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Research Article
Levonorgestrel and Desogestrel Modulate Gut Microbiota and Blood Biochemistry of Female Wistar Rats

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Abstract

Background and Objective: Hormonal Contraceptive Pills (HCPs) are commonly prescribed to avoid unwanted pregnancies, endometrial cancers, migraines and to regulate the menstrual cycle. In the present study, female Wistar rats were orally administered with Levonorgestrel (LNG) and Desogestrel (DG) for 12 weeks. Twelve-hours fasting rats were dissected, blood biochemistry, gut microbiota and histology of breast tissue were analysed. **Materials and Methods:** The study was conducted on 12 female Albino Wistar rats of similar age and weight. Two groups were treated with hormonal contraceptive medicines and one group was labelled as the negative control. Post-treatment rats were dissected and subjected to blood biochemistry and gut microbiota analysis. ANOVA and Tukey's test was applied for statistical analysis. The p-values up to 0.05 were considered significant. **Results:** Results have shown significant changes in cortisol, testosterone, lipid profile and heart/liver associated plasma enzyme markers. The study demonstrates an increase in the gut microbiota diversity in the treated samples. An increase in some pathogenic genera such as *Desulfovibrio*, *Anaerovorax*, *Oscillibacter* in the levonorgestrel was also found. The histological study of breast tissues has shown an increased fat deposit among the medicated rats. **Conclusion:** In conclusion, found a positive correlation between the use of contraceptive pills and both blood biomarkers and gut microbiota. The second generation medicine (LNG) has shown severe negative effects as compared to third-generation medicine (DG).

Key words: HCPs, gut microbiota, biomarkers, lipid profile, breast histology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hormonal Contraceptive Pills (HCPs), consisting of either progestin's or a progestogens-estrogens combination are the most commonly used contraceptives among the fertile women[1]. According to estimates, more than 100-150 M women are using HCPs worldwide[2,3]. The HCPs have been divided into three different generations, 1st generation not being used. The common representatives of 2nd and 3rd generation HCPs are levonorgestrel and desogestrel, respectively[4]. The mechanisms of HCPs include suppression in the secretion of gonadotropin resulting in the inhibition of ovulation, inhibiting sperm motility by increasing the viscosity of cervical mucus and reducing the level of luteinizing hormone[3,5]. In the majority of countries, the HCPs are available as on the counter drugs without and prescription[6]. Many non-contraceptive benefits have been associated with the use of contraceptive pills. Decreased chances of ovarian cyst formation, increased menstrual blood, better cycle control, decreased chances of endometrial and ovarian cancers are common non-contraceptive benefits[7,8]. The use of HCPs is associated with cardiovascular diseases[9], ischemic heart disease[10], altered lipid metabolism, enhanced chances of atherosclerosis[11,12]. The microbiome of the mammalian intestine is considered a physiologically active organ that consists of up to 100 trillion microbes and their genomes[13,14]. These are unique populations of bacteria, fungi and viruses which dynamically change with age and body health status[15,16]. Symbiotically living in the host, the microbiota participates in the regulation of exogenous substances like medicine[17] and hazardous substances to maintain the status of human health[18,19]. The understanding of the interaction of gut microbiota with the drugs has renovated the perception of microbiota and their importance. The enzymes from microbiota are plausible intermediate targets to change the drug metabolism[20,21]. The metabolites from gut microbes enter the bloodstream and affect the physiology of vital organs, any change in the bacterial diversity will result in the change in metabolic reactions[22]. The metabolites from the intestinal microbial community contribute a significant share in the mammalian bloodstream and interfere with the hormone, nervous and immune systems[23-25]. The association of gut microbiota modifications with the onset of various diseases including cancer[26,27], Cardiovascular Diseases (CVD)[28,29] and Inflammatory Bowel Diseases (IBD)[30] is well established. The present study has evaluated the effect of HCPs on the ecology of gut microbiota, blood biomarkers and breast histology of female rats.

MATERIALS AND METHODS

Study area: The animals were purchased and subjected to medicines from October 20, 2019 to January 20, 2020. The blood biochemistry was evaluated and compared immediately after the animal dissection. DNA was isolated from the stool samples during February, 2020 and NGS based detection of microbial species and data analysis was commercially conducted from Macrogen Korea.

Animals and ethical approval: Age-matched, 1.5 month old 12 female Wistar rats with a weight of 100-120 g were purchased from King Fahad Medical Research Centre (KFMRC) at King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. The study protocol was approved by the ethical research committee of the Faculty of Science at King Abdulaziz University under agreement number (Reference No. 442-16). The second and third generation hormonal contraceptive pills, levonorgestrel (LNG) and desogestrel (DG) were purchased under the trade names of Emkit DS 1.5 mg and Desofam 0.15 mg, respectively manufactured by Zafa Pharmaceutical Laboratories Private Limited, Karachi, Pakistan.

Experimental design: The experiment was carried out following approved guidelines. The rats were acclimatized for a week in the polypropylene shoebox cages (50×25×20 cm) with dry hardboard bedding retained in the lab adjusted at 22±3°C, 55±5% humidity and 12 hrs a day/night cycles with a normal diet. The animals were divided into 3 groups, each group having 4 animals was housed in a separate cage. Group-I was untreated or normal control, Group-II was subjected to 350 µg of LNG[31], Group-III was treated with 150 µg of DG per kg of body weight per day[32]. The drug was administered orally as an aqueous suspension using sterilized plastic droppers. All groups were subjected to the above environmental and drug dose conditions for 12 weeks. The drug dose was varied according to the weekly increase in body weight of animals.

Dissection and sampling: The rats were kept starving for 12 hrs, dissected under mild anaesthesia administered in a closed glass chamber filled with ether vapours. Blood samples were collected by cardiac puncture method collected and saved in sodium citrate, potassium EDTA coated and clot activator gel tubes. The blood serum/plasma was isolated by centrifugation, identification code, collection date/time of was recorded and samples were stored at -80°C. Three replicates

of each sample were prepared and stored. Stool samples were collected in sterile plastic containers immediately after dissection, tagged with their identifications and stored at -80°C until further processing. Three replicates of each sample were prepared and stored.

Lipid profile: Lipid profiles including Total Lipids (TL), Total Cholesterol (TC), Total Triglycerides (TG), High-Density Lipoprotein (HDL), Low-Density Lipoproteins (LDL), Very Low-Density Lipoproteins (Vldls) were determined.

Liver function tests (LFTs): The plasma activity of Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were commercially analyzed from Healthcare Diagnostics and Research Centre, 704-Kamran Block, Iqbal Town, Lahore, Pakistan.

Cortisol and testosterone: Rat specific cortisol ELISA Kit, catalogue number LS-F10025 (LifeSpan BioSciences. The USA). The method provided by the manufacturer was adopted. Testosterone was commercially analyzed from Healthcare Diagnostics and Research Centre, 704-Kamran Block, Iqbal Town, Lahore, Pakistan.

DNA extraction from stool samples: QIAamp Fast DNA Stool Mini Kit (QIAGEN-catalogue No. 51604) was used for DNA isolation. The procedure described by the manufacturer was followed with modifications for better DNA yield. Briefly, 200-300 mg of faecal sample was homogenized in 1000 µL ASL lysis buffer by vortexing for 2-3 min in a labelled 2 mL sterilized Eppendorf tube. Homogenate was incubated for 15 min in the water bath adjusted at 85°C. After every 5 min of incubation, each sample was mixed well by vortex for 15 sec. The heat-treated homogenate was centrifuged at 14000×g for 5 min and residue discarded. About 600 µL clear reddish yellow supernatant was transferred to a fresh 2 mL Eppendorf tube, 25 µL proteinase K, 200 µL AL solution was added, mixed well by vortex for 15 sec and incubated in the water bath adjusted at 70°C for 10 min. To the sample above, 600 µL of absolute ethanol was added and mixed well by the short vortex. Each sample was added to the column provided with the kit and centrifuged at 14000×g for 1 min. DNA bound to the column was washed with 600 µL of AW1 and AW2 wash buffers, respectively. After the removal of the AW2 wash buffer, the empty column was dried by centrifugation for 2 min at the above conditions. Finally, the columns were placed in labelled sterilized 1.5 mL Eppendorf tubes, 150 µL elution buffer was added to each column, incubated for 10 min at room temperature and centrifuged as above for 1 min. The eluted DNA sample was evaluated both quantitatively and qualitatively by Nanodrop and agarose gel electrophoresis methods.

Microbiome analysis by NGS and 16S RNA based taxonomic preparations: Gut microbiome was commercially evaluated from Macrogen Korea (10F, 254 Beotkkot-ro, Geumcheon-gu, Seoul Republic of Korea) using Next-Generation Sequencing (NGS) and data were analyzed. High-quality sequence reads were selected and retained by looking at both, the quality of score (average Phred score >20) and length of reads (>250 bp). Based on the similarities and variations of 16S rRNA sequences, the taxonomic levels were assigned as follows: >97% similarity was placed in the same species, >94% similarity placed as the same genus, >90% similarity as same family, >85% similarity as same order, >80% similarity as a same class and >75% similarity as the same phylum. Bacterial compositions were plotted to make the group differences visible in Fig. 1.

Statistical analysis: The results are described concerning the Mean±SD (Standard Deviation). Statistical analysis of biochemical analysis was performed by one-way analysis of variance analysis (ANOVA) which was followed by Tukey's test (t-test) by using Prism 6.0 GraphPad software. Value of $p<0.05$ was counted to be statistically significant. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, when compared with the control group. Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to identify the significant difference between bacterial taxa among different groups. Heat map and generated by Prism 6.0 GraphPad software (GraphPad Software, San Diego, USA).

Histopathological examination of breast tissue: Samples (breast tissues) for autopsy were isolated from each group of animals, then quickly preserved in the solution the formalin with 10% buffer solution of phosphate. Subsequently, samples were mounted and fixed in paraffin. These fixed samples were used for the final histopathological examination. These samples were sliced in 5 µm thick sections and paraffin was removed from samples and then stained with eosin-hematoxylin dye. Prepared specimens were observed for fat deposition, swelling of cells, change in the architecture of cells, tubular dilatation, ballooning, necrosis ad edema of cells in control as well as all treatment groups.

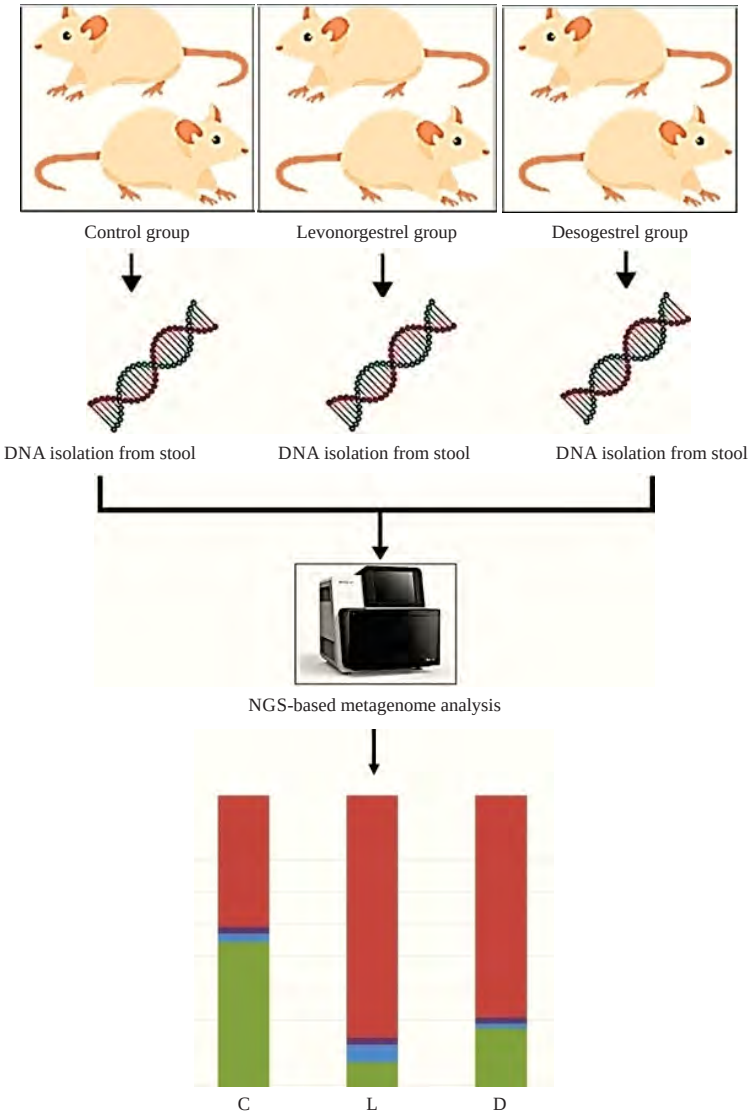

Fig. 1: DNA was isolated from the stool of each rat after 12 weeks of treatment by hormonal contraceptive medicines and application of NGS-based metagenome analysis

RESULTS

Level of cortisol and testosterone: In Group-II animals, after the treatment with LNG, the level of cortisol significantly ($p<0.01$) increased to 96.9±.10.02 nM in comparison to Group-I normal control animals 74.3±2.45 nM. The cortisol level of Group-III animals after the treatment with DG was also significantly ($p<0.05$) increased 86.3±3.13 nM as compared to the normal level of Group-I animals 74.3±2.45 nM in Fig. 2. After the treatment with LNG, the level of testosterone was significantly ($p<0.01$) decreased to 0.10±.0.01 ng mL^{-1} in Group-II animals, as compared to Group-I animals 0.14±0.04 ng mL^{-1}. The testosterone level of Group-III animals after the treatment with DG was also slightly decreased 0.13±.0.01 ng mL^{-1} significantly ($p<0.05$) as compared to the normal level of Group-I animals in Fig. 3.

Changes in the lipid profile: The level of TL was increased significantly ($p<0.01$) to 468.1±20.45 mg dL^{-1} in Group-II animals after the treatment with LNG, as compared to Group-I animals 388.2±18.11 mg dL^{-1}. TL level of Group-III animals after the treatment with DG was also increased 410.5±.19.56 mg dL^{-1} significantly ($p<0.05$) as compared to the normal level of Group-I animals. The level of TC was increased significantly ($p<0.001$) to 98.4±4.65 mg dL^{-1} in

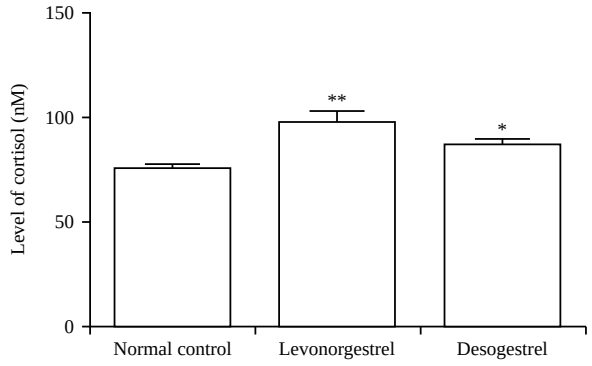

Fig. 2: Effect of hormonal contraceptives on blood cortisol level

Values are expressed as Mean±SEM (n = 4), #Groups as compared to normal control, *Groups as compared to disease control, *p<0.05, **p<0.01 and ***p<0.001

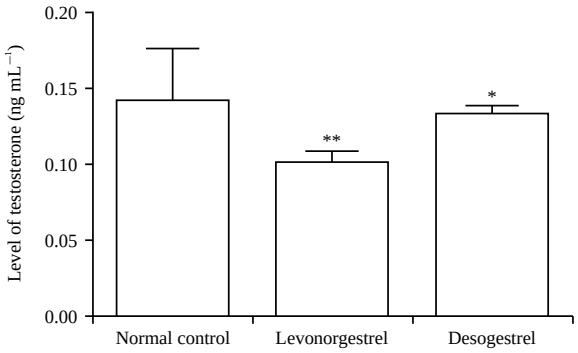

Fig. 3: Effect of hormonal contraceptives on blood testosterone level

Values are expressed as Mean±SEM (n = 6), #Groups as compared to normal control, *Groups as compared to disease control, *p<0.05, **p<0.01 and ***p<0.001

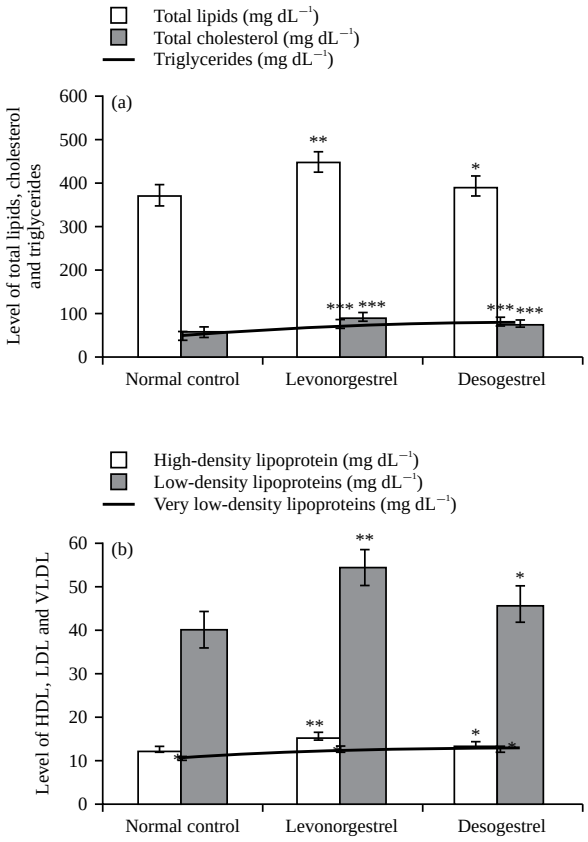

Fig. 4(a-b): Effect of hormonal contraceptives on lipid profile, (a) Effect of hormonal contraceptives on total lipids, total cholesterol and triglycerides and (b) Effect of hormonal contraceptives on HDL, LDL and VLDL

Values are expressed as Mean±SEM (n = 4), #Groups as compared to normal control, *Groups as compared to disease control, *p<0.05, **p<0.01, ***p<0.001 and ns: Non-significant

Group-II animals after the treatment with LNG, as compared to Group-I animals 66.3±4.22 mg dL^{-1}. TC level of Group-III animals after the treatment with DG was also increased 83.1±.3.91 mg dL^{-1} significantly ($p<0.001$) as compared to the normal level of Group-I animals. After the treatment with LNG, the level of TG was increased significantly ($p<0.001$) to 83.2±2.76 mg dL^{-1} in Group-II animals as compared to Group-I animals 56.1±2.98 mg dL^{-1}. The level of TG in Group-III animals after the treatment with DG was also increased 89.6±.2.67 mg dL^{-1} significantly ($p<0.001$) as compared to the normal level of Group-I animals Fig. 4a. The HDL level was increased significantly ($p<0.01$) to 16.1±0.32 mg dL^{-1} in Group-II animals after the treatment with LNG, in comparison to Group-I animals 13.2±0.24 mg dL^{-1}. The level of HDL of Group-III animals after the treatment with DG was also increased by 14.2±.0.45 mg dL^{-1} significantly ($p<0.05$) as compared to the normal level of Group-I animals. The level of LDL was increased significantly ($p<0.01$) to 57.3±0.55 mg dL^{-1} in Group-II animals after the treatment with LNG, in comparison to Group-I animals 42.1±0.32 mg dL^{-1}. The level of LDL of Group-III animals after the treatment with DG was also increased 48.1±.0.51 mg dL^{-1} significantly ($p<0.05$) as compared to the normal level of Group-I animals. The VLDL level was increased significantly ($p<0.05$) to 13.2±0.16 mg dL^{-1} in Group-II animals after the treatment with LNG, in comparison to Group-I animals 11.1±0.19 mg dL^{-1}. The level of HDL of Group-III animals after the treatment with DG was also increased by 13.4±.0.15 mg dL^{-1} significantly ($p<0.05$) as compared to the normal level of Group-I animals in Fig. 4b.

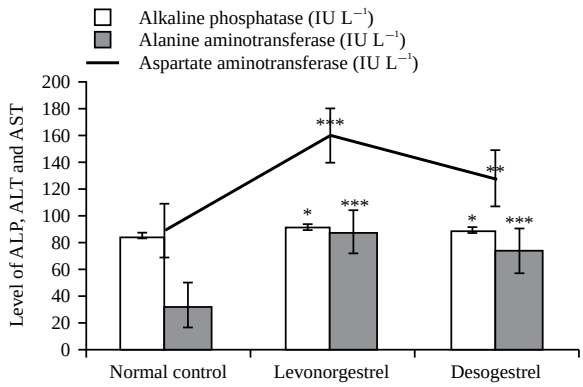

Fig. 5: Effect of hormonal contraceptives on liver function test
Values are expressed as Mean±SEM (N = 4), #Groups as compared to normal control, *Groups as compared to disease control, *p<0.05, **p<0.01, ***p<0.001 and ns: Non-significant

Liver biomarker enzymes: ALP level was increased significantly (p<0.05) to 93.4±1.48 IU L^{-1} in Group-II animals after the treatment with LNG, in comparison to Group-I animals 86.6±1.56 IU L^{-1}. The level of ALP of Group-III animals after the treatment with DG was also increased by 90.3±.1.66 IU L^{-1} significantly (p<0.05) as compared to the normal level of Group-I animals. After the treatment with LNG, the level of ALT was increased significantly (p<0.001) to 89.5±1.33 IU L^{-1} in Group-II animals in comparison to Group-I animals 34.1±0.59 IU L^{-1}. The level of ALP of Group-III animals after the treatment with DG was also increased by 75.8±.0.28 IU L^{-1} significantly (p<0.001) as compared to the normal level of Group-I animals. AST level was increased significantly (p<0.001) to 161.5±4.59 IU L^{-1} in Group-II animals after the treatment with LNG, in comparison to Group-I animals 90.5±2.92 IU L^{-1}. The level of ALP of Group-III animals after the treatment with DG was also increased 129.9±.3.83 IU L^{-1} significantly (p<0.01) as compared to the normal level of Group-I animals in Fig. 5.

Histopathology of breast tissue: Histopathological slides NC animals after observations revealed organized cellular architecture with no infracted fat cells, no deposition of fat around the cells in Fig. S1a-c. Further welling, change in architecture, tubular dilatation, ballooning, necrosis were also absent in NC slides. After examination of histopathological slides of LNG group animals, multiple infracted cells surrounded by fat as well as lipid-laden macrophages, swelling and abnormal architectures of cells were also found. Histology of DG group animals slides shows many infracted fatty cells surrounded by lipid-laden macrophages and inflammation.

General gut microbiota composition: All the DNA samples were processed by the quality control division and certified for good quality and quantity by Macrogen Korea. Overall, 358,659 sequences were obtained from 12 samples by next-generation sequencing targeting 16s rRNA, with an average length of 29,887.833. The observed number of Operational Taxonomic Units (OTU) was 905 in all groups. The sequences were assigned to 13 different phyla including one archaeal phylum *Euryarchaeota*, which was revealed at a minor abundance and 12 bacterial phyla that were *Actinobacteria*, *Bacteroidetes*, *Candidatus saccharibacteria*, *Cyanobacteria*, *Deferribacteres*, *Elusimicrobia*, *Firmicutes*, *Fusobacteria*, *Planctomycetes*, *Spirochetes* and *Tenericutes*. The greater number of sequencing reads relate to Bacteroidetes and Firmicutes in Fig. 6. At the class level, 18 bacterial classes have been recognized in all gut microbiota populations. *Bacteroides* and *Bacilli* were the highest abundances among all groups. At the family level, 43 bacterial families were identified in all groups. The *Prevotellaceae*, *Lactobacillacea* and *Lachnospiraceae* were present in high abundance through all groups also another family named *Ruminococcaceae* were present at a high level in the LNG group. At the genus level, 72 bacterial genera were recognized. The top abundance of genera among all groups were *Prevotella* and *Lactobacillus*. Additionally, *Schwartzia* in LNG group in Fig. S2.

Changes in the gut microbial diversity: The alpha diversity of the gut microbiota population is represented by the rarefaction of observed OTUs and estimated Chao1. The rarefaction curves of the sequence showed high sampling coverage gained in the 3 groups. The good's coverage was above 99% for all sequences in the 3 groups. The rarefaction with observed OTUs in Fig. S3 and estimated Chao1in Fig. 7 showed that the LNG group and DS group had richer than the control group. Principal coordinate analysis that represents beta diversity revealed that the gut microbiota of each group clustered together in Fig. 8.

Alterations in gut microbiota composition: The study found significant differences in gut microbiota population between the control group and both treated groups at different levels. At the phylum level, a notable increase in the Firmicutes/Bacteroidetes (F/B) ratio in the gut microbiota population of LNG and DG groups compared to the control group in Fig. 6. At the class level, the LNG treated group showed a significant increase in *Deltaproteobacteria*,

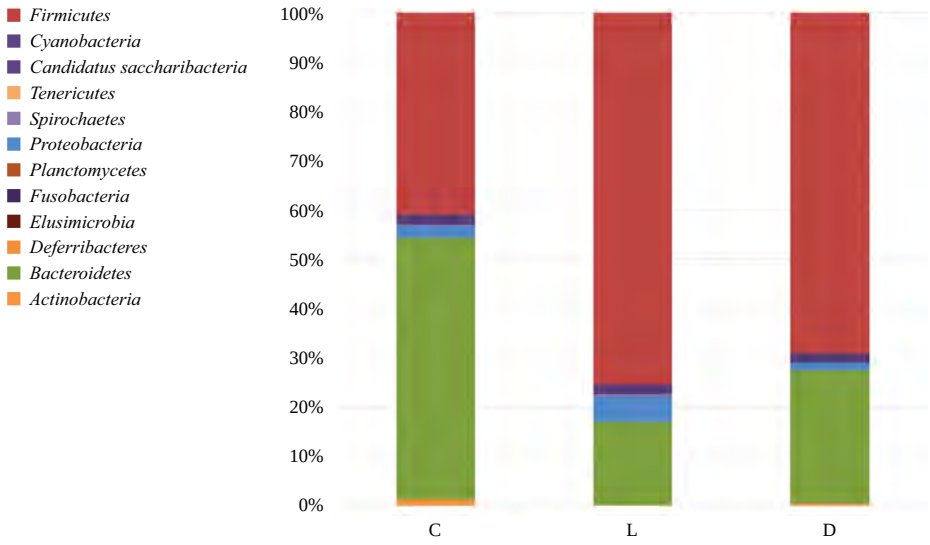

Fig. 6: Relative abundance expressed as a percentage of mainly present archaeal and bacterial phyla among different contraceptives pill treated groups

C: Control group, L: Levonorgestrel group and D: Desogestrel group

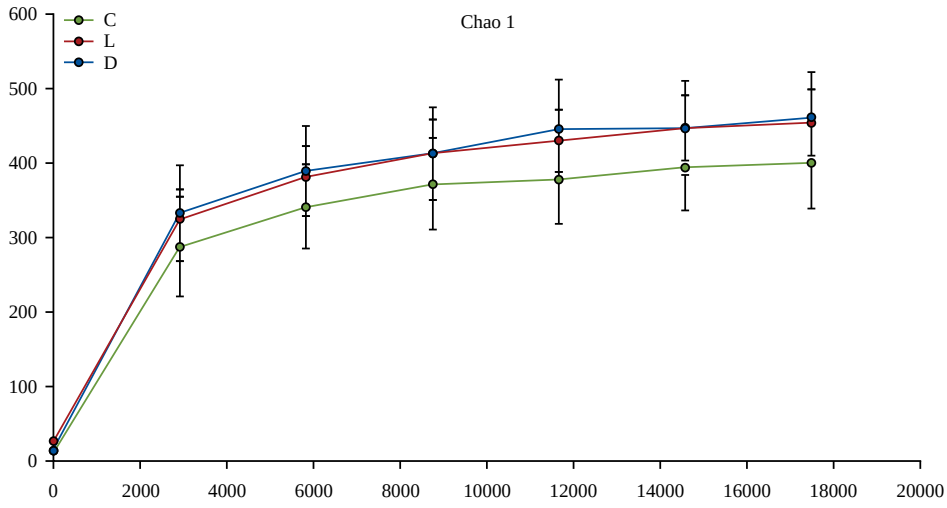

Fig. 7: Alpha diversity analysis of the sequence reads

Rarefaction curves estimated by Chao1, C: Control group, L: Levonorgestrel group and D: Desogestrel group

Clostridia and *Negativicutes* classes and a decrease in *Epsilonproteobacteria* compared to control groups in Fig. 9a-c. At the order level, observed increase in *Desulfovibrionales*, *Clostridiales* and *Selenomonadales* families, while a decreasing observed in *Campylobacter ales* family of the LNG group compared to the control group in Fig. 9d-f. At the family level, we reported that *Desulfovibrionaceae*, *Clostridiales_incertae sedis* XIII and *Ruminococcaceae* are increasing LNG group. In addition, the study reported decreasing in *Corynebacteriaceae*, *Campylobacteraceae* and *Helicobacteraceae* in the LNG group compared to the chow-diet fed group in Fig. 9g-l. Among genus level, five genera have increased *Desulfovibrio*, *Anaerovorax*, *Flavonifractor*, *Oscillibacter* and *Pseudoflavonifractor* and three genera have decreased *Campylobacter*, *Helicobacter* and *Corynebacterium* in the gut microbiota population of the LNG group as illustrated in Fig. 9m-t.

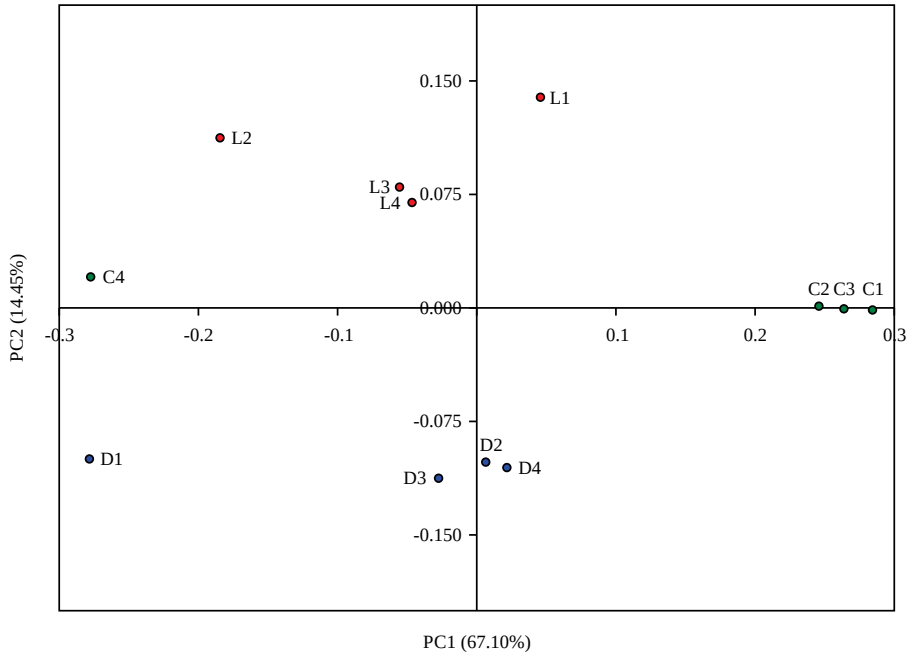

Fig. 8: Multivariate principal coordinates analysis of the composition of microbial communities between C, L and D groups
C: Control group, L: Levonorgestrel group and D: Desogestrel group

DISCUSSION

Having more than 100 million users worldwide, HCPs are considered as one of the most influential drugs at present[33]. These drugs have an effective role in birth control and family planning and are also prescribed for irregularities in the menstrual cycle, menstrual and endometrial pain, fibroids and migraines[34-36]. In parallel, the use of HCPs has also been associated with many health problems including sexual dysfunction, inflammatory bowel disease[5], heart diseases[37] and breast cancer[38]. The present study describes the effect of the long term application of second and third-generation HCPs on the blood biomarkers and the gut microbiota in female Wistar rats.

Major hormone alterations during puberty, pregnancy or menopause are considered as the critical points for an increased mood disorder, anxiety and depression among females[39,40]. Clinical manipulations of ovarian hormone levels by the use of HCPs are therefore questionable[41]. Cortisol is stress associated steroidal hormone synthesized in the adrenal gland[42,43]. A normal balanced level of cortisol is essential for health. The prolonged elevated level of cortisol leads to various disorders including Cushing syndrome. Cushing syndrome is associated with rapid weight gain in the abdomen, chest and face, increased blood pressure, flushed face and osteoporosis[44]. Increased levels of cortisol can also lead to loss of libido and menstrual cycle in women[45]. Further depression and anxiety may arise with an increased level of cortisol[46]. In this experiment, after the treatment with LNG and DG, the level of cortisol was significantly increased ($p<0.01$ and $p<0.05$) as compared to normal control rats (Fig. 2). The results are following similar human clinical studies[47]. The level of testosterone was decreased in the rats treated with both LNG and DG. However, the decrease was more significant in the rats treated with LNG (Fig. 3). Testosterone is considered a male hormone but females also produce small amounts of testosterone in the ovaries that are obligatory for their reproductive health[48]. The decline in androgenic female hormones has been associated with the long term use of HCPs[49,50]. The low level of testosterone like androgens in females is associated with premature ovarian insufficiency leading to female reproductive dysfunction[51,52]. However, future studies with variable doses of new generation HCPs can lead to a better conclusion.

Post-HCPs treatment, the lipid profile was altered, the total blood lipid content, cholesterol and triglycerides were elevated post HCPs treatment (Fig. 4). The elevation of lipid profile, body mass index and diastolic blood pressure have been reported in females using HCPs which can enhance the risk of cardiovascular diseases[53,54]. A significant increase in the

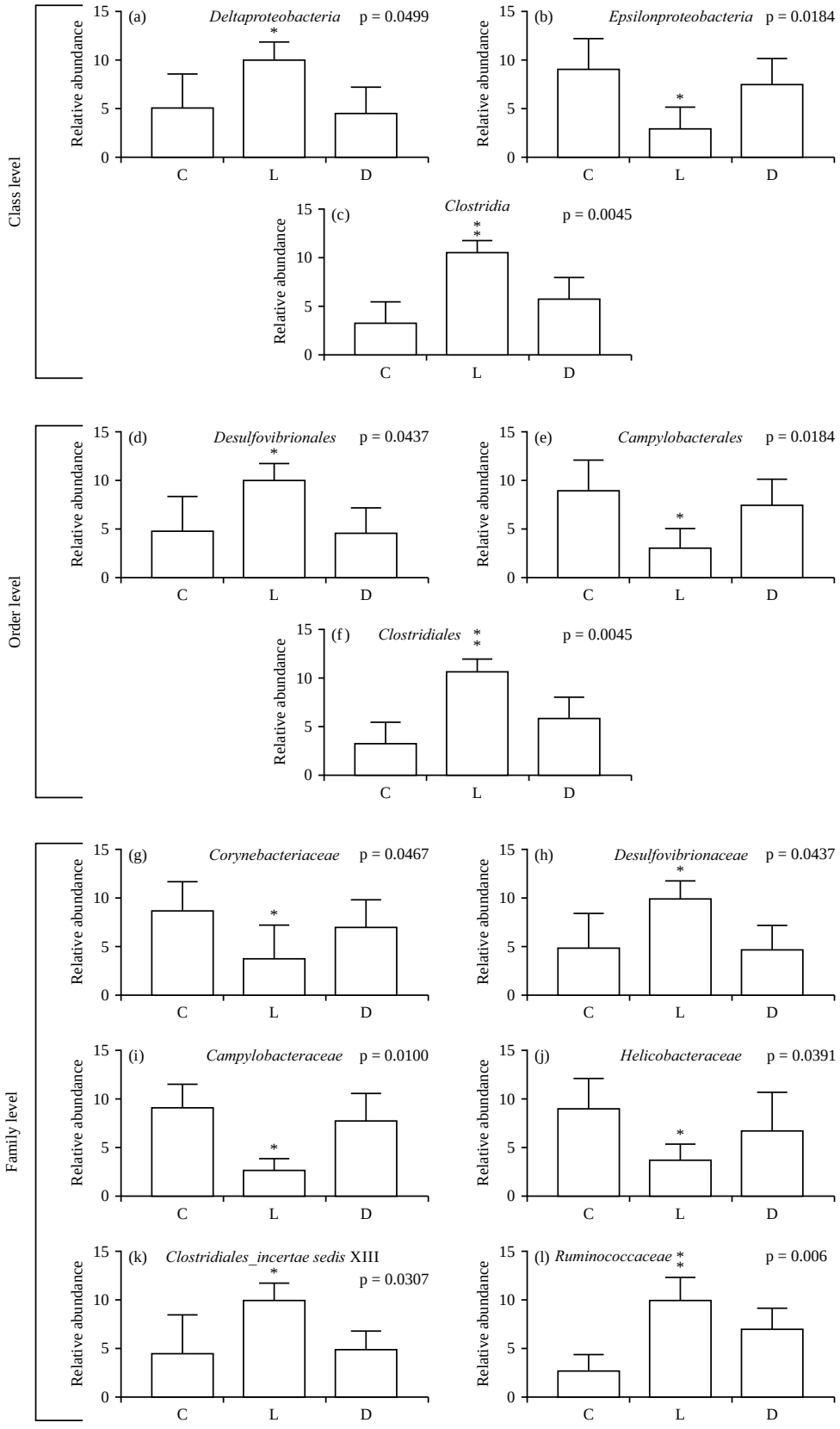

Fig. 9(a-t): Continue

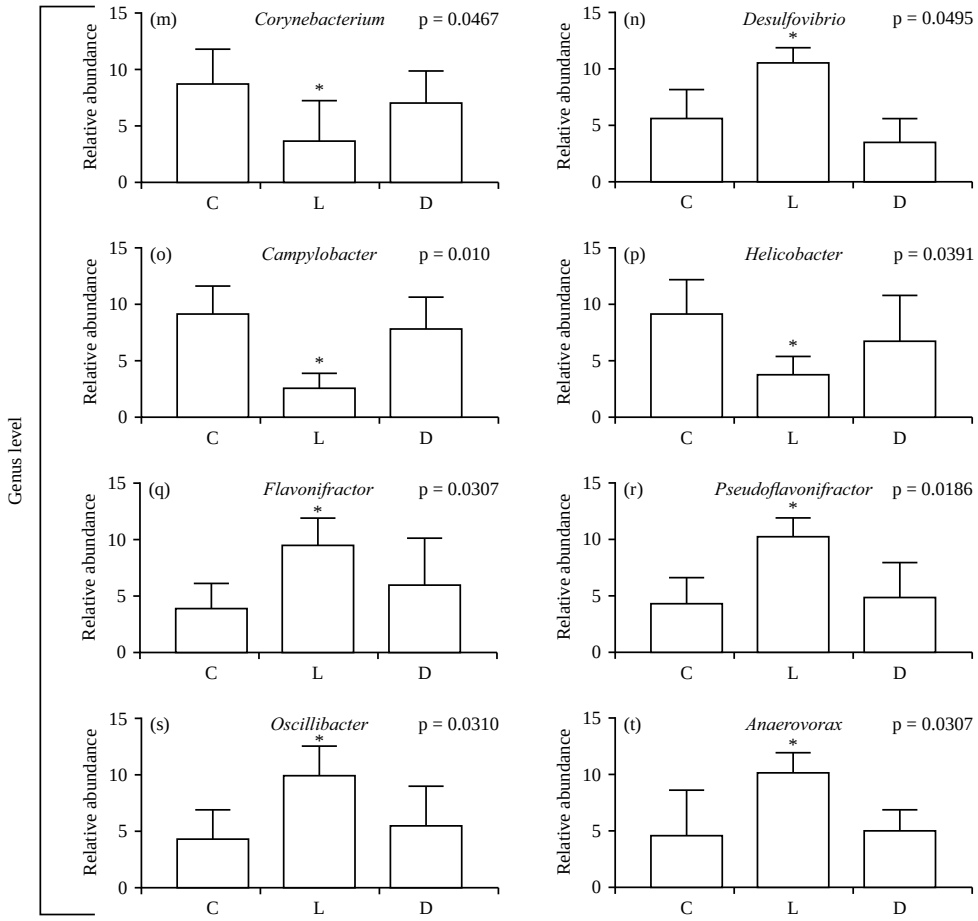

Fig. 9(a-t): Relative abundance expressed as a percentage of the significant difference between C, L and D groups with a p-value at different levels, the changes in, (a) *Deltaproteobacteria* class, (b) *Epsilonproteobacteria* class, (c) *Clostridia* class, (d) *Desulfovibrionales* order, (e) *Campylobacterales* order, (f) *Clostridiales* order, (g) *Corynebacteriaceae* family, (h) *Desulfovibrionaceae* family, (i) *Campylobacteraceae* family, (j) *Helicobacteraceae* family, (k) *Clostridiales_incertae sedis* XIII family, (l) *Ruminococcaceae* Family, (m) *Corynebacterium* genus, (n) *Desulfovibrio* genus, (o) *Campylobacter* genus, (p) *Helicobacter* genus, (q) *Flavonifractor* genus, (r) *Pseudoflavonifractor* genus, (s) *Oscillibacter* genus and (t) *Anaerovorax* genus

C: Control group, L: Levonorgestrel group and D: Desogestrel group

plasma levels of heart-specific biomarkers such as alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase was found in the present study (Fig. 5). Current findings are in correlation with the previous studies where an increase in the plasma levels of AST and ALT has been reported[55,56]. However, the results are contrary to the studies that indicate a decrease in the ALP level[55]. The elevated levels of AST and ALT are advocating some sort of physiological stress to the liver or heart cells in the female rats subjected to the HCPs. The presence of multiple infracted cells surrounded by fat as well as lipid-laden macrophages, swelling and abnormal architectures of cells in the LNG group, indicates long term severe side effects of oral contraceptive hormone levonorgestrel (Fig. S1). At a later stage, calcifications, fibrosis and squamous metaplasia may occur. Further, a biopsy test is generally recommended for differentiation of fat necrosis from carcinoma cells. Histopathology of levonorgestrel and desogestrel treated animals indicates regular use of these hormones may lead to induction of breast cancer.

Overall 358654 gut microbes were detected based on fragment length of at least 250 bp or above. The accuracy of data was reported up to or above 99%. Studies with an accuracy above 95% are reported in the literature[57]. Gamma

diversity was found for 905 OTUs in all groups. The gut microbiota composition is different between individuals. However, the mainly two members are *Firmicutes* and *Bacteroidetes*[58]. The study found 12 different bacterial phyla in our samples among all groups. The majority of phyla were *Firmicutes* and *Bacteroidetes* (Fig. 6). At the genus level, we observed 72 different bacterial genera and the main two genera among all groups were *Prevotella* and *Lactobacillus* (Fig. S2). The rarefaction with observed OTUs and estimated Chao1 showed that the LNG group and DG group had richer more than the C group (Fig. 7 and S3). In 2018, a study had reported that the diversity of gut microbiota represented by observed OTUs and Chao1 increased in postmenopausal women with breast cancer compared to healthy postmenopausal women[59]. Beta diversity showed that gut microbiota of LNG and DG groups clustered closer to each other and farther than the control group, which indicate that the gut microbiota had been affected, by the contraceptive pills (Fig. 8). It was reported that the ratio of *Firmicutes* to *Bacteroidetes* increased in obese and high risk of diabetes rats compared to lean. In this study, the (F/B) ratio has increased in the gut microbiota population of both LNG and DG groups. *Desulfovibrio* is a gram-negative genus that belongs to the *Proteobacteria* phylum. It has been reported that enhanced *Desulfovibrio* is associated with IBD through its ability to reduce sulphate and release hydrogen sulphide a cytotoxic compound that damage the epithelium cells in the gut[60,61]. In addition, it has been demonstrated that patients with symptomatic ischemic attacks showed increasing in *Desulfovibrio*[62]. The Levonorgestrel treated samples in this study exhibit an obvious increase in *Desulfovibrio* genera in the LNG group compared to the control group (Fig. 9n). *Anaerovorax* is a gram-positive genus that belongs to *Firmicutes* phylum. The function of this genus is still not completely understood. However, A single study observed that *Anaerovorax* abundance has a positive correlation with blood glucose[63]. Another study found increasing in the *Anaerovorax* genus in IBD subjects relative to healthy subjects[64]. The current stud found a significant in increasing in *Anaerovorax* in the LNG group relative to the control group (Fig. 9t). *Oscillibacter* is a gram-positive genus belonging to *Firmicutes* phylum. It has demonstrated that patients with stroke have a higher abundance of *Oscillibacter* in their gut[62]. In the present study, we demonstrate that *Oscillibacter* was in higher abundance in the LNG group compared to the control group (Fig. 9s). *Flavonifractor* is a genus that belongs to the Bacteroidetes phylum and it has been observed that this genus can induce inflammation and oxidative stress in the host via cleavage of the flavonoid C-ring and degeneration of querctin[65]. In this study, we found an increase in the *Flavonifractor* genus in the LNG treated group (Fig. 9q).

CONCLUSION

According to current findings, a positive association exists between the application of HCPs and changes in the gut microbiota, breast tissue histology and blood biomarkers for hypertension, compromised liver function, stress, anxiety and cardiovascular diseases. The second generation medicine has shown more significant effects on the blood biochemistry and gut microbiota as compared to 3rd generation medicine.

SIGNIFICANCE STATEMENT

This study discovered the blood biochemistry, gut microbiota and histology of breast tissue. LNG has shown severe negative effects as compared to the DG. This study will help the researchers to uncover the critical areas of Hormonal Contraceptive Pills. That many researchers were not able to explore. Thus a new theory on orally administered with Levonorgestrel and Desogestrel in female Wistar rats may be arrived at.

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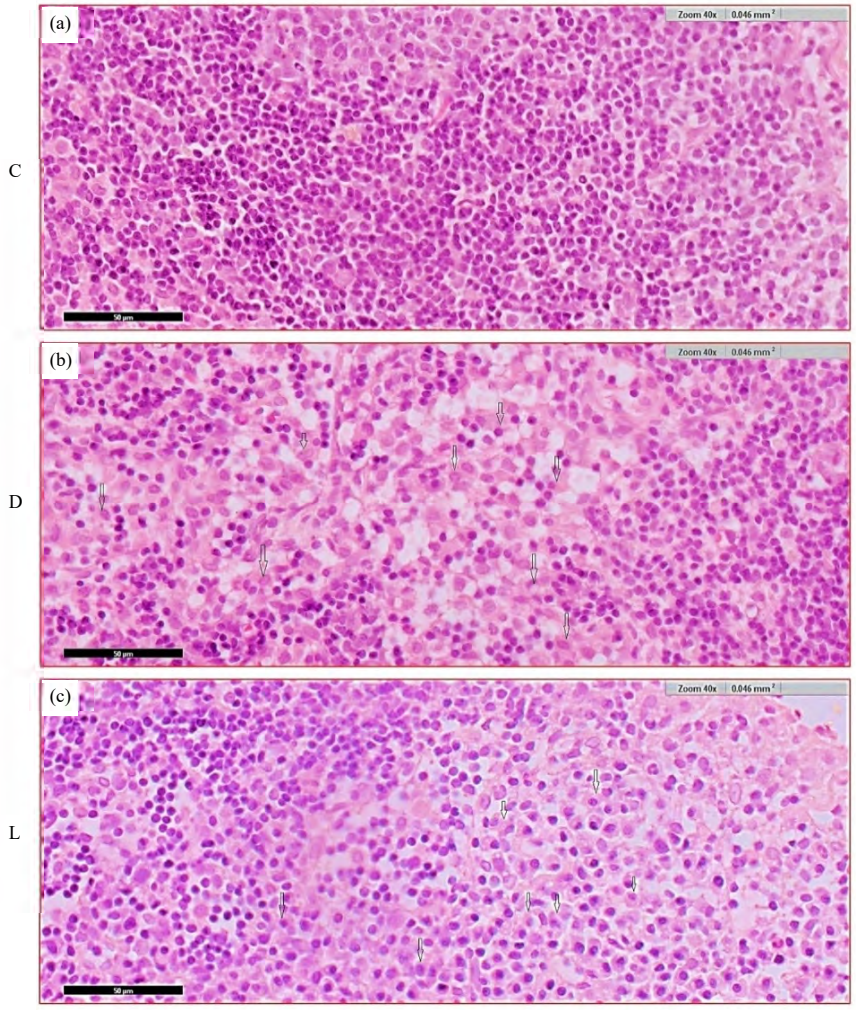

Fig. S1(a-c): Histopathological effects of HCPs on the breast tissues

C: Organized cellular architecture with no infracted fat cells or deposition of fat around cells, D: Many infarcted fatty cells surrounded by lipid-laden macrophages. In the later stage, calcifications, fibrosis and squamous-metaplasia may occur. Further, a biopsy test is generally recommended for differentiation of fat necrosis from carcinoma cell, L: Multiple infarcted cells surrounded by fat as well as lipid-laden macrophages. In a later stage, Calcifications, fibrosis and squamous-metaplasia may occur. Further, a biopsy test is generally recommended for differentiation of fat necrosis from carcinoma cell

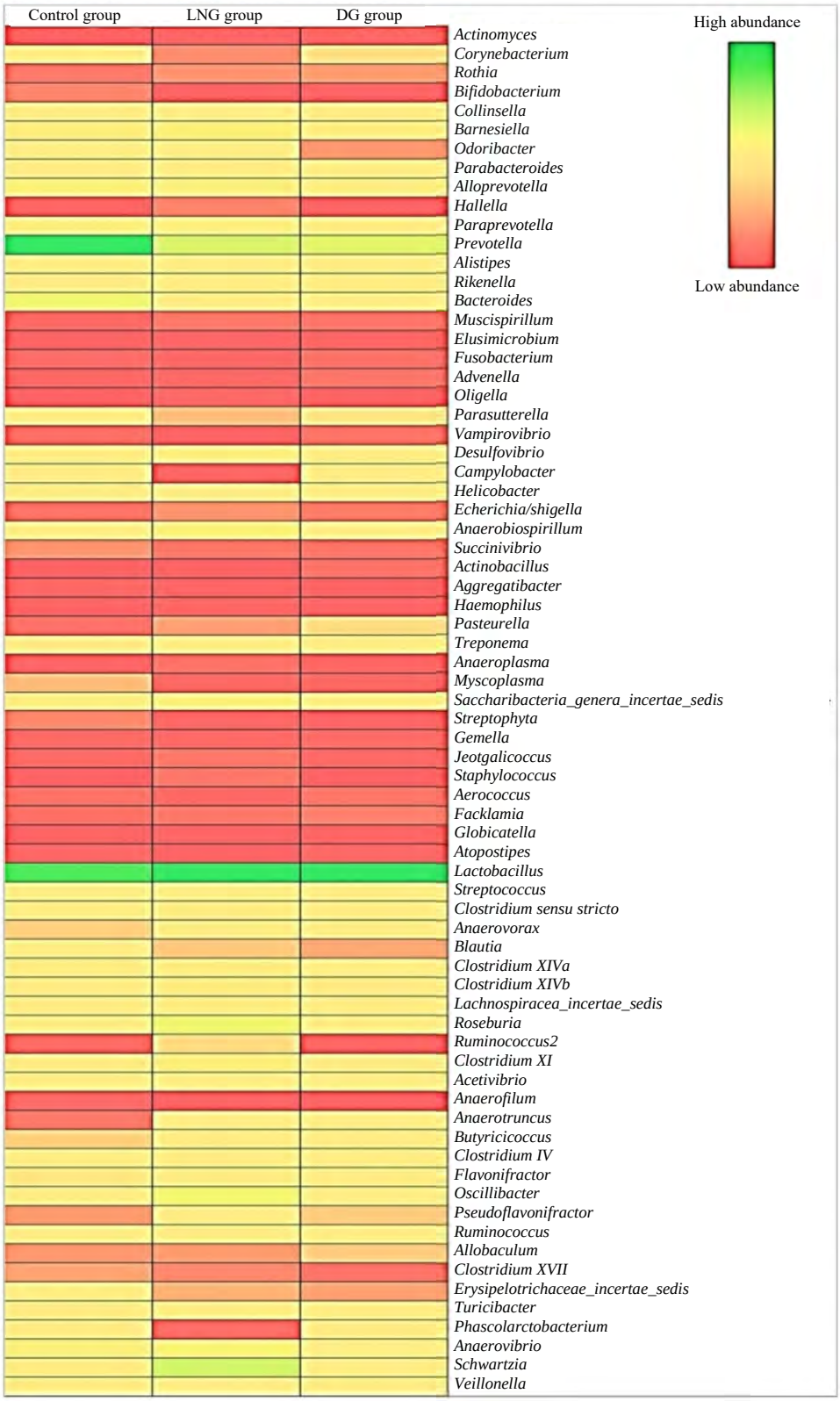

Fig. S2: Heat map shows the abundance of gut microbiota

Rows represent bacteria genus and columns represent experimental groups. LNG: Levonorgestrel group and DG: Desogestrel group

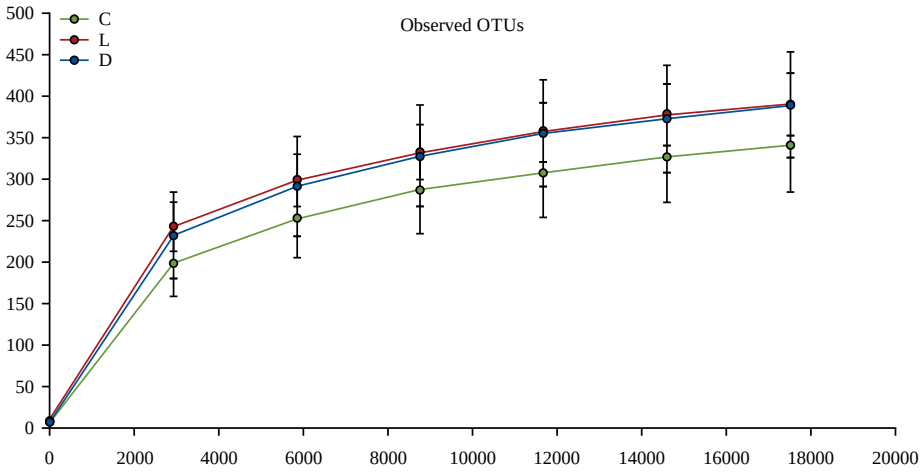

Fig. S3: Alpha diversity analysis of the sequence reads

Rarefaction curves of observed OTUs, C: Control group, L: Levonorgestrel group and D: Desogestrel group