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Polycystic Ovary Syndrome a Non-Communicable Disease of Women Affected by Lifestyle, Stress, and Vaginal Dysbiosis

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Abstract

Background: Hectic lifestyle, oxidative stress, obesity, and delayed childbearing cause many women's diseases worldwide. One of the diseases related to the reproductive system is "vaginal dysbiosis (VD)" an unbalanced microbiota, which paves the way for opportunistic microorganisms that cause health disorders and lead to various gynecological problems, such as Polycystic Ovary Syndrome (PCOS), and infertility. The study of women's lifestyle and VD in PCOS patients is one of the objectives. **Methods**: Vaginal swab samples from PCOS patients and healthy women volunteers were collected. The swab samples were cultured in plates containing blood-agar media. The Petri plates were sealed with Para-film to create internal anaerobic conditions and incubated in a BOD incubator. Colonies in plates were photographed on 3rd, 5th, and 7th day. The photographs were displayed on the screen and morphological observations were made for various parameters.**Results**: Results show significant differences between the two contrasting groups for seven lifestyle parameters of PCOS and healthy volunteers. The participants were between the ages of 23-40 years; of which 84.0% of PCOS were married but 80.0% did not have children (P-value<0.001). 88.0% were non-vegetarians with a fat-rich diet (P-value<0.001). The cytological observations of microbial colonies in culture plates showed morphologically different vaginal microbiota in all PCOS samples in contrast to healthy ones.**Conclusion**: All PCOS patients showed vaginal dysbiosis. Notably, the healthy bacteria were completely replaced by pathogenic bacteria. The characteristics of the pathogenic bacterial colonies were distinct and diverse in each patient, suggesting theinfluenceof PCOSon altered microbiota.

Keywords: Dysbiosis, Lifestyle, Microbiome, PCOS, Stress

Introduction:

Changedlifestylesand diverse environmental factors are causing biological stress in women globally.Consequently,women'sbodyreacts to stress by altering their metabolism and releasing excess hormones, causing many health disorders.

Specificmicrobiota exertsvital function in women's health and normal functioning. Among the human organs,significant changes in the microbial patternoccur in the gut and vagina (Haahr TJ et al., 2016).The composition of the human vaginal microbiome is critical for maintaining the first line of defence against pathogenic intruders (Boskey ER et al., 1999). The landscape of the vaginal microbiome has been reported to be influencedbysocioeconomic conditions, country of origin, promiscuity, hormonal status, and many other confoundingfactors. The inner surface of the vagina is inhabited by a diverse microbial population that includes pathogenic, non-pathogenic, and maximally beneficialmicrobes-thechanges in the composition of vaginal microbiota can cause various gynecological diseases. A healthy vaginal microbiome contains several strains of Lactobacillus that have unique properties to support vaginal health (Greenbaum et al., 2019).Lactobacillushelps maintain the vaginal microenvironment by producing lactic acid, hydrogen peroxide (Hillier et al., 1993), and other chemicals that may target unwanted bacteria (Jang et al., 2019). The acidic environment (pH 3.5-4.5)prevents the growth of harmful bacteria, which requires a pH >4.5 (Godha et al., 2018). Among the diseases that mark the changes in the vaginal microbiota is PCOS.It is one of the most common reproductive metabolic disorders, significantly affecting the biological functionalities of ovaries. It is highly complicated with the key clinical manifestations of hyper-androgenemia, oligo/anovulation, and polycystic ovary morphology (Dumesic et al., 2015).AlarminglyPCOS incidences are increasing and affecting one in every 10 women globally andshockingly the incidence in Indian women has been reported to be one in every 5 women (Franks, 2006).

An advanced stage of polycystic ovarian disease (PCOD) is a medical condition in which the woman's ovaries produce immature or partially mature eggs and over time these become cysts in the ovaries (Bhumika, 2019),thecondition called PCOS. Some risk factors that probably induce PCOS are diet, hectic lifestyle, genetics, obesity, medication, infections, and hormonal imbalance. PCOS can lead toanovulation, where ovaries stop releasing eggs and follicles convert into cysts. PCOS patients reportedheterogeneous clinical manifestations with varied phenotypes. The main cause for the onset of PCOS is not fully understood, but it affects women with premenopausal conditions andcan vary between individuals. Furthermore, women with PCOS have a higher incidence of insulin resistance, periodontitis, and vaginitis compared to healthy women (Azziz, 2018).High insulin levels can hinder ovulation and increase the amount of testosterone produced by the ovaries (Stener-Victorin and Deng, 2021).

Although many investigations have reported specific changes in the intestinal flora in PCOS patients,very little systematic research to date has been carried out on associations between type of diet, vaginal microbiome, and PCOS. Few reports indicate thata stressed lifestylecanmodulate the hormonal pattern considerably,andhave a drasticeffect on vaginal microbiota. The condition of

unbalanced microbiota in the vagina may pave the way for opportunistic microorganisms to colonize and cause manyreproductive diseases (Donders et al., 2000) that includes PCOS. Vaginal dysbiosis cancause multiple gynecological problems that lead tobacterial vaginosis (BV)a polymicrobial disorder characterized by an increase in the vaginal pH over 4.5 (Caillouette, 1997). The onset of BVsymbolizes thedepletion of lactobacillus, whichencouragesthe overgrowth of several facultative and obligatory anaerobic bacteria (Eschenbach, 1993 and Ness et al., 2001).However, there is no standard methoddeveloped to diagnose vaginal flora alterations in womenwith PCOS (Nugent et al., 1991).

Though, the vaginal microbiome isa critical concern, yet largely overlooked. Very fewstudies have evaluated the relationship among lifestyle factors,vaginal dysbiosis, and PCOS. Understanding theinterrelationsamongthese factorsis essential toprovidingdiagnosis and effective medical treatment. Thus, the present study attempted to determine the possible inter-interrelationsamong vaginaldysbiosis, and lifestyle parametersofPCOSpatients in comparison with healthy women. During this study, the major emphasis was given to characterizingmicrobial colonies cytologically in the vaginal microbiota to understand the nature of altered vaginal microbiomes.

Materials and Methods:

Case-control studieswere conducted, in which the microorganisms settling in the vaginal area were compared between two groups of women including PCOS diagnosed and the healthy control volunteers. The vaginal swab samples were collected by the women volunteers admitted to the ARCHISH IVF diagnostic center, in Bangalore, under the supervision of a practicinggynaecologist.

StudyDesign and Sample Collection

Collection of vaginal swab samples from women volunteers: to begin, a copy of the survey sheet was prepared and distributed to each participant.They have been asked to fill in the datasheetby ticking against the matching parameters. The information collected was analyzed statisticallyand comparisons between two contrasting groups were calculated for the significance.The vaginalsamples were collected aseptically bysterile portable swab sticksfrom participants.The swabsticks with the samplesenclosed in portable sterile 2 ml glass tubes having screw capswere immediately transported to the research laboratory for further investigation.

Morphological characterization of microbial colonies: For each sample tube,one ml of nutrient media was added to the vials,and gently mixedto release the microbes into the media. Inoculated100 □l of test sampleontoaPetri dishcontaining solid blood-agar medium, and was spread evenly.The inoculated Petridisheswere sealed around the sides of the plates tightly with Para-filmtape to

createan anaerobic condition and prevent contamination.The culture plates were incubated in a BOD incubator(set at 37 ^{O}C andRH 85.0 %).

Digital recording of microbial colonies in theculture plates: On the 3rd day, the plates were checked for contamination and formation of observable microbial colonies. The colonies in the culture plates were photographed onthe 3rd day, 5th day, and 7th day respectively,this was to include the slow-growing bacterial colonies.

Onscreen scoring of microbial colonies with a digital floating scale: Scoring themicrobialcolonies in culture plates at different incubation timingsmanually,is tedious and may not be accurate. Thus, a simple technique is followed tocount and measurecolony sizes on photographsdisplayed on the screen. Firstly,a transparent plasticscale is scanned and the 90 mm length of the scale is calibrated by stretching to the plate diameter of 90 mm.This floating can be moved easily from one photomicrograph to another with the help of a cursor. The sizes of the colonies relative to the scale length were recorded as shown in the diagram (Figure 1). This technique for determining colony diameter and numberswas very accurate and convenient.

Cytological analysis of microbial colonies: The Cyto-morphological characterization of microbial colonies was performed by displaying the photographs ona computer screen, the details of the colonies were well-visible and wereconvenient to analyze. The parameters recorded includedthe number of colonies per plate, different types of colonies, colour, size, colony surface,periphery, etc.

Estimation of bacterial growth rate by UV-spectrophotometer analysis: Unique bacterial colonies isolated from each test sample were cultured individually in liquid broth medium, and the bacterial population density was estimated on the 5th day by spectrophotometer absorbance set at 650 nm.

Results:

Analysis of the survey data collected from volunteers: The information related to the lifestyle parameters obtained from PCOS and healthy women volunteers and their group differences are presented in Table 1.It consistsof group percentages,mean, standard deviation betweenthe contrasting groups, and significance level (P-value)among the groupscalculated by using Pearson's chi-squared test.

Table 1. Pair-wise comparisons between groups of lifestyle parameters of 25 PCOS women volunteers

Variables [a]	**Groups**	**No.**	**%**	$\bar{x}$	**S.D.**	**p-value**
Age	23-29 vs.	9	36.0	50	19.8	<0.05 [b]
	30-40	16	64.0			
Marital status	Married vs.	4	16.0	50	48.1	<0.001 [b]
	Unmarried	21	84.0			
Children	Children vs.	5	5.0	50	42.4	<0.001 [b]
	No children	20	20.0			
Diet	Vegetarian vs.	3	3.0	50	53.7	<0.001 [b]
	Non-veg.	22	22.0			
Thyroid	Thyroid vs.	4	4.0	50	48.1	<0.001 [b]
	Non-thyroid	21	21.0			
Diabetes	Diabetes vs.	0	0.0	50	70.7	<0.001 [b]
	Non-diabetes	25	25.0			
Medications	Medication vs.	1	1.0	50	65.1	<0.001 [b]
	No medication	24	24.0			

[a]Among the meaningful independent variables, seven groups were compared one by one, and the results were meaningful.

[b]Statistically significant

Observation of culture plates at different incubation periods: The microbial colonies in culture platesin situwere photographed on the 3rd day, 5th day, and, 7th day for all the 30 samples (25 PCOS +5 healthy women volunteers). The photomicrographs were used torecord colony numbers and sizes by displayingthem on the computer screen at a convenience(Figure 1). The photographs in Figure 2 are colonies in situ taken onthe 7th day for all the test samples cultured on solid blood agar plates.

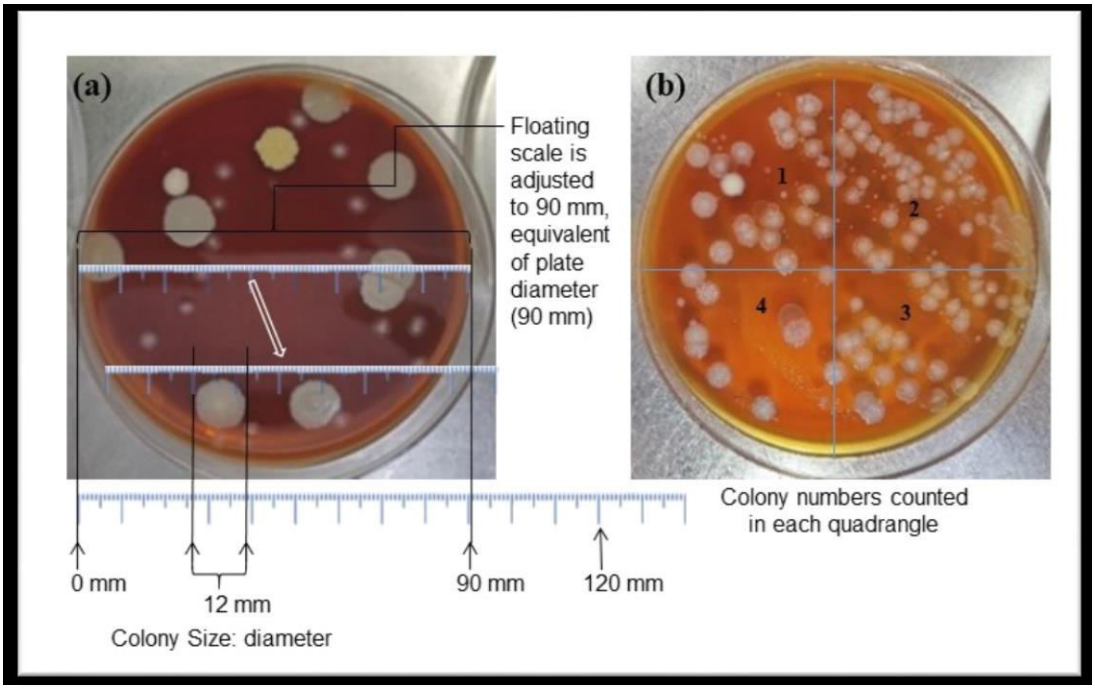

Figure 1 Measurement of colonies number and size digitally on digital photomicrographs using a digital floating scale, as described in the materials and methods

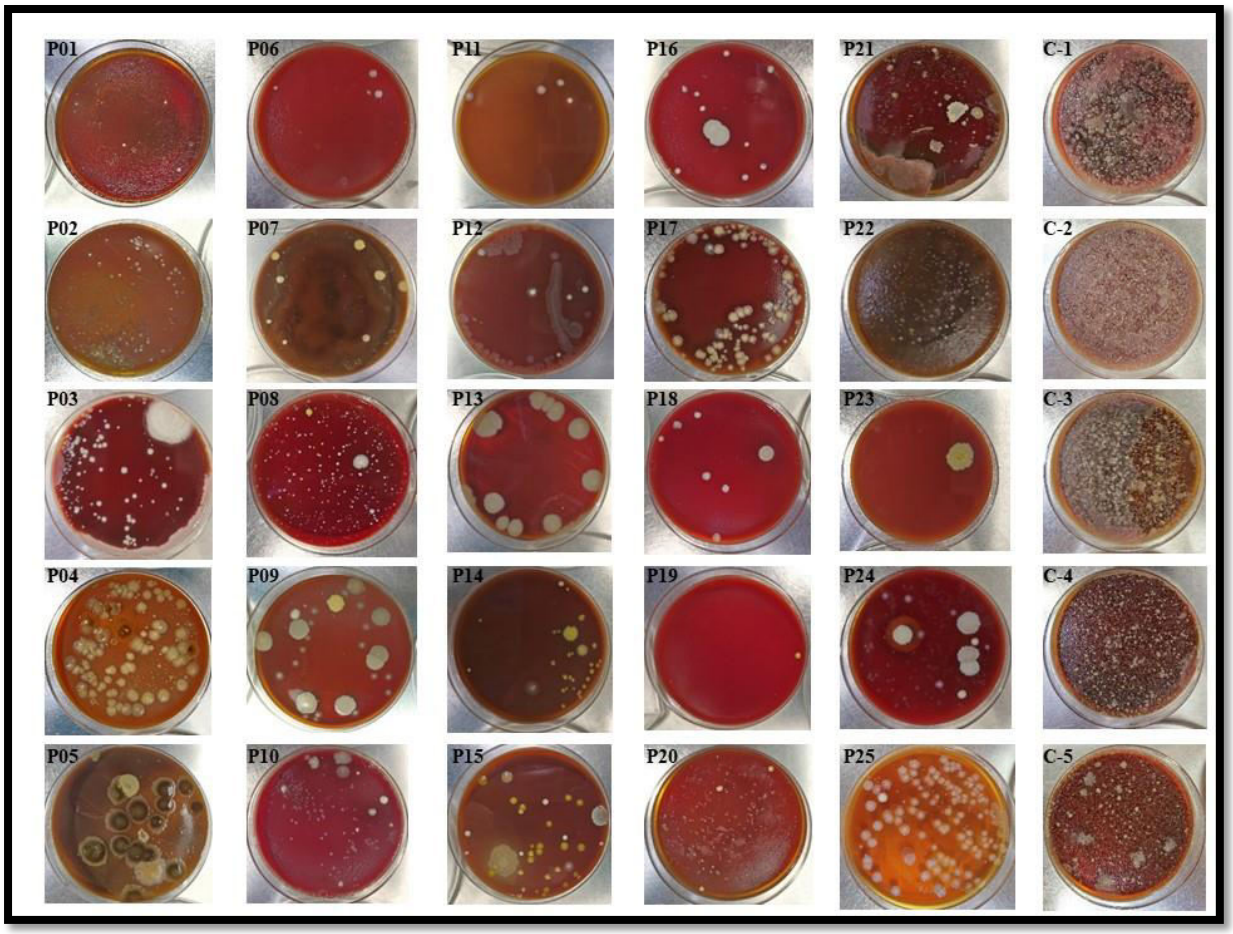

Figure 2 The photomicrographs of PCOS patient cultures P1→P25 and C1-C5 healthy volunteers taken at 7th day after culture initiation in blood agar culture media.

Morphological characterization of the colonies: The morphological characterizations of microbial colonies grown on blood-agar mediawerescored on the photomicrographs for various parameters. The observational data with statistical analysis are presented inTables 2-4. The colony numbers for each group, mean standard deviation and confidence intervals are mentioned in Table 2,whereas the colony types are noted in Table 3.Thedistribution of different colonies and colonysizes (mm)for each plate were also observed.

Table 2. Number of colonies per plate of 25 PCOS and 5 healthy women vaginal swab samples.

	PCOS patients			Healthy controls		
Day	3rd	5th	7th	3rd	5th	7th
Range	1-112	1-126	1-133	267-293	356-384	382-397
$\bar{x}$	35.24	43.24	53.76	281.8	368.8	389.4
S.D.	40.17	44.09	46.25	10.85	10.35	6.07
p-value	<0.05	<0.001	<0.01	NS	NS	NS

Statistically significant when p-value< 0.05, p-value< 0.001, and non-significant (NS) if p-value> 0.05

Table 3. Colony types per plate of PCOS and healthy women vaginal swab samples.

Groups	PCOS patients			Healthy controls		
Day	3rd	5th	7th	3rd	5th	7th
Range	1-5	1-5	1-5	1-2	1-2	1-2
$\bar{x}$	1.68	1.80	2.04	1.4	1.6	1.6
S.D.	1.11	1.08	1.10	0.5477	0.5477	0.5477
p-value	NS	NS	NS	NS	NS	NS

Statistically significant when p-value < 0.05, p-value < 0.001, and non-significant (NS) if p-value > 0.05.

Isolation of Unique microbial colonies in PCOS patientsamples: Since the colony types observed were highly diverse and unique to each person, the distinctive colonytypes were isolated, and culturedfor further analysis (Figure 3).

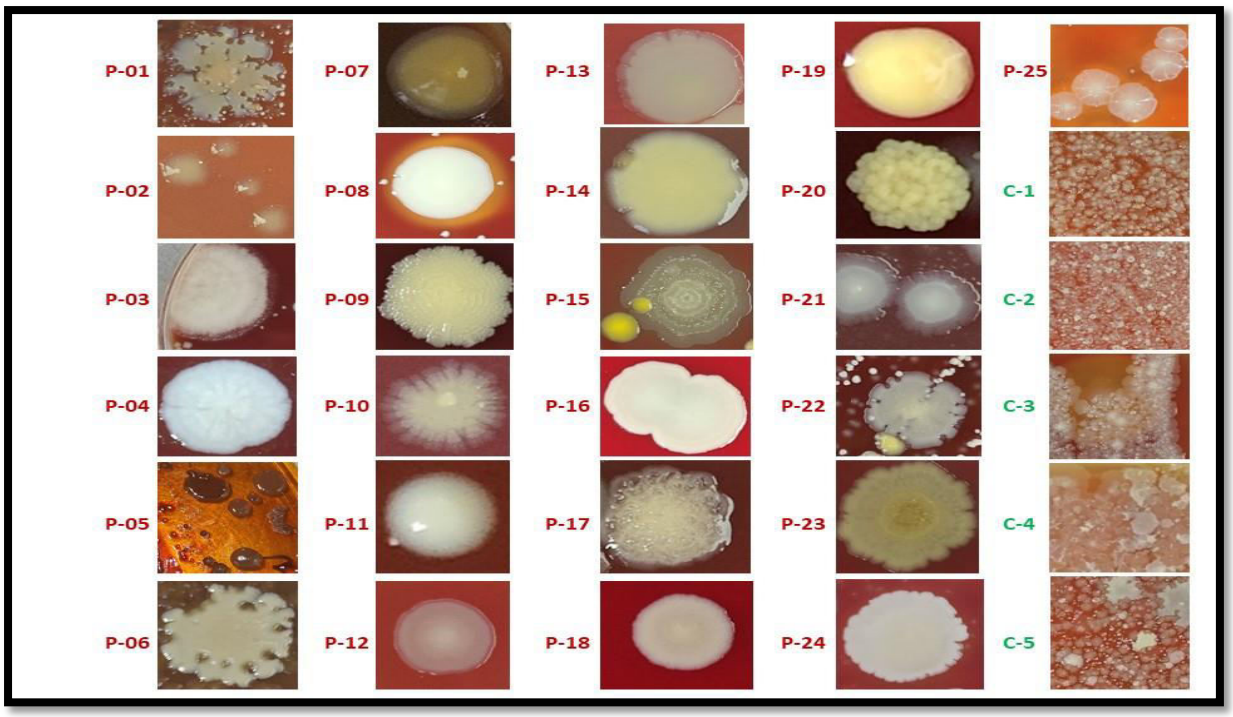

Figure3 Distinctive bacterial colonies isolated from each test sample from both PCOS patients and healthy women volunteers.

Estimation of bacterial growth rate:

Unique bacterial colonies were isolated from each test sample and cultured individually in a liquid broth medium, and the bacterial density on the 5th day estimated by a spectrophotometer is presented in Figure 4.

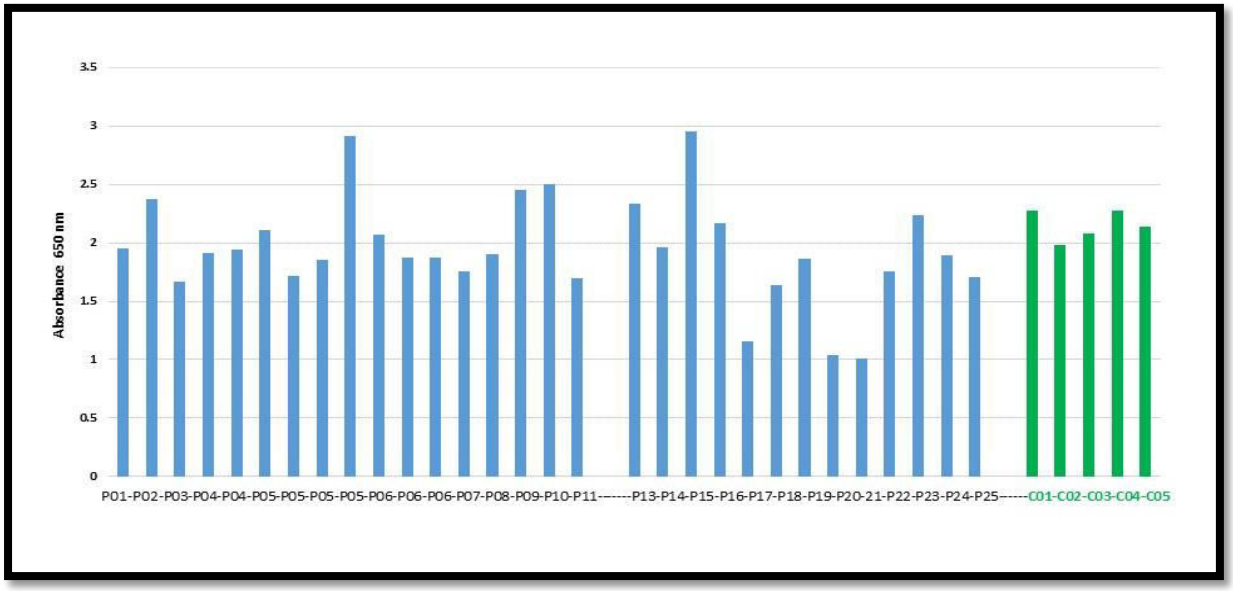

Figure 4 Theestimate of the growth rate of distinctive bacterial colonies isolated from PCOS and healthy women volunteers, by UV spectrophotometer (set at 650nm).

Discussion:

In this study, we have conducted investigations on lifestyle parameters and their cytological profiling of vaginal swab samples obtained fromPCOS andhealthy women volunteers. PCOS affects an estimated 10.0%–15.0% of reproductive-aged women globally and more than 70.0% of them are undiagnosedright time. The growing prevalence of PCOS, along with its significant impact on women's health, has ledto extensive research globally to unravel its underlying mechanisms and develop effective management strategies (Lu et al., 2021).

Undoubtedly diet plays a crucial role in maintaining the balanced microbial population in various parts of the human body and seems to be one of the foremost factors determining the occurrence of PCOS (Kshetrimayum, 2029). Other than gut microbiota; vaginal microbiota has been highly influenced by a type of diet and stress, which can cause many diseases related to the reproductive system. Often growing interest was in understanding the impact of gut microbiota and short-chain fatty acids (SCFAs) on metabolic and hormonal disorders, including PCOS. Surprisingly, the results from this study show 88.0% of PCOS were non-vegetarians, P-value<0.001 (Table 1). Revealing foods with high-fat content and a sedentary lifestyle contributes to obesity, and in turn,obesitypromotes bacterial dysbiosis and PCOS. As reported by many sources, high-fat and protein-richfoods can alter the composition of the gut microbiome.In turn, the gut microbiomes regulate the level of estrogens, and the estrogens moderate the vaginal microbiome. In addition, the gut microbiome can access the birth canal, since they are very closely situated. Thus, the abnormal gut microbiomes and vaginal microbiomes might affect each other in PCOS patients, including regulating the composition of the microbiome and causing hormonal imbalance.

During this investigation, the PCOS patients studied were in the age group between 23-40 years indicating PCOS is a major reproductive health issuesince women arestill in their childbearing age. The higher percentages of PCOS patients belonged tothe 30 to 40 years(p-value<0.05) age group. This is the age at which most women are alarmedabout not getting pregnant. Thus, this condition may persuade them to go for a clinical diagnosis and may seekIVF help. In support of this, though 88.0% of PCOS women were married 80.0% of them didnot have children (Table 1). Despite beingstill atchildbearingage due tothe onset of PCOS,they might have become infertile. As reported elsewhere, in most PCOS women'sovaries,the normal follicles have converted into ovarian cysts causing infertileconditionsandfailure to get pregnant.PCOS patients may also develop many other health disorders, including vaginitis, hyperandrogenism, and eventually ovarian cancer.

Interestingly, all the PCOS women studiedwere non-diabetic since they are below40 yearsof ageand probably they may be inthe early phase of hyperinsulinemia. This condition may elicit higher apatite andan increased tendency to overeat. Insulin resistance develops when an excess of insulin is

produced in response to elevated blood sugar concentrationsdue to overeating glycaemic and fat-rich foods that lead to obesity.Over-accumulation of insulin in the body maytrigger many alterations in differentbody organs, among them the induction of type-2 diabeteslately,and the appearance of abnormal hormonal patterns. Allied to this, a stressed lifestyle and increased oxidative stress are suspected as the major culprits to augment the PCOS.Women under medication particularly have been shown to have a deleterious effect on the hormonal pattern and induction of PCOS. Though96.0% of PCOS patients studiedwere not under the medication, they still developed PCOS indicating initiation of PCOS could be due to multiple factors.

Our studies reveal that PCOS women have a higher incidence of vaginitis (100.0%) compared to healthy women. Fewer studies to date have focused on investigating the composition of vaginal flora in culture plates and characterizing the colonies on a cytological basis. The diversity and number of colonies observed in each PCOS patient culture plate revealed high microbial diversity (p-value<0.001). Morphologically, many distinct types of colonies across the samples were identified (Figure 2), and the number of colonies per patient drasticallydifferedwithdiverse colony sizes and growth rates. The composition of the human vaginal microbiome is critical for maintaining the first line of defense against pathogens (Boskey et al., 2001).The microbial growth in the culture plates varied with time and patients. An abnormal microbiome composition observedin the PCOS patients (Figure 2)revealed that changes in vaginal microbiota may be associated strongly with such pathological conditions as bacterial vaginosis, infertility, and adverse pregnancy outcomes faced by the women (Fethers et al., 2012).

The microbiota community is now well-acknowledged as complex ecosystemsexist in almost all parts of the human body and playa vital role in regulating homeostasisviadifferent metabolic pathways (Robertson et al., 2019).Studies on the microbiome in human health usually focusedmaximally on the gut, but not much on the vaginal microbiota. That's in part because there are cultural factors like taboos surrounding female genitalia and sexuality that limit open, honest conversations about vaginal symptoms (Girishma et al., 2018).Alterations in gut microbiota composition (gut dysbiosis) observed in individuals with PCOSsuggest that dysbiosis may contribute to the development of PCOS by affecting hormonal regulation, insulin sensitivity, and inflammation.The combined actions of several factors can trigger pathologically significant shifts in the microbiota of a vagina. The threshold levels required to trigger dysbiosis largely depend on the bacterial groups (Figure 3) effect and their lifestyle risk factors. In healthy women, the vaginal microbiome is predominantly populated by a Lactobacillusgenus. However, a host environment and lifestyle factors can upset this optimal microbial makeup (Gupta, 2021).

Regrettably, till today, very few reports describe the direct role of vaginal dysbiosis on PCOS supportinga type of microbial colonies with

cytologicalobservations. All PCOS patients displayed varied microbiomes. Microbial coloniesinPCOSvaried from 1 to 133(p-value <0.001) in number and more than one type of colonies. In contrast, the healthy cultures contained >394 colonies per plate and the diversity was minimal (less than two types, P-vale>0.05). The results point-out thatin PCOS patients, normally growing healthy bacteria were completely replaced by pathogenic bacteria. In PCOS samples the microbial colony sizeswere significantly bigger varying by 1-39 mm in diameter indicating different bacterial species withdiverse growth rates. In contrast, the sizes of the bacterial colonies in healthy sampleswere less than 2 mmin size (p-value >0.05). Vaginal dysbiosis, characterized by the loss of Lactobacillus dominance and increase of microbial diversity ofpathogenic bacteria, is closely related to gynecological diseases, thus, investigatingvaginal microbiota composition is significant and promising in the treatment of gynecological diseases. Creating an environment that promotesLactobacillusdominance is generally considered aneffective method tomaintain a healthy vagina (Maet al., 2012) and prevent reproductive disorders.

Towards future research, some other point to be seriously considered is do cysts inthe ovary have a direct role in vaginal dysbiosis or if abnormal microbiota induces PCOS? The research findings indicate that PCOS has a role in vaginal dysbiosis since all the PCOS patient's cultures displayed radicalchanges in vaginal microbial composition. Ifpolycystic ovary hasany influence onvaginal dysbiosis or vice versa, then what specific factors altered the vaginal microbiota, and whichfactorssuppresslactobacillus growth is important to know. Changes in the vaginal microbiome and PCOS have strong associations, which may be responsible for ovarian disease and infertility. Further, researchis needed to identifythe roles of the different microbes within this dysbiosis community, to identify the mechanisms through which they contribute to health or disease, and to figure out how to bring a healthy microbiome back into balance (normalcy).Vaginal dysbiosis has long been a taboo subject socially, but studying and optimizing the vaginal microbiome could be a game changer for women's health.

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