive effects on the immune response by reducing the frequency, duration and symptoms of common cold infections [4,7]. It is also genetically very similar to another strain, *Lactiplantibacillus plantarum* 299v, that has been evaluated in students during examination induced stress [2]. In the study with the probiotic strain *Lactiplantibacillus plantarum* 299v, students with an upcoming academic exam consumed either the probiotic or placebo for 14 days and at day 15, the cortisol level in the saliva was significantly lower in the probiotic group compared to the placebo group.

Cortisol is a stress-related hormone, elevated as a response to stress and considered an objective biological marker of stress. Relevant markers for acute stress are also C reactive protein (CRP), interleukin IL-6 and IL-10, tumor necrosis factor (TNF)-α and other compounds coupled to inflammation [36].

Fractalkine (CX3CL1) is a chemokine that is prominent in the intestinal epithelium [43], making it interesting to measure in relation to probiotics. A receptor (CX3CR1) that can bind fractalkine has been demonstrated in natural killer (NK) cells, monocytes and T cells. Fractalkine can be either membrane-bound or soluble and the soluble form is formed by cleavage of the membrane bound form by proteases ADAM10 and ADAM17 [24]. ADAM10 is responsible for constitutive shedding, whereas ADAM17 activity increases cleavage in response to cell activation. It has previously been shown that fractalkine is involved in the majority of inflammatory diseases, where the increased expression of fractalkine results in progression of the diseases. For example, fractalkine has been shown to be involved in atherosclerosis and cardiovascular diseases, as it contributes to the plaque development and rupture [24]. ADAM17 is also involved in the shedding of CD163 from the cell surface during an inflammatory reaction [12]. The scavenger receptor CD163 is expressed on macrophages and the expression is induced by glucocorticoids [29]. The level of the soluble form (sCD163) is increased in low-grade inflammation [42] as well as during use of antidepressants [41]. In the present study we measured the level of soluble fractalkine and sCD163 during acute stress and investigated if the plasma levels were affected by intake of probiotics.

2. Method

2.1. Participants

Healthy males and females aged 19–35 years, were recruited through advertisements at Lund University. The subjects were asked to have a high stress level in everyday life and has been doing so for the past six months. Interested subjects were sent a link to a web-based screening form where they filled in the Shirom-Melamed Burnout Questionnaire (SMBQ) to evaluate if they had a high stress level for a longer time or not (SMBQ score ≥ 3.75). If the subject fulfilled all inclusions and exclusion criteria, they obtained more information about the study and if they were still interested an inclusion visit (visit 1) was scheduled. Exclusion criteria were a body mass index > 30, pregnancy, previous or ongoing contact with health care due to stress-related problems, known physical (diabetes, pulmonary or cardiovascular disease, celiac disease, thyroid problems, gastrointestinal disease) or mental illness, consumption of psychotropics, beta blockers, asthma or rheumatoid drugs, steroid drugs or creams containing cortisone. Furthermore, participants were excluded if they had consumed any antibiotics three months, or probiotics two weeks prior to start of intake of study product. Intake of other probiotics during the study was also not allowed. The participants were informed that intake of antibiotics during the study was not allowed and would lead to direct exclusion.

2.2. Study design

This was a single-center, randomized, double blind, placebo-controlled clinical study including 70 healthy test participants that had chronic stress. The participants consumed either a probiotic strain (10^{10} cfu/day) or a placebo product. The study duration was 6 weeks divided in two periods: 1) A run-in period of two weeks and 2) an intervention period of 4 weeks. Visits were made before the run-in period (visit 1, inclusion) and in the end of the study after 4 weeks intervention when the TSST was made (visit 2, intervention day 30 $\pm$ 3). The study was performed between September 2017 and April 2018 at the Department of Food Technology, Engineering and Nutrition (visit 1) and at the Department of Design Studies (visit 2) at Lund University, Lund, Sweden. Before any study related procedures, all participants gave written informed consent, which specifically indicated that participation was voluntary and could be terminated at any time without giving any reason. The study was approved by the regional ethical review board at Lund University (Dnr. 2017/402), Sweden, registered on ClinicalTrials (NCT03284905) before the first test participant was included, and followed the Declaration of Helsinki.

2.3. Study product

The study product consisted of capsules containing either the probiotic bacteria *Lactiplantibacillus plantarum* HEAL9 at a concentration of 10^{10} cfu/capsule or placebo capsules without the bacteria. The filler used in the capsules was maize starch and magnesium stearate was used as anti-caking agent. Both the probiotic and the placebo capsules consisted of a white powder and had similar appearance, texture and taste. The probiotic mixture contained traces of soy. The study product was prepared in labeled packages of 40 capsules per participant and labeling was done according to the corresponding randomization list. The randomization list was computer-generated with a block size of four to either LPHEAL9 or a placebo product (1:1) by an external statistician. The study product was packed externally, and specific personnel not involved in the study were responsible for the labeling. All capsules needed were handed out at visit 1 and study personnel provided the participants with study product in the order they were randomized. The study was double-blind, and the participants and personnel involved in the study did not know which product (LPHEAL9 or placebo) that was distributed. The study product was taken once daily in connection with breakfast by chewing the capsule and swallowing the entire content. This was done for extra compliance measurements, as saliva samples were taken before and at the end of the four-week study. At the end of the study (visit 2), participants were asked to hand in the remaining capsules that were counted to verify that the right number of capsules was ingested. The participants were also instructed to note daily intake of study product in the study diary.

2.4. Stress induction

The Trier Social Stress Test (TSST) was used to induce social stress in laboratory settings [28] at visit 2. In the present study the TSST was performed in a virtual reality environment (V-TSST) according to [25] and conducted in the afternoon (1 pm to 5 pm) to avoid diurnal fluctuations of cortisol. Briefly, the test participant was asked to hold a

speech and perform an arithmetic task in front of a committee. The committee consisted of three animated actors who showed no emotional responses to the test participant, making the situation very stressful. The V-TSST was created with a CAVE™ system (Cave Automatic Virtual Environment, Electronic Visualization Laboratory at the University of Illinois) with three rear-projected walls (4 m × 3 m), and one floor projection. For 3D-vision, passive stereoscopy was used. Two virtual rooms were created using the software Autodesk1 3 ds Max1 9 and EON professional 5.5 (EON Reality, Inc.): a waiting room including a table, pictures on the walls, two chairs, a small table and a door on the opposite wall. Behind the door there was a room with one woman and two men (designed by the company AXYZ design) constituting the committee [25,48]. Comments and instructions from the committee were given by prerecorded voices, in accordance with the standard TSST protocol [28]. Comments were activated by the test leader with a remote keyboard invisible to the test participant. For example, if the participant had difficulties continuing the presentation, a member of the committee told him that "you have time left," or "please continue, I will tell you when your time is up". The V-TSST has been shown to evoke reliable cortisol and cardiovascular responses in healthy females and males [14,25,26]. Since the cortisol level varies during the menstrual cycle, the female participants were, if possible, performing the TSST in their luteal phase.

2.5. Procedure

Before the test, participants were told that they were going to perform an acute stress test in a virtual reality environment and that saliva and blood would be sampled, but no other details were specified. Some of the participants knew each other, but all were explicitly told not to tell anyone about the details of the experimental procedure since that knowledge could affect the outcome [25]. Participants did not consume food, drinks or use nicotine 2 h before the V-TSST. On arrival to visit 2, saliva was sampled, and a nurse applied an indwelling catheter (1.1 × 33 mm, B Braun Melsungen AG, Germany) in the arm. The participant then rested for 50 min before the V-TSST was carried out [1]. The SMBQ, the state form of the State-Trait Anxiety Inventory (STAI-S) and a gut function questionnaire were completed during this time (see below for information about the questionnaires). To avoid variations, the same member of the technical staff took care of, informed, and performed the V-TSST for all participants, without knowing if the participants had taken LPHEAL9 or placebo product. The V-TSST was carried out with the sequence of conditions described by Jönsson et al. [25] but recovery was prolonged to 60 min [32]. The setting of the V-TSST was thus: an initial condition of rest for 5 min (BASE), 5 min to prepare the speech (PREP), 5 min of speech (SPEECH), 5 min for the arithmetic task (counting backwards from 1687 in steps of 13) (MATH), followed by 60 min of recovery during rest (R + 10, R + 20, R + 30, R + 40 and R + 60). At the end of the recovery period, the state form of STAI-S was completed again and questions about gut function were asked.

2.6. Questionnaires

2.6.1. Shirom-Melamed burnout questionnaire (SMBQ)

The SMBQ consists of 22 items that estimate four dimensions of burnout syndrome: burnout, tension, listlessness, and cognitive weariness [40]. The SMBQ global score is represented by the mean of the four dimensions. The Swedish translation has previously been validated [18,34]. SMBQ was measured at visit 1 (inclusion) and at the day when the TSST experiment took place (visit 2).

2.6.2. Spielberger state and trait anxiety inventory (STAI-S)

The state scale of STAI (STAI-S) was assessed at visit 2 before BASE and after 60 min of recovery to estimate the participants' experiences of V-TSST. At the second assessment, the instructions were slightly changed, from "answer the questions how you feel right now" to "answer to the questions based on how you felt during the V-TSST". The time point for the second assessment was 60 min after the TSST since we did not want to disturb the participants more than necessary during the recovery period, and also to be able to compare the results with prior studies.

2.7. Measurement of efficacy and safety

The primary endpoint was to assess whether intake of LPHEAL9 could counteract elevated levels of cortisol in participants that were exposed to acute stress. The secondary endpoints included inflammation markers, the gut permeability, heart rate, heart rate variability, abdominal pain, flatulence and bloating in participants that were exposed to acute stress.

2.7.1. Heart rate (HR)

Electrocardiography (ECG) and respiration were recorded at 1 kHz using the ML866 Power Lab data acquisition system and analyzed using its software Chart 5 (ADInstruments Pty Ltd.) and MATLAB (MathWorks, Inc., Natick, MA). ECG was assessed using disposable electrodes (Lead II Einthoven) and respiration using a strain gauge over the chest. Mean Heart Rate (HR) was analyzed for 5 min in each condition: BASE, PREP, SPEECH, MATH, and during the four subsequent resting periods (R + 10,… R + 40), i.e. 8 conditions [25].

2.7.2. High frequency heart rate variability (HF-HRV)

R-R intervals were transformed to a tachogram (ms) and linearly interpolated at 4 Hz. The data were further linearly detrended and high-pass filtered (second order Butterworth filter, 0.10 Hz) to eliminate fluctuations below the respiratory frequency. For each 5-min sequence, HRV power spectra were calculated for 17 segments of 128 points (32 s) with 50% overlap, using a fast Fourier transform (1024 points) following the application of multiple peak matched windows. The peak matched multiple windows (PM MW) method optimizes the mean square error of the spectrum estimate when the spectrum can be expected to include peaks [20,22]. The PM MW method has been shown to give reliable results for the HRV spectrum [19,21]. The integral of the power spectrum was studied in the high frequency (HF) region (0.12–0.4 Hz) that is related to respiration [5]. The data were log transformed (ln) to approach a normal distribution. The respiration measures were used to ensure that the respiratory rate was within the HF range. The same timepoints as for HR was applied for HF-HRV.

2.7.3. Gut function

At visit 1, basal questions about the gut function were asked. Further assessment of the gut function by filling in a VAS scale (0–10) for abdominal pain, flatulence and bloating was done three times; once at visit 1 and twice at visit 2 (before and after the TSST).

2.7.4. Biochemical analyzes

2.7.4.1. Lactobacilli. Oral lactobacilli were analyzed in order to verify intake of LPHEAL9 versus placebo. Saliva from the participants was collected before (visit 1) and after treatment (visit 2). Saliva and 2x Hogness Freezing Media (9.8 mM K_2HPO_4, 2.9 mM KH_2PO_4, 10.2 mM $C_6H_5Na_3O_7$ * 2 H_2O, 2.0 mM $MgSO_4$ * 7 H_2O, 24.2% glycerol) were mixed in equal amounts before frozen and stored at −80 °C. Abundance of lactobacilli was evaluated by plate count. The samples were diluted and spread on Rogosa agar plates (Oxoid, England) and the plates were incubated at 37 °C for 72 h under anaerobic conditions (2.5 L, AnaeroGen, Oxoid). The detection limit was 2.9 $\log_{10}$ (cfu/mL).

2.7.4.2. Cortisol, zonulin and inflammation markers. Peripheral venous blood was collected eight times; twice before the TSST (BASE, PREP), directly after the challenge (TSST; SPEECH + MATH) and five times during the recovery phase (R + 10 min, R + 20, R + 30, R + 40 and

R + 60). Serum was separated at room temperature at 2000 x g for 10 min and plasma was kept in ice bath and separated at 4 °C at 2000 x g for 10 min within 30 min after sampling, using EDTA as anticoagulant (Eppendorf Centrifuge 5702R). Serum and plasma samples were frozen on dry ice and then stored at −80 °C until further analyzes.

Plasma cortisol levels were determined with a one-step competition assay with the Electrochemi-luminescence Immunoassay (ECLI) detection technique based on ruthenium derivate and was conducted by Labmedicin Skåne (University and regional laboratories in Region Skåne, Sweden), with a detection limit of 1.5 nmol/L. CRP was also analyzed by Labmedicin Skåne using an immune turbidimetric analysis (detection limit 0.6 mg/L). Zonulin was analyzed in serum using an ELISA method (K5601, Immundiagnostik AG, Bensheim, Germany), detection range 0.25–64 ng/mL. This method has since we used it been found not to measure zonulin [46]. The company has thus renamed what is analyzed to zonulin family peptides (ZFP) and therefore the results are presented as this. The cytokines were analyzed in plasma using multiplex technology (U-PLEX human 7-plex (fractalkine, IFN-γ, IL-10, IL-1β, IL-6, IL-8, TNF-α), MesoScale Discovery, Rockville MD, US), with detection limits 102 pg/mL, 1.7 pg/mL, 0.14 pg/μL, 0.15 pg/mL, 0.33 pg/mL, 0.15 pg/mL and 0.51 pg/mL, respectively. Since fractalkine was significantly affected by intake of LPHEAL9 a *post hoc* analysis of soluble CD163 was performed with ELISA (DY1607, R&D Systems, Minneapolis, US), according to manufacturer's instructions. All samples were diluted 1:200 before analysis and have a reported detection limit of 156 pg/mL.

2.7.5. Safety

Adverse events were recorded during visit 2 after 4 weeks intervention by asking the participants and by checking the participant's diary where adverse events should be filled in. The focus was on gastrointestinal related adverse events.

2.8. Statistics

All correctly included and randomized participants that underwent the TSST were included in the full analysis set (FAS) and all randomized participants that consumed at least one capsule of the placebo or study product were included in the safety set. In order for the participants to be included in the per-protocol set (PPS) they had to fulfill the following criteria: The TSST was carried out day 30 ± 3 of the intervention, at least 80% of the study product had been consumed, no antibiotics were consumed during the study and no other probiotic products had been consumed after the start of the study (single intake allowed). Prior to breaking the codes, the definitions of the PPS were reviewed and the decisions for inclusion of participants were documented.

The statistical analysis was done based on repeated measures ANOVAs with CONDITION as repeated factor and intervention (LPHEAL9 and placebo) as between group factor. Thus, an analysis of changes over time (CONDITION) was also included. AGE, BMI, gender and the initial SMBQ scores were used as covariates and entered stepwise. Covariates that did not contribute significantly to each model were removed and were not reported. When needed, variables were transformed with ln, $\log_{10}$ or sqrt but for ease of interpretation, untransformed data is presented. To meet the assumption of sphericity Greenhouse-Geisser correction was carried out and reported with corrected *p*-values, non-corrected *df* and ε. $\eta^2_{partial}$ was reported as effect size ($\eta^{2_{partial}}$ = 0.01, 0.06, >0.14 were interpreted as small, medium and large effect size, respectively). Significant omnibus effects were followed up with polynomial contrasts. The BASE level was used as the baseline. Outliers with deviation larger than 3 SD from the mean were removed in each group before the analysis: cortisol (placebo, n = 2; LPHEAL9, n = 2); fractalkine (placebo, n = 6; LPHEAL9, n = 2); sCD163 (placebo, n = 2); IL-6 (placebo, n = 3; LPHEAL9, n = 2); IL-8 (placebo, n = 1; LPHEAL9, n = 1); TNF-α (placebo, n = 3); IL-10 (placebo, n = 1; LPHEAL9, n = 2); IL-1β (placebo, n = 3; LPHEAL9, n = 1); zonulin family peptides (placebo, n = 1); HR and HF-HRV (placebo, n = 2).

The correlation between fractalkine and sCD163 levels at each time point was analyzed with Spearman's correlation test. A comparison between the LPHEAL9 and the placebo group using single time-points was also done as an exploratory analysis using the Wilcoxon rank sum test. The baseline was in this case calculated as the mean of the BASE and PREP values. Change from baseline within a group at each time point was evaluated with Wilcoxon signed rank test. No outliers were removed. The change in gut function before and after the TSST was analyzed using Wilcoxon rank sum test (comparison of intervention groups) or with Wilcoxon signed rank test (comparison before/after within a treatment). The same statistical methods were used to compare the levels of lactobacilli in saliva, SMBQ global score and STAI-S. Statistical analyzes were performed in IBM SPSS version 25.0 and for all analyzes, α was set to 0.05.

2.8.1. Determination of sample size

The primary endpoint was the cortisol level during the TSST. The power determination was based on a medium-sized effect, f = 0.25, a power of 80% and α = 0.05. The number of levels for the repeating factor varied for the different measures. For example, zonulin family peptides were measured on three occasions and cortisol on eight occasions during the TSST. The power analysis was therefore based on three repeated measurements (since an increased number of measurements will lead to a lower number of participants). To be able to identify a GROUP (2) * CONDITION (3) interaction, 14 individuals were needed. However, usually the spherical requirements are not met and thus the degree of freedom had to be corrected for with a factor of 0.5, leading to 22 participants per group. The study sample was further increased to in total 60 since cortisol levels are not increased as a response to the TSST in approximately 20% of the population. Due to dropouts, 10 more participants were included, ending up with totally 70 participants.

3. Results

3.1. Participants

One hundred and eighty-six (186) individuals were screened among which 70 were eligible and included into the study (Fig. 1). Seven individuals did not complete the study. One person terminated the study before consumption of any study product and four of the excluded participants did not return their diaries and safety could not be evaluated. Sixty-five (65) participants were therefore included in the safety set and 63 participants in the FAS. Among the seven excluded from FAS, three of the participants consumed antibiotics between the time of inclusion and the TSST, two of the participants could not come on the test day, one withdrew the consent and one of the participants reported an adverse event and did not want to continue. Five of the participants were excluded from the PPS due to consumption of study product more than 33 days and one participant was excluded due to non-comparable TSST (had to wait for 45 min before the TSST could be done), leaving 57 participants in the PPS. The intake of study product (compliance) was good, ranging from 92 to 107% and thus no participant had to be excluded from the PPS due to a compliance lower than 80%. There was no significant difference in compliance between the two groups (mean intake in the LPHEAL9 group 98.6% and in the placebo group 99.2%). Few participants consumed probiotics regularly prior to visit 1 (4 of 70, 6%).

The FAS population of 63 participants had a mean age of 24.8 years and a mean BMI of 22.2 kg/m^2 (Table 1). Most of the subjects were students (90%). No differences were seen between intervention groups in demographic data at inclusion. All presented efficacy results are for

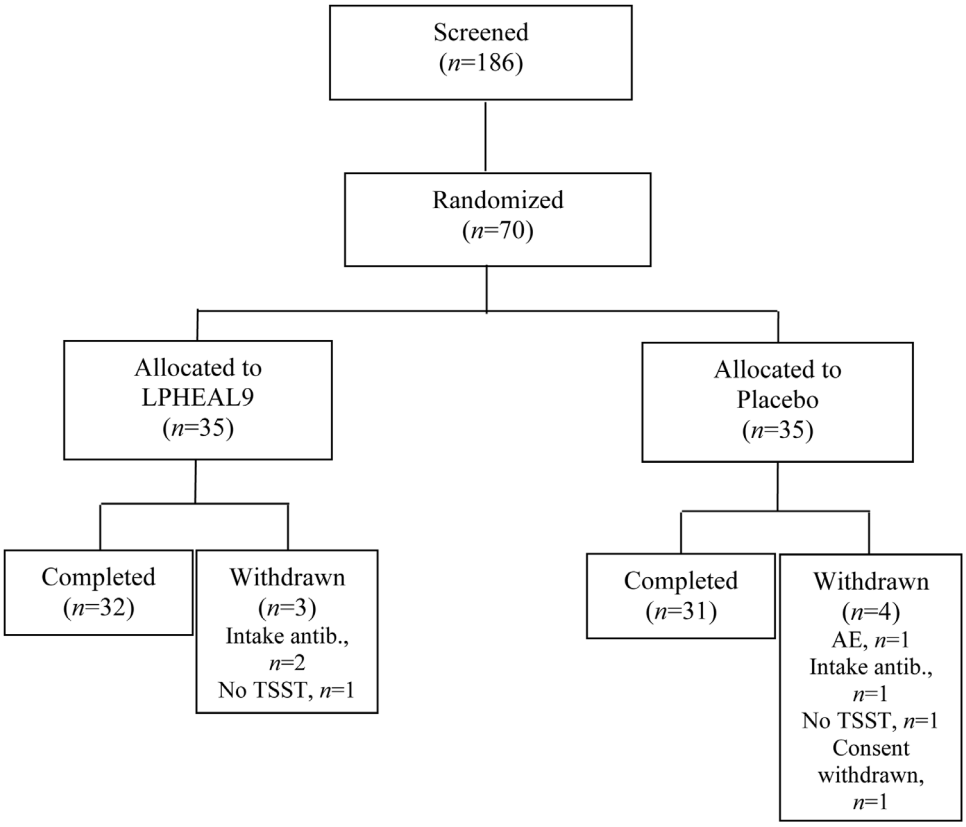

Fig. 1. Diagram presenting disposition of participants.

Table 1
Summary of demographic data at inclusion (mean, range).

	FAS LPHEAL9 (n = 32)	FAS Placebo (n = 31)	PPS LPHEAL9 (n = 30)	PPS Placebo (n = 27)
Females	63%	71%	63%	70%
Age, year	25.3 (19–35)	24.3 (19–32)	25.4 (19–35)	23.8 (19–32)
BMI, kg/m^2	22.4 (19–29)	21.9 (18–26)	22.2 (19–29)	21.9 (18–26)

No significant differences at inclusion between intervention groups.

the FAS, if not otherwise specified.

3.2. Counts of lactobacilli in the saliva

At the start before intervention 76% of the participants had detectable levels of lactobacilli in the saliva. The counts of lactobacilli in saliva were increased significantly in the group that consumed LPHEAL9, from a median value of 7.3 $\log_{10}$ cfu/mL before the intervention to 9.3 $\log_{10}$ cfu/mL after the intervention. For the placebo group the median lactobacilli value was similar before and after the intervention (4.6 and 5.3 $\log_{19}$ cfu/mL, respectively). After four weeks of intake, the level of lactobacilli in saliva was significantly higher in the LPHEAL9 group compared to the placebo group ($p < 0.0001$).

3.3. SMBQ global score and state-trait anxiety inventory (STAI-S)

At inclusion, the mean (*SD*) SMBQ value was 4.79 (0.63) in the LPHEAL9 group and 4.66 (0.62) in the placebo group. After four weeks of intervention the SMBQ global score was reduced significantly in both groups to 4.13 (0.76) in the LPHEAL9 group and to 4.18 (0.90) in the placebo group, but no significant difference between the groups was found. About 1/3 of the participants had a SMBQ global score below 3.75 after the intervention, and thus were not classified as highly stressed at this timepoint. Both the LPHEAL9 and the placebo group reported an induced acute stress effect, measured by STAI-S, related to the TSST ($p < 0.0001$).The mean scores (*SD*) for STAI-S before and after V-TSST were in the LPHEAL9 group 40 (7.0) and 52 (11.0), respectively, and in the placebo group, 38 (9.5) and 52 (9.6), respectively.

3.4. Cortisol levels

Cortisol was the primary endpoint in the study and a main effect of CONDITION, $F(6, 342) = 21.42$, $p < 0.0001$, $\eta^2 = 0.27$, $\epsilon = 0.409$, showed that the cortisol level increased after stress induction and after 10 min of recovery, and then slowly decreased as a function of time, linear contrast $F_{linear}(1, 57) = 18.72$, $p < 0.0001$, $\eta^2 = 0.25$, quadratic contrast $F_{quad}(1, 57) = 24.35$, $p < 0.0001$, $\eta^2 = 0.30$, and cubic contrast $F_{cubic}(1, 57) = 23.10$, $p < 0.0001$, $\eta^2 = 0.29$ (Fig. 2). No significant differences in cortisol levels between the groups were found.

Including participants that reacted to the TSST with increased cortisol levels (77% of the participants, LPHEAL9 $n = 23$, placebo $n = 21$, PPS) showed a significant increase in cortisol levels compared to baseline, 0 and 10 min after the TSST, for both groups. For the placebo group it was also significantly increased compared to before the test at 20 and 30 min. There was no significant difference between groups at any time point but at 10 min after the test there was a trend for a lower cortisol level in the LPHEAL9 group compared to the placebo group ($p = 0.07$).

A *post hoc* analysis was made on participants that did not have chronic stress on the day the TSST was made (SMBQ < 3.75, 33% of the participants, LPHEAL9 $n = 9$, placebo $n = 12$). This analysis showed a significantly lower cortisol level 10 min after the TSST ($p = 0.025$) for the LPHEAL9 group and there was a trend for a lower cortisol level also 30 min after the test ($p = 0.058$) compared to the placebo group.

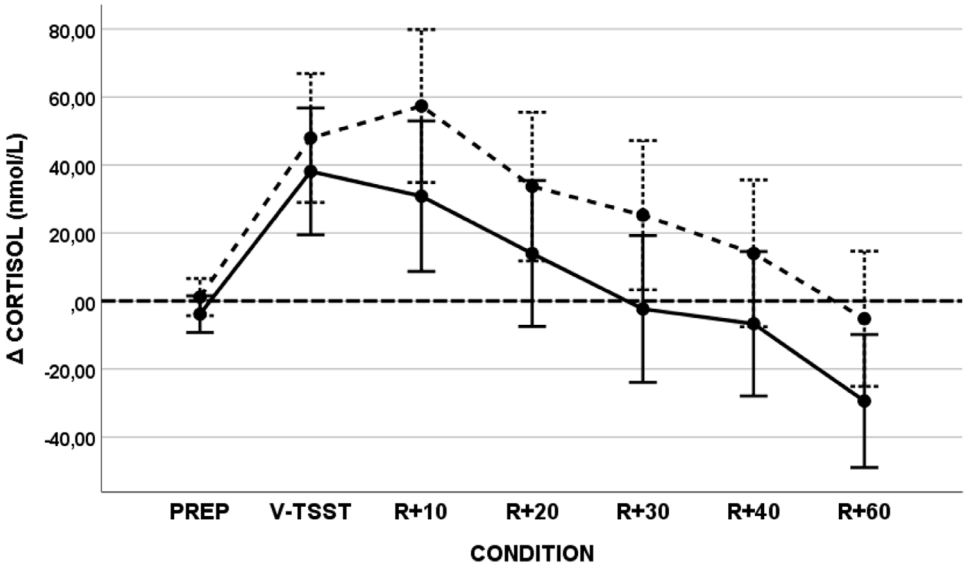

Fig. 2. Change in cortisol levels (nmol/L) after the TSST (mean ± *SE*), LPHEAL9 (—), Placebo (- -).

3.5. Inflammatory markers and zonulin family peptides

The level of soluble fractalkine was significantly affected by the stress test and increased and peaked 10 min after the TSST and then gradually decreased during the following 20 min (Fig. 3). After that the level increased again during the remaining recovery, $F(6, 318) = 14.21$, $p < 0.0001$, $\eta^2 = 0.21$, $\epsilon = 0.737$, $F_{linear}(1, 53) = 7.12$, $p < 0.010$, $\eta^2 = 0.12$, and $F_{cubic}(1, 53) = 39.61$, $p < 0.0001$, $\eta^2 = 0.43$. A main effect of GROUP was found, $F(1, 53) = 9.41$, $p = 0.003$, $\eta^2 = 0.15$. Compared to baseline, soluble fractalkine increased more in the placebo group, and remained over the levels of fractalkine in the LPHEAL9 group. The CONDITION*GROUP interaction effect was not statistically significant.

A *post hoc* analysis of soluble CD163 was made since it is released by ADAM17, the same protease that releases fractalkine. A main effect of CONDITION generally showed that sCD163 values decreased after the TSST to the end of the recovery, $F(6, 354) = 9.51$, $p < 0.0001$, $\eta^2 = 0.14$, $\epsilon = 0.862$, together with $F_{linear}(1, 59) = 28.68$, $p < 0.0001$, $\eta^2 = 0.33$, and $F_{cubic}(1, 59) = 14.97$, $p < 0.001$, $\eta^2 = 0.20$ (Fig. 4). Also the GROUP*CONDITION interaction was significant, $F(6, 354) = 2.56$, $p = 0.026$, $\eta^2 = 0.04$, $\epsilon = 0.862$, $F_{quad}(1, 59) = 4.90$, $p = 0.03$, $\eta^2 = 0.08$, and $F_{cubic}(1, 59) = 9.48$, $p = 0.003$, $\eta^2 = 0.14$. After baseline the placebo group's sCD163 values decreased at preparation and then increased and peaked after stress induction. After that the values gradually decreased as a function of time until 40 min of recovery, when a second increase followed. The sCD163 values for the LPHEAL9 group increased during preparation and then decreased during recovery until 30 min of recovery and continued to stay about the same levels during the rest of recovery. No other significant effects were found. Comparing single time points showed that the sCD163 level was significantly lower in the LPHEAL9 group compared with the placebo group at timepoints 0, 10, 20 and 60 min after the test. There was a significant correlation between fractalkine and sCD163 levels, 10 and 20 min after the TSST ($p = 0.001$ and 0.012, respectively).

The results for IFN-γ showed a significant main effect of CONDITION $F(6, 348) = 10.66$, $p < 0.0001$, $\eta^2 = 0.16$, $\epsilon = 0.702$, $F_{quad}(1, 58) = 4.87$, $p = 0.031$, $\eta^2 = 0.08$, and $F_{cubic}(1, 58) = 41.40$, $p < 0.0001$, $\eta^2 = 0.42$. After an initial small decrease, the IFN-γ level increased and reached the highest level 10 min after stress induction, after which it decreased below the baseline levels. No other significant

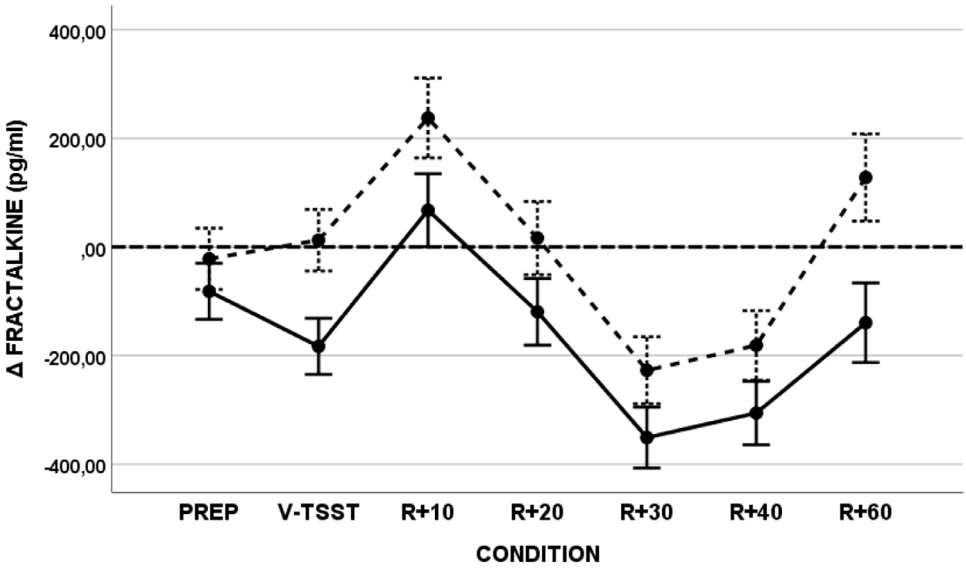

Fig. 3. Change in soluble fractalkine levels (pg/mL) after the TSST (mean ± *SE*), LPHEAL9 (—), Placebo (- -).

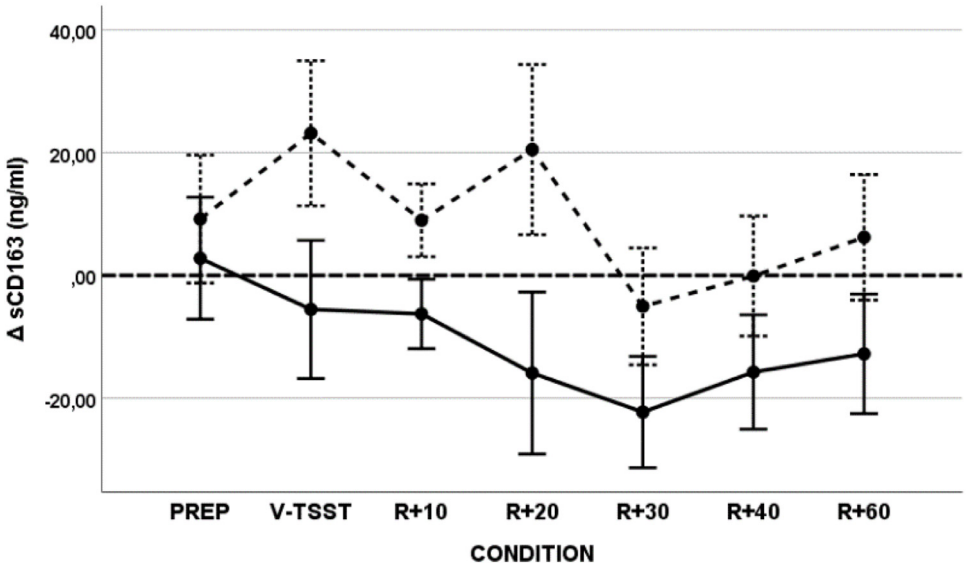

Fig. 4. Change in sCD163 levels (ng/mL) after the TSST (mean ± *SE*), LPHEAL9 (—), Placebo (- -).

effects were found. The main effect of CONDITION for TNF-α was significant, $F(6, 348) = 18.38$, $p < 0.0001$, $\eta^2 = 0.24$, $\epsilon = 0.819$. After a small decrease at preparation and TSST, TNF-α levels increased and peaked after 10 min of recovery. Then the levels gradually decreased until 40 min of recovery when a rapid increase occurred, $F_{linear}(1, 58) = 8.32$, $p = 0.005$, $\eta^2 = 0.13$, $F_{quad}(1, 58) = 7.96$, $p = 0.007$, $\eta^2 = 0.12$, and $F_{qubic}(1, 58) = 64.61$, $p < 0.0001$, $\eta^2 = 0.53$. No other significant effects were found.

Also the results for IL-6 showed a significant main effect of CONDITION, $F(6, 330) = 86.74$, $p < 0.0001$, $\eta^2 = 0.61$, $\epsilon = 0.502$, $F_{linear}(1, 55) = 173.70$, $p < 0.0001$, $\eta^2 = 0.76$, and $F_{cubic}(1, 55) = 19.77$, $p < 0.0001$, $\eta^2 = 0.26$ (Fig. 5). After preparation IL-6 levels starts to increase rather steep. After 10 min of recovery the level continuous to increase, but less steep, until 40 min of recovery where it increased steeper again. However, after the initial SMBQ scores was included as a covariate the effect of CONDITION completely vanished, $F(6, 324) = 1.05$, *n.s*. Generally, initial SMBQ scores covaried positively with IL-6 values, $F(1, 54) = 4.74$, $p = 0.034$, $\beta = 0.284$, 95% CI [0.004; 0.105].

A main effect of CONDITION was found for IL-8, $F(6, 354) = 9.92$, $p < 0.0001$, $\eta^2 = 0.14$, $\epsilon = 0.831$. During the preparation condition IL-8 levels were slightly lower than during baseline. Then IL-8 levels increased at TSST and then decreased until 30 min of recovery, followed by a second increase after 40 min of recovery, $F_{quad}(1, 59) = 14.79$, $p < 0.001$, $\eta^2 = 0.20$, and $F_{cubic}(1, 59) = 16.35$, $p < 0.0001$, $\eta^2 = 0.22$. Also the main effect of GROUP was significant, $F(1, 59) = 4.26$, $p = 0.043$, $\eta^2 = 0.07$. The IL-8 levels were increased more in the LPHEAL9 group than in the placebo group and remained higher during the rest of recovery. However, comparing single time points showed no significant difference between the groups and the CONDITION*GROUP interaction effect was not significant.

For IL-1β only 0.2% of the analyzed samples had a level above the median detection limit (0.15 pg/mL) and changes during the TSST were minor. The obtained levels of IL-10 were also low and only 9% of the samples had a value above the median detection limit (0.14 pg/mL). The IL-10 level did not change during the TSST (mean change 0.00 for both groups at all time points). About half of the participants had a CRP level below the detection limit at baseline (<0.60 mg/L) and small non-significant changes during the TSST were observed for both groups. Zonulin family peptides were measured at baseline and 10 and 60 min

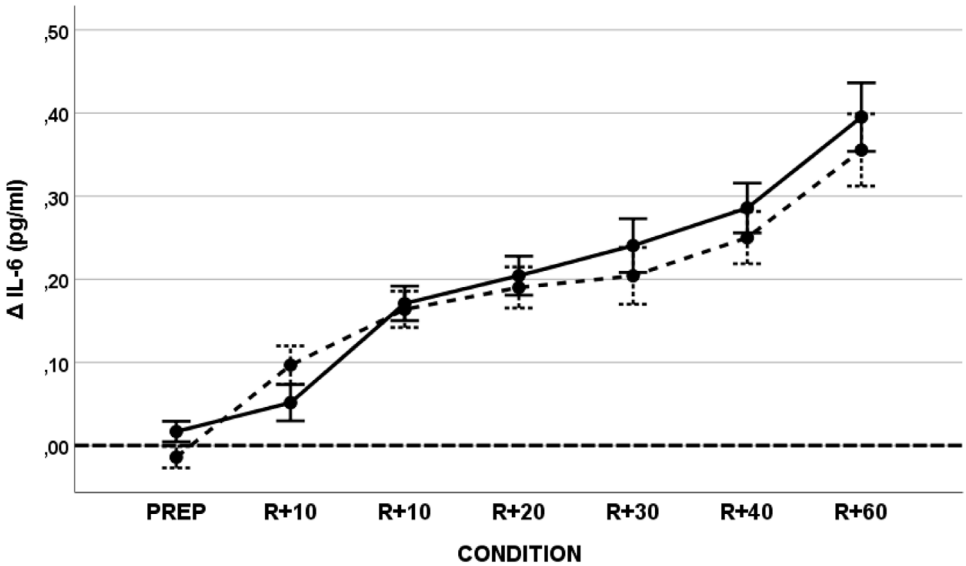

Fig. 5. Change in IL-6 levels (pg/mL) after the TSST (mean ± *SE*), LPHEAL9 (—), Placebo (- -).

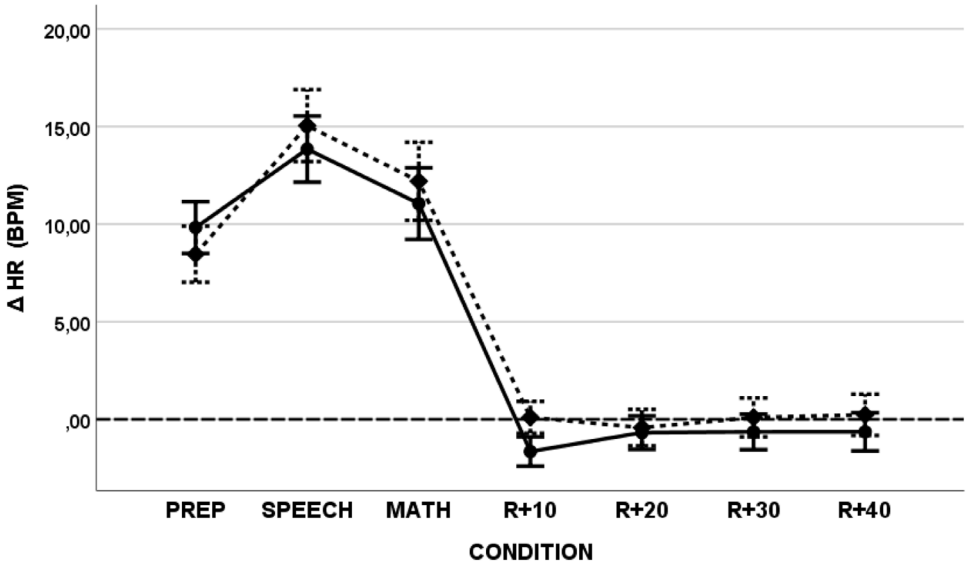

Fig. 6. Change in HR (BPM) during and after the TSST (mean ± *SE*), LPHEAL9 (—), Placebo (- -).

after the test. At baseline the mean zonulin family peptide level was 38 and 39 ng/mL for the placebo and LPHEAL9 group, respectively, and small non-significant increases were observed 10 and 60 min after the test, with no difference between the groups.

3.6. Heart rate (HR) and heart rate variability (HF-HRV)

The main effect of CONDITION was significant for heart rate, $F(6, 330) = 107.49$, $p < 0.0001$, $\eta^2 = 0.66$, $\epsilon = 0.44$, $F_{linear}(1, 55) = 157.37$, $p < 0.0001$, $\eta^2 = 0.74$, $F_{quad}(1, 55) = 18.94$, $p < 0.0001$, $\eta^2 = 0.26$, $F_{cubic}(1, 55) = 115.22$, $p < 0.0001$, $\eta^2 = 0.68$ (Fig. 6). After baseline HR increased and peaked at SPEECH where after it decreased and remained at baseline levels. Also for heart rate variability, the main effect of CONDITION was significant, $F(3, 330) = 6.12$, $p = 0.001$, $\eta^2 = 0.10$, $\epsilon = 0.46$, $F_{linear}(1, 55) = 8.09$, $p = 0.006$, $\eta^2 = 0.13$, and $F_{quad}(1, 55) = 13.32$, $p = 0.001$, $\eta^2 = 0.20$. HF-HRV decreased during PREP, SPEECH and MATH, increased during recovery the first 10 min and then it slightly decreased during the rest of recovery. No significant differences between the groups were found for HR and HF-HRV.

3.7. Gut function and gastrointestinal related adverse events

Abdominal pain, flatulence and bloating were evaluated three times (VAS 0–10); at inclusion before intake of study product and at the end of the intervention period, before and after the TSST. The symptoms were reduced during the intervention period in both groups and further reduced when comparing levels before and after the TSST. The mean value (*SD*) for abdominal pain for all participants at inclusion was 2.5 (2.2) and it was reduced to 1.6 (1.6) after the intervention and further reduced to 0.6 (0.8) after the TSST. The corresponding values for flatulence and bloating were similar (at inclusion 3.3 (1.8) and 2.9 (2.2), after the intervention 2.8 (2.1) and 2.3 (1.8), and after the TSST 0.9 (1.4) and 0.9 (1.1), respectively). No significant differences between the groups were found.

Gastrointestinal adverse events were reported by 21 participants (32%). Most of the events were mild and possibly related to intake of the study product. There were no differences between the groups in reported gastrointestinal adverse events; in the LPHEAL9 group 9 participants reported 16 events and in the placebo group 12 participants reported 20 events. The most common gastrointestinal adverse event was abdominal pain (8 events), followed by bloating (7 events) and rumbling stomach and nausea (5 events each).

4. Discussion

The Trier Social Stress Test (TSST) is one of the most widely used laboratory stress protocols. Studies have shown that cortisol as well as subjective stress responses to the TSST are significantly associated with acute stress responses in real life [23].The stress induction in the present study worked well since many of the stress and inflammation markers were affected significantly by the V-TSST (cortisol, fractalkine, sCD163, IFN-γ, IL-6, IL-8, TNF-α, heart rate, heart rate variability). This is in line with earlier studies [25,32]. The mean scores for STAI-S before and after the test were also comparable to an earlier TSST study [32].

The global scores for SMBQ before the intervention were comparable to those earlier observed in a TSST study on highly stressed subjects (mean value 4.64) [32].

Cortisol is suggested to be an objective biological marker of stress [51] and it was the primary endpoint in the study. Plasma cortisol correlates well with the level of cortisol in saliva [32] and therefore plasma cortisol was measured since blood was sampled to avoid further disturbance for the subject during the TSST by also sampling saliva. The cortisol level increased after the TSST in both groups but no significant difference between the groups was found. However, the cortisol level for the LPHEAL9 group was lower compared to the level for the placebo group, 10 min after the test, and this difference persisted throughout the 60 min recovery phase. As far as we know, this is one of the first times that TSST has been used to evaluate the effect on cortisol and inflammation markers after intake of probiotics. Thus, there was no earlier study to base the number of participants to be included in the study and if more participants had been included it may have been possible to detect a significant difference in cortisol levels between the groups. Another factor influencing the result was that some of the participants had a higher cortisol level before compared to after the TSST. Since the participants had chronic stress it is not surprising that some of them already had high cortisol levels when they arrived for visit 2 and when they learned more about the stress test it actually reduced the stress and thus led to decreased cortisol levels. For these participants it was therefore difficult to evaluate the effect of intake of LPHEAL9 after the TSST and only including subjects that experienced increased cortisol levels, resulted in an almost significant difference between the LPHEAL9 and placebo group, 10 min after the TSST ($p = 0.07$). One further factor that influenced the result was that some of the participants did not have chronic stress at the day the TSST was made. An analysis of these participants showed that intake of LPHEAL9 before the stress test led to a significantly lower cortisol level in

comparison with intake of placebo. This indicates that participants not experiencing chronic stress at the day of the TSST could be protected from high cortisol levels during acute stress by intake of probiotics.

Chronic stress may diminish the physiological reactions to acute stress. For example, the more stress full life events that had been reported by children, the smaller was the cortisol increase after TSST [3] and low socioeconomic status and living in a more urban area were also related to blunted cortisol reactivity in adolescents after a stress task similar to TSST [13]. Stressful life events can also give a smaller cardiovascular response after TSST [16]. We have used V-TSST earlier to study subjects with low- and high (chronic) stress ([32]. The definition for having high (chronic) stress was the same as in this study (SMBQ $\geq$ 3.75) and for having low stress, the subject had a SMBQ score $\leq$ 2.75. In the study by Linninge et al. [32], the subjects with chronic stress had, in contrary to the studies listed above, a significantly higher increase of the cortisol level compared to the subjects with low stress. In the present study, the TSST led to significant increased levels of many stress markers but since we do not have a group with low stress it is difficult to evaluate if the subjects had a blunted stress response or not.

It is known that acute stress over-activates the immune system, leading to an imbalance between inflammatory and anti-inflammatory responses [33,44]. This imbalance may lead to development of diversified stress-related diseases such as cardiovascular diseases, neurodegenerative diseases and cancer. Stress also increases the response of the gastrointestinal system to inflammation and may reactivate previous inflammation and accelerate the inflammation process [52]. For example, chronic stress may increase the risk of IBD, which may be exacerbated by acute psychosocial stress. IBS, which in part can have an inflammatory origin, is also associated with stress.

The inflammatory stress marker IL-6 increased significantly for both groups after the TSST and the increase was especially pronounced at the end of the one-hour recovery period, with no significant difference between groups. A similar time course of the IL-6 levels after TSST has also earlier been shown in a study including low or highly stressed males with no difference between the test groups [32]. The IL-6 level probably increases even further since the increase was pronounced at the end of the recovery period and to better follow changes in IL-6 after TSST, IL-6 should have been analyzed for a longer period than for 60 min after the test. This was also indicated in a meta-analysis of 29 studies where from >10 min to 120 min after the stress test, the increase in IL-6 was significant at all time points, with the highest level found at 90 min after the test [36].

To our knowledge, the effect of an acute stress test on the level of soluble fractalkine has not been studied previously. As fractalkine receptors are also expressed by all macrophages in the central nervous system [31], soluble fractalkine would have a significant effect on the CNS if able to pass the blood-brain barrier. It is well known that fractalkine modifies the inflammatory reactions of microglia [8] and stress is known to be an inducer of neuroinflammation despite its historically been considered as anti-inflammatory [15]. Studies in mice have shown that fractalkine signaling affects coping behavior in stress-tests by fractalkine as mediator in metabolic-, hormonal- and behavioral responses to acute as well as chronic stress [50]. The level of fractalkine in our study was significantly affected by the stress test and the level was lower in the LPHEAL9 group compared to the placebo group after the test (p = 0.003). The expression of mRNA for fractalkine in intestinal cells (Caco-2 cells) after addition of heat killed bacteria has earlier been investigated [47]. It was found that *Lacticaseibacillus rhamnosus* GG and *Lacticaseibacillus casei* did not increase the expression of mRNA for fractalkine while *Escherichia coli* significantly induced the expression. Fractalkine is produced in response to different inflammatory stimuli such as IL-1β, TNF-α, IFN-γ and LPS and has been shown to be involved in different conditions like atherosclerosis, cancer, rheumatoid arthritis and IBD, which adds to its complexity as inflammatory mediator [24,45]. The decreased level of soluble fractalkine in the present study after intake of LPHEAL9 indicates a reduced inflammatory response in the body during acute stress. If the higher circulatory fractalkine levels observed in the placebo group will pass the blood-brain barrier and affect behavior and coping in humans still needs to be proven.

Soluble CD163 was significantly lower after the acute stress test in the LPHEAL9 group compared to the placebo group and a correlation between the fractalkine and the sCD163 level was found 10 and 20 min after the test. The result for sCD163 thus confirmed the shown changes in fractalkine levels. Soluble CD163 has been shown to be increased in serum of critically ill patients, in chronic inflammatory and infectious diseases [6]. Today there are no reports that suggests a typical reactivity pattern of sCD163 and fractalkine in acute stress reactions. However, both CD163 and fractalkine are cleaved and released in the circulation by ADAM17. This is the same protease that releases membrane bound TNF-α from the surface during inflammation and it is reasonable to assume that sCD163 and fractalkine would follow the pattern for TNF-α in stress [35,9]. However, due to the complexity of synergy and redundancy in cytokine signaling this need to be further investigated in future studies.

It was difficult to evaluate the effect of intake of LPHEAL9 on several inflammation markers after TSST since the cytokine levels were low and many of the samples had a level that were below the detection limit. This was especially valid for IL-1β and IL-10 but also for CRP, TNF-α and IFN-γ. This is not surprising since the participants in the study were young and healthy. The method used to measure the cytokines (multiplex) also has higher detection limits compared to if each cytokine had been analyzed separately with for example ELISA.

The effect of two other *Lactiplantibacillus plantarum* strains; DR7 and P8, to alleviate stress and anxiety has earlier been evaluated [11,30]. Both studies looked at the long-term effects of probiotic intake in the same target group as in the present trial but they did not include an acute stress test but instead measured fasting levels of different stress markers. In the DR7 study, one hundred and eleven adults with moderate stress were given the probiotic bacteria (10^9 cfu/day) or placebo and intake of DR7 gave a significantly lower blood cortisol level as well as a higher concentration of IL-10 compared to placebo after 12 weeks intervention. The P8 (10^{10} cfu/day) study included one hundred and three moderately stressed adults and also ran over a period of 12 weeks. The primary outcome was stress levels assessed via questionnaires, but also blood samples were taken. Intake of P8 for 12 weeks significantly decreased stress scores, including anxiety, as well as reduced levels of IFN-γ and TNF-α, while no significant effect on cortisol levels was seen, compared to placebo. Thus, as in the present study, these studies showed effects on inflammatory markers after intake of *Lactiplantibacillus plantarum* strains in moderately stressed subjects.

The intention was to measure zonulin, but after the analyzes had been carried out we found that the specific kit used had been changed to instead measure zonulin family peptides. They are structurally and possibly functionally related to zonulin and shown to be significantly increased in patients with diabetes and obesity and to correlate strongly with markers of the lipid and glucose metabolism [46]. However, no change in serum zonulin family peptides were observed during the TSST challenge. In an earlier trial, the zonulin family peptide level was found to be increased ten minutes after the stress induction and then decreased one hour after TSST [32]. This was, however, the case after age was included as a covariate. There were no difference in the mean age of the participants between that study and the present study (mean age 24.6 and 24.8 years, respectively), but the zonulin family peptide levels were higher at baseline in the earlier study with a mean value of 50 ng/mL compared to a mean value of 39 ng/mL in the present study and this may have affected the result.

The gut function (abdominal pain, flatulence, bloating) improved during the intervention period and also during the stress test in both groups but there was no difference between the groups. An earlier study [32], showed that subjects with chronic stress experienced more gut

dysfunction in general (65% of the subjects) and they also had more problems during the TSST than low stressed subjects.

The study has some limitations that have to be taken into consideration. One limitation is that the study is most likely underpowered since we used a medium effect size to calculate the required sample size, and the results should therefore be interpreted with caution. Also considering that some of the measurements have not been used before in response to acute psychosocial stress induction, the study should be regarded as a pilot study. One further limitation is that there were no blood samples taken before start of the intervention. Thus, there were no possibility to analyze if the study participants had any change in cortisol levels or inflammation markers over time due to intake of LPHEAL9. It should also have been valuable to have done the TSST before and after the intervention but due to the design of TSST, it is difficult to repeat the stress test. This is because once an individual knows the test procedure, acute stress will not be evoked in the same way [25]. That is, the cortisol stress response habituates quickly.

In conclusion, the study is the first to show that intake of LPHEAL9 gave significantly lower plasma levels of two inflammatory markers (soluble fractalkine and CD163) after an acute stress test compared to placebo. Based on the novelty of the results it is relevant to perform additional larger studies to confirm the effect of intake of probiotics on inflammation during acute stress.

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Author contribution

G.Ö. participated in the study design, planned the work, interpreted data, and wrote the first version of the manuscript, M. Hi. was involved in the selection and analysis of the inflammatory markers and participated in the manuscript preparation, M. He. performed the study, was test leader in V-TSST, analyzed the lactobacilli and participated in the manuscript preparation, C.M. was involved in the V-TSST and participated in the manuscript preparation, J.E. participated in the study design and approved the manuscript, S.A. designed the study and participated in the manuscript preparation, P.J. designed the study, performed the physiological and statistical analyzes, interpreted data, and participated in the manuscript preparation.

Declaration of Competing Interest

The authors declare no conflict of interest related to this publication. G.Ö. and C.M. are employed by Probi AB.

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