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Abstract

Previously, the role of decreased biodiversity of gut microbiota in polycystic ovary syndrome (PCOS) was demonstrated, but the objective criteria for assessing the representation of microorganisms, associated with are limited. A total number of 175 premenopausal women (26 women with PCOS and HA and 149 women without HA, including 19 healthy controls) were recruited during the Eastern Siberia PCOS Epidemiology and Phenotype (ESPEP) Study (2016-2019). Methods included a questionnaire survey, clinical examination, pelvic U/S, blood and feces sampling. Gut microbiome was analized by high-throughput sequencing of the V1–V3 of the variable regions of the 16S rRNA gene (Illumina MiSeq, USA). Amplicon libraries of 16S rDNA were processed using the QIIME2 bioinformatics pipeline. All data were analyzed using R 3.6.3. The gut microbiocenosis in women with HA was characterized by higher representation, predominantly, of *Lactobacillus* and a less prevalence of Clostridia class. For *Faecalibacterium, Christensenellaceae_R-7_group*, and *[Eubacterium] eligens group* the cut-offs values of their relative presence, associated with HA, were estimated as: ≤0.043%, ≤0.039%, and ≤0.02%, respectively. Conclusions: HA in PCOS is associated with a less prevalence of Clostridia class gut microorganisms, predominantly. The threshold values proposed may be useful to justify the administration of probiotics.

Keywords: androgens; hyperandrogenemia; polycystic ovary syndrome; PCOS; gut microbiota; amplicon metasequencing

1. Introduction

Polycystic ovary syndrome (PCOS) is the most frequent cause of hyperandrogenic disorders in premenopausal women and associated with metabolic syndrome and increased cardiovascular risk [1,2]. In recent years, the role of gut microbiota in the pathogenesis of PCOS as well as the relationship between microbiome and cardio-metabolic disorders become the subjects of active study [3–10]. Available data on gut microbiota biodiversity primarily support a decrease in the values of at least one of the alpha diversity indices in PCOS, while the scientific findings regarding the diversity differences between non-androgenic and hyperandrogenic women are contradictory [3,5,11–21].

Taking into account the published data, it seems important to determine which specific microorganisms are responsible for the decrease in the biodiversity of the gut microbiome. Currently, some of bacterial species are considered markers of gut dysbiosis associated with PCOS [14,22–29]. A significant decrease in the number of certain bacteria, including potential probiotics, has been reported in PCOS patients [10,14]. At the same time, the different groups of authors consider the role of the same bacterial species controversial [29,30].

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Our study objectives were to create the quantitative criteria for assessing the representation of gut microorganisms, negatively associated with HA, which could be useful for justifying patient's management.

2. Results

2.1. Main Characteristics of Premenopausal Women

The main characteristics of the examined women depending on the presence or absence of hyperandrogenemia are presented in Table 1. By anthropometric characteristics, women with hyperandrogenemia did not differ significantly from women in the comparison group in general and healthy women in particular. The distribution of women with HA included in the main group (group 1, n=26) by phenotype was as follows: with phenotype A – 11/26 (42.3%), B – 6/26 (23.1%), C – 9/26 (34.6%), $p > 0.05$. When assessing the main characteristics of PCOS in the groups of women examined, consistently higher values of ovarian volumes and the number of follicles were found in hyperandrogenemia, as well as a significantly higher proportion of women with hirsutism, oligo-ovulation (OA), and polycystic ovarian morphology (PCOM) according to ultrasound (Table 1).

Table 1. Main characteristics of premenopausal women with HA, without HA, and in the control group.

Parameter	Group 1 (*n* = 26) 1	Group 2 (*n* = 149) 2	Controls (*n* = 19) 3	*p*#
	M ± SD u Me (IQR)			
Height, cm	163 ± 8.2 165 (158; 169)	164 ± 6.0 165 (160; 168)	163 ± 6.0 160 (158; 166)	0.62 1–2 0.78 1–3
Weight, kg	71.5 ± 17.0 68.4 (62.8;78.5)	71.4 ± 15.0 68.7 (60.5;78.8)	65.7 ± 12.5 65.7 (53.4;74.0)	0.96 1–2 0.27 1–3
BMI, kg/м^2	26.9 ± 6.1 25.9 (22.4; 31.3)	26.4 ± 5.4 25.8 (22.0; 29.0)	24.6 ± 3.5 25.1 (21.3; 27.4)	0.70 1–2 0.22 1–3
WC, cm	80.7 ± 13.5 76.5 (72.5; 89.5)	79.6 ± 12.7 78.0 (70.0; 86.0)	76.0 ± 8.9 76.0 (68.5; 82.5)	0.76 1–2 –
SBP, mm Hg	125 ± 14.0 125 (115; 133)	124 ± 13.2 124 (113; 132)	117 ± 11.7 114 (110; 124)	0.64 1–2 –
Right ovary volume, cm^3*	11.6 ± 5.0 10.8 (9.1; 13.5)	10.8 ± 10.9 8.5 (5.9; 11.7)	6.4 ± 1.7 6.1 (5.0; 7.3)	**#0.016**1–2 **#0.000**1–3
Left ovary volume, cm^3	8.6 ± 3.3 8.6 (6.7; 9.5)	9.4 ± 8.5 7.4 (5.5; 10.6)	6.2 ± 2.0 6.4 (4.7; 7.2)	#0.4861–2 **#0.009**1–3
FNPO, right ovary	11.4 ± 3.8 12.0 (9.0; 14.0)	9.1 ± 4.3 8.0 (6.0; 12.0)	6.6 ± 3.1 6.0 (5.0; 7.5)	**#0.006**1–2 **#0.000**1–3
FNPO, left ovary	10.6 ± 3.2 12.0 (8.0; 13.0)	8.4 ± 4.0 7.0 (5.0; 12.0)	6.5 ± 2.5 6.0 (5.0; 8.0)	**#0.003** 1–2 **#0.000** 1–3
LH, mIU/ml	13.0 ± 11.2 10.0 (6.3; 15.5)	7.6 ± 7.4 5.6 (3.2; 9.2)	6.5 ± 5.2 5.8 (3.9; 6.9)	**0.00**1–2 **0.00**1–3
FSH, mIU/ml	5.9 ± 1.8 5.9 (5.0; 7.2)	5.8 ± 5.4 5.1 (3.7; 6.4)	5.7 ± 1.8 6.1 (4.4; 6.8)	**0.04** 1–2 0.79 1–3
PRL, mIU/ml	322 ± 161 291 (233; 435)	336 ± 189 286 (215; 408)	296 ± 132 248 (189; 418)	0.95 1–2 0.73 1–3
TSH, IU/l	1.6 ± 0.8 1.6 (0.9; 1.9)	1.8 ± 1.6 1.5 (1.1; 1.9)	1.5 ± 0.7 1.6 (1.0; 1.9)	0.76 1–2 0.94 1–3

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AMH, ng/ml	6,8 ± 5,8 4,6 (2,5; 8,6)	4,5 ± 5,0 2,7 (1,0; 6,1)	2,8 ± 2,1 2,0 (1,3; 3,6)	**0,01**[1–2] **0,00**[1–3]
17-OH-P, nmol/l	5.4 ± 3.2 4.9 (2.7; 7.5)	5.3 ± 3.5 5.0 (2.6; 7.3)	3.9 ± 2.8 3.3 (2.0; 5.6)	0.72 [1–2] 0.11 [1–3]
TT, ng/dl	62.8 ± 28.8 55.6 (44.5; 81.1)	27.7 ± 14.7 26 (17.9; 36.5)	23.5 ± 11.8 25.6 (16.2; 28.4)	**< 0.001** [1–2] **< 0.001** [1–3]
SHBG, nmol/l	65.3 ± 52.9 40.3 (31.3; 89.8)	78.9 ± 51.3 69.7 (43.1; 99.3)	89.1 ± 46.9 68.7 (59.3; 108)	**0.032** [1–2] **0.018** [1–3]
FAI	5.3 ± 3.8 4.6 (2.2; 6.7)	1.6 ± 1.2 1.3 (0.8; 2.2)	1.1 ± 0.8 1.0 (0.5; 1.4)	**0.00** [1–2] **0.00** [1–3]
		n/N (%)		
HA	26/26 (100.0 %)	0/149 (0.0 %)	0/19 (0.0 %)	**##0.000** [1–2] **##0.000** [1–3]
Hirsutism	10/26 (38.5 %)	19/149 (12.7 %)	0/19 (0.0 %)	**##0.003** [1–2] **##0.002** [1–3]
OA	17/26 (65.4 %)	49/149 (32.9 %)	1/19 (5.3 %)	**##0.004** [1–2] **##0.000** [1–3]
PCOM	20/26 (76.9 %)	67/149 (45.0 %)	3/19 (15.8 %)	**##0.002** [1–2] **##0.000** [1–3]

– Mann–Whitney U Test; ## – Chi-square ($\chi 2$) test. Abbreviations: WC is waist circumference, BMI is body mass index, SBP – systolic blood pressure, FNPO is follicle number per ovary, PCOM – polycystic ovarian morphology, TSH – thyroid-stimulating hormone, LH – luteinizing hormone, FSH – follicle-stimulating hormone, AMH – anti-Mullerian hormone, 17OHP – 17-hydroxyprogesterone, TT – total testosterone, SHBG – sex-hormone-binding globulin, FAI – free androgen index, DHEAS – dehydroepiandrosterone sulfate.

2.2. Gut Microbiota in Premenopausal Women with HA

Firstly, we characterized the gut microbiota of the examined women at the phylum, class, and genus taxonomic levels. As shown in Table 2, the representation of the Bacteroidota phylum was statistically significantly higher in the HA group as compared to the controls; whereas, the Bacillota phylum was detected significantly less frequently in the HA group than in the control group. At the same time, we showed a statistically significantly lower representation of the Clostridia class in the main group compared to the control group. Quantitative characteristics of the proportions of other represented classes in the gut microbiota of women in the compared groups did not differ significantly. We did not detect Verrucomicrobiota in the gut microbiota of women with HA, whereas this phylum was present, albeit slightly, in the gut of women with the absence of HA. The proportions of the Pseudomonadota, Actinomycetota, Fusobacteriota, Candidatus Melainobacteriota, and Desulfobacterota phyla in the gut microbiota of women in the compared groups did not differ quantitatively.

Table 2. Gut microbiota composition in premenopausal women with HA, without HA, and in the control group.

Taxon Name / Taxonomic Level	**Group 1 (*n* = 26)** **1**	**Group 2 (*n* = 149)** **2**	**Controls (*n* = 19)** **3**	***p*#**
		M ± SD Me (IQR)		
Bacteroidota / phylum	42.7 ± 24.7 41.7 (25.0; 66.4)	36.3 ± 24.4 36.8 (12.6; 56.7)	26.7 ± 17.0 28.3 (12.1; 36.6)	0.23[1–2] **0.04**[1–3]
Bacillota / phylum	52.8 ± 25.6 50.9 (31.4; 73.4)	58.4 ± 26.1 56.7 (37.0; 83.4)	70.2 ± 18.7 69.8 (59.7; 85.3)	0.29[1–2] **0.03**[1–3]
Clostridia / class	42.0 ± 22.0	49.9 ± 25.5	63.1 ± 18.3	0.14[1–2]

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	32.7 (27.5; 55.7)	47.1 (28.2; 69.8)	64.1 (50.5; 80.7)	**0.00**$_{1–3}$
Catenibacterium / genus	0.05 ± 0.1 0.0 (0.0; 0.03)	0.02 ± 0.08 0.0 (0.0; 0.00)	0.01 ± 0.03 0.0 (0.0; 0.00)	**0.02**$_{1–2}$ **0.04**$_{1–3}$
Faecalibacterium / genus	0.07 ± 0.12 0.03 (0.01; 0.05)	0.13 ± 0.17 0.06 (0.02; 0.18)	0.23 ± 0.23 0.14 (0.07; 0.30)	**0.03**$_{1–2}$ **< 0.001**$_{1–3}$
Christensenellaceae_R-7_group / genus	0.01 ± 0.01 0.00 (0.00; 0.01)	0.03 ± 0.06 0.01 (0.00; 0.03)	0.04 ± 0.06 0.02 (0.00; 0.07)	**0.28**$_{1–2}$ **0.03**$_{1–3}$
Lactobacillus / genus	0.01 ± 0.04 0.0 (0.0; 0.00)	0.00 ± 0.01 0.0 (0.0; 0.0)	0.001 ± 0.004 0.0 (0.0; 0.0)	**0.01**$_{1–2}$ **0.15**$_{1–3}$
[Eubacterium] eligens group / genus	0.003 ± 0.01 0.0 (0.0; 0.00)	0.004 ± 0.01 0.0 (0.0; 0.004)	0.007 ± 0.02 0.00 (0.0; 0.01)	**0.21**$_{1–2}$ **0.02**$_{1–3}$
Oscillospirales UCG-010 / genus	0.00 ± 0.00 0.0007 (0.0; 0.003)	0.0031 ± 0.01 0.001 (0.0; 0.0043)	0.01 ± 0.01 0.004 (0.0004; 0.00898)	0.77$_{1–2}$ **0.04**$_{1–3}$
Acidaminococcus / genus	0.00076 ± 0.001 0.0 (0.0; 0.001)	0.0012 ± 0.00604 0.0 (0.0; 0.0)	2e–05 ± 7e–05 0.0 (0.0; 0.0)	0.04$_{1–2}$ 0.19$_{1–3}$
Delftia / genus	0.0001 ± 0.00034 0.0 (0.0; 0.0)	0.0004 ± 0.00172 0.0 (0.0; 0.00026)	0.0002 ± 0.00027 0.0 (0.0; 0.00046)	**0.04**$_{1–2}$ **0.02**$_{1–3}$
Ruminococcaceae_Incertae Sedis / genus	0.00021 ± 0.00085 0.0 (0.0; 0.0)	0.0005 ± 0.00144 0.0 (0.0; 0.00041)	0.00026 ± 0.00053 0.0 (0.0; 0.00015)	**0.04**$_{1–2}$ 0.40$_{1–3}$
Oxalobacter / genus	0.00057 ± 0.00098 0.0 (0.0; 0.00073)	0.00029 ± 0.00094 0.0 (0.0; 0.0)	2e–05 ± 9e–05 0.0 (0.0; 0.0)	**0.02**$_{1–2}$ **0.02**$_{1–3}$

– Mann–Whitney U Test.

When assessing the gut microbiocenosis of the examined women at the genera level, in the group with HA *vs* women without HA, a statistically significant increase in the relative abundance was noted for *Catenibacterium* and *Lactobacillus* (class Bacilli), and for *Oxalobacter (*class Gammaproteobacteria). At the same time, in HA group, *Faecalibacterium* and *Ruminococcaceae_Incertae Sedis* (class Clostridia), *Acidaminococcus (*class Negativicutes), *Delftia* (class Gammaproteobacteria), were significantly less represented.

In the control group, the significant increase was found compared to the main group in the relative abundance of *Faecalibacterium*, *Christensenellaceae_R-7_group*, *[Eubacterium] eligens group*, and *Oscillospirales UCG-010* (class Clostridia), as well as *Delftia* (class Gammaproteobacteria). Along with this, in HA, a statistically significantly higher representation of *Catenibacterium* (class Bacilli) was observed compared to the control group.

Then, we assessed the degree of relationships of microbiome characteristics and hormones levels in the total group of examined women. When analyzing associations of gut microbiota at the phylum, class, and genus levels, with serum androgens, no strong associations were found (Table 3).

Table 3. Statistically significant correlations of representatives of the gut microbiocenosis with hormones in the examined women (n = 175).

Parameters	***r*s**	***p***
Phylum / hormones		
Bacteroidota & AMH	0.23	< 0.001
Bacillota & AMH	–0.22	< 0.001
Pseudomonadota & DHEAS	–0.16	0.03
Actinomycetota & AMH	–0.2	0.01
Candidatus Melainobacteriota & FSH	–0.17	0.03
Class / hormones		

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Clostridia & TT	–0.15	0.05
Clostridia & FAI	–0.15	0.04
Clostridia & AMH	–0.31	< 0.001
Negativicutes & AMH	0.15	0.05
Gammaproteobacteria & DHEAS	–0.15	0.05
Alphaproteobacteria & SHBG	–0.17	0.03
Coriobacteriia & AMH	–0.19	0.01
Actinobacteria & AMH	–0.16	0.04
Genus / hormones		
Faecalibacterium & FAI	–0.16	0.03
Christensenellaceae_R-7_group & FAI	–0.16	0.03
[Eubacterium] eligens group & TT	–0.16	0.03
[Eubacterium] eligens group & FAI	–0.17	0.02
[Eubacterium] eligens group & AMH	–0.23	< 0.001
Delftia &FAI	–0.21	0.01
Ruminococcaceae_Incertae Sedis & FAI	–0.16	0.03
Oxalobacter & AMH	0.2	0.01

However, at the phylum level, a weak negative correlation was recorded between Pseudomonadota and DHEAS, and at the class level, a negative associations were found between the representation of the class Clostridia, testosterone levels, and FAI. Furthermore, a negative association was found between Gammaproteobacteria and DHEAS.

When assessing the gut microbiota at the genus level, a negative relationship was found between *Faecalibacterium*, *Christensenellaceae_R-7_group*, and *[Eubacterium] eligens group* with FAI, suggesting that these microorganisms may have potential protective effects against HA. *Delftia* and *Ruminococcaceae_Incertae Sedis* had also demonstrated the weak negative associations with FAI, but their quantitative representation in the gut microbiome of the examined women was significantly lower than that of the above-mentioned microorganisms.

Then, we used data from women with PCOS and HA and a comparison group (without HA), and applied ROC analysis to determine threshold values for the relative abundance of gut microorganisms, potentially suitable to support the probiotics administration in PCOS patients (Table 4).

Table 4. Cut-off values for the relative abundance of significant, as HA markers, microorganisms in the gut microbiocenosis.

Microorganism	Cut-off (95% CI)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Faecalibacterium	0.043 (0.043;0.043)	0.631 (0.520; 0.743)	0.604 (0.523; 0.685)	0.731 (0.538; 0.885)
Christensenellaceae_ R-7_group	0.039 (0.039;0.039)	0.566 (0.459; 0.674)	0.215 (0.154; 0.282)	1.000 (1.000; 1.000)
[Eubacterium] eligens group	0.002 (0.002;0.002)	0.577 (0.477; 0.677)	0.356 (0.282; 0.430)	0.846 (0.692; 0.962)

According to the data obtained, if any value is below the established threshold (relative amount of Faecalibacterium ≤0.043%, Christensenellaceae_R-7_group ≤0.039%, and [Eubacterium] eligens group ≤0.002%, this can be considered as a basis for prescribing probiotic drugs to the patient.

3. Discussion

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women, associated with reproductive disorders and multiple comorbidities. Recently, the relationship

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between gut microbiota, PCOS and cardio-metabolic comorbidities was reported by many authors [3–10].

Modern molecular diagnostic methods based on high-throughput sequencing (meta-sequencing) of bacterial 16S rRNA genes are currently used not only in research projects and clinical trials but also in genetic laboratories providing services to the public. This methodological approach allows for the qualitative and quantitative identification, according to various estimates, of between 100 and 300 genera and approximately 500 species, including uncultured microorganisms. Analysis of amplicon library meta-sequencing data is of great interest for identifying changes in the relative quantitative composition of the microbiota, which in turn are associated with the characteristics of pathological changes in patients. Assessing not only qualitative but also quantitative changes in the composition of the intestinal microbiome may be a non-invasive way to identify dysbiosis associated with PCOS and hyperandrogenemia in women of reproductive age.

Kirillova E. et al. (2023) [8] demonstrated that the gut microbiota in PCOS is characterized by a decrease in species richness and an altered balance of microbial communities, which is most pronounced in the reduced abundance of symbiotic species of *Clostridium*, *Bacteroides*, and a number of other bacteria that provide intestinal colonization resistance. The authors also reported that there is an increase in the population of opportunistic species of *Clostridium* and *Staphylococcus*, which produce exotoxins associated with chronic subclinical inflammation, excess adipose tissue, and the development of insulin resistance. According to the authors, a high level of intestinal colonization with commensal microorganisms of the *Clostridium leptum* group (>9 Lg GE/g) can be expected to result in higher efficacy of metformin therapy. A drawback of this study is that the authors have not established the role of decreased prevalence of the following microorganisms: *Faecalibacterium*, *Christensenellaceae_R-7_group*, and [*Eubacterium*]_*eligens*_*group* in HA in women with PCOS, and have not elaborate threshold values for their abundance that are prognostically significant for HA.

Previously, we demonstrated the significant decrease in alpha diversity of the gut microbiome compared with healthy women without any signs of PCOS, and then developed criteria for assessing alpha diversity using cut-off points for the most significant indices can be useful for monitoring the results of different therapeutic interventions (prebiotics, probiotics, etc.) in hyperandrogenic phenotypes of PCOS [18,20].

Nevertheless, it was important to find the specific microorganisms which are responsible for the decrease in the biodiversity. In current study, we have established the cut-offs for the relative amount of *Faecalibacterium*, *Christensenellaceae_R-7_group*, and *[Eubacterium]_eligens_group* – the microorganisms negatively correlated with androgens, and less represented in gut microbiome of women with HA. We consider the obtained data as useful for justifying the prescription of probiotics to women with PCOS and hyperandrogenemia.

Producers of short-chain fatty acids (SCFA), including butyrate producers, actively maintain the gut barrier and its permeability and their reduced abundance might be associated with increased CVD risk among women with PCOS [31]. A hypothesis called DOGMA (dysbiosis of gut microbiota) with microbiological paradigm in PCOS etiology [32] suggested that gut dysbiosis increased the gut mucosal permeability and lipopolysaccharides (LPS) from opportunistic colonic bacteria enter the blood circulation and contact the cardiomyocytes and cardiac fibroblasts. These patterns lead to an increase in cytokines such as IL-1, IL-6, IL-22, IL1-β, and TNF-α [31,33,34]. It was also shown that IL-1, IL-6, and TNF-α are higher in women with PCOS, thus explaining their increased risk of CVD and the possible protective role of a healthy gut microbial community. *Faecalibacterium* is one of the major butyrate producers in the gut and it is significantly depleted not only in women with PCOS [35,36], but also in obesity, cardiovascular pathologies, and diabetes [37–39].

Other SCFA producers include representatives of the *Christensenellaceae_R-7_group* and *[Eubacterium]_eligens_group*. A decrease in their quantitative indicators has been determined in various pathologies, both infectious [40] and non-infectious [41–44]. Researchers note different intestinal microbiota profiles in patients who respond and do not respond to cancer treatment [43,44]. Chen HH et al. noted that differences in the structure and composition of the gut microbiota were

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observed in lung cancer patients who did not show a therapeutic response to treatment [43]. They noted that these patients had higher α-diversity of the microbiota, but lower β-diversity compared to patients who responded to treatment. In particular, *Christensenellaceae_R-7_group* and *Bacteroides_stercoris* were more common among non-responders [43]. Liu L et al. assessed the prognostic potential of the gut microbiome in relation to the response to neoadjuvant immunochemotherapy in esophageal squamous cell carcinoma [44]. The authors showed that treatment-responding patients had higher levels of SCFA-producing bacteria, such as *Faecalibacterium*, *Eubacterium_eligens_group*, *Anaerostipes*, and *Odoribacter*, while non-responding patients had an increased abundance of *Veillonella*, *Campylobacter*, *Atopobium*, and *Trichococcus*. Novielli P et al. used machine learning (ML) classifiers integrated with explainable artificial intelligence (EAI) methods to identify microbiome biomarkers predictive of colorectal cancer and adenomatous lesions [45]. The authors showed that taxa belonging to the *Fusobacterium* and *Peptostreptococcus* genera were associated with an increased risk of colorectal cancer, while the *Eubacterium eligens group* was identified as a reliable negative predictor. Taken together, these results demonstrate that our target taxonomic groups of *Faecalibacterium*, *Christensenellaceae_R-7_group*, and *[Eubacterium]_eligens_group*, may be useful biomarkers for HA in PCOS. We emphasize the potential of microbiome analysis for developing interpretable, noninvasive tools for predicting and managing the risk of developing HA in PCOS.

The strengths of our study. All study materials were obtained from the well phenotyped participants who were recruited from the unselected, medically unbiased population. In our study, we used a highly efficient method (LC-MS/MS) to measure testosterone levels, which is critical for the qualitative assessment of hyperandrogenemia. We also conducted a search on the composition of the gut microbiota, exploring the modern molecular diagnostic methods based on high-throughput sequencing (meta-sequencing) of bacterial 16S rRNA.

A limitation of our study. The microbiome-associated markers of hyperandrogenemia in premenopausal women with polycystic ovary syndrome were identified during the cross-sectional study, and more prospective studies are needed to investigate prognostic value of our findings.

4. Materials and Methods

We performed this research as a sub-study of cross-sectional, multicenter, institution-based Eastern Siberia PCOS Epidemiology and Phenotype (ESPEP) Study, conducted in the Irkutsk region and the Republic of Buryatia (Eastern Siberia, Russian Federation) from 2016 to 2019 (ClinicalTrials.gov ID: NCT05194384) [46].

A total of 175 premenopausal women (26 women with HA – Group 1, and 149 women without HA (Group 2)) were recruited during the annual employment medical assessment. All women from Group 1 were diagnosed with PCOS, hyperandrogenic phenotypes A, B, C, according to Rotterdam criteria, 2003.

The inclusion and exclusion criteria for ESPEP study were previously reported [46]. For sub-study, we also excluded women with antibiotics intake within one month before recruitment. For PCOS diagnosis, we used Rotterdam Criteria (2003): a presence of any two of three criteria – hyperandrogenism, oligo/anovulation, and polycystic ovarian morphology – with no conditions with similar symptoms (hyperprolactinemia, hypothyroidism, 21-hydroxylase-deficient non-classic congenital adrenal hyperplasia (NC-CAH), premature ovarian failure) [47].

Among the women without HA (Group 2) we formed the Control group – a subset of clinically healthy women (n = 19). Inclusion criteria for the Control group were as follows: the absence of signs of PCOS, regular menstrual cycles (21–35 days), modified Ferriman–Gallwey score (mF-G) < 3, absence of alopecia and acne, ovarian volume < 10 cm^3, and follicle number per ovary (FNPO) < 12. Exclusion criteria for controls were: history of chronic diseases, BMI < 18 or ≥ 30 kg/m^2, elevated blood pressure, or abnormal levels of androgens, fasting glucose, prolactin (PRL), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), or 17-hydroxyprogesterone.

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Methods included a questionnaire survey, anthropometry with body mass index (BMI) calculation (weight (kg)/height (m^2)), vital signs, modified Ferriman – Gallwey (mF-G) scoring, gynecological examination, lab tests, and pelvic ultrasound (US). Pelvic US was performed using the portable ultrasound scanner Mindray M7 (MINDRAY, China) with a transvaginal (5.0–8.0 MHz) and transabdominal (2.5–5.0 MHz) probe.

Laboratory Methods

4.1. Androgen Assessment

Blood serum total testosterone (TT) was measured using liquid chromatography–mass spectrometry (LC-MS/MS, Shimadzu LCMS-8060, Japan). Sex hormone–binding globulin (SHBG) was measured using ELISA Alkor-Bio test kits (Russia) and Elx808 microplate photometer (USA). The free androgen index (FAI) was calculated as: FAI = (TT/SHBG) × 100. Dehydroepiandrosterone-sulfate (DHEAS) was assessed using immunochemiluminescence kits (Siemens HealthCare Diagnostics, Germany) and Immulite 1000 analyzer (Siemens Health Care Diagnostics Inc., USA). Hyperandrogenemia was defined by elevation above the normal reference ranges for at least one of: TT, FAI, or DHEAS. The upper normal levels (UNL) for androgens were previously determined from the 98th percentiles for these parameters in the healthy controls and reported [48].

4.2. Other Hormonal Methods

TSH, LH, FSH, prolactin (PRL) and 17-OH progesterone were determined using competitive solid-phase ELISA test kits (Alkor-Bio, Russia) on the Elx808 analyzer (USA). Anti-Müllerian hormone (AMH) was measured using Beckman Coulter kits on ELx808 (USA).

4.3. Methods for Gut Microbiota Studying

Fecal sampling, genomic DNA isolation and high-throughput sequencing of the V1–V3 variable regions of the 16S rRNA gene was described previously [18]. Amplicon libraries of 16S rDNA (Bioproject PRJNA899143) were processed using the QIIME2 bioinformatics pipeline to conduct a comparative metagenomic study [49]. Amplicon sequencing variants (ASV) were generated using the DADA2 algorithm, which allows detection, correction, and filtering of amplicon errors and chimeric sequences [50]. The resulting representative sequences were used to determine their taxonomic classification using the sklearnbased Naive Bayes classifier trained on the SILVA v138 with 99 % 16S rDNA full-length database [51].

4.4. Statistical Analysis

Sample size calculations for the total population were based on the following formula: n = [(Z2 1–α p(1−p)]/D2, where n – individual sample size, Z1–α = 1.96 (when α = 0.05), p – assumed PCOS prevalence according to the previously published data, and D – absolute error.

The data were collected using Research Electronic Data Capture (REDCap) [52]. Outliers were identified during the Exploratory Data Analysis using the box-plot and 3σ methods. Managing missing data: in our research dataset, there were two types of missing data – missing completely at random (MCAR) and missing at random (MAR). We recorded all missing values with labels of "N/A" to make them consistent throughout our dataset. Pairwise deletion was used when the dataset was analyzed. To estimate the assumption of the normal distribution of our datasets, we performed a formal statistical test – the Shapiro–Wilk test. Chi-square (χ2) was used for frequency data. We used a Student's t-test to compare the mean values of the data with an independent sample, which followed a normal distribution, or a Mann–Whitney U-test to compare the ratio between two groups in another case. A correlation analysis was conducted with the calculation of the nonparametric Spearman rank correlation coefficient -r. The strength of the relationship was assessed as follows: $0.01 \le r \le 0.29$ – weak relationship; $0.3 \le r \le 0.69$ – moderate relationship; $0.7 \le r \le 1.00$ – strong relationship between the parameters. The statistical significance was established at $p < 0.05$. Statistical analysis

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included ROC-analysis for cutoffs development. All data were analyzed using R 3.6.3 (a free software environment for statistical computing and graphics).

5. Conclusions

Hyperandrogenemia in premenopausal PCOS patients is associated with statistically significant increase in the representation of class Bacilli in the gut microbiome, with a less prevalence, predominantly, of Clostridia class gut microorganisms.

The proposed cut-offs values for the relative abundance of marker microbes, associated with hyperandrogenemia, may be useful to justify the probiotics administration and monitoring the effectiveness of treatment in PCOS patients. However, prospective studies are needed to test the effectiveness of the proposed approach to assessing the state of the gut microbiome in relation to hyperandrogenic disorders.

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Institutional Review Board Statement: The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki (2013) and approved by the Local Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (Protocol No. 2.1, date of approval – February 24, 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Abbreviations

The following abbreviations are used in this manuscript:

AMH	anti- Müllerian hormone
BMI	body mass index

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DHEAS	dehydroepiandrosterone sulfate
DOGMA	dysbiosis of gut microbiota
ESPEP	Eastern Siberia PCOS Epidemiology and Phenotype Study
FAI	free androgen index
FNPO	follicle number per ovary
FSH	follicle-stimulating hormone
HA	hyperandrogenemia
LC-MS/MS	liquid chromatography–mass spectrometry
LH	luteinizing hormone
LPS	lipopolysaccharides
MCAR	missing completely at random
MAR	missing at random
mF-G	Ferriman – Gallwey score
NC-CAH	non-classic congenital adrenal hyperplasia
OA	oligoovulation
PCOS	polycystic ovary syndrome
PCOM	polycystic ovarian morphology
PRL	prolactin
REDCap	Research Electronic Data Capture
SCFA	short-chain fatty acids
SBP	systolic blood pressure
SHBG	sex-hormone-binding globulin
TSH	thyroid-stimulating hormone
TT	total testosterone
UNL	upper normal levels
U/S	ultrasonography
WC	waist circumference
17OHP	17-hydroxyprogesteron

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