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Sex, pain, and the microbiome: The relationship between baseline gut microbiota composition, gender and somatic pain in healthy individuals

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ABSTRACT

Background and Aim: Relative to men, women present with pain conditions more commonly. Although consistent differences exist between men and women in terms of physiological pain sensitivity, the underlying mechanisms are incompletely understood and yet could inform the development of effective sex specific treatments for pain. The gut microbiota can modulate nervous system functioning, including pain signaling pathways. We hypothesized that the gut microbiota and critical components of the gut-brain axis might influence electrical pain thresholds. Further, we hypothesized that sex, menstrual cycle, and hormonal contraceptive use might account for inter-sex differences in pain perception.

Methods: Healthy, non-obese males (N = 15) and females (N = 16), (nine of whom were using hormonal contraceptives), were recruited. Male subjects were invited to undergo testing once, whereas females were invited three times across the menstrual cycle, based on self-reported early follicular (EF), late follicular (LF), or mid-luteal (ML) phase. On test days, electrical stimulation on the right ankle was performed; salivary cortisol levels were measured in the morning; levels of lipopolysaccharide-binding protein (LBP), soluble CD14 (sCD14), pro-inflammatory cytokines were assessed in plasma, and microbiota composition and short-chain fatty acids (SCFAs) levels were determined in fecal samples.

Results: We observed that the pain tolerance threshold/pain sensation threshold (PTT/PST) ratio was significantly lesser in women than men, but not PST or PTT alone. Further, hormonal contraceptive use was associated with increased LBP levels (LF & ML phase), whilst sCD14 levels or inflammatory cytokines were not affected. Interestingly, in women, hormonal contraceptive use was associated with an increase in the relative abundance of *Erysipelatoclostridium*, and the relative abundances of certain bacterial genera correlated positively with pain sensation thresholds (*Prevotella* and *Megasphaera*) during the LF phase and cortisol awakening response (*Anaerofustis*) during the ML phase. In comparison with men, women displayed overall stronger associations between i) SCFAs data, ii) cortisol data, iii) inflammatory cytokines and PTT and PST.

Discussion and conclusion: Our findings support the hypothesis that the gut microbiota may be one of the factors determining the physiological inter-sex differences in pain perception. Further research is needed to investigate the molecular mechanisms by which specific sex hormones and gut microbes modulate pain signaling pathways, but this study highlights the possibilities for innovative individual targeted therapies for pain management.

Abbreviations: EF, early follicular; LF, late follicular; ML, mid-luteal; PTT, pain tolerance threshold; PST, pain sensation threshold; LBP, lipopolysaccharide binding protein; sCD14, soluble cluster of differentiation 14; TNF- α , Tumor Necrosis Factor alpha; IFN- γ , interferon gamma, IL-1 β , interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8.

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1. Introduction

Pain is described as an unpleasant sensory and emotional event, often associated with underlying tissue injury. Acute pain usually lasts for a short period of time following injury, allowing for tissue healing and usually resolves on its own (Fong and Schug, 2014). In contrast failure to resolve or repeated nerve stimulation can result in sensitization, impaired central nervous system (CNS) mechanisms and chronic pain (Voscopoulos and Lema, 2010). Chronic pain is a significant healthcare challenge as it confers a substantial burden on individuals, employers, health care systems, and society in general (Breivik et al., 2013, Driscoll et al., 2021).

Physiological pain threshold is influenced by biopsychosocial factors, such as biological sex differences. In general, laboratory tests demonstrate that women exhibit greater pain sensitivity compared with men (Mogil, 2012). Differences in pain perception (tolerance & threshold) between adult men and women are greatest for electrical and pressure stimuli. In contrast, heat pain thresholds are highly variable when assessing differences between men and women (Riley et al., 1998, Meyer-Frießem et al., 2020). Interestingly, in a meta-analysis including only healthy children, a greater number of studies demonstrated no significant sex differences in pain perception; however, for those of age > 12y, females report higher pain intensity in response to the cold pressor test compared to males (Boerner et al., 2014). These data suggest that the onset of puberty and the associated hormonal changes may contribute to sex bias in pain sensitivity in teenagers and possibly adults (Boerner et al., 2014).

The general pathway of pain sensation is regulated by the peripheral nervous system, which senses the variations of sensory perceptions in the peripheral organs and conveys this information to the brain through the nerves and ganglia situated outside of the spinal cord (Yam et al., 2018). In turn, CNS processes the information and allows perception of pain. Among the factors that regulate pain pathways, the gut microbiota and its related metabolites have been recently recognized to play critical roles (Brenner et al., 2021a, Brenner et al., 2021b, Lagomarsino et al., 2021).

Within the gastrointestinal (GI) tract, a unique combination of different communities of microorganisms exists (O'Mahony, 2017). Gut microbes actively participate in shaping and maintaining host physiology and organ function (Clarke et al., 2014). Interactions between the gut microbiota and the brain are mediated through the immune, neural, and neuroendocrine pathways of communication within the microbiota-gut-brain axis (Cryan et al., 2019). Gut bacteria are active producers of metabolites such as short-chain fatty acids (SCFAs) that play a key role in preserving intestinal epithelial barrier integrity (O'Riordan et al., 2022), and in modulating the intestinal and peripheral immune system thus protecting the nervous system from inflammation (Erny et al., 2021).

Gut microbiota composition of a healthy individual is influenced from birth via mode of delivery, lactation, genetic factors, and throughout life by diet, lifestyle, medication, environmental factors, GI function, and stress (Lach et al., 2020, O'Mahony et al., 2017, Ratsika et al., 2021, Rinninella et al., 2019). Changes to the gut microbiota are associated with alterations of the intestinal permeability as well as low-grade inflammation and metabolic dysfunction (Cani et al., 2012, Longo et al., 2020). Microorganisms or microbial-derived molecular profiles are able to activate macrophages and increase levels of pro-inflammatory cytokines. Administration of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria increases several cytokines (tumour necrosis factor (TNF)- α , interleukins (IL) 6, 8 and 10), cortisol and influences pressure pain threshold (Wegner et al., 2014) and electrical pain threshold (de Goeij et al., 2013, Hijma et al., 2020). Hence, increased systemic levels of bacterial components may initiate reduction of pain thresholds and contribute to pain through activation of inflammatory and stress-related pathways.

The microbiota and manipulation of it have been associated with

several types of pain, including visceral pain (O'Mahony et al., 2014); Tramullas et al., 2021, Luczynski et al., 2017 postoperative pain (Brenner et al., 2021a), inflammatory pain (Amaral et al., 2008), migraine (van Hemert et al., 2014), interstitial cystitis (Braundmeier-Fleming et al., 2016), as well as autoimmune-related pain in rheumatoid arthritis (McLean et al., 2015). Sex differences are noted in the composition and diversity of the gut microbiota (Jaggar et al., 2020), with clear effects of gonadectomy and hormone replacement on relative abundance of gut bacterial taxa, and microbial-derived products such as bile acids (Markle et al., 2013, Org et al., 2016). Gut microbiota involvement in the aetiology of somatic pain disorders or in gating somatic pain sensitivity and pain tolerance in healthy individuals is understudied. Sex-specific associations in pain symptoms of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients have been identified (Wallis et al., 2016) and in male ME/CFS patients, the bacteria from the genus *Eubacterium* were negatively correlated to pain symptoms, whereas *Lactobacillus* and *Streptococcus* were positively correlated to pain symptoms (Wallis et al., 2016). In contrast, *Streptococcus* was negatively correlated to pain symptoms in female ME/CFS patients (Wallis et al., 2016).

Whether specific microbiota taxa are related to physiological pain thresholds in healthy individuals remains to be investigated. Here, we examined gut microbiota diversity and the association of microbial taxa with electrical pain sensitivity in healthy adult male and female volunteers. Understanding the mechanisms that regulate pain sensitivity is important for developing new effective and potentially sex specific therapies for pain disorders.

2. Materials & METHODS

2.1. Study population

Students and employees of University College Cork (UCC) were recruited. The following inclusion criteria were used: 18–35 years of age, regular menstrual cycle (28 ± 4 days), body mass index (BMI) < 30 kg/m² and otherwise healthy (Supplementary Table 1). All subjects were initially screened through an online survey. Exclusion criteria were: use of psychotropic drugs and beta-blockers within the last 3 months, use of analgesic medication 24 h before measurements, neurological disease, diabetes mellitus, GI disease (e.g. inflammatory bowel diseases, irritable bowel syndrome), hormonal treatment during the past three months (with exception of hormonal contraceptive in this sub-group). In this study, 15 non-obese men and 16 non-obese women were included. Eight women used oral contraceptives and one woman used a Nuova ring (etonogestrel / ethinyl estradiol vaginal ring). Seven women did not use oral contraceptives. Each participant was asked not to engage in strenuous physical activities, sleep regular hours and refrain from alcohol the day before the experiment. Smoking was not allowed for a period of 2 h before the test session. This pilot study was approved by the local Ethical Committee of University College Cork (UCC; ECM 03/2020 PUB). All participants provided written informed consent.

2.2. Beck's depression inventory

The Beck's depression inventory (Beck et al., 1961) was completed by 21 subjects of whom 19 answered all questions. Only fully completed questionnaires were used for further analyses.

2.3. Hospital visits and sample collection

Male subjects were invited to attend the Department of Neurophysiology, Cork University Hospital once, whereas females were invited to attend three times across a single menstrual cycle. Females were asked to keep a diary in which they recorded their menstruation cycle for a period of 3 months prior to the study. Self-reported menstrual cycle days

Table 1

Proinflammatory cytokines levels were not affected by sex differences, menstrual cycle, or hormonal contraceptive use. Plasma TNF- α , IFN- γ , and IL-8 concentrations of males, and females (using hormonal contraceptives y/n) during the EF, LF, ML phase. Kruskal Wallis test and Friedman test with Dunn's post-hoc tests were performed to assess differences between selected groups. Abbreviations: HC = hormonal contraceptive users; nHC = non-hormonal contraceptive users. EF = early follicular phase, ML = mid-luteal phase, TNF- α = tumour necrosis factor α , IFN- γ = interferon γ , IL-8 = interleukin 8. N = 15 for males. For females contraceptive users N = 8 for EF; N = 9 for LF and N = 9 for ML. For females no-contraceptive users N = 7 for EF; N = 6 for LF and for ML.

Cytokine (pg/ml)	Males	Females HC			Females nHC		
		EF	LF	ML	EF	LF	ML
TNF- α	2.611 ± 0.130	2.460 ± 0.158	2.418 ± 0.183	2.200 ± 0.169	2.327 ± 0.144	2.368 ± 0.161	2.311 ± 0.125
IFN- γ	6.146 ± 1.793	8.079 ± 1.946	6.288 ± 1.164	4.550 ± 0.677	4.491 ± 0.646	3.318 ± 0.281	4.127 ± 0.545
IL-8	4.117 ± 0.394	5.453 ± 0.442	4.372 ± 0.447	4.484 ± 0.588	4.217 ± 0.486	4.710 ± 0.422	4.395 ± 0.341

2–7, 9–13, and 18–23 were defined as early follicular phase (EF), late follicular (LF) phase, and mid-luteal (ML) phase, respectively (Reed and Carr, 2000). During the test visits, subjects brought saliva collected that morning and stool samples, which were subsequently stored at -80°C . Furthermore, an electrical stimulation test was performed, and after the test blood samples were collected.

2.4. Saliva sample collection

Subjects were asked to collect saliva samples upon awakening (Sample 1) and 30 (Sample 2), 45 (Sample 3) and 60 (Sample 4) minutes after awakening in a *salivette* through chewing on a cotton swab for 90 s and transferring the bud from the mouth directly into the tube, without using hands/fingers. Next, they were instructed to place the *salivettes* directly into a refrigerator, or into plastic container with an ice pack prior to refrigerator placement. Additional notes included instructions not to brush teeth throughout the collection process, drink or eat anything prior to Sample 1, drink or eat anything 15 min prior to Sample 2, 3 and 4.

2.5. Stool sample collection

Subjects were asked to collect stool samples within 24 h prior to the visit. Samples were collected in a plastic container. After bowel movement performance, a lid containing an anaerobic gas producing sachet (ThermoFisher Scientific, Massachusetts, USA) was secured to the plastic container. Subjects were asked to store samples in a cool area prior to attendance at the hospital.

2.6. Neurophysiological assessments of pain

Quantitative sensory testing was performed by a trained clinician. The pain sensation threshold (PST) to transcutaneous constant current electrical stimulation was assessed on subjects lying supine in a warm, quiet environment using Neuropack-S neuro-diagnostic stimulator with disposable surface electrodes (Natas Medical Instruments Inc, California, USA). PST and pain tolerance threshold (PTT) were recorded during electrical stimulation characterized by manual 5 mA current increase (up to maximum 100 mA) at the right ankle just below the medial malleolus. Stimulations were set at 1 Hz, duration of 200 μs . If two threshold values differed by $> 10\%$ between runs, testing was repeated until three consecutive thresholds were recorded, each within 10%. Data of one subject could not be recovered, hence data of 30 subjects instead of 31 were used for further analysis.

2.7. Blood sample collection

Venous blood was collected in EDTA tubes (Greiner Bio-One™ Vacuette™) after completion of the neurophysiology assessment. Blood samples were centrifuged, plasma was separated and stored at -80°C .

2.8. Biochemical analyses of plasma

All plasma aliquots were centrifuged at 1000G for 10 min at 4°C prior to analysis. Duplicate lipopolysaccharide-binding protein (LBP) and soluble cluster of differentiation 14 (sCD14) level measurements were performed on plasma samples using commercially available ELISAs for free human LBP (Enzo Life Sciences, New York, USA) and sCD14 (R&D systems, Minneapolis, USA). All procedures were carried out according to manufacturer's instructions.

Plasma levels of pro-inflammatory cytokines; interferon gamma (IFN- γ), IL-1 β , IL-6, IL-8 and TNF- α were determined using a V-PLEX MULTI-SPOT assay system (Mesoscale Discovery, Maryland, USA) according to manufacturer's instructions. EDTA plasma samples were analysed in duplicate. IL-1 β and IL-6 data were excluded from further analysis, as most sample concentrations were below standard curve range or detection range.

2.9. Fecal DNA extraction and Illumina 16S rRNA gene sequencing

Faecal DNA was extracted, followed by Illumina MiSeq sequencing and bioinformatics analysis. A 0.25 g aliquot of faecal sample was added to 1 mL lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and 4% sodium dodecyl sulphate (SDS)) together with 0.4 g of zirconia beads. The samples were homogenized for 3 min using the Biospec Minibeadbeater at max speed. The homogenized samples were then heated to 70°C for 15 min and then centrifuged at 16,000xg for 5 min. The supernatant was then removed, fresh lysis buffer added and the bead beating, heating and centrifugation steps repeated. The nucleic acids were then precipitated using 10 M ammonium acetate, followed by addition of isopropanol. The nucleic acids were then pelleted, washed and resuspended in 1X TE buffer. Subsequently, removal of RNA, protein and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit along with the wash buffers and elution buffer, and DNA was stored at -20°C .

The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). The DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCA-GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC). Each PCR reaction contained DNA template, 5 μl forward primer (1 μM), 5 μl reverse primer (1 μM), 12.5 μl 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), PCR grade water to a final volume of 25 μl . PCR amplification was carried out as follows: heated lid 110° , $95^{\circ}\text{C} \times 3\text{mins}$, 25 cycles of $95^{\circ}\text{C} \times 30\text{s}$, $55^{\circ}\text{C} \times 30\text{s}$, $72^{\circ}\text{C} \times 30\text{s}$, then $72^{\circ}\text{C} \times 5\text{mins}$ and held at 4°C . PCR products were visualized using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100 V). Successful PCR products were cleaned using AMPure XP magnetic bead-based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5 μl) to index each of the

samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix, 10 µl PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were visualized using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit. Samples were then pooled in an equimolar fashion. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 × 250 cycle kit, following standard Illumina sequencing protocols.

250 base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME Version 1.9.0. Singleton removal (to ensure a minimum of 2 copies to be counted), denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST (PyNAST: python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release v123. Data were not rarefied/normalized for further analysis. Richness and α-diversity indices were generated in QIIME Version 1.9.0. Principal component analysis was performed on centred log-ratio transformed (clr) values using the ALDEx2 library (Fernandes et al., 2014).

2.10. Faecal short-chain fatty acids analysis

Faecal supernatant and standards for SCFAs analysis were prepared as reported below. Briefly, faecal samples were mixed with 5 mL milliQ/gram, acidified to pH ± 2–3, spun and filtered (0.2 µm). The concentration of SCFAs was determined by gas chromatography flame ionization detection (GC-FID) using a Varian 3500 GC system, fitted with a ZB-FFAP column and a flame ionization detector. Helium was supplied as the carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 100 °C, and was maintained for 0.5 min, and then raised to 180 °C at 8 °C/min and held for 1.0 min, before being increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5.0 min. The temperatures of the detector and the injection port were set at 250 °C and 240 °C, respectively. Samples were run in duplicate and the injected sample volume was 0.5 µl. Blanks were measured in between samples to ensure there was no potential carryover from previous samples. Peaks were integrated using Varian Star Chromatography Workstation version 6.0. Subsequently, peaks were quantified and concentrations of acetate, propionate, isobutyrate, n-butyrate, isovalerate and valerate were calculated. Isobutyrate, isovalerate and valerate were excluded from further analysis, since some samples had concentrations < 0.1 mM (lowest standard) for these SCFAs. Acetate concentrations > 10 mM (greatest standard) were not excluded. Duplicate samples with a CV > 15% for acetate were repeated. One repeated duplicate had a CV of 16% for acetate and was included. CVs of all duplicates were < 15% and < 10% for propionate and n-butyrate, respectively. A correction was made for the solution (milliQ and 5 M HCl) that was added to the faeces and moles SCFAs/gram wet feces were calculated.

2.11. Cortisol awakening response

Saliva samples were centrifuged at 1000G for 10 min at 4 °C prior to analysis. Duplicate cortisol level measurements were performed in saliva samples using a cortisol ELISA kit (Enzo Life Sciences, New York,

USA). All procedures were carried out according to manufacturer's instructions. Area under the curve (AUC) of the cortisol awakening response was calculated using the following formula:

$$\text{AUC} = 30 * ([\text{cort}]^{t=0\text{min}} + [\text{cort}]^{t=30\text{min}}) / 2 + 15 * ([\text{cort}]^{t=30\text{min}} + [\text{cort}]^{t=45\text{min}}) / 2 + 15 * ([\text{cort}]^{t=45\text{min}} + [\text{cort}]^{t=60\text{min}}) / 2.$$

2.12. Statistical analyses

Kruskal-Wallis and Friedman tests with Dunn's post-hoc tests were performed to determine differences between groups or within groups using Graphpad Prism 5. Comparisons with a p-value ≤ 0.05 were considered as statistically significant different.

For statistical analysis of the microbiota, Kruskal-Wallis tests were performed using R-studio and Friedman tests were performed using SPSS version 21. False discovery rate (FDR) corrected p-values were calculated for all taxa (phylum, family, genus) that were observed for a certain comparison by using the Benjamini-Hochberg procedure. An FDR-adjusted p-value < 0.1 was considered as acceptable for Dunn's post-hoc testing using Graphpad Prism 5. For the Dunn's post-hoc test, comparisons with p-values ≤ 0.05 were considered as statistically significant different.

Spearman's rank correlations of bacterial genera and other parameters (faecal SCFA, intestinal permeability markers, inflammatory markers, and cortisol) were performed in R-Studio. Two fungal families were removed from the data set. Microbiome count tables were transformed using the centered-log ratio transformation before any statistical test. FDR adjusted p-values were calculated for all correlations that yielded a correlation coefficient. Correlation coefficients and FDR adjusted p-values were calculated separately for "males only" and "females only" using the HALLA framework (Ghazi et al., 2021). Correlations between all the host physiological parameters (except the microbiota) were performed in the same way as the genera correlations described above.

3. Results.

3.1. Age-matched, normally menstruating females score higher for Beck's depression inventory than men.

Age, smoker's status, native English language rate were evaluated through online questionnaires (Supplementary Table 1). We demonstrated that Beck's depression inventory score was significantly greater in normally menstruating females compared to males (data not shown). Of the recruited population, two females visited twice instead of three times (Fig. 1). Raw data/samples of 59, 61, 50, 60 and 58 subjects were available for the neurophysiological assessments, and for the analyses of the faecal microbiota, faecal SCFAs, blood plasma markers and salivary cortisol, respectively (Fig. 1).

3.2. Women display a lesser ratio of pain tolerance threshold (PTT) to pain sensation threshold (PST) compared to men.

The assessment of sensory pain did not show any significant differences in PST or PTT between males and females nor in females using hormonal contraceptives and non-users (Fig. 2A and B). In categorizing women as contraceptive users or non-users, significantly lesser PTT/PST ratios were demonstrated for the EF phase of contraceptive users (p ≤ 0.05) and the ML phase of non-users (p ≤ 0.05) compared to males (Fig. 2C). However, when we combined all women, we did observe a significantly lesser PTT/PST ratios in all phases of the menstrual cycle (EF, ML = p ≤ 0.01; LF = p ≤ 0.05) compared to males (Fig. 2D).

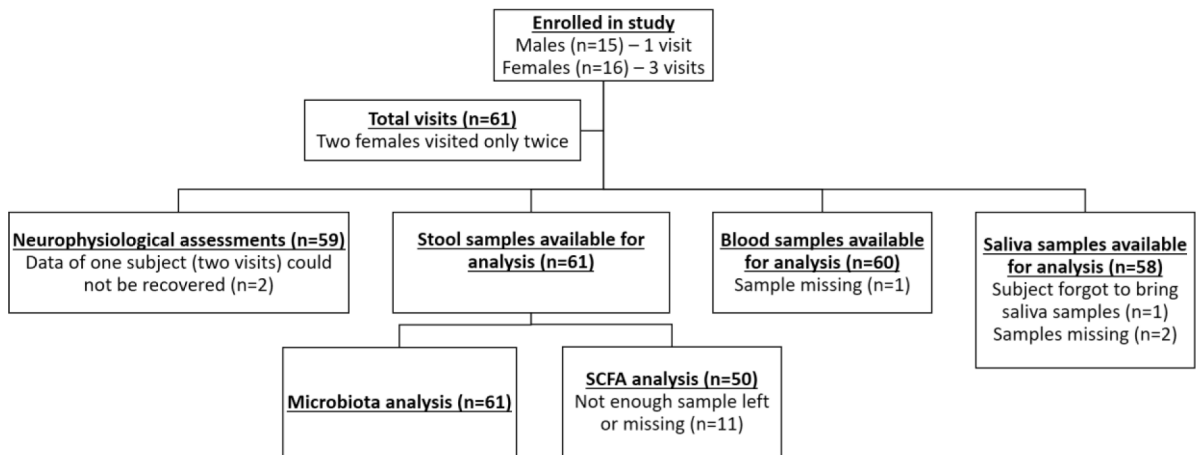


Fig. 1. Flowchart showing the number of participants/ samples (stool, blood, saliva) and neurophysiological assessments (pain) available for analysis.

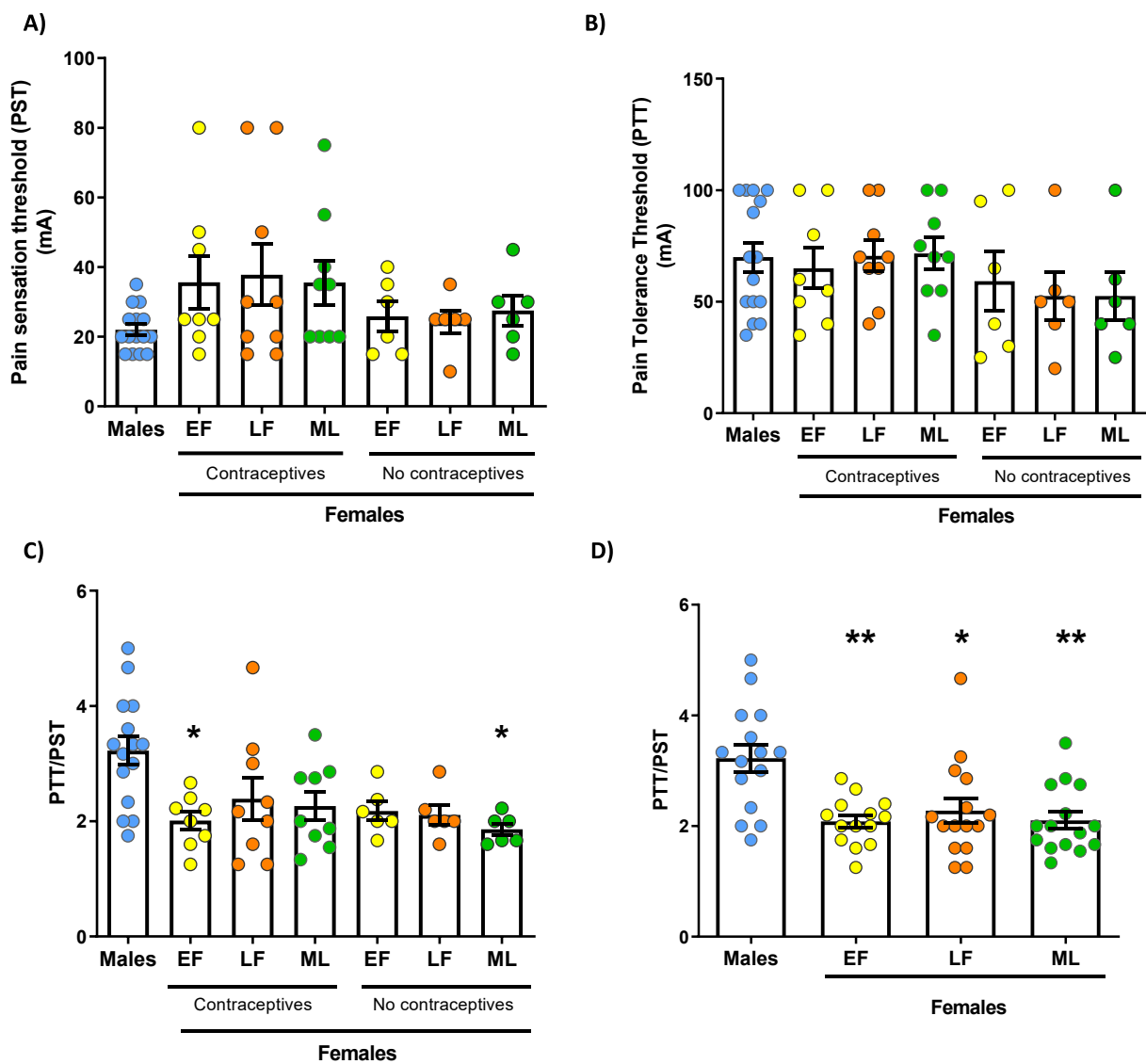


Fig. 2. The ratio of electro-cutaneous pain tolerance threshold (PTT)/pain sensation threshold (PST) is lesser in females than males. PST (panel A), PTT (panel B), and ratio PTT/PST (panels C and D) were determined in males, female and contraceptive users and no contraceptive users. Kruskal Wallis test and Friedman test with Dunn’s post-hoc tests were performed to assess differences between selected groups, and differences across the menstrual cycle. $**p \leq 0.01$, $*p \leq 0.05$ vs Males. Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid luteal phase. N = 15 for males. For females contraceptive users N = 8 for EF; N = 9 for LF, and N = 9 for ML. For females no-contraceptive users N = 6 for EF, LF and ML.

3.3. Microbiota diversity and bacterial taxonomic abundance are not influenced by sex differences, menstrual cycle, or hormonal contraceptive use

To evaluate whether sex, menstrual cycle or hormonal contraceptive use was associated with microbiota composition, richness/ α or β -diversity indices and relative abundance of bacterial taxa was determined. The number of reads and operational taxonomical units (OTUs) per sample were variable, whereas the joining efficiency/quality was high (in general over 90%) (Supplementary Table 2). We investigated potential differences in Chao1, Shannon's diversity, and Simpson's diversity yet no differences were observed for these α -diversity indices (Supplementary Table 3) or β -diversity between men and women (contraceptive users or not) and across the menstrual cycle (Fig. 2 Figure 3 A-D). In Fig. 3D, the PCA shows the temporal fluctuation of the microbiome compositions in females across the three phases of the menstrual cycle, indicated by the black lines.

When evaluating differences in relative abundance group distributions for phylum, family and genus, no significant differences were observed overall between males and females (3 phases) or due to contraceptive use (Fig. 4).

3.4. Hormonal contraceptive use is associated with increased relative abundance of *Erysipelatoclostridium*, but not faecal SCFAs levels

Across the menstrual cycle of contraceptive users and normally menstruating females, significant differences were observed for 10 and 5 taxa, respectively (Supplementary Tables 4 and 5). However, only for genus *Erysipelatoclostridium* a FDR lower than 10% was demonstrated; hormonal contraceptive use was associated with increased relative abundance of *Erysipelatoclostridium*, at least for the LF phase (Fig. 5).

Then we investigated whether SCFAs content in the fecal wet mass was associated with sex difference, menstrual cycle or hormonal

Table 2

Sex differences, menstrual cycle and hormonal contraceptive use did not influence cortisol awakening response. Salivary cortisol levels upon awakening, 30, 45 and 60 min after awakening, % salivary cortisol increase during the first 30 min after awakening, cortisol awakening response area under the curve and % negative cortisol awakening responses. Friedman tests with Dunn's post-hoc tests were performed to assess differences between selected time points within a group. Kruskal Wallis and Friedman tests with Dunn's post-hoc test were performed to assess differences between selected groups * $p \leq 0.05$ vs $t = 0$ min, # $p \leq 0.05$ vs $t = 30$ min; HC = hormonal contraceptive users, nHC = non-hormonal contraceptive users, EF = early follicular phase, LF = late follicular phase, ML = mid luteal phase. N = 10 for males. For females contraceptive users N = 7 for EF; N = 8 for LF and for ML. For females no-contraceptive users N = 7 for EF and for ML; N = 4 for LF.

Cortisol levels (pg/ml)	Males	Females HC			Females nHC		
		EF	LF	ML	EF	LF	ML
Awakening	1851	2005	2344	2968	2006	2441	2025
	± 274	± 317	± 311	± 455	± 328	± 373	± 221
Awakening + 30 min	2243	2999*	3083	2815	2944	2152	2617
	± 474	± 488	± 435	± 327	± 281	± 146	± 293
Awakening + 45 min	2470	2577	3021	3018	2804	2040	2599
	± 188	± 465	± 536	± 523	± 297	± 355	± 334
Awakening + 60 min	2137	2317#	2891	2698	2676	1853	2411
	± 353	± 460	± 534	± 506	± 339	± 353	± 278
Cortisol increase	32.6	48.8	32.2	0.5	89.5	-2.4	41.0
	± 19.5	± 10.9	± 12.0	± 9.7	± 49.2	± 20.9	± 25.7
0–30 min (%)							
Area under the curve (arbitrary unit * 1000)	131	154	172	173	158	130	146
	± 15	± 26	± 25	± 24	± 13	± 6	± 10

contraceptive use. We observed no association of these factors with total SCFA (Fig. 6) or for each separate SCFA, fecal acetate, propionate and butyrate levels (Supplementary Table 6).

3.5. Hormonal contraceptive use is associated with increased levels of LBP, but not sCD14 and inflammation

To investigate whether menstrual cycle, hormonal contraceptive use, or sex differences are associated with changes in intestinal permeability and/or inflammation, we assessed the plasma concentration of LBP and sCD14, two well-recognized markers of immune activation upon gut microbial translocation (Keane et al., 2021). LBP levels for females using hormonal contraceptives were higher compared to those of males ($p \leq 0.01$) and female non-users (LF = $p \leq 0.05$, ML = $p \leq 0.01$), except for the EF phase (Fig. 7). No differences were noted for sCD14 (data not shown).

To assess whether sex differences, menstrual cycle or hormonal contraceptive use influenced inflammatory state, plasma levels of TNF- α , IFN- γ and IL-8 were determined. In this study, we did not observe any differences in plasma TNF- α , IL-8 or IFN- γ concentration between males and females, across the menstrual cycle nor between hormonal contraceptive users and those not using hormonal contraceptives (Table 1).

3.6. Sex differences, menstrual cycle and hormonal contraceptive use did not influence cortisol awakening response

The increase in measured cortisol that occurs ± 30 min after awakening is defined as the cortisol awakening response (CAR). When looking at the CAR profile of all included subjects and time points ($n = 51$), we observed a significant increase of salivary cortisol levels 30 min after awakening compared to levels at awakening, (Supplementary Fig. 1) 60 min after awakening a significant decrease in cortisol levels was demonstrated compared to the peak (30 min) (Supplementary Fig. 1). This profile was not observed in the separate groups, except for the EF phase of females using hormonal contraceptives (Table 2). In addition, no significant differences were observed between groups/menstrual cycle phases for 1) cortisol levels at a specific time point, 2) cortisol increase during the first 30 min after awakening and 3) cortisol output during the first hour after awakening (area under the curve) (Table 2). Of special interest, for the LF phase of normally menstruating females, a mean negative cortisol awakening response was observed (Table 2). We also observed relatively high variation in mean cortisol levels at awakening and cortisol increase across the menstrual cycle (measurements on three different days), especially in females using hormonal contraceptives.

3.7. In women, the relative abundance of specific bacterial genera correlates with pain sensation thresholds, proinflammatory cytokines and CAR

Next, we investigated if there is a relationship between the gut microbiota and the levels of SCFAs, indirect markers of gut permeability (LBP and sCD14), proinflammatory cytokines, HPA-axis functioning and electrical pain thresholds in males and females.

In particular, we focused specifically on relative abundance of bacterial genera that correlated with other physiological parameters in males and females. We did not find any significant correlations for males only (data not shown). For females ($n = 46$ visits), we found several significant correlations between genus abundance and the other parameters in the LF and ML stages of the menstrual cycle (Fig. 8A and B). In the LF phase, PST and PTT positively correlated with bacteria from the genus *Prevotella* 7 or *Megasphaera*, respectively (FDR-corrected p value: $p < 0.0001$). Moreover, Spearman correlation analysis showed a negative significant correlation between the genus *Eggerthella* and the intestinal permeability marker sCD14 (FDR-corrected p value: $p < 0.0001$), and between the genus *Rothia* and TNF- α (FDR-corrected p

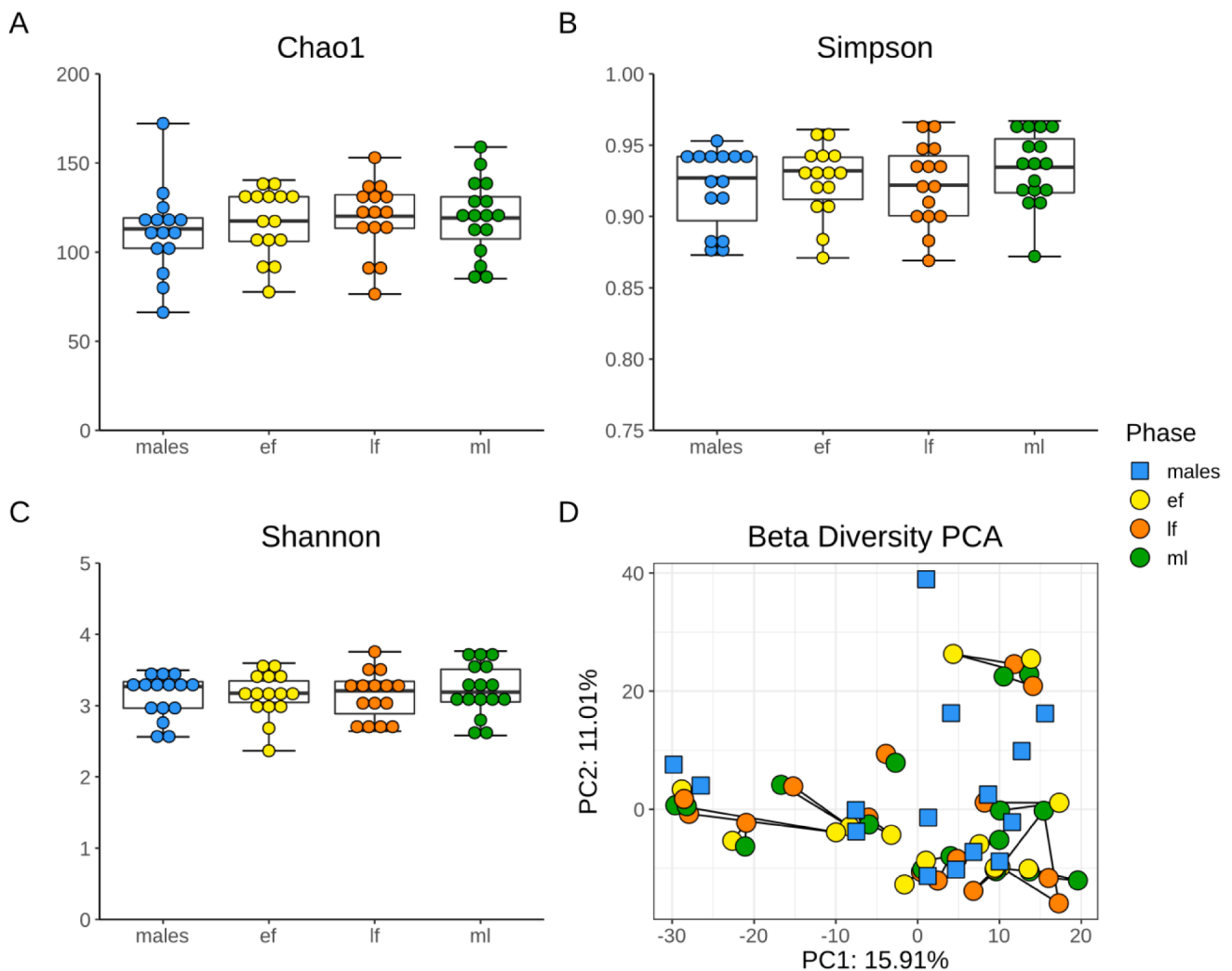


Fig. 3. Sex differences, menstrual cycle, and hormonal contraceptive use did not influence Chao1 diversity (panel A), Simpson diversity (panel B) and Shannon diversity (panel C) of the microbiota from fecal samples of male and female across the menstrual cycle. Principal Component Analysis (PCA) showing no difference in β -diversity of the microbiota from fecal samples of males and females across the menstrual cycle (panel D). Black lines display the link of the same subject based on the stage of the estrous cycle. Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid luteal phase. N = 15 for males. For females N = 15 for EF and for LF; N = 16 for ML.

value: $p < 0.0001$) in this stage of the cycle (Fig. 8A).

With regard to the ML phase of the menstrual cycle, we observed a positive correlation between the increase of salivary cortisol levels within the 30 min after awakening and the bacteria from the genus *Anaerofustis* (FDR-corrected p value: $p < 0.0001$), and a negative correlation between the decrease of cortisol level 60 min after awakening and the genus *Ruminiclostridium 5* (FDR-corrected p value: $p < 0.001$). Additionally, in this phase of the menstrual cycle the genus *Lachnospiraceae* UCG-005 was found negatively correlated with plasma levels of IL-8 (FDR-corrected p value: $p < 0.0001$) (Fig. 8B).

3.8. Cortisol awakening responses, pain sensitivity thresholds and SCFAs present greater associations in females as opposed to n males

Lastly, we investigated potential associations between pain sensitivity thresholds, cortisol awakening responses and plasma levels of LBP, sCD14, and inflammatory cytokines or stool levels of SCFAs for males (Fig. 9A) and Supplementary Table 7) and females (Fig. 9B) and Supplementary Tables 8) apart. In males, we found three significant (FDR-corrected) correlations within the SCFAs data which we do not deem overly interesting (Fig. 9A). In females, all SCFAs data, a great part of the cortisol data, some inflammatory markers, and PTT and PST

significantly correlated within each dataset (FDR-corrected p -value < 0.05 , < 0.01 Fig. 9B). Potentially the most interesting finding was that IL-8 correlated positively with PST in females (FDR-corrected p -value < 0.05 ; Fig. 8B).

4. Discussion

A large body of literature, indicates that women are more susceptible to pain than men in terms of frequency, severity, and duration of pain syndromes (Rustøen et al., 2004). Here we investigated important differences in pain sensitivity and their association with baseline gut microbiota composition in healthy male and female subjects. While our focus is on acute pain here, this study provides a significant baseline dataset to investigate further pain syndromes between the sexes.

Currently available therapies for pain syndromes are often unsatisfactory (Brandon-Mong et al., 2020), may lead to addiction or respiratory depression which are common adverse effects from chronic opioid use (Chen et al., 2021). These side effects are also more prevalent in women rather than men (Bartley and Fillingim, 2013). Since there is an urgent need for new analgesic therapeutics, research strategies are exploring alternative treatments for pain management, including the use of gut microbial manipulation with probiotics to treat visceral pain

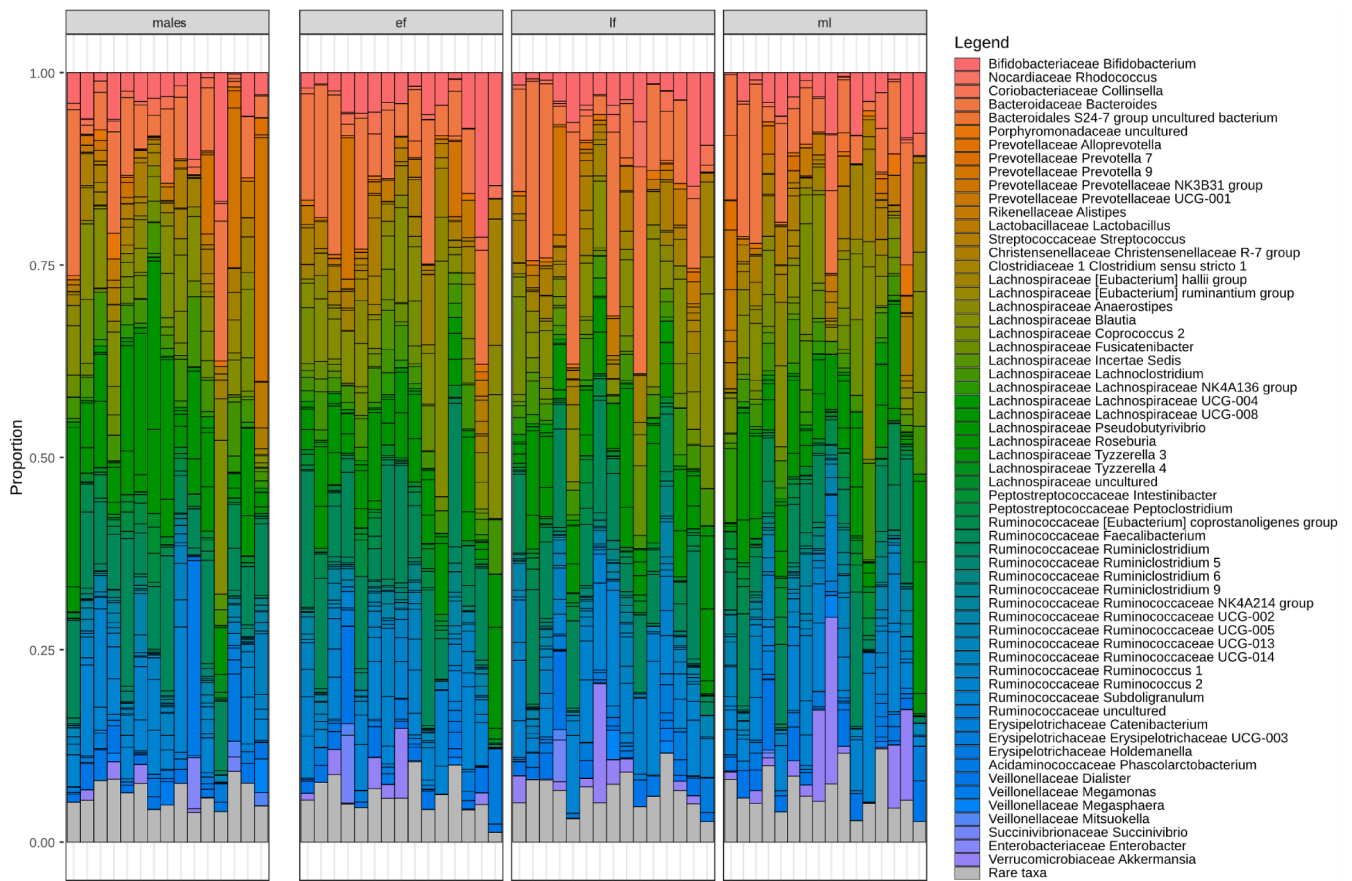


Fig. 4. Sex differences, menstrual cycle, and hormonal contraceptive use did not influence the relative abundance of the gut bacteria. Stacked bar plots showing relative abundance of the most abundant bacterial taxa per subject per time point. Rare taxa are defined here as never being present in >2% in any sample at genus level. $N = 15$ for males. For females $N = 15$ for EF and for LF; $N = 16$ for ML.

associated with functional gastrointestinal disorders showing promise (Brandon-Mong et al., 2020) (Ford et al., 2018). In this regard, our pilot study is the first to show that healthy women have a lower ratio of PTT/PST in response to electrical stimulation compared to men and that somatic pain thresholds, cortisol awakening levels, and hormonal contraceptives are associated with specific bacterial taxa of the gut microbiota in women, but not in men.

As a primary outcome, males and females were assessed for somatic pain in response to transcutaneous electrical stimulation of the right ankle. In the test, we evaluated the PST, or the minimum intensity of a stimuli (e.g., increase of electric current) at which the individual perceives pain, and the PTT, which indicates the maximum intensity of a stimuli that an individual can tolerate (Fillingim et al., 2009, Kanner, 2009). No differences were observed either for PST or for PTT between males and females, and in females using hormonal contraceptives and non-users. Studies have shown that factors influencing electrical stimulation tests include age, subcutaneous fat thickness, and culture (Al-Harthi et al., 2016, Guirro et al., 2015, Maffioletti et al., 2011). Guirro et al. demonstrated that sensory and motor thresholds at 50 Hz were higher for elderly men and women compared to respective young men and women when electrically stimulating the flexor muscle bellies of the wrist and fingers (Guirro et al., 2015). For electrical quadriceps muscle stimulation, sensory thresholds were negatively correlated to subcutaneous fat thickness, whereas motor threshold was positively correlated to subcutaneous fat thickness (Maffioletti et al., 2011). An advantage of our study is that relatively young men and women were included (19 to 33 years old). However, subjects had different backgrounds and cultures, and their ankle subcutaneous fat thickness was not considered yet we did not include individuals with a BMI over 30.

Due to the fact that electrical stimulation studies are not consistently performed on one particular body site, this obstructs researchers for making comparisons among electrical stimulation studies. Though, we suggest that culture and fat percentage could impact the variability within groups for PST and PTT (Seno et al., 2019). It is noteworthy that PST and PTT were relatively stable across the menstrual cycle in our study, with the exception for a few women using contraceptives, whose PST showed a trend toward an increase compared to normally menstruating females and to males. In contrast, Teepker et al. observed a significantly higher z-score for electrical pain threshold in healthy normally menstruating women on days 14 and 22 compared to day 1 of the menstrual cycle when performing electrical stimulation on the left volar forearm (Teepker et al., 2010). De Brito Barbosa et al. only showed a difference in pain sensation threshold between the follicular and premenstrual phase when applying 2500 Hz in phases of 100 μ s to the wrist and fingers of women using hormonal contraceptives. For phases of 20 and 50 μ s, and for normally menstruating women, there were no differences observed across the menstrual cycle (Barbosa Mde et al., 2013). We then evaluated whether the ratio of PTT/PST was influenced by sex differences, hormonal contraceptive use, or menstrual cycle phase. Similar ratios have been used in electrical stimulation studies (Chien et al., 2009). The noted significantly lower PST/detection threshold ratios at all measured body sites in volunteers suffering from whiplash compared to healthy volunteers (Chien et al., 2009). PTT/PST represents the proportionate increase in electric current that can be tolerated relative to that at which pain is first perceived (Kanner, 2009). This may be a more valid way to express pain tolerance threshold, since ratio calculations might correct for the density of afferent nerve fibres, and the conductivity through cutaneous and subcutaneous masses. We

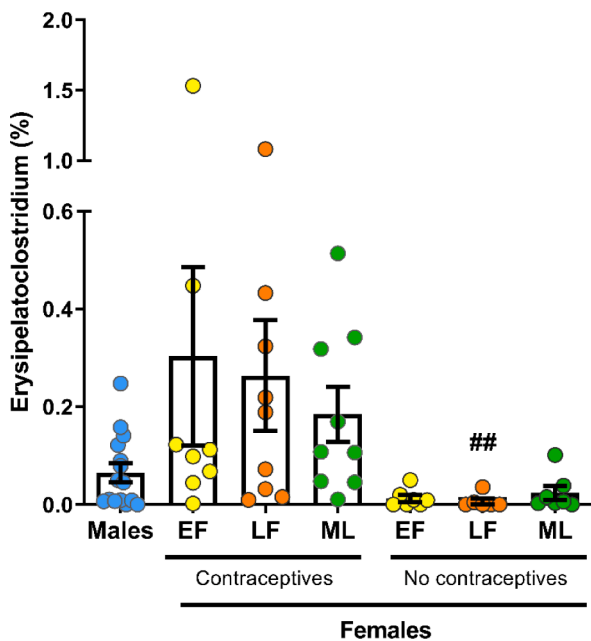


Fig. 5. Hormonal contraceptive use is associated with increased relative abundance of *Erysipelatoclostridium*. Relative abundance of *Erysipelatoclostridium* in faecal contents of males, and females (using hormonal contraceptives y/n) across the menstrual cycle. KW test and Friedman test with Dunn’s post-hoc tests were performed to assess differences between selected groups. ## $p \leq 0.01$ vs hormonal contraceptive users (LF). Abbreviations: KW = Kruskal Wallis, EF = early follicular phase, LF = late follicular phase, ML = mid luteal phase. N = 15 for males. For females’ contraceptive users N = 8 for EF; N = 9 for LF and for ML. For females no-contraceptive users N = 7 for EF and for ML; N = 6 for LF.

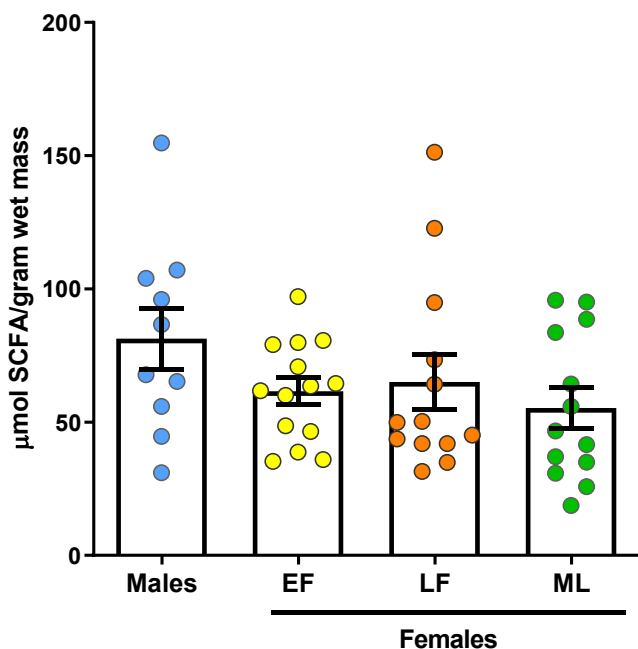


Fig. 6. Sex differences, menstrual cycle and hormonal contraceptive use did not influence SCFAs levels in faecal wet mass. SCFAs content (sum of acetate, propionate and nbutyrate) in faecal wet stool of males, and females across the menstrual cycle. Kruskal Wallis test and Friedman test with Dunn’s post-hoc tests were performed to assess differences between selected groups. Abbreviations: KW = Kruskal Wallis, EF = early follicular phase, LF = late follicular phase, ML = mid luteal phase. N = 15 for males. For females N = 14 for EF; N = 13 for LF and for ML.

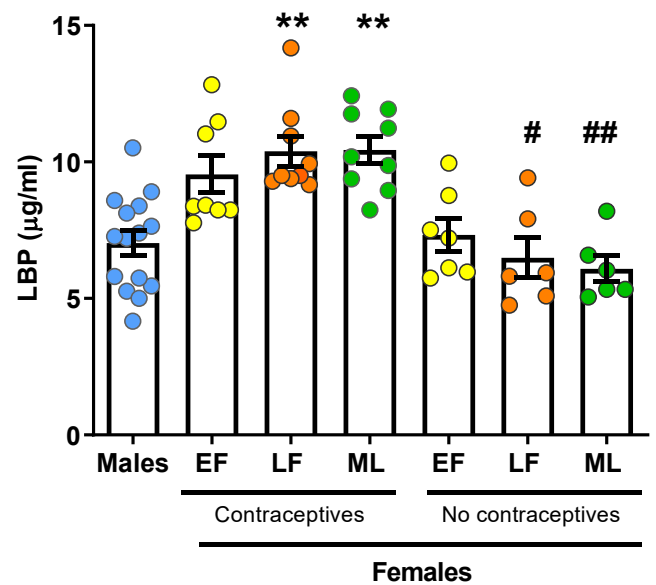


Fig. 7. Hormonal contraceptive use is associated with increased systemic levels of LBP. Plasma LBP concentrations of males, and females (using hormonal contraceptives y/n;) across the menstrual cycle. Kruskal Wallis test and Friedman test with Dunn’s post-hoc tests were performed to assess differences between selected groups. ** $p \leq 0.01$ vs Males; # $p \leq 0.05$, ## $p \leq 0.01$ vs hormonal contraceptive users (same phase). Abbreviations: EF = early follicular phase, LF = late follicular phase, ML = mid luteal phase, LBP = lipopolysaccharide-binding protein. N = 15 for males. For females contraceptive users N = 8 for EF; N = 9 for LF and for ML. For females no-contraceptive users N = 7 for EF; N = 6 for LF and for ML.

appreciate that the calculation of ratios in this study is slightly influenced by the fact that there was a maximum threshold of 100 mA. Two males and three females (per phase) exhibited a pain tolerance threshold of 100 mA, and it might be the case that these subjects could tolerate a greater electrical current stimulation. Though, we do not think that this maximum threshold has caused a false positive test outcome. PTT/PST ratio was overall lower in females than in males. When women were stratified in contraceptive users or not, significantly lower PTT/PST ratios were demonstrated only for the EF phase of contraceptive users and the ML phase of non-users. We suggest that the fact that no significantly lower PTT/PST ratios were shown for the other time points can be again ascribed to the relatively low sample size in this pilot study.

Recent studies support a role for the gut microbiota in modulating pain pathways, through the direct stimulation of nociceptive neurons, or by means of microbial-derived metabolites that can interact with several families of receptors responsible for pain perception (Lagomarsino et al., 2021). Factors that influence composition of the gut microbiota of an healthy individual include sex (Valeri and Endres, 2021) and use of oral contraceptives (Eyupoglu et al., 2020, Bharadwaj et al., 2015). Our analyses didn’t show any effect of sex, menstrual cycle or contraceptive use on the bacterial diversity or the relative abundance of the principal bacterial taxa. While other studies have also noted no significant correlation with sex, significant positive correlations for estrogenic medication were demonstrated for α -diversity (genus level), and a negative correlation was observed for ‘drospirenone and ethinylestradiol’ medication for Pilon evenness (genus level), but not for the other α -diversity indices (Falony et al., 2016).

The finding of the increase of the relative abundance of *Erysipelatoclostridium* in women contraceptive users may suggest that hormonal contraceptive use can stimulate growth of this specific genus. Species belonging to this genus include *Erysipelatoclostridium ramosum*, *cocleatum*, *spiroforme*, *saccharogumia* (Yutin and Galperin, 2013).

Changes in the abundance of bacterial taxa belonging to the

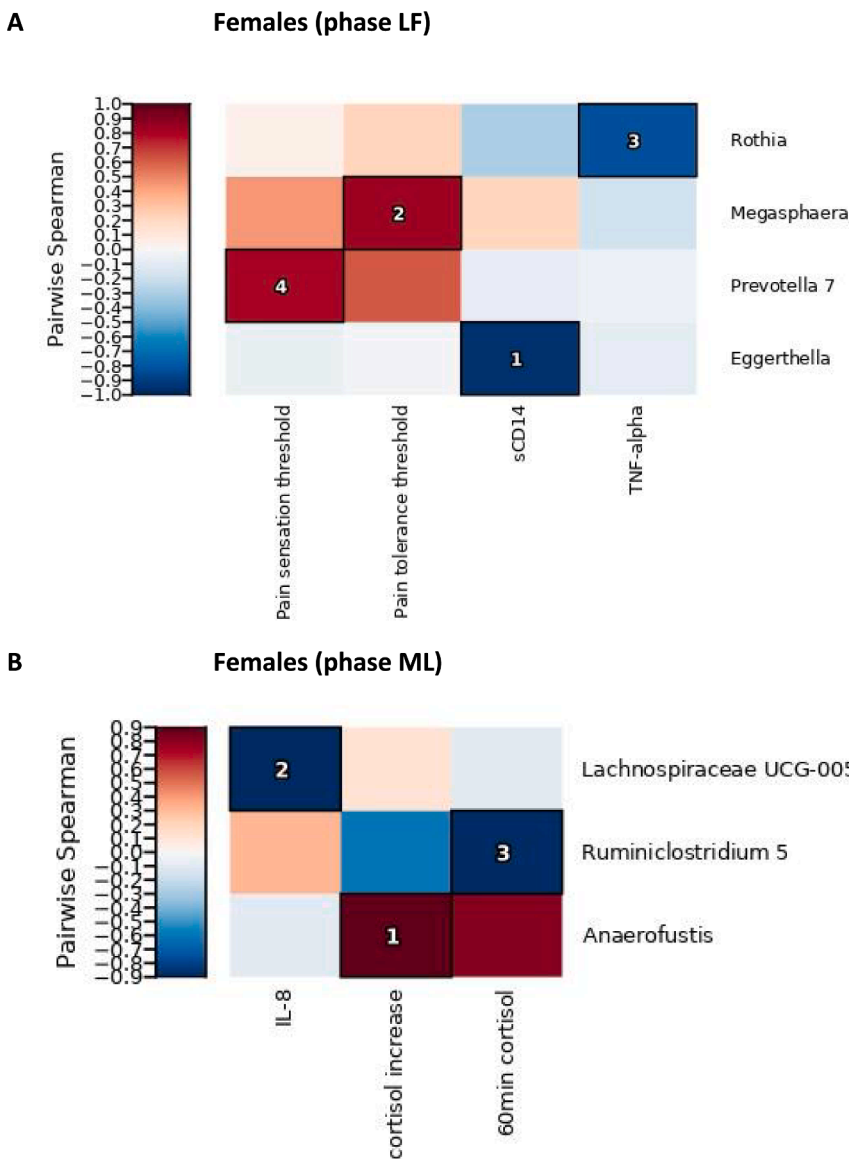


Fig. 8. Overview of the more highly prevalent/abundant genera that correlated with the parameters investigated in females across the menstrual cycle. Hallagram showing correlations between genera and physiological biomarkers statistically significant after FDR correction per phase of the menstrual cycle. Spearman correlation coefficients are expressed in colors: positive (red), negative (blue). White numbers mark the rankings of the strengths of the correlation: 1 = strong correlation, 3 = less strong but significant correlation. Abbreviations: sCD14 = soluble CD14, IL8 = interleukin 8, TNF = Tumor Necrosis Factor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Firmicutes, such as *Erysipelatoclostridium*, could underline the presence of alterations in the intestinal epithelial permeability and possible low-grade inflammation (He et al., 2020). In our study, hormonal contraceptive use was associated with increased LPB plasma levels in ML and LF phases of the menstrual cycle. LPB is a protein that is synthesized (amongst others) by the liver and intestinal epithelial cells and has a dual role in controlling inflammatory responses, since it can increase cytokine release in response to LPS (relatively low LPB levels) and neutralization of LPS (relatively high levels of LPB) (Gutsmann et al., 2001). It was previously demonstrated that estriol injection in rats strongly increased LPB mRNA in the liver (Ikejima et al., 1998). Furthermore, estrogen receptor (ER)- α was identified as the key receptor for estrogen-induced LPB mRNA expression in the liver using ER- α and ER- β knock-out mice models (Chisamore et al., 2012). Taking these findings into account, our data suggest that hormonal contraceptive use may increase systemic LPB levels through a hormone-dependent mechanism and not through an increase of intestinal permeability. This hypothesis is strengthened by the fact that no differences were seen between contraceptive users and non-contraceptive users in the EF phase; oral contraceptive users normally do not ingest hormonal contraceptive pills during the first days of the follicular phase, suggesting that LPB level increases may be reversible on a short-term basis.

Plasma levels of LPB and/or sCD14 are often associated with low grade systemic inflammation in several conditions (Beers et al., 2020, Keane et al., 2021), including pain (Kudoh et al., 2020). However, we did not note any difference in pro-inflammatory cytokine levels between males and females, across the menstrual cycle and between contraceptive users and those not using contraceptives.

Production of the stress hormone cortisol is under regulation of the HPA axis. Systemic levels of cortisol fluctuate during the day, with relatively high levels in the morning (i.e. CAR) and low levels in the evening/night. To date, CAR is widely used as an HPA-axis functional marker, as a blunted CAR has been associated with several disorders (e.g. psychological or immunological disorders) (Fries et al., 2009). We aimed to investigate differences in HPA-axis functioning between our participant groups. Therefore, morning salivary cortisol levels were determined to gain insight in the CAR profile. An advantage of measuring cortisol in saliva is that collection is non-invasive. Further, cortisol found in saliva is unbound, meaning that it reflects the systemic availability of free 'active' cortisol, which is not the case when looking at total cortisol in serum or cortisol hair. This could have impacted the stability of cortisol awakening response parameters. Elder et al. demonstrated in a highly controlled environment that total cortisol secretion and the profile of the CAR were relatively stable, but the

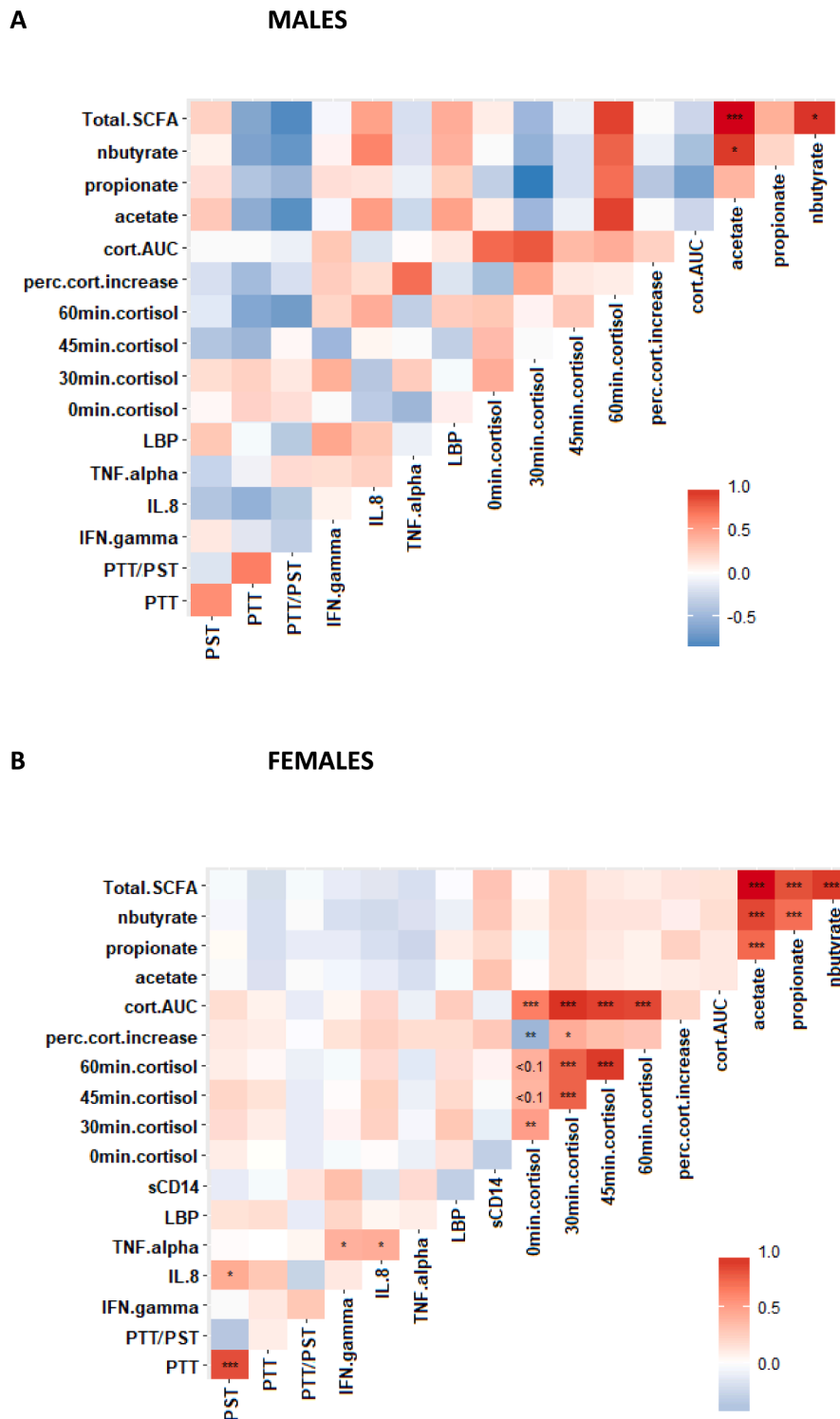


Fig. 9. Correlation plots of males (A) and females (B). Spearman correlation coefficients expressed in colors, positive (red), negative (blue). *FDR-corrected p-value < 0.05, **FDR-corrected p-value < 0.01, ***FDR-corrected p-value < 0.001. FDR were calculated separately for men and women. Abbreviations: sCD14 = soluble CD14, PST = pain sensation threshold, PTT = pain tolerance threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

magnitude of cortisol increase and awakening cortisol levels were not as stable (Elder et al., 2016). Carlsson et al. investigated the prevalence of negative CAR on workdays and days off in a healthy study population. They demonstrated a 26% prevalence of negative CAR (19% and 38% for workdays and days off, respectively) (Carlsson Eek et al., 2006). In our study we did not show an effect of sex, contraceptive use, or menstrual cycle on CAR parameters.

Correlation analyses provided some preliminary insights into sex differences in the relationship between baseline gut microbes and some host physiological parameters. In women, bacteria from the genera *Prevotella* and *Megasphaera*, previously associated with weight loss (Louis et al., 2016), diet (Jain et al., 2018), vaginal inflammation (van Teijlingen et al., 2020), and perinatal HIV infection (Kaur et al., 2018), correlated positively with electrical pain thresholds in a specific phase

(LF) of the menstrual cycle, suggesting a potential role for these bacteria in modulating hormone-dependent pain sensitivity. In the same phase of the cycle, we identified the genera *Eggerthella* and *Rothia* negatively correlated with intestinal permeability factors and inflammation, respectively. Another interesting finding is the positive correlation between bacteria from the genus *Anaerofustis* and the increase of salivary cortisol levels within the 30 min after awakening in the ML phase of the menstrual cycle. In a recent study performed in rats, the relative abundance of bacteria from the genus *Anaerofustis* was found significantly increased in animals susceptible to inescapable electric stress, whereas it did not change in those resilient (Zhang et al., 2019). On the contrary, cortisol level 60 min after awakening was negatively correlated with the genus *Ruminiclostridium* 5 in women during the ML phase. These preliminary findings suggest the presence of a sex-dependent baseline bacterial signature in the physiological functioning of the HPA axis, that could have important implication in stress-related pathological conditions, such as chronic pain.

5. Limitations

The main limitation of the present study is the small number of subjects enrolled in the trial. For instance, the lack of differences in PTT or PST across the menstrual cycle observed in our study is most likely due to the low sample size. Changes in the gut microbiota of female hormonal contraceptive users may be due to other factors not assessed in this study. Age, ethnicity, dietary habits, and use of xenobiotics (i.e., hormonal contraceptives) can influence the composition of gut microbial community, and a much larger sample size may help detect the contribution of each factor in shaping the gut microbiota as well as host inflammatory profile.

The CAR analysis presents some limitations. The prevalence of negative CAR observed in this study was up to 27%. Several factors could underlie this high prevalence. The actual time of awakening could have differed from the time that was stated as awakening, thereby shifting the cortisol awakening levels towards levels of awakening + 30 min. Bad sleep quality (e.g. caused by traffic noise) or a toilet visit during the night might be underlying factors associated with negative CARs in this study, since these may have initiated a CAR already during the night. Additionally, adherence to sampling times is an important aspect. A limitation of our study is that we did not collect the actual awakening and sampling times. Kudielka et al. investigated sampling compliance using electronic monitoring devices. 26% of the participants failed to collect at least 1 out of 6 saliva samples at the correct time of the day, and this had a significant impact on the circadian cortisol profile (Kudielka et al., 2003).

We acknowledge that the low sample number may have obstructed us from showing significant correlations between the gut microbiota and host stress or pain outcomes; however, our results demonstrate a relationship between somatic sensory pain and stress response in healthy women, in which the baseline gut microbiota seems to play a role.

6. Conclusions

This is the first study to highlight the connections between the gut microbiota and 1 sex differences in physiological pain perception. Further studies will help to elucidate how sex interacts with host factors and the gut microbiota and how these relationships modulate somatic pain sensitivity, for example, by performing this investigation in a larger cohort and by addressing sex-dependent variations in pain responses to mechanical, thermal, and electrical stimuli. Despite its limitations, this piece of research does set a foundation for identifying new somatic pain biomarkers and for developing individual therapeutic interventions for pain.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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