

## Propolis effects on dysbiosis of polycystic ovarian syndrome (PCOS) induced Wistar rats



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### ABSTRACT

**Introduction:** Polycystic ovarian syndrome (PCOS) is an endocrinopathy that has two major risk factors, genetics and environment. Tremellen and Pearce proposed a new theory of Dysbiosis of Gut Microbiota (DOGMA) as an environmental factor that can be modified by probiotics. Propolis acts as a probiotic in diabetic rats and has been proven to change gut microbiota dysbiosis. This study evaluated whether propolis extract can modify dysbiosis in PCOS animal models compared to metformin.

**Methods:** This was a post-test-only control group design randomized control trial with 30 Wistar rats divided into five groups: control, PCOS, PCOS with metformin, PCOS with propolis extract, and PCOS with metformin plus propolis extract. PCOS induced by daily 100 mg/kg.bw testosterone propionate injection intramuscularly for 28 days. The feces in the caecum were extracted after the rats were sacrificed and analyzed for Firmicutes/Bacteroidetes ratio, alpha diversity, and beta diversity.

**Result:** Firmicutes abundance in the control group was the lowest, and Bacteroidetes abundance was lowest in the propolis group, although it's not significantly different. Between groups, Firmicutes/Bacteroidetes ratio, alpha diversity, and beta diversity were not significantly different. Testosterone concentration and preantral follicle count in the propolis and metformin groups improved compared to the PCOS group ( $p=0,01$  and  $p=0,025$ , respectively).

**Conclusion:** Propolis cannot modify dysbiosis in PCOS-induced rats, but it reduces total testosterone concentration and preantral follicle counts.

**Keywords:** polycystic ovarian syndrome, propolis, dysbiosis, Firmicutes/Bacteroidetes ratio.

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### INTRODUCTION

Polycystic ovarian syndrome has three major clinical manifestations, including anovulation or oligo ovulation, disturbance of follicle maturation, which causes several preantral follicles to exceed 12 follicles in each ovary and clinical or biochemical hyperandrogenism.<sup>1</sup> Diagnosis of PCOS is made if two of three major clinical manifestations are found based on Rotterdam Criteria.<sup>2</sup> PCOS prevalence is 4% to 12% and causes ovulation dysfunction in 75-85% of patients, which causes reproductive dysfunction (infertility).<sup>3</sup> PCOS has no defined etiology, but several risk factors were contributed, including genetic and environmental factors. The new environmental factor proposed by Tremellen and Pearce was dysbiosis of gut microbiota (DOGMA), which involves obesity and food intake.<sup>4</sup>

DOGMA is a condition where gut

microbiota has an abnormality involving the amount and composition of bacteria in the host. Dysbiosis is caused by obesity, high fat intake, high sugar, and low fiber, which causes hyperinsulinemia that leads to PCOS, which is related to the growth of many pathogenic bacteria rather than beneficial bacteria. The pathogen bacteria produce lipopolysaccharide (LPS), which can induce the natural immune system to enter the guts and systemic circulation. LPS exits from the gut lumen to blood circulation and induces metabolic endotoxemia. This endotoxin will induce proinflammation cytokines production of tumor necrosis factor (TNF) alpha that can cause insulin resistance. Hyperinsulinemia will lead to abnormal ovarian condition and function, increasing androgen production, hindering normal ovulation process and caused inhibition on antral follicle growth and maturity, and changing ovarium morphology.<sup>5,6</sup>

Dysbiosis can be determined by

analyzing alpha and beta diversity or using a simple measurement such as Firmicutes/Bacteroidetes ratio. Firmicutes and Bacteroidetes are the major phylum of gut microbiota; the other bacteria is only 10%. The firmicutes/bacteroidetes ratio has a normal value of one. If Firmicutes is abundant and Bacteroidetes is decreasing, obesity can lead to PCOS, and a Firmicutes/Bacteroidetes ratio of less than 1 usually determines inflammatory bowel syndrome (IBD).<sup>7</sup> Alpha diversity is defined by four values/index: Observed, Chao1, Shannon, and Simpson. Alpha diversity assesses bacteria richness in each subject, and beta diversity is used to compare between groups.<sup>8</sup>

Food intake, probiotics, and medication can modify gut microbiota. Metformin is a medicine widely used for PCOS patients with insulin resistance.<sup>9</sup> It can modify gut microbiota by increasing the abundance of the *Halomonadaceae* family. *Halomonas magadiensis* can inhibit the production of

TNF due to LPS produced by *Escherichia coli*. Metformin is also increasing *Akkermansia muciniphila*, which is rarely found in people with obesity, insulin resistance, and diabetes. *A. muciniphila* acts as an immunomodulator, which can delay the onset of type 1 diabetes.<sup>10</sup> Even though it has been proven to provide many benefits, metformin is still considered off-label therapy, and patient compliance with taking the drug is usually low, so other alternatives are needed that are more acceptable to patients.<sup>9</sup>

Probiotics have been proven to modify the gut microbiota feasibly. As one of the probiotics, propolis can modify the gut microbiota to a better state, improve tight junction and gap junction, and have a hypoglycemic effect. Xue M et al. found that diabetic-induced rats treated with propolis had a gut microbiota composition similar to nondiabetic rats in the control group.<sup>8</sup> Propolis is easy to find in Indonesia and widely used as a supplement to many health problems. Still, there is no publication yet about the effect of this propolis on gut microbiota dysbiosis in PCOS. There are large similarities between the gut microbiota communities in humans and rodents at the phylum level. Thus, rodents are a suitable model for investigating gut microbiota dysbiosis.<sup>11</sup> This study used an animal model to control food intake that is hard to control in humans. Nevertheless, this study aimed to investigate the effect of propolis on gut microbiota dysbiosis using alpha diversity, beta diversity, and Firmicutes/Bacteroidetes ratio.<sup>12</sup>

## METHODS

This research design was a post-test-only control group design and a randomized control trial in thirty healthy female Wistar rats. Inclusion criteria were healthy female rats aged 8 weeks and had 75 – 125 grams of body weight. Exclusion criteria were rats with anatomical disability, with drop out criteria being death and severe complications due to the treatments. This research was approved by the Health Research Ethics Committee of the Faculty of Medicine Diponegoro University, Indonesia (No. 138/EC/H/FK-UNDIP/XII/2021). Wistar rats were randomized into 5 groups: control (C), PCOS (P), PCOS with metformin administration (PM),

PCOS with propolis extract administration (PP), and PCOS with metformin plus propolis extract administration (PMP). All rats were given a standard meal (10.3% fat, 65.5% carbohydrate, and 24.2% protein) and water ad libitum. They were kept in a room temperature of 25°C with a 12h light/12h dark cycle. PCOS was induced by daily testosterone propionate injection with a dosage of 10 mg/kg/bw (Wonderindo Pharmatama, Jakarta, Indonesia) intramuscularly for 28 days, starting at the estrous cycle.

Propolis extract was used from HDI Bee Propolis produced by CC Pollen Co., Phoenix, AZ, USA. This extract contained total flavonoid 3.85% b/b and total phenol equivalent gallic acid 2.93% b/b. 100 mg bee propolis has 3.85 grams of flavonoids and 5.86 grams of phenols. The Propolis extract dose was 240 mg/kg×bw and the metformin dose was 90 mg/kg×bw. The last group treatment was propolis extract (120 mg/kg/bw) and metformin (45 mg/kg/bw). All treatments were given for 4 weeks, from day 29 until day 56.

Feces in the caecum were extracted on day 57 for qPCR analysis after the rats had been sacrificed. Caecum samples were homogenized, and the caecum bacterial DNA was extracted using the Favor Prep™ Stool DNA isolation Kit (Favorgen Biotech, Ping-Tung, Taiwan, China). A Maestrogen nanodrop machine calculated DNA concentration. RT-PCR amplification and detection were performed with the CFX96 Real-Time System. A triplicate PCR reaction was conducted on 20 µL (total volume) mixture of 10µL Thunderbird SYBR Green Real-Time PCR Master Mix (TOYOBO Co. Ltd., Osaka, Japan), 2µL each of the specific primers at a concentration of 0.3µM, DNA template and nuclease-free water (NFW) were adjusted to a volume of 20µL. The temperature settings used for amplification were 50°C for 2 minutes for 1 cycle, 95°C for 10 minutes for 1 cycle, 40 cycles at 95°C for 15 seconds, annealing temperature was 60°C for 30 seconds, and then followed by 45 seconds at 60°C then 1 cycle at 72°C for 5 minutes.

ARISA (automated ribosomal intergenic spacer analysis) was used to analyze the microbial community. It measures the length heterogeneity of the

bacterial rRNA operon 16S–23S intergenic spacer to estimate microbial richness and diversity. This procedure was started with diluting PCR master mix reagents, primers, and DNA isolate. The following procedure was to vortex the mixture for 15 seconds and prepare the thermocycler machine. The cycle and temperature settings were set as follows: 1 cycle at 98°C for 45 seconds, 30 cycles at 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 1 cycle at 72°C for 5 minutes. PCR results were read on capillary gel electrophoresis to check fragments using electrophoresis using agarose gel with a concentration of 1.5% and sent for further analysis.

Blood sampling for total testosterone serum concentration was obtained and measured using an enzyme-linked immunosorbent assay (ELISA) kit. Vaginal smears were obtained at day 1 and day 57 and then evaluated microscopically with Giemsa staining for estrous cycle determination. Ovarian tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, mounted on a glass slide, stained with hematoxylin/eosin, and analyzed by microscope—number of follicles, especially preantral follicles defined ovarian morphological changes.

Statistical analysis was conducted using the One-Way ANOVA test to examine the study's findings with a 95% confidence level and  $p < 0.05$ , followed by a Post-hoc test to determine the significance of the differences between each treatment. Kruskal-Wallis and Mann-Whitney U tests were used if the data was not distributed normally. SPSS Version 26 will be used to analyze each piece of data. Beta diversity analysis was using microbiome analysis.

## RESULTS

PP group had the highest abundance of Firmicutes phylum ( $7.41 \pm 0.52$  log CFU) and the lowest abundance of Bacteroidetes phylum ( $4.77 \pm 1.88$  log CFU). Firmicutes/Bacteroidetes ratio in the C group was the lowest ( $1.06 \pm 0.71$ ), and the highest was in the PP group with  $2.22 \pm 2.07$  (Table 1). The statistical analysis of all groups was not statistically different ( $p=0.685$ ).

**Table 1. Firmicutes/Bacteroidetes ratio of research groups**

	C	P	PM	PP	PMP	p
<b>Firmicutes</b> (log CFU)	4.88±2.69; 5.26 (1.86-7.76)	6.51±1.34; 6.59 (4.16-8.16)	6.85±0.50; 7.05 (5.99-7.37)	7.41±0.52; 7.40 (6.72-8.21)	6.49±2.0; 7.09 (2.38-8.04)	0.326 <sup>*</sup>
<b>Bacteroidetes</b> (log CFU)	5.05±0.94; 5.33 (3.55-5.99)	5.04±0.64; 5.08 (4.05-5.75)	5.25±0.63; 5.31 (4.51-6.06)	4.77±1.88; 5.24 (1.04-6.19)	5.24±0.9; 5.59 (3.52-5.99)	0.922 <sup>*</sup>
<b>Firmicutes/ Bacteroidetes Ratio</b>	1.06±0.71; 1.04 (0.31-1.98)	1.28±0.14; 1.30 (1.03-1.46)	1.31±0.09; 1.31 (1.17-1.44)	2.22±2.07; 1.41 (1.8-6.43)	1.26±0.4; 1.29 (0.48-1.87)	0.738 <sup>*</sup>

Data in the table was mean±standard deviation; median (minimum-maximum)

\*Kruskal-Wallis test; statistically significant if p-value less than 0.05

**Table 2. Alpha diversity of research groups**

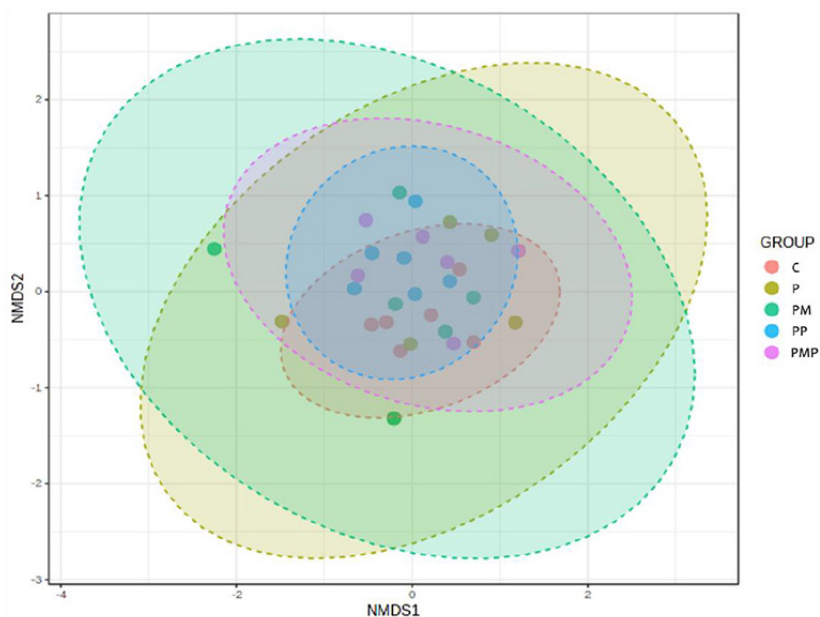
	C	P	PM	PP	PMP	p
<b>Observed</b>	10.8±1.94; 10.5 (8-13)	7.7±3.33; 9 (3-11)	8±5.06; 7.5 (3-15)	7.2±1.17; 7 (6-9)	8.7±5.24; 6.5 (5-19)	0.486 <sup>*</sup>
<b>Chao1</b>	10.3±0.50; 10 (10-11)	8.5±3.15; 8.5 (3-12)	6.7±3.39; 6.5 (3-11)	7.7±3.33; 7.5 (3-13)	8.8±5.12; 6.5 (6-19)	0.603 <sup>*</sup>
<b>Shannon</b>	1.6±0.32; 1.6 (1,2-2)	1.1±0.61; 1.3 (0-1,6)	1.1±1.02; 1.2 (0-2.4)	1.2±0.21; 1.3 (0.8-1.5)	1.5±0.7; 1.4 (0.7-2.5)	0.549 <sup>*</sup>
<b>Simpson</b>	0.8±0.06; 0.8 (0,7-0,9)	0.7±0.22; 0.8 (0.3-0.8)	0.7±0.12; 0.8 (0.5-0.8)	0.7±0.16; 0.7 (0.4-0.9)	0.8±0.17; 0.7 (0.4-0.9)	0.898 <sup>**</sup>

Data in the table was mean±standard deviation; median (minimum – maximum)

\*ANOVA test; \*\*Kruskal Wallis test; statistically significant if p-value less than 0.05

Alpha diversity could be seen with four parameters: Observed, Chao1, Shannon, and Simpson: observed species, and Chao 1 value analyzed species richness. The observed species value in the C group was the highest compared to other groups, but the difference was not significant ( $p=0.286$ ). The PM group had a higher value than the PP group. Chao1 value in the C group was the highest among the groups, but there was no significant difference. ( $p=0.603$ ). Shannon index and Simpson index were used to define diversity. Shannon index in the C and PMP groups was higher than that of the other groups, although the differences among groups were not significantly different ( $p=0.549$ ). The Simpson index, closer to 0, defined abundant species, but there was no difference in this index among groups ( $p=0.898$ ), which meant the abundant species in all groups were the same (Table 2).

Beta diversity was analyzed using a microbiome analyst on a website using the NMDS ordination method because the data and the PERMANOVA statistical method were not normally distributed. The statistical test results showed that the difference in beta diversity between



**Figure 1.** Beta diversity between research groups showed no significant difference between research groups. (C: control group; P: PCOS group; PM: PCOS with metformin treatment; PP: PCOS with propolis extract treatment; PMP: PCOS with metformin and propolis extract group treatment)

research groups was not significantly different (F-value = 1.7041, R-squared = 0.21424, p-value = 0.002 and NMDS stress = 0.18216); PERMANOVA test (Figure 1).

The total testosterone concentration of the PP group was the lowest among research groups ( $1.6±1.36$  ng/ml) and even lower than the C group (Table 3). PP and

**Table 3. Clinical manifestation of PCOS of research groups**

	C	P	PM	PP	PMP	p-value
<b>Total testosterone (ng/ml)</b>	2.9±2.01; 2.7 (0.8-5.8)	7.1±4.07; 5.6 (2.6-13.1)	2.7±3.25; 1.6 (0.5-9.2)	1.6±1.36; 1.2 (0.4-3.8)	8.6±5.1; 6.4 (4.2-17.9)	0.004*
<b>Preantral follicles</b>	8.7±5 9 (0-14)	17.3±5.6 16 (11-27)	9.3±4.7 9 (3-16)	14.5±6.1 15 (6-21)	18.2±5.2 18 (11-27)	0.010**
<b>Anovulation</b>	4	6	4	4	2	0.235***

Data in the table is mean±standard deviation; median, minimum–maximum, except anovulation.

\*Kruskal-Wallis test; \*\*ANOVA test; \*\*\*Fisher Exact test; statistically significant if p-value less than 0.05

MP groups had lower total testosterone concentrations than P and PMP groups. Significant differences in total testosterone concentration were found between C and P group ( $p=0.037$ ), P and PM group ( $p=0.025$ ), P and PP group ( $p=0.010$ ), PM and PMP group ( $p=0.025$ ) also in PP and PMP group ( $p=0.001$ ). All animal models in P groups had an anovulation cycle. PMP group only had two rats that had an anovulation cycle. Fisher Exact test results showed that the anovulation status between research groups was not significantly different; thus, the ovulation status between study groups was similar ( $p=0.235$ ). Morphological abnormalities of the ovary were assessed based on the number of follicles, especially preantral follicles. The lowest number of preantral follicles was the C group ( $2.7\pm1.6$ ), while the highest was the P group ( $17.3\pm5.6$ ). The number of preantral follicles between research groups differed significantly ( $p=0.010$ ). Significant differences in the number of preantral follicles were found between the C group and P group ( $p=0.009$ ), the P group and PMP group ( $p=0.015$ ), and between the PM group with PMP group ( $p=0.008$ ). PM group had the lowest number of preantral follicles among the three treatment groups.

## DISCUSSION

This study determined gut microbiota dysbiosis by firmicutes/Bacteroides ratio, alpha diversity, and beta diversity. The ratio of Firmicutes/Bacteroidetes demonstrates the most significant proportion of phylum in gut microbiota. Firmicutes can ferment carbohydrates and lipids, extract and absorb energy from food, and thus contribute to weight gain, leading to obesity. Obesity leads to

insulin resistance, which results in PCOS. Bacteroidetes produce short-chain fatty acids (SCFA) such as propionate and acetate. Propionate, an appetite inhibitor, participates in hepatic gluconeogenesis and reduces the synthesis of fatty acids and cholesterol. Acetate stimulates the hepatic synthesis of lipids that leads to dyslipidemia, promoting the secretion of insulin and ghrelin by the pancreas and the gastric mucosa, leading to increased fat storage and appetite, contributing to obesity.<sup>13</sup>

The use of propolis to modify gut microbiota has been widely studied in diabetes mellitus mice with hyperglycemia, hyperinsulinemia, chronic inflammation, and dysbiosis, where these conditions are also found in PCOS and are one of the pathogenesis of PCOS.<sup>14</sup> Diabetes and PCOS patients have similar pathogenesis where patients usually have high sugar, high fat, and low fiber diet, which leads to gut microbiota dysbiosis. Dysbiosis leads to low-grade inflammation that results in insulin resistance. Insulin resistance in PCOS will lead to hyperandrogenism and other clinical manifestations of PCOS. Modifying gut microbiota with probiotics, prebiotics, and synbiotics will improve bacteria diversity and composition, hyperandrogenism, polycystic ovarian morphology, and anovulation.<sup>4</sup> Polyphenols, a significant component in propolis, have a prebiotic effect, which can modify gut microbiota dysbiosis. Propolis also improves the gut microbiota imbalance through bactericidal activity. Xue M et al. found that propolis could modify gut microbiota dysbiosis in diabetic-induced rats in terms of shifted gut microbiota diversity to normal but not the abundance of specific bacteria.<sup>8</sup> Research about the effect of propolis on dysbiosis in diabetes

patients was quite common. Still, there's no publication about the propolis effect on dysbiosis in PCOS patients (searched by PubMed, ScienceDirect, and Web of Science). However, there are two research about the propolis effect on hormonal and metabolic factors in PCOS.

Metformin modifies intestinal microbiota dysbiosis by improving intestinal permeability through GLP-1 (glucagon like peptide-1) and SCFA production. The use of metformin in various studies on diabetes mellitus patients showed that the results of the number of Bacteroidetes phyla were different in each study and were inconsistent, as were the results of the number of Firmicutes phyla, but alpha and beta diversity were not studied.<sup>15,16</sup> Metformin is also used to modify gut microbiota in PCOS. Xue J et al. reported that metformin changed the genus composition of gut microbiota, such as Bifidobacterium and Helicobacter. This gut microbiota alteration leads to PCOS manifestations improvement besides the anti-inflammation role of metformin.<sup>17</sup>

Firmicutes phylum relative abundance in all PCOS-induced rat groups (P, PM, PP, PMP) was higher than in the control group. Bacteroidetes relative abundance in the propolis group was the lowest among the study groups. This result is the same as Lin et al. research, where Firmicutes phylum relative abundance was higher in PCOS and Bacteroidetes phylum relative abundance was decreased in mice of dehydroepiandrosterone-induced PCOS with a high-fat diet.<sup>18</sup> Jobira et al. reported that obese adolescents with PCOS had reduced Bacteroides and increased Prevotella (Bacteroidetes phylum). Firmicutes phylum relative abundance in PCOS was consistently higher than non-PCOS, but Bacteroidetes phylum

relative abundance has different results in several research.<sup>19</sup> Letrozole-induced PCOS rats in the study by Zhao et al. had a relative abundance of Firmicutes, Verrucomicrobia, Bacteroidetes, Actinobacteria, and Proteobacteria phyla compared with the healthy control group.<sup>20</sup> Qi et al. found that *Bacteroides vulgatus* was significantly higher in women with PCOS than in healthy women. Bacteroidetes phylum is relatively abundant. The difference between researches is caused by different data collected because there are data with phylum only, and the others provide specific genus or species of this phylum.<sup>21</sup> The association between the Firmicutes/Bacteroidetes ratio and PCOS may vary between specific populations, age groups, genders, environmental, genetic factors, and other phyla, which may also play an important role. Magne et al. stated that the Firmicutes/Bacteroidetes ratio may not be a robust indicator of microbiome dysbiosis associated with obesity thus, it may not be suitable to define gut microbiota dysbiosis in PCOS, especially in lean PCOS.<sup>13</sup> Improving gut microbiota dysbiosis with different dietary supplements usually contributes to restoring the normal gut microbiota. However, propolis as prebiotics have not yet ameliorated gut microbiota dysbiosis in PCOS.

Alpha diversity analysis was used to evaluate species diversity in each sample. The Chao1 and observed indexes are used to see the number of types of bacteria in one individual or sample. In contrast, the Shannon and Simpson indices are diversity indices where a decreasing Shannon index value indicates reduced diversity. In contrast, a Simpson index value close to 1 means the dominance of one or two bacteria. In this study, alpha diversity was assessed using the observed Shannon, Simpson, and Chao1, which showed that the results did not differ significantly between research groups.<sup>22</sup> Species richness and bacterial diversity in the PMP group were better than all PCOS-induced rat groups (P, PM, PP) even though they did not resemble the control group and were not significantly different. Xue M et al. reported that alpha diversity in diabetic-induced rats given propolis treatment did not differ from those

without treatment, although beta diversity is significantly better.<sup>8</sup> Alpha diversity in female mice with a high fat diet and 300 mg/kg.bw propolis improved significantly after 9 weeks of treatment.<sup>23</sup> Chien et al. reported species richness (Chao1 and Fisher alpha index) in male mice fed high fat diet and propolis extract, but the species evenness among groups was not different. This study also found that a combination of propolis and metformin failed to improve gut microbiota species richness and evenness.<sup>24</sup>

The R value assesses beta diversity. If the value is weak, the differences between groups are not significantly different. The R-value is declared valid if the p-value is <0.004 and the NMDS stress is <0.2, which indicates that this analysis can recognize differences between groups. NMDS is used because the data distribution is not normal. Beta diversity between research groups was also insignificantly different, but what is interesting is that the effect of propolis on each mouse was almost uniform. This shows that the impact of propolis therapy on each individual is more uniform than metformin therapy. Propolis extract, metformin, and a combination did not lower Firmicutes/Bacteroidetes ratio in PCOS-induced rats. The variation between different research groups was insignificant, possibly due to the duration and dose of each therapy's inability to change the intestinal microbiota that experienced dysbiosis. Propolis could improve intestinal microecology disorder, but it's not proven effective in PCOS.

Propolis extract and metformin were found to be equally effective for reducing testosterone levels in PCOS rats, although does not affect gut microbiota dysbiosis. These results follow research by Abbasi et al., who found propolis extract treatment could reduce total testosterone levels in PCOS patients. Propolis extract reduces testosterone levels by reducing fasting glucose and fasting insulin levels, which contribute to reducing the condition of hyperinsulinemia in rats with PCOS, which will affect the production of SHBG levels, ultimately reducing serum testosterone levels.<sup>25</sup> Metformin reduces total testosterone concentration by inhibiting SHBG production in the liver. Mishra et al. reported similar results

on PCOS women with metformin treatment for 12 weeks, where metformin ameliorated hyperandrogenism measured by free androgen index.<sup>26</sup> Propolis reduced the number of preantral follicles compared to metformin. Metformin improves polycystic ovary morphology by reducing preantral follicle number through insulin action, reducing androgen production. Bafrouei et al. reported that metformin treatment in the PCOS rats model improves follicle morphology disorders in the follicles in the ovary.<sup>27</sup> Sapmaz et al. found that propolis treatment at doses of 50 mg/kg and 150 mg/kg for ten days can reduce the number of follicles compared to PCOS mice without therapy.<sup>28</sup> Propolis extract's effect on preantral follicle count is may be due to its ability to its hypoglycemic role that could improve insulin resistance that leads to average production of androgen.

This study has limitations, such as only examining the phylum level of gut microbiota dysbiosis analysis. Many genus and species in one phylum have different roles that will affect gut microbiota. The estrous cycle in this research was measured only on day one and the last day of research, which resulted in undefined anovulation in research groups. However, PCOS diagnosis can still be established using Rotterdam criteria, which require only two of three criteria. Further research about dysbiosis should conduct with instruments that can examine not only the phylum of bacteria but also the genus or species level for a better depiction of gut microbiota, such as next-generation sequencing (NGS) and also daily vaginal smear to define estrous cycle in PCOS animal model accurately.

## CONCLUSION

Propolis extract and metformin treatment cannot modify gut microbiota dysbiosis, as measured by the Firmicutes/Bacteroides ratio, alpha diversity, and beta diversity. Dose, administration route, and treatment period may affect propolis role in gut microbiota modulation. Propolis extract could ameliorate PCOS manifestations, including reducing total testosterone concentration and preantral follicle count. Further research about dysbiosis should conduct with instruments that can examine not only the phylum of bacteria

but also the genus or species level for a better depiction of gut microbiota, such as next-generation sequencing (NGS) and also daily vaginal smear to define estrous cycle in PCOS animal model accurately.

## DISCLOSURES

### Funding

The authors are responsible for the study funding without a grant, scholarship, or other resources.

### Conflict of Interest

We declare that there were no conflicts of interest in this study.

### Author Contribution

All of the authors equally contributed to the study.

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