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Expected and Unexpected Outcomes of Probiotics and Metformin Interventions in Polyendocrine Metabolic Ovary Syndrome (PMOS, former PCOS): Findings from a Randomised Placebo-Controlled Trial

Veronika Tandl
Medical University of Graz
Valentin Borzan
Medical University of Graz
Regina Riedl
Medical University of Graz
Barbara Brunnhofer
Medical University of Graz
Olivia Trummer
Medical University of Graz
Barbara Obermayer-Pietsch
barbara.obermayer@medunigraz.at
Medical University of Graz

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Abstract

Background: Polyendocrine Metabolic Ovary Syndrome (PMOS, formerly known as Polycystic Ovary Syndrome, PCOS) is a complex endocrine disorder characterised by hyperandrogenism, oligo-or anovulation, metabolic disturbances and polycystic ovarian morphology. Depending on symptom profile and treatment goals, management commonly includes lifestyle interventions, oral contraceptives, and/or insulin-sensitizing medication. Because gut microbiome alterations are common features in PMOS, this study explores dietary probiotic supplements as a potential therapeutic approach.

Methods: A mono-centric, double-blind, randomised phase IV trial assessed a 6-month probiotic intervention versus placebo and open-label metformin (positive control). Recruitment occurred from November 2020 to March 2023, mainly during the COVID pandemic. Primary outcome was a change in serum free testosterone. Secondary outcomes included hormonal, metabolic, nutritional and gut-microbiome parameters. Within-group effects (baseline to 6 months) and between-group effects (comparisons of treatment effects) were analysed using t-test, Mann-Whitney U-test, and ANCOVA as appropriate.

Results: Of 112 participants screened, 102 were randomised (32 probiotic, 36 placebo, 34 metformin). The target sample size of n= 60 per group was not achieved. The primary endpoint was evaluable in 79 participants (25 probiotics, 27 placebo, 24 metformin). Changes in serum free testosterone were not different between the probiotic and placebo group (least square mean difference, LSMD = 0.500, 95% confidence interval, CI-0.047 to 1.047, p=0.072). Neither intervention significantly changed serum free testosterone after 6 months. Beneficial effects included a reduction in menstrual cycle length (probiotics: $\Delta = -40.1 \pm 55.0$ days, $p = 0.003$; metformin: $\Delta = -15.1 \pm 30.4$ days, $p = 0.025$). Supplemented vitamin D levels increased in the probiotic group ($\Delta = 9.82 \pm 8.32$ ng/mL, $p < 0.0001$). Across clinical, hormonal, metabolic, nutritional and gut microbiome assessments, no treatment group showed a consistent pattern of improvement; instead, both probiotic and metformin interventions were associated with less favourable changes in several metabolic outcomes during the study period.

Conclusions: While testosterone outcomes did not differ in this study, probiotic intervention significantly improved menstrual cyclicity. However, the lack of improvement of hormonal and metabolic parameters even in the metformin treated group suggests that additional factors, e.g. nutritional and lifestyle changes during the COVID-19 pandemic period might have limited treatment responsiveness across all interventions and warrants further investigation and replication.

Trial registration: EudraCT number: 2020-000228-20, 2020-08-28. Clinicaltrials.gov identifier: NCT04593459, date: 2020-10-12.

Introduction

Polyendocrine Metabolic Ovary Syndrome (PMOS - up to May 2026 known as/referred to as Polycystic Ovary Syndrome, PCOS), is the new name for a complex endocrine-metabolic disorder affecting 10–20%

of women worldwide throughout lifetime (1). PMOS has a complex pathophysiology including genetic (2), but also environmental, nutritional and intrauterine factors and shows significant clinical heterogeneity. Current definitions include at least two out of three features: hyperandrogenism (HA), oligo- or anovulation (OM), and/or polycystic ovarian morphology (PCOM), following the exclusion of other androgen-excess disorders based on the 2003 Rotterdam Criteria (1). As part of its complex systemic nature, PMOS can be accompanied by exacerbations of the metabolic risks including diabetes type 2, gestational diabetes and other pregnancy complications in case of successful conception, but also unfulfilled child wish, which might in turn aggravate psychological disturbances and body discontent and/or misconception.

Metabolic comorbidities in PMOS may range from hyperinsulinemia and insulin resistance (IR) to type 2 diabetes mellitus (T2DM), and the metabolic syndrome – therefore, including all features which significantly increase risks for obesity and cardiovascular diseases (3–5). Hyperandrogenism plays a pivotal role in this metabolic dysfunction, linking directly to insulin resistance (6). In turn, elevated insulin levels stimulate ovarian theca cell androgen synthesis, thereby promoting PMOS symptomatology by increasing androgen levels, e.g. both total and free testosterone concentrations (6, 7), but also adrenal androgens such as androstenedione (ASD) and dehydroepiandrosterone (DHEA) as part of hormonal diagnostic profiles in PMOS.

Importantly, gut microbiome dysregulation has been shown to contribute to these many symptoms in recent years and is thought to be part of PMOS' pathogenesis and vice-versa (8–10). PMOS-typical changes relate mainly to decreased diversity of bacterial microbiome composition, with an impact on their specific metabolites, e.g. bile acids or short-chain fatty acids, but certainly include also fungi, viruses, and archaea that remain to be further elucidated, as well as their specific functions to modulate host metabolism and potentially reproductive function.

Particularly with the advent of next-generation sequencing technologies, a growing body of evidence has emerged, highlighting the role of the gut microbiome and its complex interplay with host metabolism, especially with a focus on hormone regulation. This connection between metabolic health and the gut microbiome extends to PMOS, where several features of gut microbiome dysbiosis have been observed. These include reduced alpha diversity, shifted relative abundances of microbial taxa, and altered profiles of microbiota-derived metabolites (10–12).

As there is currently no uniform therapy for PMOS, clinical management is primarily symptomatic and tailored to the priorities of the affected woman. Therapeutic goals may include fertility management, metabolic improvement (including weight loss and insulin sensitization), or cosmetic concerns related to dermatological symptoms. Standard treatment options include lifestyle modifications, combined oral contraceptives and anti-androgens, but to a large part also insulin sensitizers (13). One of them is metformin, used in PMOS treatment for decades, and generally validated for its ability to mitigate hyperinsulinemia – albeit as an off-label drug in PMOS, despite its presence in the international guidelines. By lowering circulating insulin levels, metformin subsequently reduces ovarian androgen

production, thereby ameliorating a range of PMOS symptoms. However, side effects like gastrointestinal issues may occur (14). In turn, changes of gut microbiota during metformin intake have been described (15).

Interventions targeting the gut microbiome in PMOS to modulate and restore gut microbiome homeostasis are promising but remain controversial (8). While several studies have evaluated the efficacy of probiotic and synbiotic supplementation in PMOS women, results have been heterogeneous (16). Reported beneficial outcomes include the normalization of gut microbiome composition, restoration of gut barrier integrity, enhanced insulin sensitivity, improved lipid profiles, anti-inflammatory effects, and reduction of hyperandrogenism (17), but studies and inclusion/outcome criteria are very heterogeneous and frequently not covering microbiome and gut permeability data. PMOS parameters of interest are not only cycle abnormalities (18), but e.g. an improved sexual function or psychological changes in individual women with PMOS (19, 20). Of note, network structure diagrams showed some influence of probiotics and vitamin D in relation to HOMA-IR that might be of interest for further studies (21).

However, several studies are poorly defined or multimodal and/or include only small groups of PMOS women and might therefore not be extendable to many affected women.

In the present study, we aimed to further investigate the potential benefits of probiotic supplementation in PMOS in well-defined, large groups. We conducted a double-blind, randomized controlled trial comparing probiotic intervention against both placebo and metformin treatment over a six-month period.

A comprehensive panel of endocrine, metabolic, nutritional and gut-microbiome outcome parameters was measured and analysed, with the primary outcome defined as the change in serum free testosterone. The study protocol has been previously published (22).

Methods

This study followed the procedures outlined in a previously published, detailed protocol (22) and is in compliance with the CONSORT 2025 criteria (www.consort-spirit.org). Key elements and deviations are briefly summarized here.

Study design

This was a mono-centric, phase IV, randomized clinical trial with a double-blind probiotic/placebo comparison and an additional open-label metformin arm, conducted at the Endocrinology Outpatient Clinic of the Division of Endocrinology and Diabetology, Department of Internal Medicine, Medical University of Graz, Austria, between November 2020 and March 2024. The trial compared a 6-month probiotic intervention to placebo (primary comparison) and included an open-label metformin arm as a non-inferiority control.

Participants

Eligible were pre-menopausal, female adults (18–45 years), with PMOS diagnosed according to the Rotterdam criteria. Key exclusion criteria included other causes of hyperandrogenism, current pregnancy or breastfeeding, recent antibiotic use, use of systemic steroids or hormonal contraceptives within 6 months, and chronic gastrointestinal disease. Full inclusion/exclusion criteria are reported in the published protocol. The participants provided written informed consent at the screening visit, before starting the trial.

Randomization

PMOS women were first randomised in a 2:1 ratio to either a double-blind group or an open-label metformin group using permuted block randomization with block size 15. This open randomization is performed via Randomizer (www.randomizer.at) developed at the Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz. A second 1:1 randomisation step allocated participants within the double-blind arm to either the probiotic group or the placebo group. The second randomization as well as the allocation concealment is performed by Winclove Probiotics B.V, Netherlands. The published study protocol details randomization and blinding methods (22).

Intervention

The dietary probiotic supplement used (a modified version of the commercially available OMNi-BiOTiC® metabolic product; Winclove Probiotics B.V., Amsterdam, The Netherlands, provided by Institut AllergoSan GmbH, Graz, Austria) contained seven bacterial strains: *Lactobacillus salivarius* W57, *Lactobacillus casei* W56, *Lactobacillus rhamnosus* W71, *Lactococcus lactis* W58, *Enterococcus faecium* W54, *Lactobacillus plantarum* W62 and *Lactobacillus acidophilus* W22 and 2000 IU cholecalciferol per daily sachet; excipients included corn starch, maltodextrin, fructo-oligosaccharides, galacto-oligosaccharides, polydextrose, plant proteins, potassium chloride, magnesium sulfate, manganese sulfate and lactose.

The visually and organoleptically matching placebo consisted of corn starch, maltodextrin, potassium chloride, magnesium sulfate and manganese sulfate.

Participants received enough sachets to cover the intervention period while using 1 sachet per day.

The metformin group received extended-release metformin (Glucophage®, Merck GmbH, Darmstadt, Germany) starting at 500 mg once daily for 7 days, then 500 mg twice daily thereafter.

Participants were instructed to record any significant health and diet changes, and the first day of each menstrual cycle.

Timeline

Screening procedures included written informed consent, medical history, baseline questionnaires, and blood and urine sampling. Participants completed stool collection in specific sample containers at home before the baseline visit. Baseline visit 1 (within 4 weeks of screening) comprised randomization and distribution of study medication.

After 24–26 weeks of intervention, participants returned for visit 2, at which all baseline assessments were repeated. Additional protocol-specified procedures (e.g., isoflavone challenge, gut permeability assays, endoscopic biopsy sampling, or FACS analyses of B cell subtypes), were performed in the trial, however, are beyond the scope of the present manuscript and will be reported separately.

Objectives and outcome parameters

The primary endpoint was change in serum free testosterone (free T) from baseline to 6 months.

Further secondary endpoints included:

- Measures of energy metabolism:

HOMA-IR and Matsuda indices derived from an oral glucose tolerance test,

incretins (leptin, glucagon-like peptide 1 (GLP-1), glucagon, peptide YY (PYY)) and

modified food frequency questionnaires (33)

- Hormonal panel:

Anti-Müllerian hormone (AMH), androstenedione (ASD), follicle-stimulating hormone (FSH), luteinizing hormone (LH), dehydroepiandrosterone sulfate (DHEAS), 17α-hydroxy-progesterone (17-OHP), 17β-hydroxy-estradiol (E2), total testosterone (total T), 25(OH)vitamin D (25(OH)D)), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3), free tetraiodothyronine (fT4)

- Hirsutism:

Modified Ferriman–Gallwey score (mFG)

- Anthropometrics:

Body-mass-index (BMI), waist-to-hip ratio (WHR)

- Markers of inflammation:

C-reactive protein (CRP)

- Gut microbiome composition:

16S rRNA sequencing of stool samples

- Lipid profiles:

Low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides (TGs), lipoprotein(a) (LPA)

Only the outcomes relevant to the present analysis are described here; additional protocol-defined endpoints will be presented separately. Side effects were documented at each visit.

Laboratory measurements

Serum hormones and clinical chemistry were measured at the Endocrinology Lab Platform and the Clinical Institute of Medical and Chemical Laboratory Diagnostics at the Medical University Graz, Austria.

Microbiome analysis

Stool microbiome composition was analyzed by 16S rRNA gene amplicon sequencing on an Illumina platform using the primers Jac 319F_12 and Jac 806R_10. Demultiplexed and merged reads were processed in QIIME 2 using DADA2 (denoise-single) with trimming of 5 bases, truncation at 420 bp, maximum expected errors of 2, and chimera removal by consensus. Taxonomy was assigned using a naïve Bayes classifier trained on the SILVA 138 99% database. Phylogenetic trees were generated with MAFFT and FastTree. Alpha diversity (Faith's phylogenetic diversity) and beta diversity (weighted and unweighted UniFrac) were calculated after rarefaction to 650 reads per sample. This depth, which retained 31.42% observations in 86.03% samples, was chosen to keep a meaningful sample size for each timepoint and treatment group.

Sample size

The target sample size was 60 participants per arm (total n = 180), based on pilot data anticipating a mean difference in free testosterone of − 0.6 pg/mL between probiotic and placebo, a common SD of 1.1 pg/mL, 80% power and two-sided α = 0.05 (allowing 10% dropout). This recruitment target was not reached during the course of the trial, and the achieved sample size is reported in the results section.

Statistical analysis

Statistical analyses were performed on the intention-to-treat population using SAS v9.4. Continuous data are described by mean ± standard deviation (SD), median and minimum and maximum unless otherwise stated. Categorical data is summarized using frequencies and percentages. Changes from baseline to month 6 were compared between groups (probiotic vs. placebo and probiotic vs. metformin) using analysis of covariance (ANCOVA), with change from baseline as the dependent variable and baseline value and treatment group included as covariates. If ANCOVA assumptions were not met, t-tests or

Mann–Whitney U tests were used as appropriate. Treatment effects for between-group comparisons are expressed as least-squares mean differences (LSMDs) with 95% confidence intervals (CIs).

Long-term effects of probiotic intervention were explored separately for the groups by linear models including the measurements at baseline and follow up visits. Menstrual cycle length was compared between groups with Mann-Whitney-U tests and within groups with Wilcoxon signed-rank tests. Microbiome comparisons (alpha and beta diversity) used established QIIME2 workflows. All statistical tests were two-sided, with $p < 0.05$ considered significant. Full statistical methods and a predefined statistical analysis plan (SAP) were prepared prior to unblinding.

Data collection

Clinical data were recorded on CRFs and entered into the Phoenix online database; laboratory results were stored in the hospital clinical information system and exported with study codes for analysis. Data monitoring was conducted by an independent monitor assigned by the sponsor. All personal data were pseudonymized and handled in accordance with applicable Austrian regulations; access to identifiable data was restricted to authorized study staff. Data are accessible at Division of Endocrinology and Diabetology, Dept. of Internal Medicine, Medical University of Graz, Graz, Austria.

Ethics approval and consent to participate, trial registration

The study was approved by the Ethics Committee of the Medical University of Graz (approval EK 32–230 ex 19/20) and cleared by the Austrian authority (BASG). The trial is registered (EudraCT 2020-000228-20; ClinicalTrials.gov NCT04593459). The authors declare that all study procedures were performed in accordance with relevant guidelines and regulations, e.g. the Declaration of Helsinki and all participants provided written informed consent.

The study design and protocol was published in 2023 (22).

Results

Recruitment of the study took place between November 2020 and March 2023, meaning the study period overlapped significantly with the global COVID-19 pandemic (Fig. 1).

A total of 112 participants attended the screening visit and completed baseline assessments (Fig. 1). Of these, 10 withdrew before randomisation, leaving 102 participants who were randomised at Visit 1. Randomisation was performed in two stages: first, a 2:1 allocation assigned 68 participants to the probiotic/placebo pathway and 34 to the open-label metformin arm. A subsequent double-blind randomisation (1:1) allocated 32 participants to probiotics and 36 to placebo.

The primary outcome analysis set was reduced to 25 participants in the probiotic group, 27 in the placebo group, and 24 in the metformin group due to missing data (placebo: $n = 1$ at visit 1, $n = 4$ at visit 2; metformin: $n = 1$ at visit 2) and the exclusion of implausible values (placebo: $n = 1$; metformin: $n = 1$; Fig. 2, Table 2).

Baseline characteristics, including general demographics and PMOS-related parameters, were comparable across all three treatment groups (Table 1).

Table 1
Baseline characteristics of the study cohort. Continuous variables are expressed as mean ± standard deviation (SD). Reference ranges based on clinical parameters at the Division of Endocrinology and Diabetology, Medical University of Graz.

Parameter	Probiotic	Placebo	Metformin	Reference range
age [years]	26.8 ± 5.5 *n* = 32	27.5 ± 4.8 *n* = 36	27.1 ± 4.4 *n* = 34	-
Free T [pg/mL]	2.7 ± 1.2 *n* = 32	2.8 ± 1.3 *n* = 34	2.6 ± 0.8 *n* = 33	0.45–2.19
ASD [ng/mL]	4 ± 1.8 *n* = 32	3.7 ± 1.4 *n* = 35	4.1 ± 1.6 *n* = 34	0.3–3.5
DHEAS [µg/mL]	1.8 ± 0.8 *n* = 32	1.8 ± 0.7 *n* = 35	1.9 ± 0.8 *n* = 34	0.46–2.75
AMH [ng/mL]	9.8 ± 6.2 *n* = 32	8.6 ± 4.3 *n* = 35	7.9 ± 4.5 *n* = 34	0.03–13.34
FSH [µU/mL]	5.4 ± 2.1 *n* = 32	5 ± 2.1 *n* = 35	6.1 ± 2.1 *n* = 34	1.79–22.51
LH [µU/mL]	9.5 ± 6.1 *n* = 32	9.4 ± 6.5 *n* = 35	14.5 ± 20.8 *n* = 34	1.2–103.03
17-OHP [ng/mL]	1.7 ± 1.1 *n* = 32	1.6 ± 0.7 *n* = 35	1.8 ± 1.4 *n* = 34	0.35–4.13
E2 [pg/mL]	101.1 ± 127.4 *n* = 32	79.0 ± 59.4 *n* = 35	79.5 ± 84.4 *n* = 34	22.4–517
mFG score	13.8 ± 8.1 *n* = 32	13.0 ± 6.4 *n* = 36	12.0 ± 7.6 *n* = 34	< 7
cycle length [d]	72.9 ± 46.0 *n* = 24	55.1 ± 33.9 *n* = 31	54.3 ± 31.3 *n* = 28	-
regular cycle [n/total n]	5/32	10/36	5/34	-
HOMA-IR	1.96 ± 1.65 *n* = 32	1.96 ± 1.43 *n* = 32	1.79 ± 1.65 *n* = 34	-

Parameter	Probiotic	Placebo	Metformin	Reference range
BMI [kg/m²]	28.3 ± 8.8 *n* = 32	26.9 ± 7.7 *n* = 36	26.3 ± 5.6 *n* = 34	-
25(OH)D [ng/mL]	26.3 ± 17.6 *n* = 32	25.5 ± 10 *n* = 34	24.9 ± 9.1 *n* = 34	30–60
TSH [mU/L]	2.1 ± 1.5 *n* = 32	2.3 ± 1.4 *n* = 35	2.0 ± 1.0 *n* = 34	0.10–4.00

Primary Outcome: Serum Free Testosterone

Baseline serum free T concentrations did not differ among the three treatment groups (Table 1).

After 6 months of treatment, the mean free T levels were 3.0 ± 1.2 pg/mL in the probiotic group (change from baseline, Δ = +0.2 ± 1.2 pg/mL), 2.5 ± 0.9 pg/mL in the placebo group (Δ = -0.3 ± 1.4 pg/mL), and 2.3 ± 0.8 pg/mL in the metformin group (Δ = -0.3 ± 0.8 pg/mL; Table 2, Fig. 3). No statistically significant within-group changes were observed in any group (probiotic: p = 0.284; placebo: p = 0.144; metformin: p = 0.074).

Table 2
Change in serum free testosterone from baseline (BL) to 6 months of treatment (6mo) within groups. Statistical analysis using ANCOVA.

Group	Baseline mean ± SD [pg/mL]	*n* (BL)	Month 6 mean ± SD [pg/mL]	*n* (6mo)	Change (Δ) mean ± SD [pg/mL]	*n* (Δ)	*p*-value
Probiotic	2.7 ± 1.2	32	3.0 ± 1.2	25	+ 0.2 ± 1.2	25	0.284
Placebo	2.8 ± 1.3	34	2.5 ± 0.9	29	-0.3 ± 1.4	27	0.144
Metformin	2.6 ± 0.8	33	2.3 ± 0.8	25	-0.3 ± 0.8	24	0.074

Results for the primary objective, i.e. group differences (probiotic vs. placebo) in the change in serum free testosterone are presented in Table 3. The change did not differ between the probiotic and placebo groups ($\Delta\Delta$ = 0.500, 95% CI -0.047 to 1.047, p = 0.072).

However, the negative change observed in the probiotic group differed significantly from that in the metformin group (between-group comparison of Δ values: $\Delta\Delta$ = 0.636, 95% CI 0.111 to 1.161, p = 0.019; Table 3).

Table 3
Between-group comparison of change in serum free testosterone by ANCOVA as primary study outcome.

Comparison	**estimated difference (*ΔΔ*) [pg/mL]**	**lower 95% CI [pg/mL]**	**upper 95% CI [pg/mL]**	***p*-value**
Probiotic vs. Placebo	0.500	-0.047	1.047	0.072
Probiotic vs. Metformin	0.636	0.111	1.161	**0.019**

Secondary Outcomes

Hormones and Clinical Hyperandrogenism

In addition to free T, other serum hormone levels and mFG scores were assessed to evaluate PMOS-related hyperandrogenism and hormonal imbalances.

Within-group analyses demonstrated a statistically significant decrease in serum DHEAS levels in the placebo group (Δ = -0.20 µg/mL, SD = 0.48, p = 0.036; Table 4).

Table 4
Serum hormone changes from baseline to 6 months of treatment. Statistical analysis using ANCOVA. Additional results are presented in Supplementary Table 2.

Analyte	Group	Baseline mean ± SD	*n* (BL)	6 months mean ± SD	*n* (6mo)	*Δ* mean ± SD	*n* (*Δ*)	*p*-value
ASD [ng/mL]	Probiotic	3.96 ± 1.77	32	4.57 ± 2.15	25	0.54 ± 1.74	25	0.118
	Placebo	3.72 ± 1.42	35	3.43 ± 1.33	29	-0.38 ± 1.47	28	0.223
	Metformin	4.11 ± 1.59	34	3.66 ± 1.86	25	-0.59 ± 1.82	25	0.137
DHEAS [µg/mL]	Probiotic	1.75 ± 0.78	32	1.98 ± 0.96	25	0.19 ± 0.71	25	0.184
	Placebo	1.77 ± 0.66	35	1.59 ± 0.68	29	-0.20 ± 0.48	28	**0.036**
	Metformin	1.86 ± 0.75	34	2.24 ± 2.00	25	0.38 ± 1.67	25	0.265
total T [ng/mL]	Probiotic	0.41 ± 0.20	32	0.41 ± 0.16	25	0.00 ± 0.12	25	0.945
	Placebo	0.35 ± 0.10	35	0.35 ± 0.10	29	-0.01 ± 0.11	28	0.838
	Metformin	0.37 ± 0.10	34	0.33 ± 0.11	25	-0.04 ± 0.11	25	0.063

Between-group comparisons revealed that probiotic treatment was associated with a significantly greater increase in ASD levels compared to both placebo ($\Delta\Delta$ = 0.991, 95% CI 0.171 to 1.811, p = 0.019) and metformin ($\Delta\Delta$ = 1.05 ng/mL, 95% CI 0.087 to 2.012, p = 0.033; Table 5). Differences between probiotics and placebo were also observed for DHEAS levels ($\Delta\Delta$ = 0.391 µg/mL, 95% CI 0.065 to 0.716, p = 0.02), while total T changes differed between probiotic and metformin treatment ($\Delta\Delta$ = 0.057 ng/mL, 95% CI 0.003 to 0.110, p = 0.0038).

Table 5
Between-group comparisons of change in hormone parameters by ANCOVA or t-test. Additional results are presented in Supplementary Table 2.

Analyte	Comparison	estimated difference (*ΔΔ*)	lower 95% CI	upper 95% CI	*p*-value
ASD	Probiotic – Placebo	0.991	0.171	1.811	**0.019**
[ng/mL]	Probiotic – Metformin	1.05	0.087	2.012	**0.033**
DHEAS	Probiotic – Placebo	0.391	0.065	0.716	**0.02**
[µg/mL]	Probiotic – Metformin	-0.175	-0.907	0.557	0.633
total T	Probiotic – Placebo	0.032	-0.02	0.084	0.22
[ng/mL]	Probiotic – Metformin	0.057	0.003	0.110	**0.038**

No statistically significant within-group changes or between-group differences were observed for other assessed hormones, including AMH, FSH, LH, 17-OHP, E2, as well as mFG score (Tables S1, S2).

Menstrual Cycle

Study participants were asked about their menstrual cycle length and regularity.

Cycle length decreased significantly in both the probiotic (*Δ* = -40.1 d, SD = 55.0, *p* = 0.003) and metformin groups (*Δ* = -15.1 d, SD = 30.4, *p* = 0.025, Table 6), with no significant differences between treatment groups (probiotic vs. placebo p = 0.146, probiotic vs. metformin p = 0.385).

Table 6
Changes in cycle length from baseline to 6 months of treatment. Statistical analysis using Wilcoxon test.

Analyte	Group	Baseline mean ± SD	*n* (BL)	6 months mean ± SD	*n* (6mo)	*Δ* mean ± SD	*n* (*Δ*)	*p*-value
cycle length	Probiotic	72.9 ± 46.0	24	39.7 ± 38.7	20	-40.1 ± 55.0	17	**0.003**
[d]	Placebo	55.1 ± 33.9	31	47.0 ± 24.5	28	-5.2 ± 29.8	25	0.103
	Metformin	54.3 ± 31.3	28	49.0 ± 56.4	23	-15.1 ± 30.4	20	**0.025**

At baseline, 27/32 participants (84%) in the probiotic group had irregular menstrual cycles (Fig. 4). Following 6 months of probiotic intervention, 7 of these 27 participants (26%) achieved a regular cycle according to international consensus (13). One participant with a regular cycle at baseline developed

irregularity. Cycle status remained unchanged in 15 participants with irregular cycles and 2 with regular cycles. Data at 6 months were unavailable for 5 participants.

In the placebo group, 26/36 participants (72%) presented with irregular cycles at baseline. After 6 months, 5 of these 26 (19%) experienced an improvement to regular cycles, and 2 participants with initially regular cycles transitioned to irregular.

In the metformin group, 29/34 participants (85%) had irregular cycles at baseline. The intervention improved cycle regularity for 11 of these 29 participants (38%). None transitioned from regular to irregular cycles during the study period.

Energy Metabolism, Anthropometrics, Clinical Measures

The complex and varied symptomatology of PMOS lead us to investigate a broad panel of parameters related to metabolism, inflammation, thyroid, as well as general anthropometrics and clinical measures.

After 6 months of treatment, within-group analyses revealed significant changes in several metabolic parameters (Table 7).

Both the probiotic and metformin groups exhibited a significant increase in HOMA-IR (probiotic: $\Delta = 0.93$, SD = 1.09, $p < 0.001$; metformin: $\Delta = 0.35$, SD = 1.00, $p = 0.041$), accompanied by a decrease in the Matsuda index (probiotic: $\Delta = -.217$, SD = 3.54, $p = 0.002$; metformin: $\Delta = -2.10$, SD = 4.47, $p = 0.009$).

In the probiotic group alone, significant increases were observed in leptin ($\Delta = 2.3$ ng/mL, SD = 3.1, $p = 0.027$), TG ($\Delta = 14.4$ mg/dL, SD = 31.4, $p = 0.024$), and 25(OH)D levels ($\Delta = 9.82$ ng/mL, SD = 6.84, $p < 0.001$), alongside a reduction in WHR ($\Delta = -0.03$, SD = 0.05, $p = 0.001$).

The placebo group demonstrated a significant decrease in PYY levels ($\Delta = -28.9$ pg/mL, SD = 56.9, $p = 0.031$).

Table 7
Changes in energy metabolism parameters, anthropometrics and clinical measures from baseline to 6 months of treatment. Statistical analysis using ANCOVA. Non-significant findings in Supplementary Material.

Analyte	**Group**	**Baseline mean ± SD**	***n* (BL)**	**6 months mean ± SD**	***n* (6mo)**	***Δ* mean ± SD**	***n* (*Δ*)**	***p*-value**
HOMA-IR	Probiotic	1.96 ± 1.65	32	3.15 ± 2.00	24	0.93 ± 1.09	24	**< 0.001**
	Placebo	1.96 ± 1.43	32	1.88 ± 1.73	31	-0.10 ± 0.99	27	0.735
	Metformin	1.79 ± 1.65	34	2.37 ± 1.65	25	0.35 ± 1.00	25	**0.041**
Matsuda	Probiotic	7.37 ± 4.94	28	4.60 ± 2.78	23	-2.17 ± 3.54	21	**0.002**
	Placebo	6.68 ± 4.32	31	6.79 ± 4.10	26	0.07 ± 4.35	23	0.904
	Metformin	8.09 ± 4.92	31	5.65 ± 3.70	24	-2.10 ± 4.47	23	**0.009**
GLP-1 [pg/mL]	Probiotic	38.4 ± 55.3	26	79.1 ± 103.3	21	12.0 ± 103.9	18	0.108
	Placebo	46.1 ± 70.3	30	52.4 ± 61.1	32	-3.8 ± 77.8	26	0.769
	Metformin	43.1 ± 71.7	28	42.0 ± 48.7	25	-1.0 ± 60.7	20	0.973
Glucagon [pg/mL]	Probiotic	25.5 ± 18.9	13	27.7 ± 15.6	17	-0.5 ± 13.0	10	0.916
	Placebo	20.4 ± 12.2	16	19.7 ± 8.8	26	0.7 ± 11.4	13	0.862
	Metformin	22.3 ± 14.6	18	21.9 ± 12.4	21	-4.9 ± 11.9	12	0.463
PYY [pg/mL]	Probiotic	102.7 ± 74.2	26	94.3 ± 44.9	19	-14.9 ± 66.0	16	0.576
	Placebo	124.1 ± 78.7	30	104.6 ± 46.4	29	-28.9 ± 56.9	23	**0.031**
	Metformin	87.6 ± 46.1	28	119.5 ± 72.0	24	24.2 ± 83.3	19	0.063
Leptin [ng/mL]	Probiotic	15.4 ± 12.2	9	23.1 ± 14.1	12	2.3 ± 3.1	6	**0.027**

Analyte	Group	Baseline mean ± SD	*n* (BL)	6 months mean ± SD	*n* (6mo)	*Δ* mean ± SD	*n* (*Δ*)	*p*-value
	Placebo	12.3 ± 11.6	14	15.2 ± 12.0	17	2.6 ± 5.7	11	0.347
	Metformin	16.2 ± 23.7	11	13.2 ± 9.7	14	-8.2 ± 29.1	7	0.676
TG [mg/dL]	Probiotic	86.9 ± 46.4	31	108.7 ± 54.9	25	14.4 ± 31.4	25	**0.024**
	Placebo	75.0 ± 33.2	36	67.1 ± 26.1	30	-5.0 ± 21.1	30	0.127
	Metformin	70.4 ± 20.3	34	77.3 ± 30.7	25	3.3 ± 24.4	25	0.501
BMI [kg/m²]	Probiotic	28.30 ± 8.76	32	29.51 ± 9.36	25	0.19 ± 1.06	25	0.356
	Placebo	26.85 ± 7.67	36	27.47 ± 7.75	33	0.42 ± 1.42	33	0.093
	Metformin	26.33 ± 5.61	34	26.64 ± 5.80	26	-0.60 ± 1.51	26	0.058
WHR	Probiotic	0.83 ± 0.06	32	0.79 ± 0.05	25	-0.03 ± 0.05	25	**0.001**
	Placebo	0.82 ± 0.06	36	0.81 ± 0.06	33	-0.01 ± 0.05	33	0.336
	Metformin	0.81 ± 0.06	34	0.80 ± 0.06	26	-0.01 ± 0.05	26	0.209
25(OH)D [ng/mL]	Probiotic	26.29 ± 17.61	32	34.05 ± 12.95	25	9.82 ± 6.84	25	**< 0.001**
	Placebo	25.50 ± 9.97	34	26.11 ± 8.79	31	-0.49 ± 8.32	29	0.898
	Metformin	24.87 ± 9.07	34	24.83 ± 11.98	25	1.38 ± 8.71	25	0.417

Between-group analyses identified significant differences in several metabolic parameters (Table 8). The probiotic group showed significantly greater increases in HOMA-IR compared to both placebo (*ΔΔ* = 1.032, 95% CI 0.436 to 1.628, *p* = 0.001) and metformin (*ΔΔ* = 0.608, 95% CI 0.022 to 1.194, *p* = 0.042). Conversely, the probiotic group exhibited a significantly larger decline in the Matsuda index compared to placebo (*ΔΔ*: -2.253, 95% CI -2.353 to 0.958, *p* = 0.016).

Significant differences were also observed for TG levels (probiotic vs. placebo: *ΔΔ* = 23.032, 95% CI 8.5 to 37.565, *p* = 0.003) and BMI (probiotic vs. metformin: *ΔΔ* = 0.821 kg/m², 95% CI 0.069 to 1.573, *p* =

0.033). Additionally, 25(OH)D changes differed significantly between probiotic and both the placebo (t = − 5.00, df = 51.896, $p < 0.001$) and metformin ($\Delta\Delta$ = 8.446 ng/mL, 95% CI 3.94 to 12.951, $p < 0.001$) groups.

When comparing the modified food frequency questionnaires, significant changes observed for two components only: fruit/vegetable juices (reduction of consumption, p = 0.022) or mineral water/tea consumption (reduction of consumption, p = 0.008) – no report of changes for any other food items.

Table 8
Between-group comparisons of change in energy metabolism parameters, anthropometrics and clinical measures by ANCOVA or t-test (t-value and degrees freedom). Non-significant findings in Supplementary Material.

Analyte	Comparison	estimated difference ($\Delta\Delta$)	lower 95% CI	upper 95% CI	*p*-value
HOMA-IR	Probiotic – Placebo	1.032	0.436	1.628	**0.001**
	Probiotic – Metformin	0.608	0.022	1.194	**0.042**
Matsuda	Probiotic – Placebo	-2.253	-4.072	-0.434	**0.016**
	Probiotic – Metformin	-0.698	-2.353	0.958	0.400
TG [mg/dL]	Probiotic – Placebo	23.032	8.5	37.565	**0.003**
	Probiotic – Metformin	12.401	-4.327	29.129	0.143
BMI [kg/m²]	Probiotic – Placebo	-0.170	-0.853	0.513	0.620
	Probiotic – Metformin	0.821	0.069	1.573	**0.033**
25(OH)D [ng/mL]	Probiotic – Placebo	t-test	t: -5.00	df: 51.896	**< 0.001**
	Probiotic – Metformin	8.446	3.94	12.951	**< 0.001**

Gut Microbiome Composition

Gut microbiome composition was assessed using 16S rRNA sequencing.

Alpha diversity, measured by Faith's Phylogenetic Diversity, did not change significantly over the 6-month treatment period in any group (probiotics: $p = 0.3101$; placebo: $p = 0.2294$; metformin: $p = 0.2841$; Fig. 5).

Figure 5: Longitudinal changes in gut microbiome alpha diversity (Faith's Phylogenetic Diversity) from baseline (BL) to 6 months (6mo). No significant within-group changes were detected using Kruskal-Wallis test. All samples were rarefied to an even sampling depth of 650 prior to analysis.

Beta diversity analysis based on weighted and unweighted UniFrac distances showed no clustering of samples according to treatment group or time point (Fig. 6).

Safety and Adverse Events

Overall, no significant adverse effects were observed. About one third of the participants in all treatment groups reported some abdominal discomfort/transient diarrhoea at the start of the study, but no further reports during the study.

Dropouts from the study occurred in 18 participants, 3 of whom occurred in the placebo group (1 due to personal reasons, 1 due to gastrointestinal side effects, 1 to pregnancy), 7 in the probiotic group (4 due to personal reasons, 1 due to side effects, 2 due to pregnancy) and 8 in the metformin group (1 due to personal reasons, 5 due to side effects and 1 due to pregnancy). Furthermore - in the probiotic group, one pregnancy occurred before and one in the post-intervention period.

Discussion

The gut microbiome has increasingly been recognized as a modifiable factor in PMOS pathophysiology, with dysbiosis potentially contributing to metabolic disturbances, inflammation, and hyperandrogenism. In the present double-blind, randomized controlled trial, we sought to determine whether probiotic supplementation could ameliorate PMOS features, using metformin as a benchmark for therapeutic efficacy.

Though the primary endpoint, free testosterone, showed no significant overall changes, the between-group ANCOVA comparison revealed a significant difference between the probiotic and the metformin group, with a small increase in the former and a non-significant decrease in the latter group that was not expected based on the current knowledge about metformin effects in PMOS (1, 3), similar to the placebo group.

Of note, a significant shortening of the well-known oligo/amenorrhoeic cycles in PMOS women was seen in the probiotic intervention group, which was larger than in the parallel group using metformin. A similar result was reported in a recent randomized double-blind placebo-controlled probiotic trial without metformin comparison in 104 women with PMOS, where cycle shortening was the most prominent outcome compared to changes in total testosterone and other significant results such as an improvement of waist circumference and quality of life without adverse effects (18).

In addition, as no contraceptive measures were defined, four pregnancies were recorded, with two of them in the probiotic group. All of these outcomes have somewhat been expected based on the current literature (8).

However, there was a significant worsening of insulin sensitivity surrogates during the study period, which was never described in the currently available studies, as summarized in (11). There, probiotic

supplementation tended to improve anthropometrics, insulin resistance, lipid profiles, as well as biomarkers of inflammation and oxidative stress.

None of the other predefined parameters in our study, such as a variety of androgens or metabolic parameters were significantly improved by either probiotic or metformin therapy. The recorded marginal changes of variables were mainly comparable to the placebo group, or the dimension of changes did not reach clinical relevance.

On the contrary, even body weight/BMI, insulin-related parameters - with a concordant increase of HOMA-IR and a concomitant decrease of Matsuda indices - and incretins such as GLP1 and leptin tended to increase in some groups during the course of the study – sometimes even significantly - which was somehow unexpected.

Therefore, a critical limitation of this study is the recruitment timeline relative to the COVID-19 pandemic trajectory, which reduced the recruitment rate per se. Depending on their enrolment date, participants could have experienced up to three government-mandated lockdowns during the six-month intervention period, confronting them with extraordinary lifestyle disruptions (23, 24). Conversely, participants recruited later underwent their intervention during a period of relative normalization regarding the pandemic. These starkly divergent conditions, occurring within all treatment groups, likely acted as a confounding variable that may have obscured potential treatment effects and patient numbers. Meanwhile, there is a considerable number of publications, describing the acute consequences of mobility restrictions, social distancing, and the permanent perception of social alert during the COVID-19 pandemic, resulting in significant lifestyle – especially nutritional - changes all over the globe (23).

Notably, our study failed to demonstrate overall efficacy not only for the probiotic intervention but also for metformin, a well-established therapy for PMOS (14). This suggests that the profound psychosocial and physiological stressors associated with the pandemic peak may have attenuated the response to both experimental and standard treatments. Of interest, not only food choice and intake, but also other aspects of nutrition including prioritization of (unhealthy) products and cooking habits have been thought to be influenced by the movement restrictions and the social and psychological modifications induced by the pandemic (24).

However, this example might in turn be of interest to future interventions that might occur during exceptional situations. However, detailed food questionnaires did not show significant changes but might also be difficult to interpret under exceptional circumstances and might not entirely reflect dietary changes within the intervention groups.

A primary strength of this study lies in its comparative design. Probiotic intervention was evaluated against both a randomized placebo and an open-label metformin arm. This design was pivotal – in case the metformin arm had shown efficacy in comparison to the probiotic group, the conclusions would have been markedly different. The absence of positive results in both active arms points to systemic

confounders, most likely the COVID-19 pandemic, as discussed, which also severely hampered recruitment and resulted in a sample size below the target.

However, study participants and the study team were completing the planned procedures even under pandemic restrictions and generated a large amount of data despite external constraints. An important additional aspect in this context might be additional stress and psychological effects on metabolic and hormonal changes in PMOS. Psychological stress is a substantial burden in women living with PMOS including an increased risk of depression and suicidal behaviour. Therefore, psychological factors during intervention studies and the more in clinical practice should be considered and addressed (28).

Another specific strength of the study are the many research parameters measured, with a special focus on systemic incretin levels such as leptin, GLP1, glucagon and peptide YY. Further analyses of gastric and gut biopsies for local changes are ongoing, as described. To our knowledge, there are no PMOS probiotic intervention studies available that have measured incretin levels in blood – the current study might therefore serve as a basis for further investigations about incretin background and reactions within clinical studies in PMOS.

Of note, GLP-1 among other incretins has been regarded as an important modulator in the crosstalk of the reproductive and metabolic tissues, as GLP-1 receptors are present throughout the reproductive system and may be responsible for many bilateral effects, shown in animal models and some rare human studies (29). The many interferences become more evident with increasing knowledge about central hypothalamic functions (30). Given the developments in GLP-1 agonist drugs, these incretins are currently opening up a large field of potential interventions in women with PMOS (31) - open for new insights, but also inherent challenges and cautions in clinical use. The identification of women with PMOS displaying a specific multidimensional, metabolic high-risk PCOS phenotype with symptoms including central obesity, insulin resistance and metabolic dysfunction and respective medical history has been recommended as compared to women selected by BMI alone. Individualised therapy plans may therefore be important and should be supported by additional well-designed, PMOS-specific trials to close current knowledge gaps in obesity medications.

Of note, the integration of 16S rRNA sequencing represents a significant advancement over previous PMOS probiotic studies. Although this methodology holds theoretical promise for identifying mechanistic pathways, the lack of significant clinical effects in this trial limited its interpretability. Again, several expected changes on microbiome level could not be demonstrated – e.g. changes in alpha diversity or clustering in beta diversity have not been shown.

The probiotic intervention used in this study consisted of a dietary supplement powder comprising seven probiotic strains, cholecalciferol, and other ingredients listed in the methods. Because the formulation is a complex mixture, it is not possible to attribute the observed efficacy to any single component.

However, it is worth to look at vitamin D that has long been known to have some (patho)physiological associations in women with PMOS (32) and might also have its place in the complex interaction of

metabolic and fertility regulation (33). Low-dose cholecalciferol supplementation in the current study led to normal 25(OH)vitamin D serum levels in women with PMOS within the probiotic group. Initially, vitamin D levels of all participants had been comparably low including about 30% of women with overt vitamin D deficiency. We assume therefore that the effect of vitamin D supplementation could have had an additional impact on some of the changes recorded. It is noteworthy that the placebo formulation did not contain cholecalciferol, as it was intended to serve as an inert comparator, whereas cholecalciferol was considered as an essential component of the active intervention. Consequently, this study design represents also some study limitation, as the specific effects of the probiotic cannot be interpreted in complete isolation. However, measurement and sufficient vitamin D levels would be worth to be part of interventions planned in upcoming clinical studies, especially in countries with a high prevalence of vitamin D deficiency (34). Furthermore, vitamin D might be seen as one of several players in inflammatory, immunological and steroid signaling as well in the metabolic crosstalk with the microbiome and its many functions (35).

Overall, microbiome-targeted therapies continue to be of big interest to women with PMOS and their clinical care for their individual therapy goals and options (8).

There is a need for evidence-based and comparable studies in the use of probiotics and other microbiome-associated interventions to expand reliable therapy options for women with PMOS, adding the current study to the field of explorative data in view of the complex interactions between metabolic and fertility fields. With the many millions of women living with PMOS worldwide, it is worth to investigate further short- and long-term clinical treatment investigations with optimal individual approaches.

Summary

In this multistrain probiotic intervention study supplemented by vitamin D compared to placebo and a separate metformin treatment arm, a clinically relevant shortening of the menstrual cycles has been documented, but no changes in androgens or other hormonal parameters. As of an increase in BMI and metabolic profiles in all groups, the authors hypothesise, these changes might have mainly been provoked by nutritional and lifestyle changes during the COVID pandemic, which represents an important learning point for further intervention studies during challenging circumstances.

Abbreviations

17-OHP	17α-hydroxy-progesterone
25(OH)D	25(OH)vitamin D
AMH	Anti-Müllerian Hormone
ANCOVA	Analysis of Covariance

ASD	Androstenedione
BL	Baseline
BMI	Body Mass Index
CI	Confidence Interval
COVID	Corona Virus Disease
CRF	Case Report Form
CRP	C-Reactive Protein
DHEAS	Dehydroepiandrosterone Sulfate
E2	17β-hydroxy-estradiol
Faith PD	Faith’s Phylogenetic Diversity
FSH	Follicle-Stimulating Hormone
fT3	free Triiodothyronine
fT4	free Tetraiodothyronine
GLP-1	Glucagon-like Peptide 1
HA	Hyperandrogenism
HDL	High-Density Lipoprotein
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance
IR	Insulin Resistance
LDL	Low-Density Lipoprotein
LPA	Lipoprotein(a)
LH	Luteinizing Hormone
LSMDs	Least-Squares Mean Differences
mFG score	modified Ferriman-Gallwey score
OM	Oligo-- or anovulation

PCOM	Polycystic Ovarian Morphology
PCOS	Polycystic Ovary Syndrome
PHEIC	Public Health Emergency of International Concern
PMOS	Polyendocrine Metabolic Ovary Syndrome
PYY	Peptide YY
SAP	Statistical Analysis Plan
SD	Standard Deviation
T	Testosterone
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TSH	Thyroid-Stimulating Hormone
WHO	World Health Organization
WHR	Waist-to-Hip Ratio

Declarations

Ethics approval and consent to participate

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Consent for publication

All authors consented to publish the manuscript

Availability of data and materials

Data and materials can be accessed at the Division of Endocrinology and Diabetology, Dept. Internal Medicine, Medical University of Graz, Graz, Austria

Competing interests

All authors declared no competing interests

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Authors' contributions

VT (data analysis, manuscript), VB (study protocol and conduct, recruitment), RR (data analysis), BB (study conduct and recruitment), OT (manuscript), BOP (study protocol and conduct, recruitment, data analysis, manuscript); VT and VB contributed equally to the manuscript.

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Figures

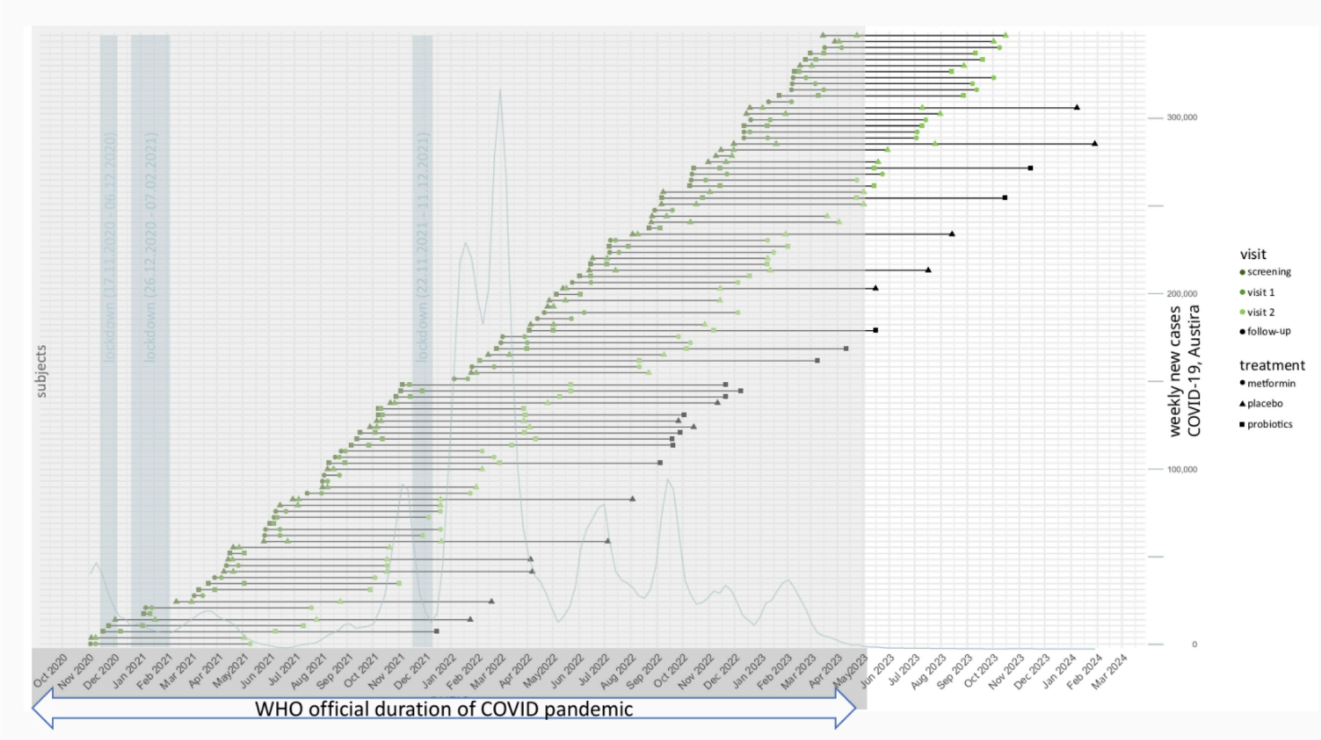


Figure 1

Study timeline. Dates for screening (dark green), visit 1 (green), visit 2 (light green) and, if applicable, follow-up (black), are given for each subject. The light blue line indicates the weekly new COVID-19 cases in Austria for the same time frame (26). The Light blue squares show government mandated lockdowns. The grey shaded months indicate the period during which the World Health Organization (WHO) declared a Public Health Emergency of International Concern (PHEIC, (25)).

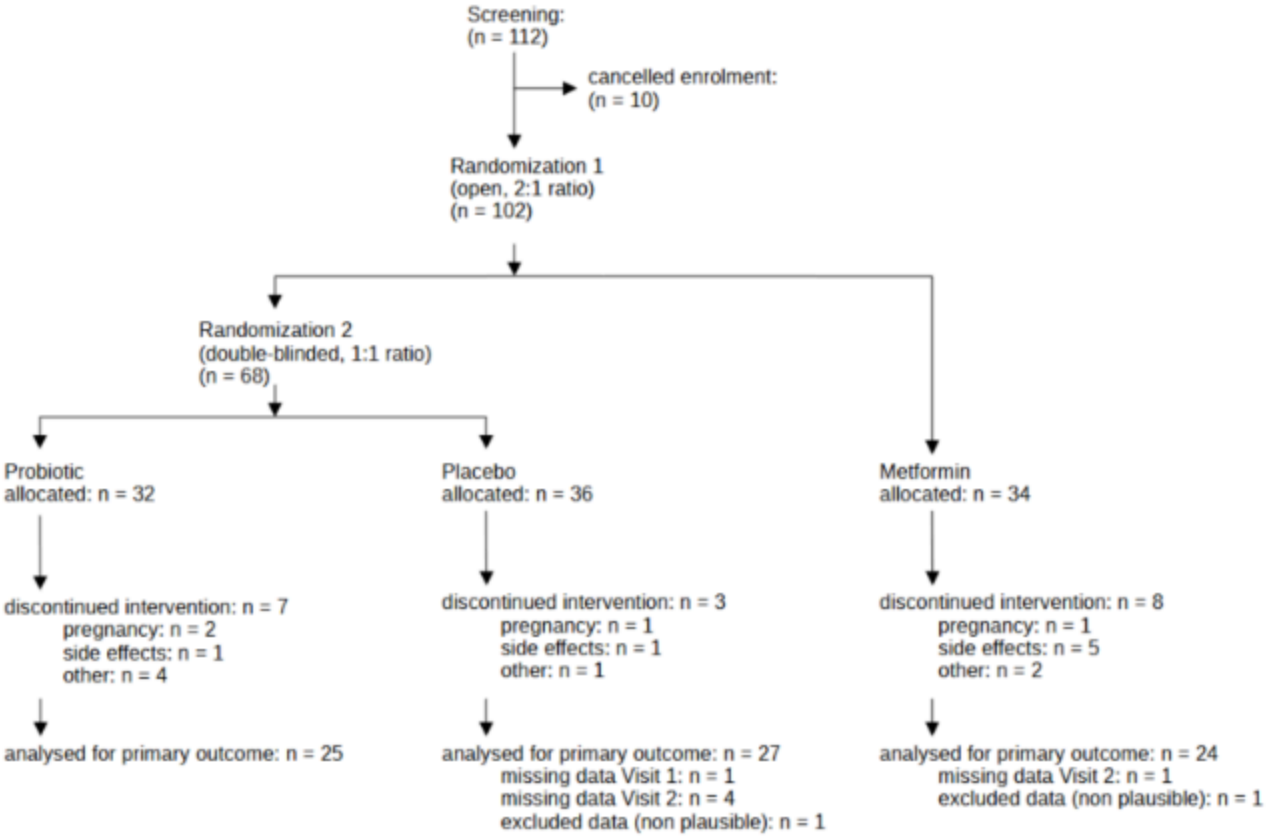


Figure 2

CONSORT Flow Diagram. Participant recruitment, randomization, and retention throughout the study. The diagram displays the number of participants screened, randomized into 3 treatment groups (probiotics, placebo, metformin), completed intervention, and analysed (primary endpoint). Reasons for discontinuation of intervention are indicated for each group.

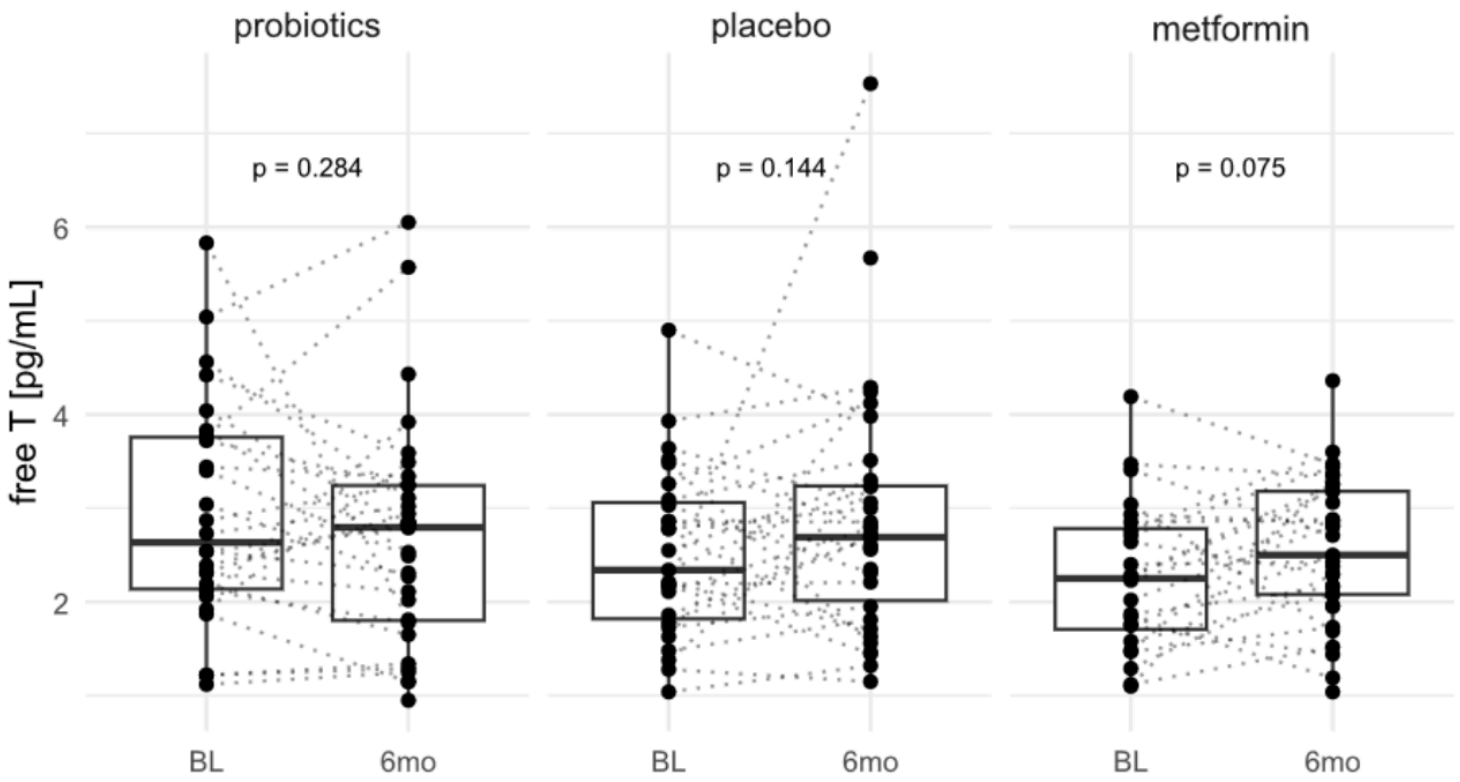


Figure 3

Box plot depicting changes in serum free testosterone (T) from baseline (BL) to 6 months of treatment (6mo) Dotted lines link paired measurements from the same participants.

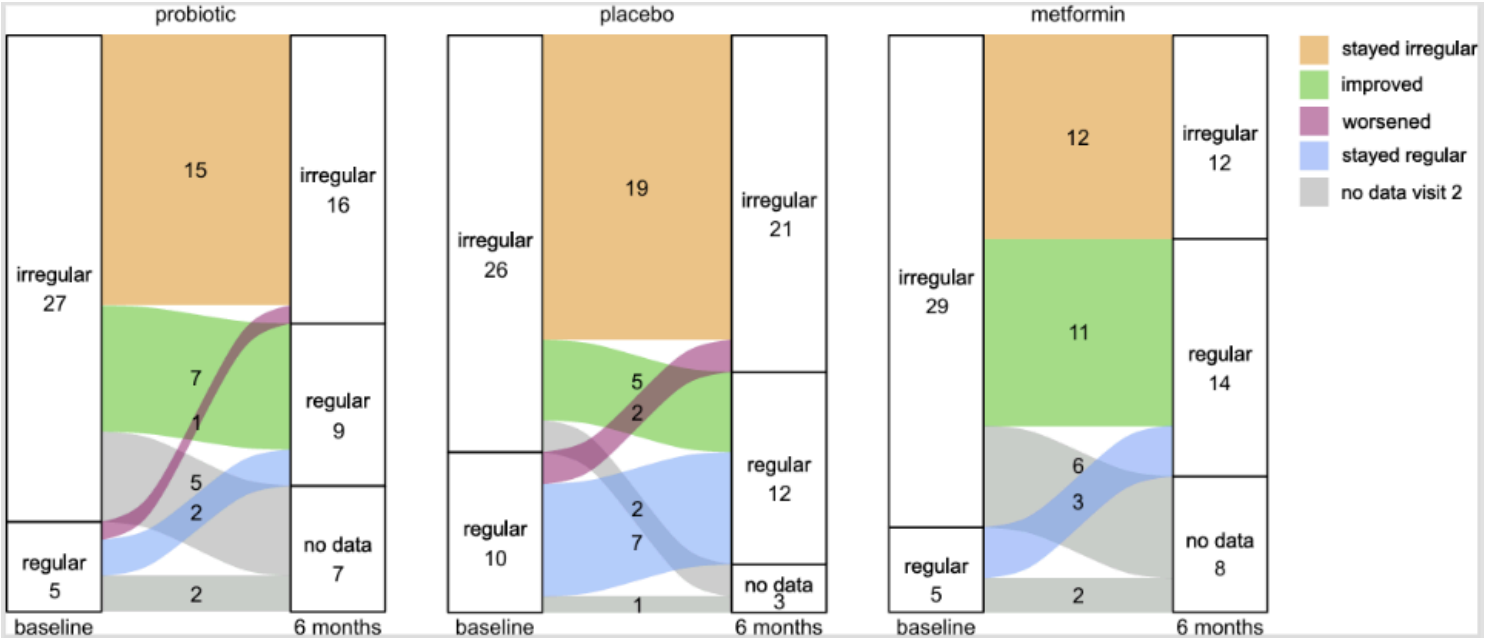


Figure 4

Cycle regularity across treatment arms. Alluvial plots illustrate transitions in menstrual cycle status from baseline to 6 months of treatment. Participants improved (irregular to regular), worsened (regular to irregular), or stayed regular/irregular. Flow widths represent participant counts (n).

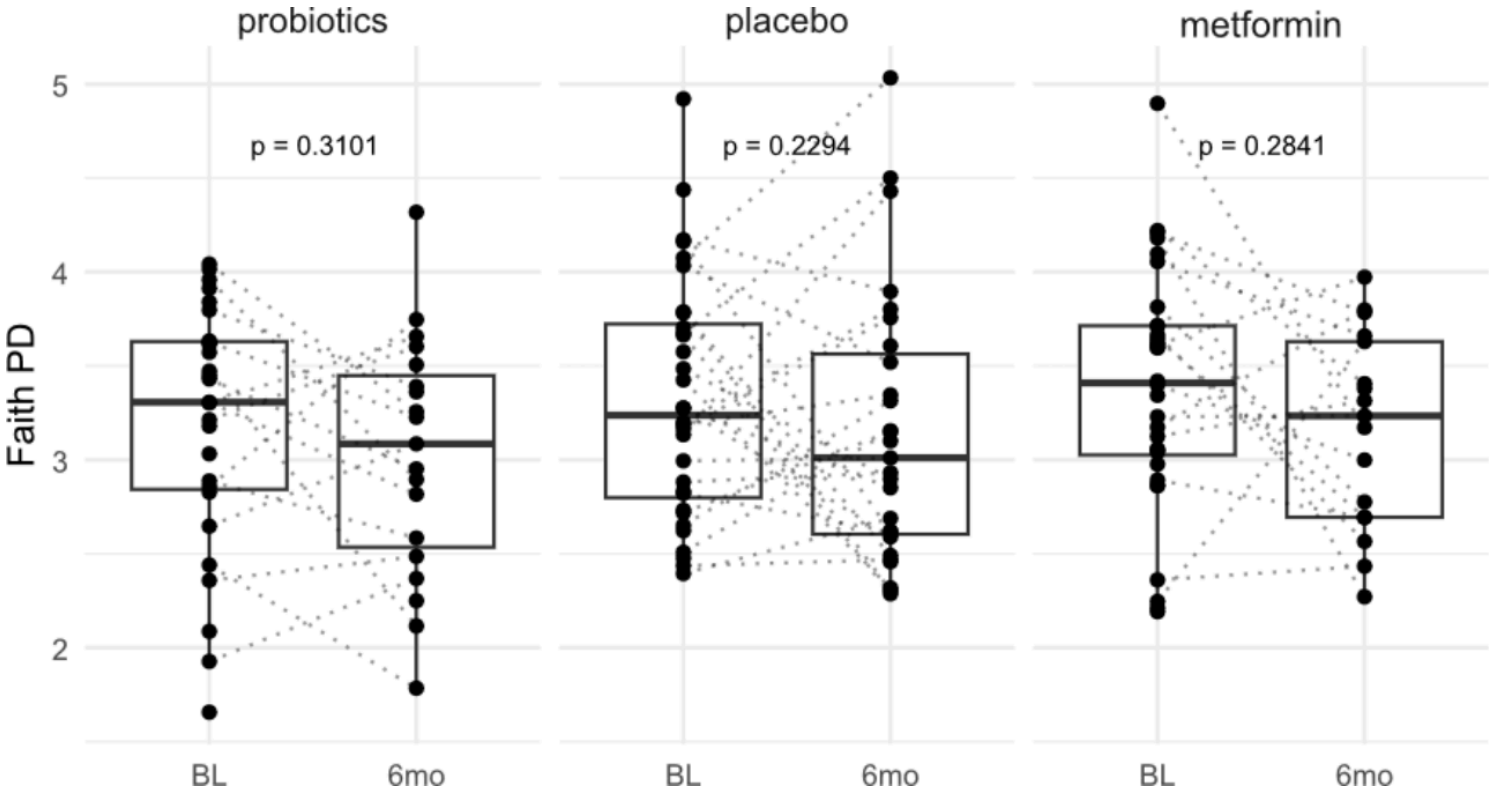


Figure 5

Longitudinal changes in gut microbiome alpha diversity (Faith's Phylogenetic Diversity) from baseline (BL) to 6 months (6mo). No significant within-group changes were detected using Kruskal-Wallis test. All samples were rarefied to an even sampling depth of 650 prior to analysis.

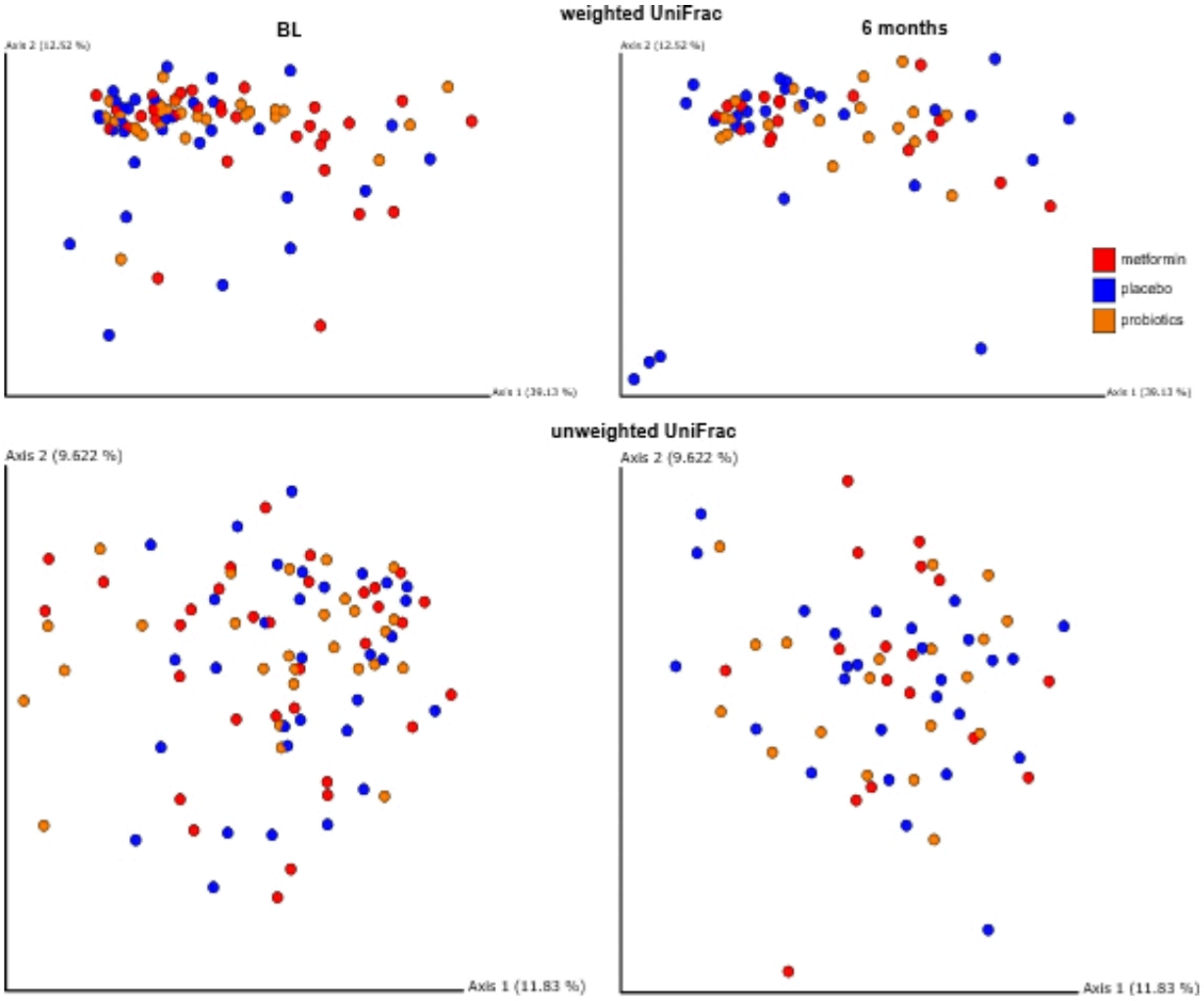


Figure 6

Beta diversity analysis of gut microbiome composition at baseline (BL) and after 6 months (6mo) of intervention, coloured by treatment group. Upper panels: weighted UniFrac; lower panels: unweighted UniFrac. All samples were rarefied to an even sampling depth of 650 prior to analysis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.docx