Gut Flora Mediated Mouse Metabolic Health Risk Caused by Dietary Exposure of Acetamiprid and Tebuconazole

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Abstract

Background:

Dietary pesticide residue is an important dietary inducement of metabolic syndrome.

Results:

Chronic inflammation, insulin resistance, obesity, and non-alcoholic fatty liver disease were induced to tested mice through long-term and low-level of acetamiprid and tebuconazole. On the basis of these phenotypes, the mouse gut flora with metabolites, host circulation metabolic profiling, and their interrelations were investigated, and host metabolic pathways were detected. Results showed that pesticide exposure differently altered the abundance of gut microbial species, such as high ratio of *Firmicutes/Bacteroidetes* and increased high lipopolysaccharide-production species. Correlation analysis between gut flora and its metabolic profiling further explained these changes and their associations. Under these influences, metabolic profiling of host serum and liver was performed, and metabolic disorders were characterized. The relationships between serum and gut flora were determined via their significantly different metabolites. Alterations to the metabolic pathways of liver were clarified to deeply explore the influences on host physiology. Host metabolic disorders were evidently released by fructooligosaccharide and fecal microbiota transplantation intervention, directly proving that gut flora is a vital medium in metabolic health risk caused by pesticide exposure.

Conclusion:

Dietary long-term and low-level pesticides threated metabolic health *via* affecting intestinal flora. Metabolism of intestinal flora and host were all stressed by the exposure, and their alterations were in close proximity. Dietary interventions mitigated metabolic diseases *via* improving disorder of intestinal flora. This work supplied theoretical bases and intervention approaches to body metabolic problems caused by pesticides exposure on the basis of gut flora.

Background

Gut flora is profoundly associated with a large diversity of diseases and human health, and it functions as an inducer and even a driving force in diseases, such as metabolic disease, mental illness, degenerative diseases, and cancers [1]. Thus, researchers view gut microbiome as a new metabolic organ and pay increased attention to it. Among the diseases, metabolic dysfunction includes a board spectrum of diseases, such as nonalcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D), obesity, and atherosclerosis [2]. These diseases and their related complications are some of the leading causes of low quality of life and even death. For example, the prevalence of diabetes in China is approximately 11%, ranking first worldwide [3]. NAFLD is an increasingly common condition that affects roughly one-third of adults in the United States [4].

Aside from poor eating habits [5, 6] and mental stress [7], dietary contamination promotes gut flora alteration and usually poses a direct hazard to gut flora [8]. It is also an essential driving force of dysfunction in gut flora. Pesticide residue is an inevitable problem in dietary contamination because pesticide is still the most effective countermeasure to ensure food supply and security. For example, the burst of locust plague worsened hunger worldwide in 2020. The pesticide residue level in food is normally kept in trace amounts; thus, it does not exert acute toxicity to the body. Long-term intake of pesticide with diet potentially threatens human health [9, 10] although the residue level is normally not out of the limit. Evidence supported that pesticides promote chronic or metabolic health problems [11], and they should not be an underestimated public health issue [12].

Pesticides were reported to affect body health and metabolism. For example, pesticides affected bile acid metabolism and led to body inflammation [11, 13]. Exposure to pesticide residue also altered the composition of gut flora, thus triggering host metabolism changes and even diseases [14]. Although the newly developed and widely used pesticides usually have low toxicity, the influence of long-term intake of low-level pesticide residue on health should not be underestimated. Moreover, combined residues usually exist in foods due to the mixed usage of various pesticides in agricultural production. The effects of combined pollution on gut flora are ambiguous due to different bioactivities. Therefore, combined pollution may bring increased uncertainty in gut flora, thus deserving further study to obtain the relationship among pesticides, gut flora, and disease.

The gut flora is of large diversity, and its metabolism alteration becomes more intricate when influenced by pesticides. Understanding these changes and developing associated analysis are the basic and effective ways to obtain the relationship among contamination, gut flora, and disease. Here, mice were exposed to a combination of pollution, low-level residue of a type of insecticide and a type of fungicide. Mice physiological changes were first observed, and then alterations in gut flora and its metabolism was investigated. The results closely linked the alterations between the gut flora and physiological changes. Gut flora and its metabolism were thoroughly explored to understand the relationships and mechanism among pesticides, host physiological changes, and intestinal microflora. Further, host circulating metabolism was analyzed, and its association with gut flora was determined. Alterations in the metabolism pathways in the host liver were also examined. Finally, interventions to the gut flora proved that dietary pesticide exposure mediated the risk of on host metabolic health.

Results

Effects of Exposure on Mouse Bodyweight

The results in Fig. S1 showed that the weight gradually increased over the course of exposure. Approximately 3 weeks later, the bodyweight difference increased extensively. Compared with the control check group (CK), acetamiprid (D), tebuconazole (W), and combination groups (DW) showed a 19.35–22.06% increase in bodyweight on average. This finding indicated that obvious obesity trends or even obesity occurred in the exposure-treated mice. Meanwhile, the bodyweight of the blank group showed no

significant difference with that of the CK group, indicating that the corn oil used as a reagent in the administration hardly affected the basal metabolism of the mice and the bodyweight difference was mainly caused by the pesticides. Bodyweight is only the phenotype of obesity, and metabolic abnormalities occurred in the physiological reactions [15]. Physiological observation and metabolic investigation could be performed in further studies.

Effects of Exposure on Glucose Tolerance and Insulin Resistance

Long-term obesity usually occurs along with insulin resistance (IR), and IR is a vital core in metabolic syndrome because it is closely related to obesity and directly causes T2D, NAFLD, and cardiovascular diseases [16–18]. Fasting blood glucose (FBG), glucose tolerance (GT), and IR are the commonly used indices to investigate the sensitivity of insulin and the status of body glucose metabolism. They are also believed to be the risk indicators of early-stage T2D[18]. The results showed that the FBG in the treated groups slightly increased but less than 6 mmol/L (Fig. 1a). The mouse plasma glucose increased rapidly after glucose intake in all the groups. Then, the glucose concentrations decreased relatively slowly in the following 90 min. The treated groups maintained a higher plasma glucose than the CK group at the end of 120 min. This finding inferred that the plasma glucose in the treated mice spent more time returning to normal, indicating that pesticide exposure lowered the capacity of the body to regulate glucose [19].

IR shows that the target cells lowered the sensitivity to insulin. In the homeostasis model assessment of IR, higher index corresponding to more serious IR. The test results (Fig. 1b) showed that the IR indices in the treated groups were nearly 1–3 times more than those in the CK group. Significant differences in IR index levels were observed in mice pressured using two pesticides. IR is the basis of T2D, and it is the link of many other metabolism diseases. It was considered a crucial warning of metabolic diseases. The results of the present study revealed that dietary pesticide exposure induced IR to the mice and further posed metabolic health risk to them. The effect of combined pesticides on IR was apparently higher than that of their single components, indicating that the combination exposure enhanced the health risk compared with single component. This finding may be attributed to the recognized synergism in combination exposure, which strengthened the exposure effects compared with single components.

Serum Biochemistry Analysis

IR and the disorder of carbohydrate metabolism occurred, indicating that many aspects of metabolism were affected. Serum biochemistry analysis is a commonly used method in investigating physiological changes, and it is an effective evidence in clinical diagnosis [20, 21]. Pesticide exposure obviously led to a significant increase in serum cholesterol (Fig. S2) and a decrease in high-density lipoprotein cholesterol (HDL-C) in all the treated groups. Similar increasing trends were also obtained in serum triglyceride (TG). The combined exposure group displayed significantly higher serum TG than any single exposure group. Therefore, the metabolic abnormalities caused by pesticide exposure could induce risk of cardiovascular disease and fatty liver to the body.

Liver is the largest digestive gland in the body and the main metabolic organ [22, 23], and serum biochemistry analysis is an effective method to evaluate liver function in clinical diagnosis [24]. In this work, liver function was investigated and evaluated using related indices (Fig. S3), such as aminotransferase (ALT, AST, and ALP) and serum protein (TP and ALB). Aminotransferases, especially ALT and AST, were enhanced at different degrees by pesticide exposure, indicating that minor injuries occurred in the liver. The concentration changes in serum protein were slighter in the exposure than those in aminotransferase. The evaluation results revealed that liver was slightly impaired and mild dysfunction may have occurred in the exposure [25]. Moreover, these abnormalities were found in different exposure groups, and no obvious joint effects were obtained in the combination exposure.

Evaluation of Mouse Inflammation

According to previous reports [26, 27], chronic and systemic inflammatory responses trigger the development of IR. Thus, the inflammation status of the mice was observed in this section. The levels of several important inflammatory mediators in serum are exhibited in Fig. 2. TNF-α is one of the most crucial factors in the IR formation; it works by inhibiting insulin signal transduction. A previous study reported that the antibody against TNF-α evidently improved insulin sensitivity and alleviated IR [28, 29]. Single and combination exposures led to a massive increase in TNF-α by almost 10-fold. For example, exposure to tebuconazole increased TNF-α from 35.7 EU/L (CK) to 338.10 EU/L. No significant joint action effect was obtained in the combination exposure group compared with the single-component group.

Interleukin (IL) is a large class of cell factors that exerts considerable effects to the body's inflammatory reaction and immunity. Numerous studies have shown that IL-1, IL-6, IL-8, and IL-10 exerted substantial effects on IR. Increased levels of IL-1β and IL-6 were detected in patients with T2D and obesity, and they promoted the inflammatory process. The combination exposure to pesticides showed an obvious synergy compared with its single counterpart. MCP-1 is another important promoter that acts as a kind of inflammatory chemokine and contributes to IR. Although MCP-1 did not respond intensively, a relatively significant increase of its concentration was observed. Different from these inflammatory mediators, IL-10 is a well-recognized multifunctional factor, and it was also negatively-related with IR in this work. No evident concentration differences were observed between CK and exposure groups. Overall, the observation of these cell factors indicated that dietary exposure to pesticides induced inflammation, and it is an important basis of IR.

Detection of Lipopolysaccharide and Intestinal Permeability

Lipopolysaccharide (LPS) is usually produced by the intestinal flora, and alteration to intestinal permeability enhances its transfer from the gut to the blood [30, 31]. Thus, investigating serum LPS and intestinal permeability is vital in examining LPS-induced chronic inflammation. The results in Fig. S4b displayed that increased FITC-dextran amount was observed in all the treated groups, indicating that the intestinal permeability significantly increased, especially in the tebuconazole and combined groups. Tebuconazole exhibited a more obvious effect than acetamiprid, and no significant difference was found

between tebuconazole and combined groups. Serum LPS increased in exposure groups, and a synergistic effect was obtained in the combined exposure group (Fig. S4a).

Increase of intestinal permeability and serum LPS level can promote each other. The coordination effects of combination exposure were exhibited in the results of investigation on LPS and intestinal permeability; significant differences occurred between combined and single actions. Thus, pesticide exposure increased the intestinal permeability, and the resulting enhanced transfer of harmful gut flora and metabolites to the blood led to increased physiological dysfunction, such as low-grade inflammation.

Tissue Histology

Host tissue histology showed that lipid droplets were gradually formed and NAFLD was presenting in the treated groups (Figs. 3 and 4). It was difficult to judge the severity of fatty liver in among different groups because they were in the development of NAFLD. HE staining of colon slices manifested that aggravated inflammatory cell infiltrates occurred in the treated groups, especially in tebuconazole and combined exposure group. Cell structure of intestinal villi was loosened and intestinal barrier function was impaired in acetamiprid group, and it would enhance intestinal permeability. The results proved that dietary pesticides exposure lead to NAFLD, leaky-gut, and even enterocolitis. Metabolism health risks of chronic dietary pesticides exposure were confirmed.

Effects of Pesticide Exposure on the Diversity of Gut Flora Community Structure

As mentioned above, to investigate the changes in the intestinal flora caused by pesticide exposure were necessary in this work [30, 31]. In the present study, 16S rRNA gene sequencing for microbiome analysis was used to detect the microbial communities and evaluate the effects of pesticide exposure on mouse gut flora. Together with the host, the microflora and intestinal microflora maintain microecological balance [32][33]. Structure and distribution are the most vital characters of a microbial community. The diversity of gut flora was investigated at alpha and beta levels. The results of alpha diversity (Shannon index) showed that no significant difference was found between the CK and treated groups, indicating that the species diversity of gut flora within a community was not destroyed by pesticide exposure (Fig. S5a). Nonmetric multidimensional scaling analysis is an appropriate method for calculating the beta diversity of the gut flora community. Fig. S5b illustrated that relatively different beta diversities occurred in different groups of treated mice, thus revealing that the gut flora of mice had a unique response to the exposure of different pesticides and that their communities were differently altered. The stress value of the model was 0.093, revealing that the model could stimulate the actual samples accurately.

Species Changes in Gut Flora

Similar to the reports [34, 35], approximately more than 95% of the gut flora of mice was mainly distributed in the phylum of *Bacteroidetes* and *Firmicutes* (Fig. S6a). It was reported that a low ratio of *Bacteroidetes*/ *Firmicutes* (B/F) was usually obtained in individuals who were obese, and transplanting

fecal microbes with high or low B/F was effective in making the host lose or gain bodyweight [34, 35]. In the present research, the B/F in the CK group was approximately 3-4-fold higher that in the treated groups, contributing to host obesity.

The results in Fig. 5 and Fig. S5 (c, d) showed that most significantly different gut flora species (around 18 categories) were obtained in the tebuconazole group according to LDA effect size (LEfSe) analysis when the LDA threshold was 3. The abundances of *Firmicutes* and *Bacteroidetes* were mostly obtained in the tebuconazole and control groups, respectively. In addition, the family of *Peptostreptococcaceae* and *Lactobacillaceae* showed abundant microbes in the gut, and these microbes were accumulated in the tebuconazole group. The affected groups were the combined and acetamiprid groups. A significantly different flora was only found between the tebuconazole and CK groups when the LDA threshold was 4, indicating that the gut flora was mostly affected by tebuconazole in all exposure groups. Compared with single components, the combination exposure did not perform evident synergies nor promotion effects. Further investigation was focused on the quantity alteration in the flora and its distribution in the level of family or genus.

The gut flora species of different groups at the genus level was investigated (Fig. S7). Compared with the CK group, acetamiprid enhanced the abundance of *Ruminiclostridium*, *Roseburia*, *Lachnoclostridium*, *Marvinbryantia*, *Intestinimonas*, *Rhodococcus*, and *Caulobacter*, while most of them were the species with low abundance. In the dominant species, *Alistipes, Blautia*, unidentified_*Ruminococcaceae*, and *Oscillibacter* were relatively increased and in the treated group, including harmful and beneficial bacteria. Thus, acetamiprid exposure caused varied changes to the gut flora. Compared with the CK group, *Lactobacillus*, *Klebsiella*, *Streptococcus*, *Romboutsia*, *Mitsuokella*, *Enterococcus*, and *Sphingomonas* were upgraded by tebuconazole, while *Alloprevotella, Bacteroides*, and *Muribaculum* were decreased. The intestinal microecology may be deteriorated due to the accumulation of major pathogenic bacteria. For example, the accumulation of *Enterococcus* leads to gut inflammation and lowers the butyrate-maker flora and butyrate in the gut [36]. *Klebsiella* and *Streptococcus* are also the typical infectious bacteria [37], while *Alloprevotella*, *Bacteroides*, and *Muribaculum* are anti-inflammatory bacteria that produce short-chain fatty acids (SCFAs). A decrease in these bacteria lowers the anti-inflammatory and immune capacity of the host [38–40]. Thus, dietary tebuconazole exposure exerted negative effects on the gut flora, thus disrupting the healthy gut flora of the host and posing metabolic risk to it. However, the combination exposure of pesticides exerted multiple effects on the gut flora. Harmful bacteria, such as *Helicobacter* and *Lachnospira*, and beneficial bacteria, such as *Parabacteroides* and *Akkermansia*, were all enhanced. Meanwhile, absolutely dominant bacteria, including *Alloprevotella* and *Bacteroides*, were all decreased by the exposure. Therefore, although pesticide exposure influences to the gut flora were multifaceted, disorder still obtained for the disruption to the absolutely dominant species of bacteria. Overall, tebuconazole disrupted the gut flora more than the other treatments, and no significant synergy effects were obtained from the combination exposure group. This finding was consistent with the result of LEfSe analysis. LPS produced by *Enterobacteriaceae* and *Desulfovibrionaceae* was reported to be approximately 1000-fold higher than that from other bacteria [41], and it was remarkable in causing chronic inflammation. The abundance of *Desulfovibrionaceae* in the acetamiprid, tebuconazole, and

combined groups were approximately 3.5, 1.5, and 10 times that in the CK group, and pesticide treatments significantly enhanced this abundance in the tebuconazole and acetamiprid groups. The abundance of *Enterobacteriaceae* in these groups was 5.3, 64, and 3.2 times higher than that in the CK group (Fig. S6b). They were highly accumulated by pesticide exposure, especially in the tebuconazole and combined groups, which could extremely promote endotoxin accumulation in the gut.

Metabolic Profiling of Gut Flora

Based on the gut structure [42, 43], the metabolites of the gut flora are the main media of interaction between the flora and the host through blood absorption [44]. Thus, identifying the altered metabolites of the gut flora is an essential means to investigate the effects of pesticide exposure on host physiological activity. Metabolic analysis is an effective method to obtain the changes in gut flora metabolism between the CK and exposure groups.

On the basis of the metabolites, the samples of different exposure groups were distinguished significantly only by the unsupervised learning algorithm, principal component analysis (PCA, Fig. S8). The results indicated that circulating metabolism changes and differences occurred when hosts were stressed by different pesticides. Furthermore, differential metabolites were yielded via OPLS-DA model under the following statistical conditions: VIP > 1, FC > 2, FDR < 0.05, and $p < 0.05$. In the CK, acetamiprid, tebuconazole, and combination groups, 37, 43, and 32 different metabolites (Additional File 15: Table S1.) were investigated and used for further investigation. Different metabolites were also obtained in the single and combined exposure groups to evaluate the gut flora response to pesticide exposure.

In this work, pesticide exposure involved many significant metabolite variations in the gut. Trimethylamine N-oxide (TMAO) comes from the metabolism of intestinal microflora, and it is highly related to cardiovascular diseases. It was the main risk factor of atherosclerosis [44]. In the present study, the acetamiprid and combined groups showed a considerable increase in TMAO in the gut by approximately thousands of times more than the CK group. However, the metabolites between the CK and tebuconazole groups did not significantly differ. Thus, acetamiprid was evidently an important dietary contaminant, and its exposure led to the risk of cardiovascular diseases via adjustment of the gut flora metabolism. Spermidine, the most effective polyamine in preventing lipid peroxidation, was lowered in the acetamiprid group. On the contrary, putrescine, another type of polyamine, increased to approximately 100 times when the mice were exposed to acetamiprid, thus considerably enhancing the risk of leaky gut and colitis. These significant variations in polyamines did not occur in the tebuconazole and combined groups. Imidazole propionate and imidazoleacetic acid were the metabolites of the gut flora. A significant decrease in imidazoleacetic acid was yielded in all pesticide-exposed groups. They were homolog in structure, with only a difference in CH_2. Imidazole propionate and imidazoleacetic acid were all gut flora metabolites of histidine from totally different metabolic pathways. Imidazole propionate occurred with the action of histidine ammonia lyase, while imidazoleacetic acid was the final metabolite of histamine, which was obtained from histidine treated with histidine decarboxylase. Imidazole propionate was reported to cause T2D by disrupting GT and insulin signaling [45]. No physiological function of imidazoleacetic acid was ever reported, but speculations could be made on the basis of their extremely

similar molecular structure. Extensive work is necessary to identify how gut flora works in the pathway of imidazoleacetic acid production and what determines the metabolic method of histidine.

The metabolism of tryptophan involved a number of bulk compounds, especially indole derivatives. Many of them, including 3-Indoxyl sulphate, 3-Indolelactic acid, 5-HIAA, and indole-3-acrylic acid, were significantly different metabolites distributed in the content sequence of control > acetamiprid > combination > tebuconazole. These metabolites played substantially important signaling roles in regulating the host physiological activities, such as enhancing mucosal homeostasis by alleviating intestinal permeability (possibly mediated by pregnane X receptor), suppressing appetite, secreting insulin, and slowing gastric emptying by inducing the release of glucagon-like peptide 1 in enteroendocrine L-cells [46]. A decrease in these metabolites by pesticide exposure could elevate the health risk to the host. The results suggested that tebuconazole lowered indole derivatives more than acetamiprid, and no combinatory effects were obtained. Many other gut intestinal metabolites that were proven to act on host metabolic activity were affected by pesticide exposure. For instance, 12, 13-DiHOME is a kind of gut lipid that activates the brown adipose tissue of the host, regulate fat metabolism, and lowers the host risk of heart disease and diabetes as a metabolic signal [47]. In the present work, only single-component exposure significantly decreased 12, 13-DiHOME in the host gut. Trichostatin A is a metabolite of Streptomyces, and it was reported to perform the activity of histone deacetylase inhibitor, which exhibited the effects of anticancer. The pesticide exposure of tebuconazole and acetamiprid disturbed the gut flora and decreased trichostatin A in the gut, which is not beneficial to the anticancer capacity of the host. However, this phenomenon did not occur in the combination exposure group.

Association of Gut flora with Its Metabolism

Under the pressure of pesticides, the gut flora communities were affected and the metabolites were altered correspondingly. The relevance between altered gut flora and its metabolites were analyzed, and the results are shown in Fig. 6. A high relationship ($r > 0.7$ or $r < -0.7$ and $p < 0.05$) between the flora at the genus level and its metabolites was subjected to network analysis, and their relevance is exhibited in Fig. 6.

The genera of *Lactobacillus*, *Alloprevotella*, *Alistipes*, *Roseburia*, *Enterorhabdus*, *Romboutsia*, *Faecalibacterium*, *Clostridioides*, *Sphingomonas*, *Butyricimonas*, *Desulfovibrio*, *Intestinimonas*, *Marvinbryantia*, *Oscillibacter*, *Candidatus_Arthromitus* were involved in high relevance, and *Lactobacillus* was associated with most of the metabolites. For example, it was positively related with N-(2-Acetylphenyl)formamide, 1,5-Dimethyl-4,5-dihydro-1H-pyrazole, Tyramine, 2-Butoxy-N-[2-(diethylamino)ethyl]nicotinamide, α-tocopheronic acid, 4-Aminopyridine, 1-[(9Z)-octadecenyl]-2-hexadecanoyl-sn-glycero-3-phosphocholine, N-(icosanoyl)ethanolamine, and negatively related with 1-Methyl-3,5,6-indolinetriol and 1-hexadecanoyl-sn-glycero-3-phosphoethanolamine. *Faecalibacterium* and *Sphingomonas* were all positively correlated with 3-(Methylthio)propylamine. An unknown genera of *Lachnospiraceae* was positively related with N-Acetylserotonin and Methyl indole-3-acetate, while negatively related with Indole-3-acrylic acid. Methylimidazoleacetic acid would be decreased by *Intestinimonas* and *Marvinbryantia*, while N-Acetylputrescine was totally different with it. Apart from

Methylimidazoleacetic acid, N-isopentylacetamide and 1-(4-Aminobutyl)urea were also negatively related with *Oscillibacter*. Although *Butyricimonas* and *Desulfovibrio* exerted different effects on host, they were all positively related with (S)-2-amino-6-oxopimelic acid. *Desulfovibrio* still related with it more closely than *Butyricimonas*. Gut flora and metabolites are all groups with large amounts and complexity, and their correlations are also extremely intricate. The pesticides altered the intestinal microflora, which changed the metabolism of the flora. This phenomenon mediated the effects of pesticides on host physiological activity. To explore their relationships is an effective way to investigate the effects of gut flora on host metabolic syndrome.

Effects of Exposure on Host Circulating Metabolism

Blood is the pool of circulating metabolism, and it characterizes the physiological activity of the host. Non-target metabolic profiling of serum was performed in this study to investigate the alterations in the host circulating metabolism. Different groups were distinguished in accordance with the identified metabolites, as shown in Fig. S9.

Wide varieties of significantly different metabolites were identified, mainly including amino acids and their derivatives, free fatty acids and their methyl esters, phospholipids, nucleotides, carbohydrates, hormones, and other physiological metabolic compounds (Fig. S10 and additional file 16: Table S2). Dysregulated metabolism of these compounds poses a great threat to the host health and is a remarkable risk factor of chronic metabolic diseases, such as obesity, T2D, and NAFLD. Shown in Table S3 (Additional File 17), different alterations in amino acids were observed; for example, the contents of branched-chain (valine and leucinein) and aromatic amino acids (phenylalanine) in the serum showed a variable degree of elevation in all the treated groups. Threonine was increased by pesticide exposure, and no obvious change trend was found in the amino acid derivatives. As reported, the disorders of fat and carbohydrate metabolism also exhibited a dysfunction of body physiological activities, especially in chronic metabolism diseases, such as obesity, T2D, and NAFLD. Fatty acids and methyl esters were accumulated by around 2–11-folds in the exposure groups. Interestingly, all of them were unsaturated fatty acids, such as docosadienoic acid, ocosatetraenoic acid, docosapentaenoic acid, and docosatrienoic acid. (5R)-5-[(1S)-1,2-Dihydroxyethyl]-alpha-D-lyxopyranose,5-O-alpha-L-Arabinofuranosyl-alpha-L-arabinofuranose and methyl 6-deoxy-2,3-O-isopropylidene-alpha-L-mannopyranoside were significantly increased by pesticide exposure in the treated groups by up to approximately 3.3 times. Increased contents of branched-chain amino acids, aromatic amino acids, fatty acids, and carbohydrate in serum are usually found in individuals with the abovementioned metabolic diseases. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and lyso PC (LPC) are the important phospholipid compounds in serum, and they were also affected by pesticide exposure. PC decreased in the pesticide-exposed groups, whereas LPC increased in the treated groups. Different results about these phospholipid compounds in a study on metabolic disorders were reported. The results obtained in the present work were not totally consistent with those from previous reports. Phospholipid-derived compounds undoubted played considerably important roles in physiological activities. For example, PC

decreases blood fat and peroxide and demonstrates positive effects on the liver and heart, while LPC is an important pre-inflammatory factor of arteriosclerosis [48].

Spermine was reported to have a negative relationship with T2D [49], and it showed a 0.38–0.73-fold decrease in present study. This finding may also be an evidence for the prediction of T2D. Nucleic acids cytosine adenosine, uracil, xanthine, 7-methylxanthine, and 1-methylhypoxanthine were identified, while no obvious change trends were found. As the metabolites of purine, uric acid compounds were also reported to be closely related with obesity and T2D [50]. They were remarkably elevated by metabolism disorders. 9-Methyluric acid and uric acid were identified in the serum, and they were decreased by exposure to acetamiprid but enhanced by exposure to tebuconazole. The effects of pesticide mixture of tebuconazole and acetamiprid were in the middle.

Similar to other fatty acids, prostaglandin A1 and E1 also increased in the treated groups, while their metabolite, 15-dehydro-prostaglandin E1, decreased. The physiological responses caused by this alteration were identified. 7-Alpha-hydroxy-17alpha-methyltestosterone was the detected androgen in the serum, and it decreased in the acetamiprid and combined groups but not in the tebuconazole group, indicating that dietary acetamiprid exposure also affected the reproductive endocrine system.

Association between Metabolites of Gut Flora and Host Circulating

Co-inertia analysis (CIA) was performed to find a covariation between serum metabolites and gut microbiota metabolites and further investigate whether the altered abundance of host metabolites correlated with the altered gut flora (Fig. S11) [51]. A correspondence analysis model was applied to analyze the relevance between the designed significantly different metabolites of the gut flora (yield in the relevance analysis between 16 s r DNA and gut flora metabolism) and the host circulating metabolites (Additional File 18: Table S4).

Figure S11 demonstrate that the representativeness of the CIA model was obviously reflected by the first two axes, which exhibited most of the shared features of the metabolites of gut flora and serum. High consistency was obtained between the two datasets of the metabolites of the flora and serum. The relevance of the two metabolism datasets was also significant. Correlation analysis between the metabolites of gut flora and host serum exhibited quantitative relationships of the significant compounds (Fig. 7). The trimethylamine N-oxide, N-acetylsphingosine, and betaine in the gut enhanced the leucine in the serum, while 3-(2-hydroxyethyl)-1H-indol-5-yl alpha-D-glucopyranoside, N-acetyltyramine, (13alpha)-13-hydroxyspartein-2-one, N-acetylhistamine, tyramine, and 5,6-Indolinediol lowered it. Unsaturated fatty acids, except for arachidonic acid, were mostly inhibited by 2-butoxy-N-[2-(diethylamino)ethyl]nicotinamide and alpha-tocopheronic acid. Prostaglandin comes from the metabolism of arachidonic acid, and its abundance in the serum was significantly enhanced. The results of the observation proved that in the treated groups, more arachidonic acid was metabolized into prostaglandin, and its content decreased. N-methylimidazoleacetic acid, N-acetyltyramine, pyridoxamine, 3-(2-hydroxyethyl)-1H-indol-5-yl alpha-D-glucopyranoside, and nicotinamide decreased in the gut of the

treated mice, whereas the contents of 4alpha-formyl-5alpha-cholest-8-en-3beta-ol, N-(2-hydroxyethyl)heptadecanamide, and N-acetylsphingosine increased. The above two groups of compounds affected the PCs in the host serum positively and negatively. Ceramide, spermine, and other amino acids related to the dysfunction of host metabolites all showed a strong correlation with the significantly different compounds in the gut. Correspondences between host and gut flora were identified via the metabolites, and it is getting more clear and close along with more action pathways or new compounds were identified.

Host Liver Metabolism and Gut-liver Dialogue under Pesticide Exposure

In this study, liver metabolism was profiled to investigate the effects of the altered metabolites of the gut flora caused by pesticide exposure on the host. These annotated significantly different compounds were subjected to the KEGG metabolic database to map and analyze the involved pathways, and the results are exhibited in Fig. 8 and Fig. S12. PCA showed that the different exposure groups were apparently distinguished in accordance with the metabolites (Fig. S13). The results indicated that pesticides exerted different effects on the body metabolism. These different compounds were annotated into many metabolic pathways, indicating that these metabolism pathways, mainly involving amino acid and derivative metabolism, glycerophospholipid and fatty acid metabolism, and vitamin and nucleotide metabolism, were intervened when the hosts were exposed to pesticides. Purine metabolism, glycerophospholipid metabolism, and vitamin B6 metabolism were shared by three different treated groups. Moreover, unique intervened pathways were obtained in the joint exposure group. For example, beta-alanine metabolism and glycine, serine, and threonine metabolism were intensively affected by the combined stress of tebuconazole and acetamiprid.

The intervened pathways were integrated together in accordance with the shared metabolites. In the metabolism of cholic acid, a decrease in choline was observed in the three treated groups, and choline deficiency impaired PC synthesis, very-low-density lipoprotein synthesis, and hepatic lipid export [52]. Thus, this disorder posed NAFLD risk to the host. The results were consistent with the detection of serum components. Meanwhile, increased betaine was detected, and it can be speculate that the activity of choline oxidase was enhanced because betaine was obtained from the oxidation of this enzyme. While the reasons for the enhancements of enzyme were unclear. A high content of betaine led to the condition for TMAO formulation, and the results were proven in previous observations. TMAO is a high-risk factor leading to atherosclerosis and cardiovascular diseases.

Histidine could be converted into urocanic acid or histamine via two different pathways, and an abnormality in histidine and urocanic acid was found when the host was stressed by tebuconazole. The results indicated that histidine metabolism was intensively intervened. When the body was stressed by tebuconazole, downregulated urocanic acid, upregulated histidine, and accumulation of histamine occurred. Histamine was further reacted into N-methylhistamine and imidazoleacetic acid through different enzymes. In this work, more N-methylhistamine and less imidazoleacetic acid were the results of

the alteration. More histamine was obviously oxidated into N-methylhistamine. Histamine is a type of key conductive chemical and one of the most widely studied inflammatory mediators that lead to inflammation and allergies to tissues. N-methylhistamine is the major metabolite of histamine produced by mast cells. Anaphylaxis and mastocytosis are typically associated with increased N-methylhistamine levels [53]. Upgraded N-methylhistamine was detected in the blood in the above step. Thus, the histamine and N-methylhistamine in the liver could be the significant reason behind mastocytosis and hepatitis.

The downstream nucleotide metabolites of L-Asp, L-argininosuccinate, adenylosuccinate, and CDP were upregulated in all the treated groups. On the contrary, the upstream nucleotide metabolites of beta-alanine were all downregulated. Many other metabolites were also annotated into different pathways. However, an insufficient effective abnormality was found when formulating clear pathways. This finding may be resolved by detecting and identifying more metabolites in the analysis.

Effects of Interventions on Physiology of Pesticide-exposed Mice

Fructooligosaccharide (FOS) [54], fecal microbiota transplantation (FMT) [55], and FOS + FMT were used as the intervention measures in this study. IR, body inflammation status, and physiological metabolites were all detected to evaluate the variation caused by the treatments, and the results are shown in Fig. S14 and Fig. 9.

Significant differences of IR were obviously obtained between the exposure group and the dietary treatment group. The IR index was remarkably lowered by FOS and FMT, and both of them were effective means to improve IR (Fig. S14a). FMT was more effective than FOS in improving mouse IR caused by pesticide exposure, and the insulin sensitivity was basically reverted to a normal level. FOS + FMT presented similar effects with that in single treatment of FMT, indicating that FMT played a primary role in improving IR. The rapidly responded inflammatory cytokines in the serum, namely, IL-1β, IL-6, and TNF-α, were observed after the treatments, as shown in Fig. S14b. The contents of cytokines significantly decreased in the serum, indicating that host inflammatory status was remarkably released. The effects of these treatments were in the order FOS + FMT > FMT > FOS, suggesting that the combined treatments of FOS + FMT enhanced the effects of single-factor treatments, almost returning to the normal level in IL-6 and TNF-α.

The contents of some metabolites in the gut and serum were observed (Fig. 9). SCFAs, usually C2–C4, were produced by the intestinal microflora decomposing the dietary fiber. Butyrate is the most important SCFAs in the gut, and it supplies 30% energy for the host and most of the energies for the gut flora. Moreover, butyrate was reported to be inhibiting pathogens and enhancing probiotics in the gut. Pesticide exposure obviously decreased the butyrate content in the gut, while FOS, FMT, and FOS + FMT enhanced it, of which FOS + FMT was the most effective treatment. Butyrate maintains host fullness by stimulating the vagus nerve and promotes fat oxidation to restrict host diet. A high content of butyrate prevents diet-induced obesity and increases insulin sensitivity. Moreover, it was helpful in host anti-cancer activities and in improving immunity. Thus, butyrate is one of the most vital gut flora metabolites in regulating host

metabolism. Propionic acid is effective in decreasing host cholesterol, relieving hypertension and inflammation, and reducing liver fat. However, in the present work, the exposed group showed increased propionic acid concentration in the feces, and the mechanism was not found. Overall, pesticide exposure altered the intestinal flora and its SCFAs, thus increasing the risk of metabolic syndrome in the host. TMAO is a high-risk pro-atherogenic metabolite produced by gut microbiota, and it was observed to be extremely accumulated in the above research. In terms of the association of gut bacteria with host circulating metabolites, TMAO had a high positive relationship with leucine in host serum, and the exposure enhanced TMAO and leucine. Both decreased in the serum after interventions. Moreover, TMAO was positively related with fat and fat acids in the serum. Significant unsaturated fat acids, such as C20:2 and C22:2, also remarkably decreased in the treated groups. High contents of branched chain amino acids (BCAAs), Aromatic amino acids (AAAs), leucine, phenylalanine and valine were reported to be T2D risk factors. They were also increased by pesticide exposure. In this step, they were remarkably reduced by the treatments, especially FOS + FMT. Tyramine was proven to be positively related with these metabolites, and similar alteration occurred. Ceramide was reported to be related with cerebral vascular diseases, IR, and HbA1c abnormality; it may be a new biomarker of adverse cardiovascular events [56]. Lyso PE induces inflammation and increases oxidative stress [48]. They were both the unfavorable factors in the serum increased by pesticide stress, while the conditions were improved in the intervention.

Discussion

Effects of Pesticide Exposure on Physiological Phenotype

More attention is often paid to the metabolic syndrome caused by mental stress and unhealthy diet [5–7] than contamination, and the latter is usually underestimated for its characteristics of low level and concealments [8], especially dietary pesticide residue, because it is usually taken into the circulating system directly along with the diet. Nowadays, many environmental contaminations were reported to cause obesity and T2D to the human body. For example, dietary chlorpyrifos residue caused IR and obesity.

Obesity is one of the most common manifestations of metabolic syndrome. In this work, obesity and IR were yielded when the mice were exposed to residues of acetamiprid and tebuconazole. This finding indicated that the chronic dietary pesticide residues of acetamiprid and tebuconazole affected glucose and fat metabolism although they had low toxicity and low levels. Further, synergistic effects were yielded in combination exposure, and they aggravated the intervention to the body physiological metabolism. In this work, IR and the decrease in GT indicated that abnormality occurred to carbohydrate metabolism. Metabolism of fat and protein may also be altered because they are interrelated with carbohydrate metabolism. Serum biochemistry analysis exhibited that under pesticide stress, plasma cholesterol and fat were remarkably accumulated, while the HDL-C contents were downregulated. This finding indicating that disorders occurred to fat metabolism, and it may lead to the risk of NAFLD and arteriosclerosis. Observations of aminotransferase and serum protein exhibited that liver function was partially restricted. Body inflammation is closely related with these above physiological responses[57].

Previous literature reported that air pollutants caused pneumonia by activating alveolar macrophages. Observation to inflammatory factors in the serum showed that the body was in a state of chronic inflammation when the mice were exposed to the pesticide. Host tissue histology proved that dietary pesticides exposure lead to NAFLD, leaky-gut, and even enterocolitis to the host. Metabolism health risks of chronic dietary pesticides exposure were confirmed.

Organic or inorganic dietary contamination has long been an advocating research topic. Many action mechanisms were put forward, and different assessment methods were developed. Different from other contamination, pesticides are invented on the basis of a relatively clear target, aiming to control agricultural pests and pathogens. Thus, avoiding the action target existing in higher animals, especially mammals, is one of the main objectives in designing low-toxicity pesticides. In addition, the intake of dietary pesticides is extremely low. Therefore, we speculate that effects of dietary pesticides on the body are indirect. Pesticides caused alterations to the gut flora and then led to physiological effects would be a significant action mode. Intestine is the highest density distribution part of body microbial community and main source of endogenous LPS. LPS is a major component of the cell wall of Gram-negative bacteria, and it is widely considered as the main endotoxin resulting in inflammation by activating inflammatory response genes [30, 31]. Accumulation of LPS in serum was observed in this work, and it showed that the distribution of intestinal microflora was intervened by pesticide exposure. Observation of intestinal permeability indicated that inflammation damaged the intestinal barrier and accelerated the leakage of LPS from the gut to the blood. Decreased intestinal barrier in turn increased inflammation.

Therefore, the results in this work proved that the gut flora is a significant action pathway of pesticide exposure to host metabolism health, and it was often neglected due to the low toxicity and residue level of pesticides. Long-term intake of dietary pesticide residue still poses a great threat to body metabolism health although it is a long process.

Relationships Among Gut Bacteria, Host and Metabolic Disease Risk

The results of gut flora analysis showed that the inter-population diversity of the gut flora was affected by pesticide exposure, directly proving that the intestinal microflora was a significant action target of dietary pesticide exposure. Tebuconazole performed more effects on the body than acetamiprid. A high ratio of *Firmicutes*/ *Bacteroidetes* was yielded in the exposure group, and the results were similar to those of previous reports. In the literature, *Firmicutes* was reported to regulate host sugar metabolism by releasing signal to the host to transform more blood glucose into fat, thus making the host obese. However, *Bacteroidetes* takes in less energy from the host and lowers its energy accumulation, which is beneficial for the host to prevent obesity [34, 35].

The relationships between gut flora and its metabolites were analyzed on the basis of the alterations caused by pesticide exposure. The dominant population of the intestinal microflora, *Alistips, Limnobactor, Brevundimonas*, *Enterococcus*, *and Blautia*, was involved, and the compounds used in the analysis were all significantly different metabolites with high correlation coefficient. They elevated the relevance of the

results. TMAO, 2-hydroxyhippuric acid, 3-indolelactic acid, 3-indoxyl sulphate, N-acetyltyramine, imidazoleacetic acid, 3-oxolauric acid, and pyridoxamine were involved in the analysis. Relevance analysis was an effective mean to present the combined approaches in directional intervention to the gut flora. Clarifying the relevance was helpful to confirm the intervention target. However, totally determining the decisive factors in this work remains to be a problem because identification of metabolites and intestinal microflora are usually limited and their interaction are extremely complex.

On the basis of bacterial size, multiple intestinal barriers, and host immunity, the gut flora could not easily breakthrough the intestinal barrier and directly intervene the host physiological activity in general. In contrast, the metabolites of the intestinal microflora could easily get into the blood, and they are the main media or the link of dialogue between the gut flora and the host. The compounds produced by the intestinal microflora could get into the blood and be taken to the whole body, where they participate in host physiological metabolism directly or indirectly. They could be termed as the base or link of many mechanisms, such as gut-liver and –kidney [58–61]. With the foundation of new intestinal microflora, new metabolites or new action mechanisms, increasing interactions between the intestinal microflora and the host were clarified. In addition, more diseases and metabolism disorders were found to be related with gut flora alterations.

The gut flora is closely related to more than 90% of the diseases, gut-liver axis, gut-brain axis, and gut-kidney axis are the well-acceptable medical concepts [58, 59]. With further research on the gut flora, increasing health-related intestinal microflora was discovered and studied, and they were proven to affect the host health. *Roseburia* utilized dietary fiber to produce butyrate and protect the gut barrier function and decrease host inflammation and atherosclerosis [62]. The species members of *Blautia* alleviated inflammation, IR, and obesity. *Butyricimonas* and *Intestinimonas*, important butyrate-producing bacteria, improved host health through butyrate. *Faecalibacterium* was reported as an anti-inflammation bacterium that alleviated inflammatory bowel disease and obesity. Except for the above improvements in host metabolism, the members of *Akkermansia* enhanced immunity and exerted anti-cancer effects via its metabolism. The inactivated *Akkermansia* significantly alleviated IR and even lowered cholesterol and the markers of liver inflammation. These beneficial floras were all detected in the present work, and their abundances were significantly decreased by pesticide exposure. In contrast, the effect of LPS produced by *Enterobacteriaceae* and *Desulfovibrionaceae* was 1000 times that of others[41], and the two bacteria increased up to 10 times. Moreover, *Desulfovibrionaceae* extremely promoted host gut absorption of fat. Their alterations all posed metabolic disease risk to the host due to pesticide exposure.

The host could respond to the regulation caused by the flora metabolites in the serum via physiological activity. In this work, BCAAs, AAAs, fat, fatty acids, and LPC were remarkably upgraded, while PC, PE, and androgen were downgraded. The BCAAs, AAAs, and fat in the blood are the high-risk factors of IR, T2D, and NAFLD. LPC, an inflammation-promoting compound, was reported to cause atherosclerosis. PC and PE are health-beneficial components with the function of lowering TGs and cholesterol, improving HDL, and preventing fatty liver [48]. However, they decreased in the host circulating metabolism. The regulation

of intestinal microflora could directly cause the changes, and it was a vital pathway when the host was under pesticide exposure.

The metabolites of the gut flora would enter the host circulating metabolism with blood and affect the host metabolism. As the largest digestive gland and main metabolic organ in the body, the liver leads and participates in most of the substance and energy metabolisms, involving great amounts of pathways of metabolism. Therefore, under the exposure of pesticides, liver would be the most important target organ, and to investigate metabolic profiling alterations of liver is an essential ways to clarify the health risk mechanisms of pesticides residue. In this work, the influenced pathways were enriched according to annotate the significantly different metabolites, and related health risk were explained based on these metabolic pathways. Disorder of cholic acid posed high risk to NAFLD, and high betaine probably lead to cardiovascular disease. Dysregulation of histidine metabolism induced inflammation. These results were in accordance with the above phenotype analysis. At last, colitis and NAFLD were caused by the risk of the metabolic disorders. These results verified the rationality and effectiveness of the present work. Many vital disordered metabolic pathways were clarified via metabolic profiling, which perfected the research on the process of health risk caused by pesticides. The findings were theoretical bases for the presentation of treatments or interventions to body metabolic problems. However, not sufficient pathways were clarified for the limited identification and detection of compounds, and it restricts more interpretations to the risk mechanism.

Effects of Interventions on Stressed Host

As a type of soluble dietary fiber, FOS is not digested by mammalian endogenous enzymes and not absorbed by the small intestine [54]. It is an effective proliferation factor for the gut flora and is utilized by many intestinal floras, with the function of regulating host metabolisms, improving health condition, and mitigating many diseases. In the present study, FOS possibly improved IR and alleviated the inflammatory status of the host by mainly affecting the gut flora. Gut bacteria and their metabolites were the main contents in the used water extract of feces, and they affected the physiological activities flora community [55].An apparent release to disorder of host metabolism was yielded after interventions, and it proved that FMT directly affected host metabolism *via* intervening the gut. The inflammatory status improved, and the risk metabolites in the serum decreased. Thus, FOS improved the disorder of the gut flora through the effects of proliferation on the beneficial bacteria, which rely on dietary fiber, while FMT directly changed the gut flora of the stressed mice via transplantation. The effects of FOS + FMT were the best, indicating that the transplanted intestinal microflora and supplementary dietary fiber exerted synergies on the gut flora. FOS and FMT are strong evidence that proved that alterations to the gut flora is a vital inducement and even source of health risk caused by long-term dietary exposure to low-level pesticide residue. The study also supplied clues and insights for the treatments or interventions to body metabolic problems on the basis of gut flora.

Conclusions

Gut flora is the most complex microbial ecosystem in nature, with a population 10 times that of human body cells. The flora and host are superorganisms, and the gut flora is considered as another organ. It is closely related to nearly all of the host physiological activities. In the present study, physiological phenotype and biochemical analysis indicated that chronic metabolic syndrome, IR, gut-leak, NAFLD, and obesity, occurred in mice. Starting from the clue of metabolic disorders, the reasons were returned back to gut flora. Then, the alterations in gut flora and its metabolism under exposure were observed. Many harmful intestinal microflora and risk factors of body metabolism were found, and their correlations were determined. The effects of pesticide exposure on the host physiological activities were characterized via metabolic profiling. Considering the role of metabolites in the reaction between the intestinal microflora and the host, the correlations between metabolites of gut flora and host were explored. Many disorder indicators, either beneficial or harmful, were linked with the reported risky metabolites in the gut. Several altered metabolic pathways of host circulation were identified in accordance with the different metabolites in the liver. Finally, the intervention experiment verified that the affected gut flora was the vital medium and inducement in the metabolic health risk caused by pesticide exposure. This work manifested that the gut flora was the action pathway of the host metabolic health risk caused by long-term exposure to low-level dietary pesticide residue. The findings served as theoretical bases for the presentation of treatments or interventions to body metabolic problems. This work also supplied clues and insights for the treatment or intervention to body metabolic problems on the basis of gut flora.

Methods

Animal Experiment

Three-week-old male Kunming mice were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The experiment was carried out at PONY test Co., Ltd. (Beijing, China) in accordance with its Animal Welfare Committee guidelines for laboratory animals. After 7 days of acclimation, the mice were randomly divided into six groups (eight mice/group) and subjected to treatments of W (tebuconazole in corn oil), D (acetamiprid in corn oil), DW (tebuconazole and acetamiprid in corn oil), control check (CK, only corn oil), and N (only water). Five groups of DW and two groups of control check (CK) were arranged. According to the literatures[11, 63–66] and ADI (Acceptable Daily Intake) values for mice (conversed according to human dose), mice of W, D and DW treated groups were respectively exposed at the level of 1.35, 3.15 and (1.35 + 3.15) mg/(kg Bodyweight) per day for 13 weeks, 25 μL of solution for each mouse. They were maintained under a 12:12 h light/dark cycle at 25 °C–28 °C and allowed free access to feed and water. The padding in the cages was regularly replaced per week to keep the humidity at 40–70%.

Sampling

After 12 weeks of treatment, the mice were transferred into metabolic cages at the same time of the days. Their urine and feces were collected, immediately flash frozen in liquid nitrogen, and stored at − 80 °C until processing. After 13 weeks of treatment, four combined groups (32 mice) and one CK group (eight

mice) mice were remained and fed continuously, whereas the others were euthanized by CO_2 asphyxiation and cervical dislocation. Blood was collected and centrifuged to obtain serum. Samples for hematoxylin and eosin (HE) staining of colon and liver were collected and fixed in 10% formalin. The rest of the liver was cut into several pieces, immediately flash frozen in liquid nitrogen, and stored at − 80 °C.

Measurement of Mouse Glucose Tolerance

A blood glucose meter (Accu-Check, Roche Diagnostics) was used to measure the tail-vein blood glucose levels. At approximately 90-day exposure, the GT of mice was examined. After the mice were fasted for 16 hours (water was available), they received a gavage of glucose (2 g/kg bodyweight). Blood glucose was measured at 0, 30, 60 and 120 min after the glucose loading.

Measurement of Mouse Insulin Resistance

Homeostasis model assessment was employed to calculate and estimate the IR of the mice. At the end of the exposure experiment, the mice were fasted overnight and fasting blood glucose was measured using the blood glucose meter. Then, fasting serum insulin (FINS) was determined using an ELISA kit (Mercodia) after the serum was obtained. Finally, the IR index was calculated in accordance with HOMA-IR = FBG (mmol/L) × FINS (mIU)/22.5.

Serum Biochemical Assays

Serum biochemical parameters, alanine aminotransferase (ALT), aspartate aminotransferase, total protein and albumin were assessed using a biochemical analyzer (Hitachi 7100, Japan).

Inflammatory Factor Assays

Anti- and pro-inflammatory factors were investigated to evaluate the inflammatory status of the mice. The LPS, MCP-1, IL-1β, IL-6, IL-10, and TNF-α level in serum or feces were assayed using a commercial ELISA kit in accordance with the product's instruction manual.

Evaluation of In-vivo Intestinal Permeability

For the observation of intestinal permeability, blood was obtained from the overnight-fasted mice (0 hour). Then, the mice were administered with 600 mg/kg of FITC-Dextran (MW 4000, Sigma–Aldrich) by gavage. Two hours after, the blood and urine were collected and detected via fluorometry, with a 0-hour sample as background (Ex, 490 nm; Em, 520 nm).

Histological Observation

Fixed liver and colon were paraffin-embedded, deparaffinized, rehydrated, and then stained with HE for histopathological analysis. The staining was visualized with an ECHO microscope (RVL-100), and the observations were approved by a histopathologist.

Metabolism Analysis

The alterations that occurred to the mouse physiology after exposure were investigated using metabolic techniques on the basis of high-resolution mass spectroscopy (QE plus, Thermo Fisher), and the samples involved serum, feces, and liver.

Polar and low-polar solvents were used in the analyte extraction procedures [67, 68]. For the liquid samples, four times the volume of methanol (MeOH): acetonitrile (ACN) (v: v = 1:1) was added into the serum or urine samples. Then, the mixture was blended for 1 min, incubated for 10 min at − 20 °C, and concentrated at 13000 *g* and 0 °C. Subsequently, the supernatant was filtered through 0.22 μm membranes to yield the polar extraction solution of the serum or urine samples. The system methyl tert-butyl ether: MeOH: sample (serum or urine) = 10:2:5 was employed in low-polar analyte extraction. After the same pretreatments and centrifugation were conducted, the supernatant was transferred into a new tube, blown dry with nitrogen, and reconstituted with CH_3Cl: MeOH = 1:3. The liver tissue was homogenized with pre-chilled MeOH: ACN: water (v: v = 2:2:1, 0 °C) in accordance with the proportion at 100 mg/mL. The slurry was incubated for 30 min at − 20 °C and concentrated at 13000 g and 0 °C. A similar volume of the supernatant for each sample was transferred into a new centrifugal tube and concentrated into powder by nitrogen blowing and vacuum freezing. A polar extraction solution of the liver was prepared by reconstituting the powder with MeOH: water (v: v = 4:1) and filtering through 0.22 μm membranes. The precipitate of the liver samples was suspended with pre-chilled MeOH: CH_2Cl_2 (v: v = 1:3, 0 °C) in accordance with the same proportion. Then, the remaining procedures were repeated in accordance with that in the polar extract, and this is the pretreatment for low-polar extract of liver. For different moisture contents, the mouse feces were dried by vacuum freezing. The pretreated feces were immersed in pre-chilled MeOH: ACN: water (v/v = 2:2:1, 0 °C) at 100 mg/mL and − 20 °C for 1 hour and subjected to sonication for 15 min on an ice bath. Then, polar or non-polar extracts were obtained using the same steps in the liver samples.

All the extracted samples were analyzed using high-performance liquid chromatography (HPLC)-HRMS (QE plus, Thermo Scientific) operating with BEH T3 (Waters, USA) column for the separation of polar and low-polar extracts and BEH HILIC for the polar extract. The positive and negative scan modes were separately applied for every sample. The MS parameters (electrospray ionization, ESI) were optimized and set as follows: spray voltage, 4 kV for positive mode and 3.5 kV for negative mode; sheath gas flow rate (N_2), 40 arbitrary units (a. u.); auxiliary gas flow rate (N_2), 10 a. u.; and capillary temperature, 300 °C. Data were acquired in data-dependent analysis mode (Full MS/dd-MS^2), with resolutions of 70,000 for Full MS and 17,500 for dd-MS^2. Monovalent ions with the NC (Nuclear/Cytoplasmic) in 70–1000 and AGC target of more than 10^5 were scanned in the NCE mode (15, 30, and 45). The LC methods for different samples are described in additional file 19 (Table S5).

The raw data of gut flora metabolomics from the LC-MS system were processed using a data process software (Compound Discoverer 3.1, ThermoFisher) with public (KEGG, HMDB, MMMDB, and SMDB) and commercial databases (mz Cloud). The identified metabolites and their abundance results were subjected to statistical analysis (Simca 14.1, Umetrics) for multivariate analysis of variance. Pathway

analysis was conducted to study the functional interplay among the differential metabolites by using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) and KEGG (http://www.genome.jp/kegg/).

Gut Flora Analysis

The gut flora in the frozen feces was analyzed using the 16S rRNA gene sequencing method in accordance with the procedures of Novogene Bio-information Technology Company (Beijing, China; Additional File 20: M1).

Intervention to Metabolic Disorders

After approximately 13 weeks of exposure, the four DW groups and one CK group were fed continuously for 4 weeks. The four DW groups were treated with FMT, FOS, FMT + FOS. Capsules (10–15) of fresh feces were collected into a tube with 5 mL of normal saline and pounded using glass rods. Then, the mixture was filtered (200 um), and the FMT solution was prepared. The reference for human dose [(20 g/60 Kg BW/d), 0.15 g/50 g BW/d FOS in normal saline (1.5 g/mL)] was applied to the mice. The DW groups were administered with FOS (0.15 g/50 g) and pure water (50 μL), FMT (50 μL) and FOS (0.15 g/50 g), FMT (50 μL) and pure water (100 μL), and pure water (150 μL) once every 2 days for a month by gavage. The CK group was treated with 150 uL of pure water by using the same method. At the end of the intervention, fecal and serum samples were all collected in accordance with the sampling procedures. In accordance with the analytical results of the above procedures, the changes in objective metabolites were quantitatively detected using LC-MS/MS (Q-Trap, 6500, AB Sciex).

Targeted Metabolite Profiling

Analysis was performed via ultra-HPLC coupled to an MS/MS detector (QTrap, 6500, AB Sciex) with the ESI source in positive ion mode. A Waters BEH T3 column (1.7 μm, 2.1 × 100 mm) was used for targeted compound separation, with a flow rate of 0.3 mL/min at 35 °C. The methods were shown in additional file 22 (Table S6). Multiple reaction monitoring was used to monitor the compounds. The mass parameters were as follows: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min, interface temperature, 300 °C; DL temperature, 250 °C; heat block temperature, 400 °C; and drying gas flow, 10 L/min. All the amino compound standards were purchased from Sigma-Aldrich (USA).

Correlation and Statistical Analysis

Correlation analysis among data sets was conducted based on *Spearman* coefficient and the results were exhibited by network map *via* Cytoscape (3.8.0). Differences between data groups were investigated with one-way ANOVA by SPSS (19.0). Diversity analysis was carried out on omicshare data processing platform (https://www.omicshare.com) or by R language.

Declarations

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Availability of data and materials

Authors' contributions

Jingkun Liu, Yongzhong Qian, Yanyang Xu and Jing Qiu conceived and designed the experiments. Jingkun Liu and Fangfang Zhao performed the experiments. Jingkun Liu and Fangfang Zhao and Nan Li analysed the data. Jingkun Liu and Fangfang Zhao wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All the animal procedures were carried out in agreement with the guidelines of the Animal Welfare of PONY, and approved by the Animal Experimentation Ethics Committee of Chinese Accdemic of Chinese Academy of Agricultural Sciences.

Competing interests

The authors declare that they have no competing interests.

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Figures

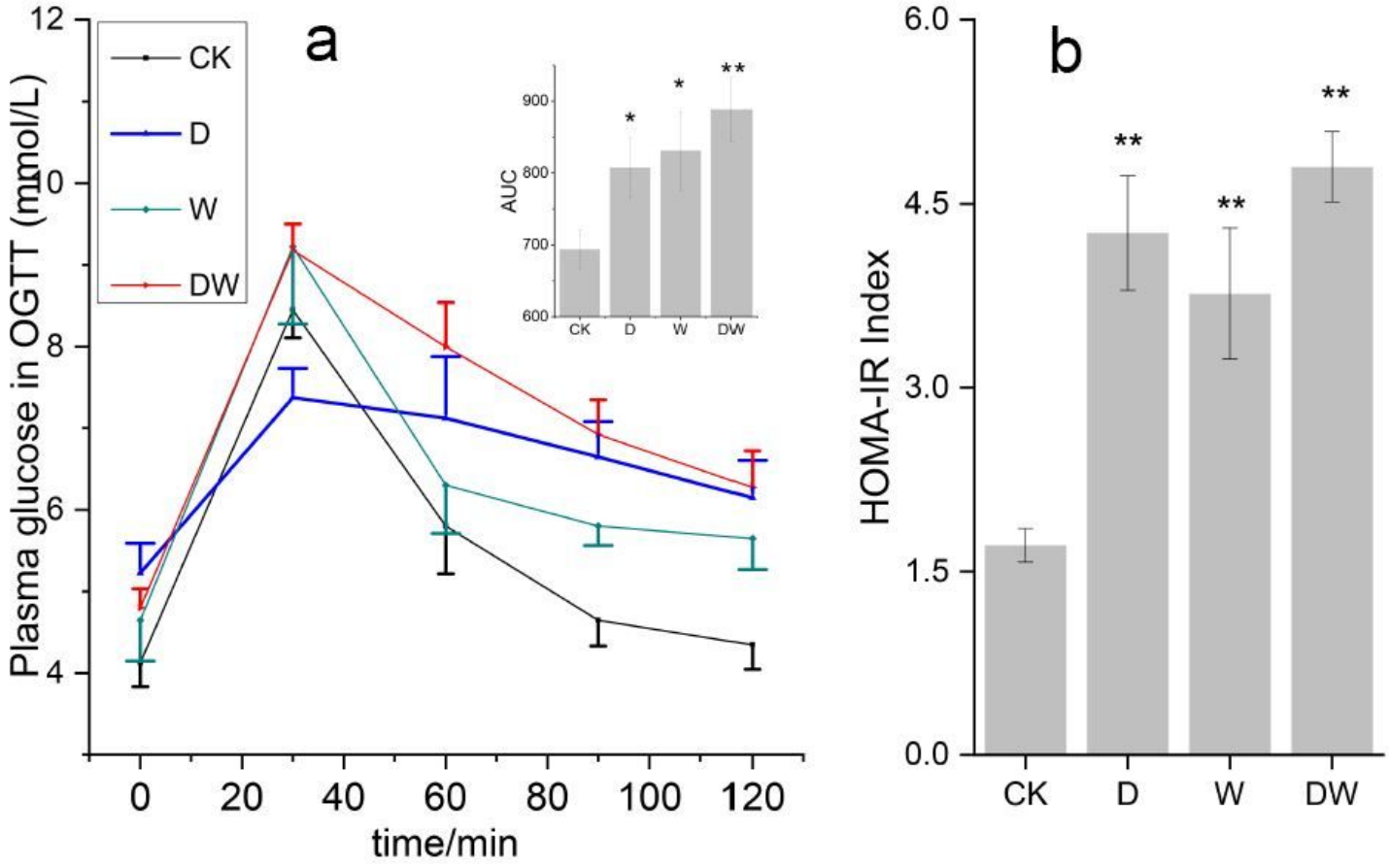
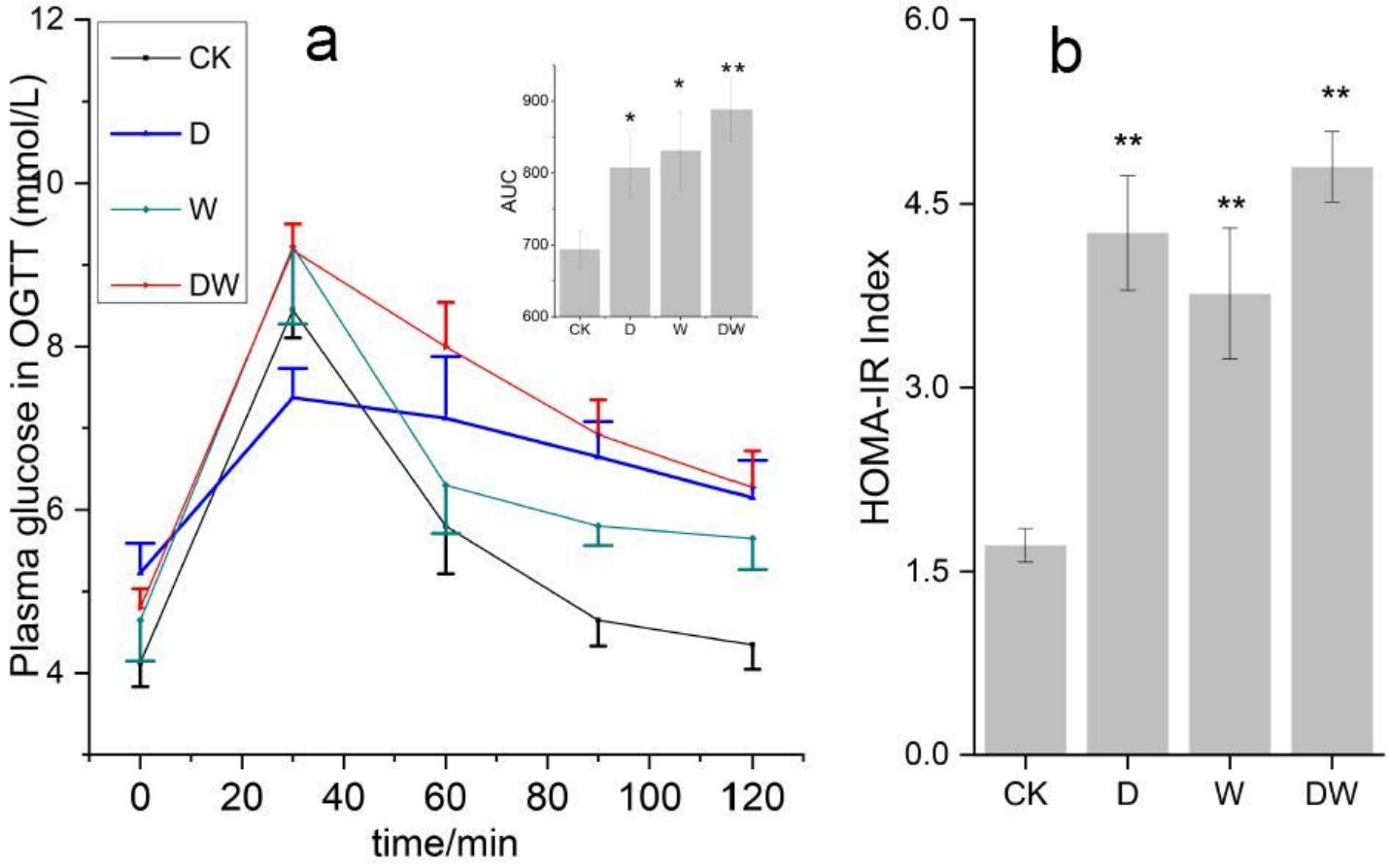


Figure 1

Effects of pesticides exposure on mice glucose tolerance (a) and IR (b) (n=4). Data are expressed as the mean ± SEM (*p < 0.05; **p < 0.01). CK: control check group, D: acetamiprid treated group, W: tebuconazole treated group, DW (or WD): combination treatment group



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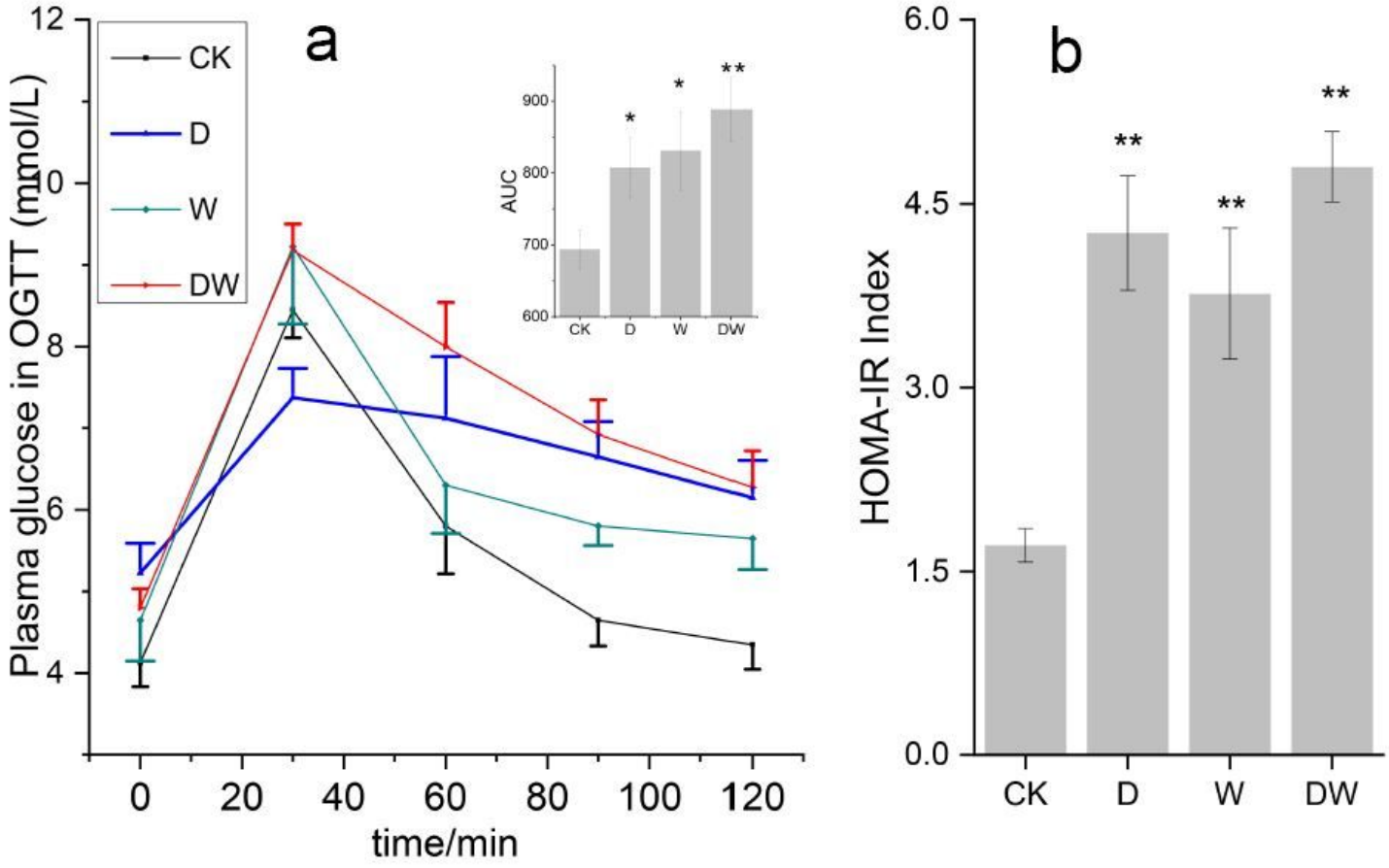

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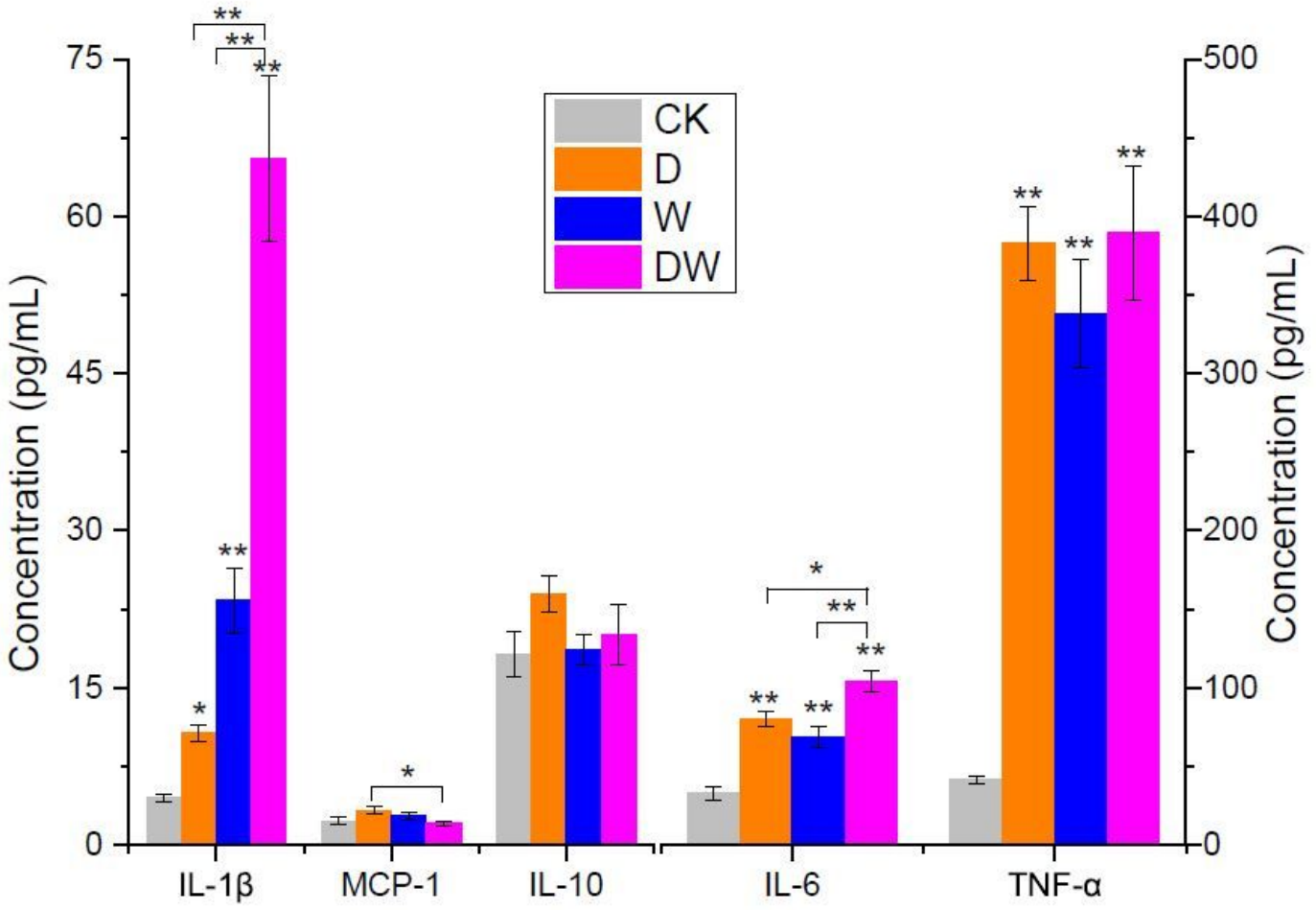
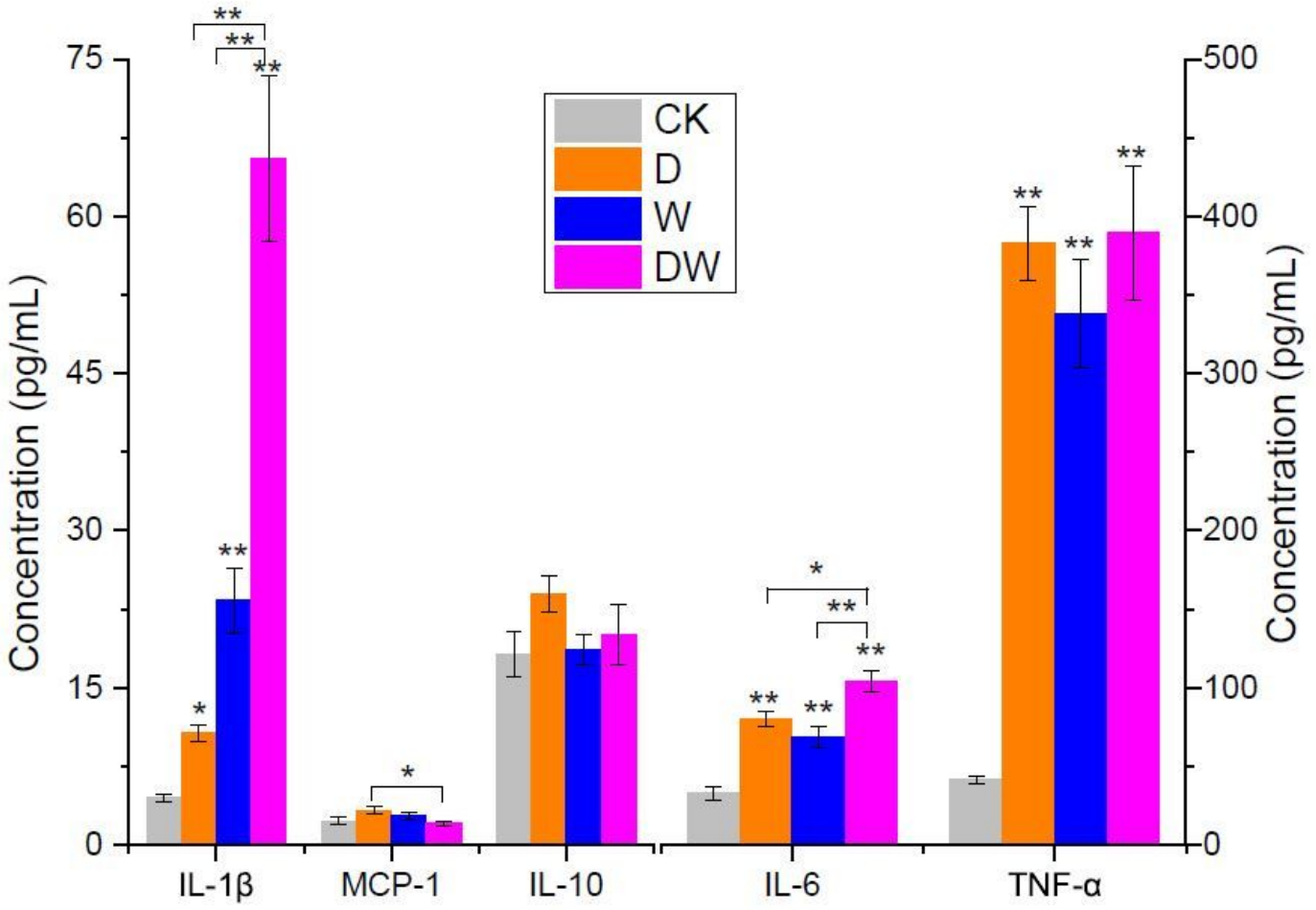


Figure 2

Concentrations of mice serum inflammation factors (n=4). Data are expressed as the mean ± SEM (*p < 0.05; **p < 0.01).



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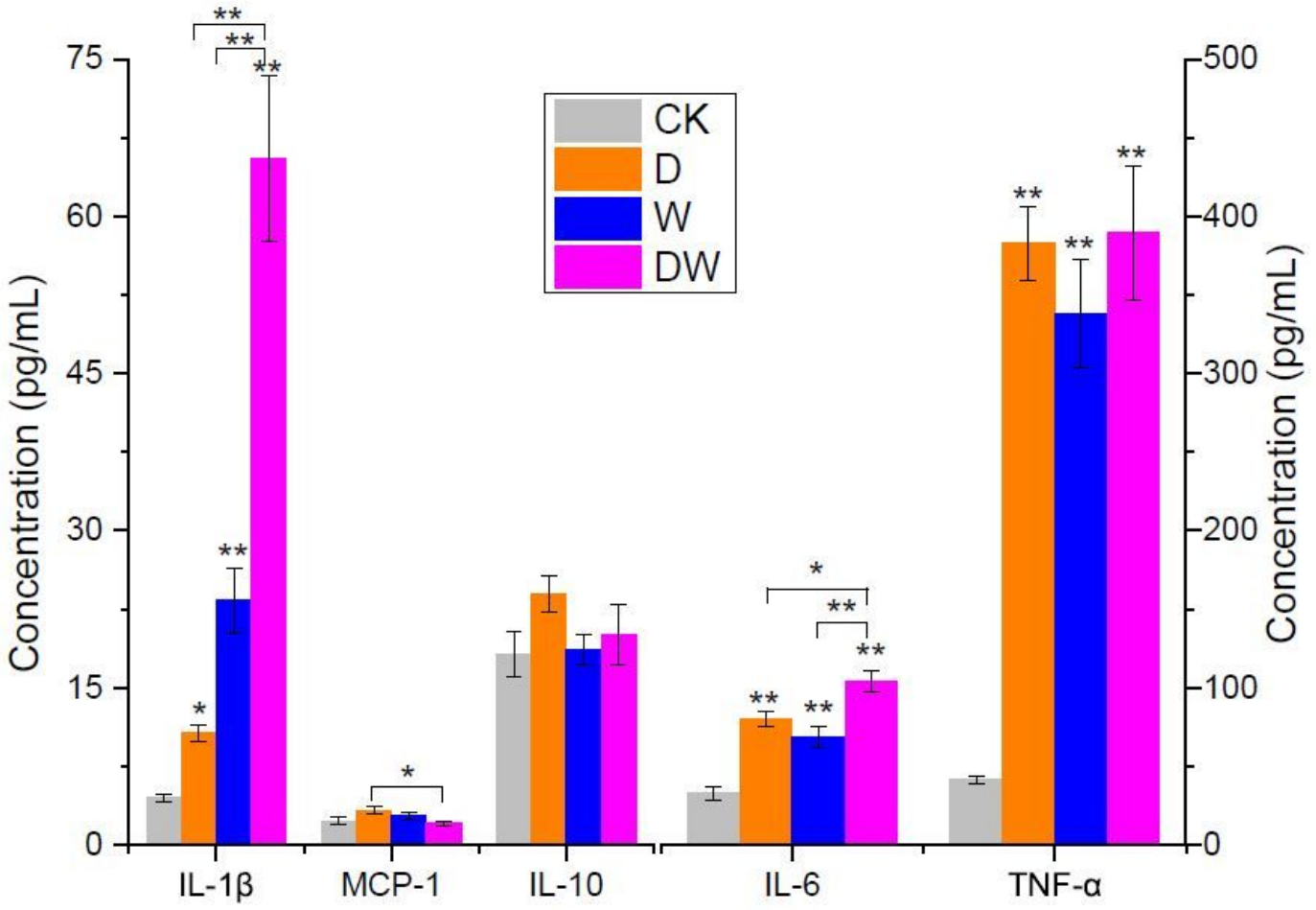

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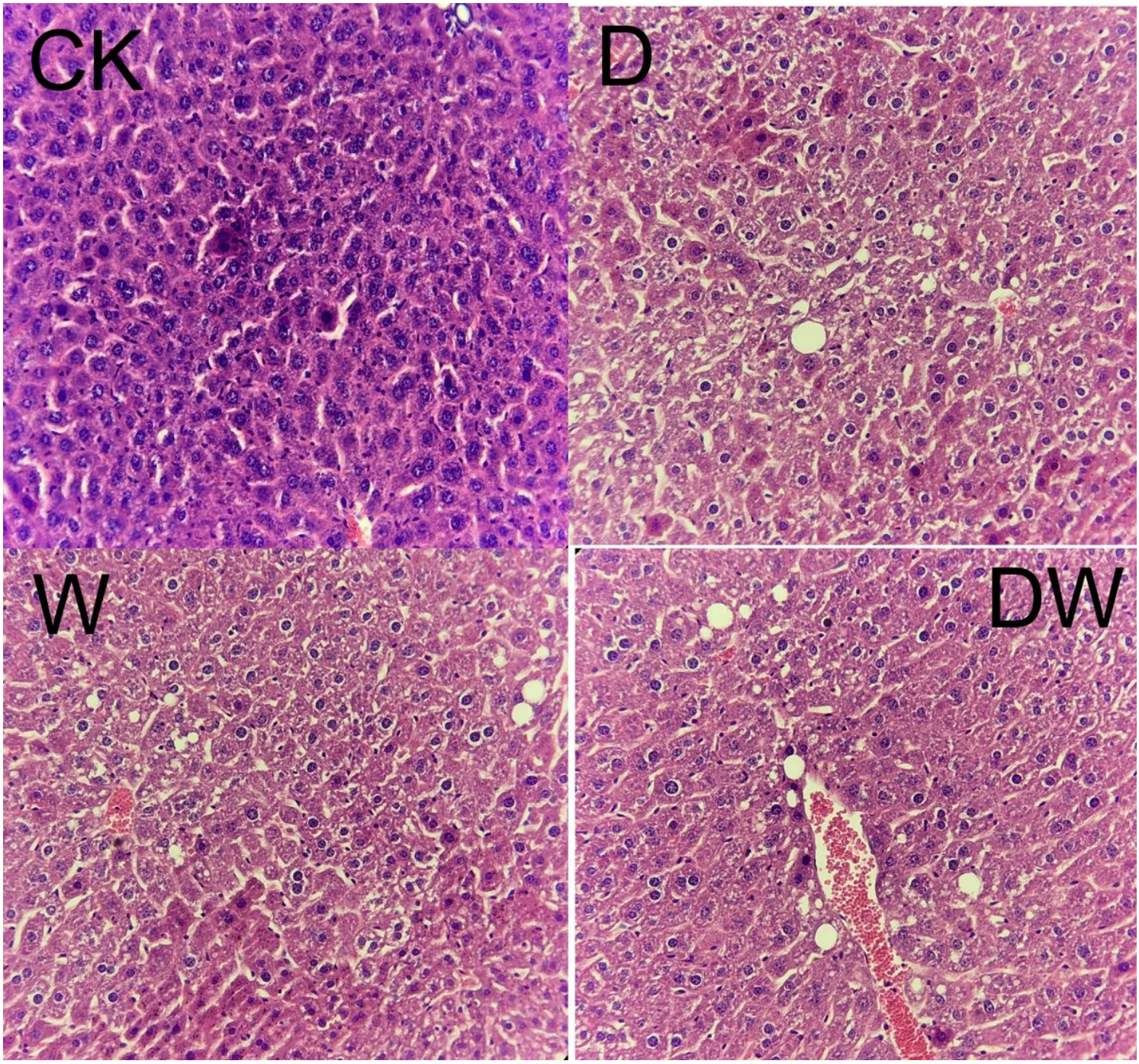
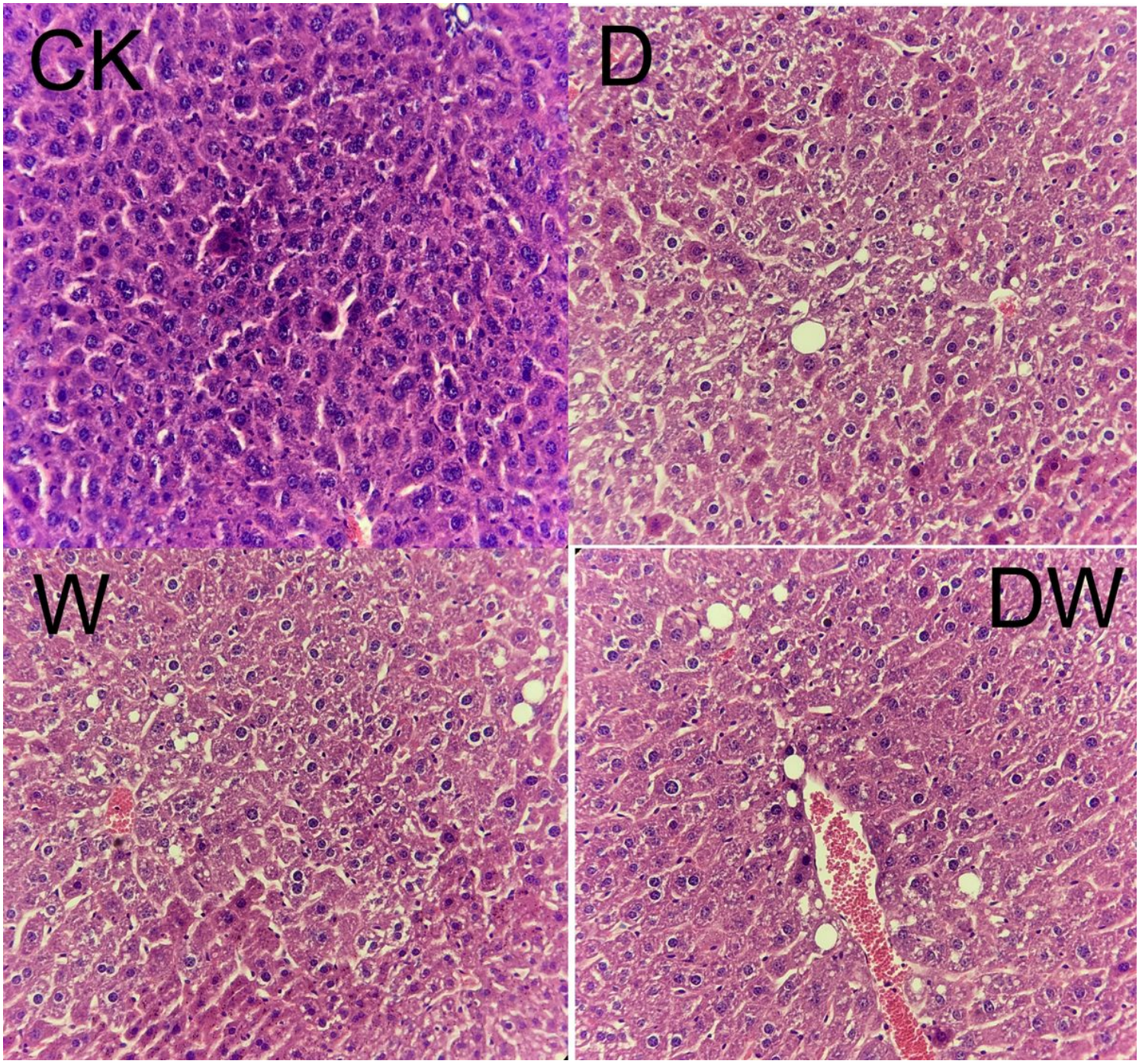


Figure 3

HE staining of mice liver sections.



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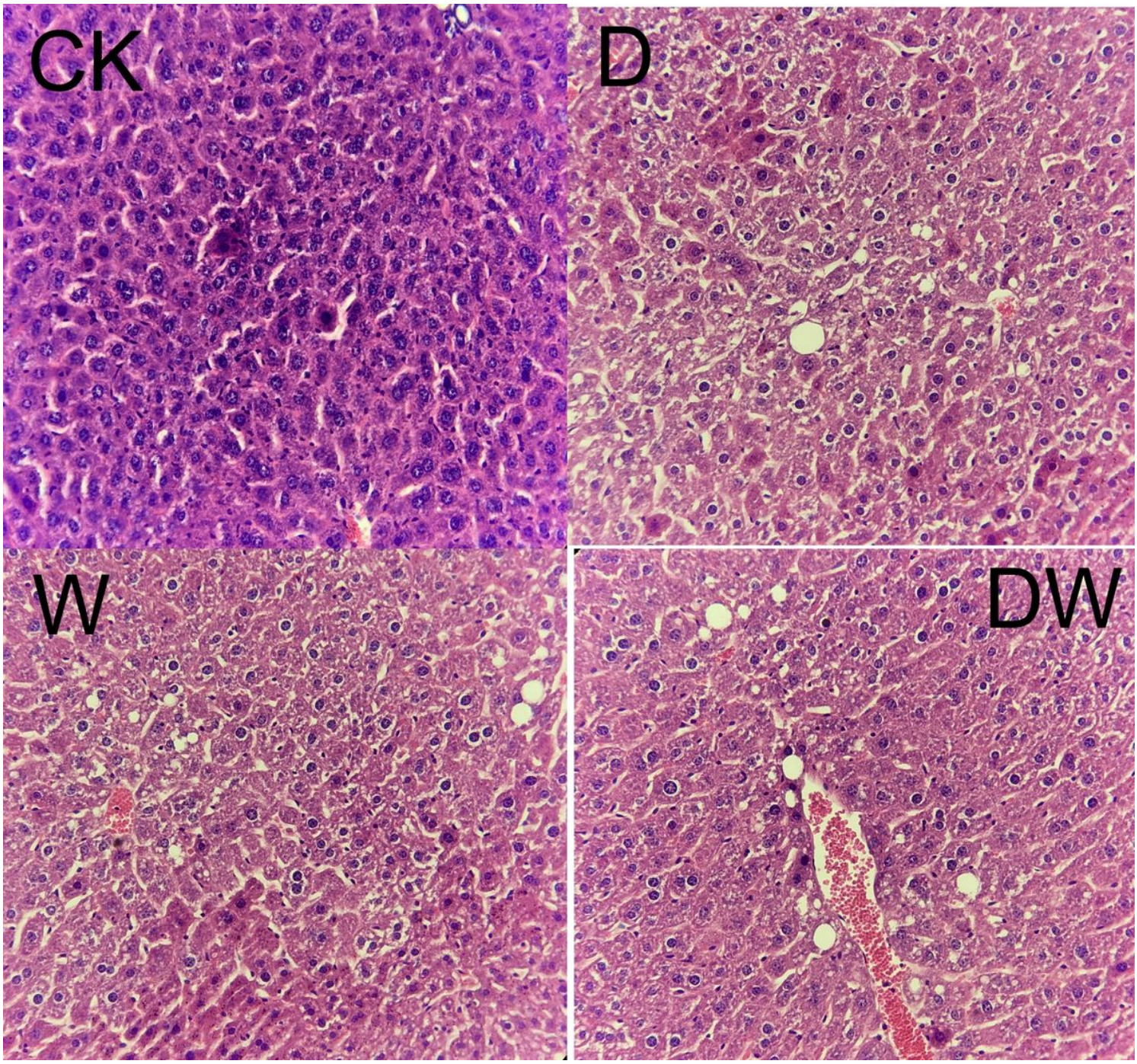

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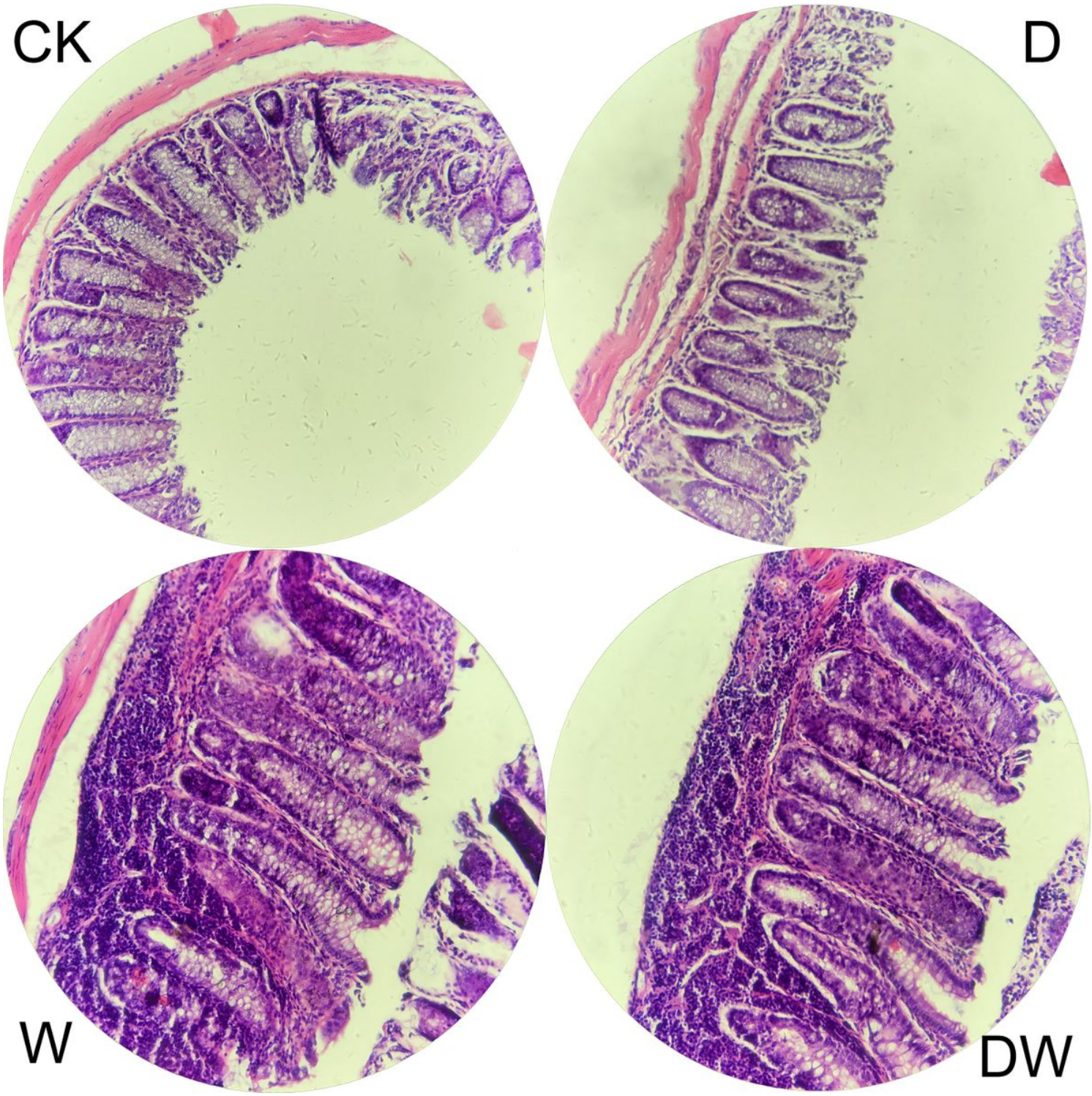

Figure 4

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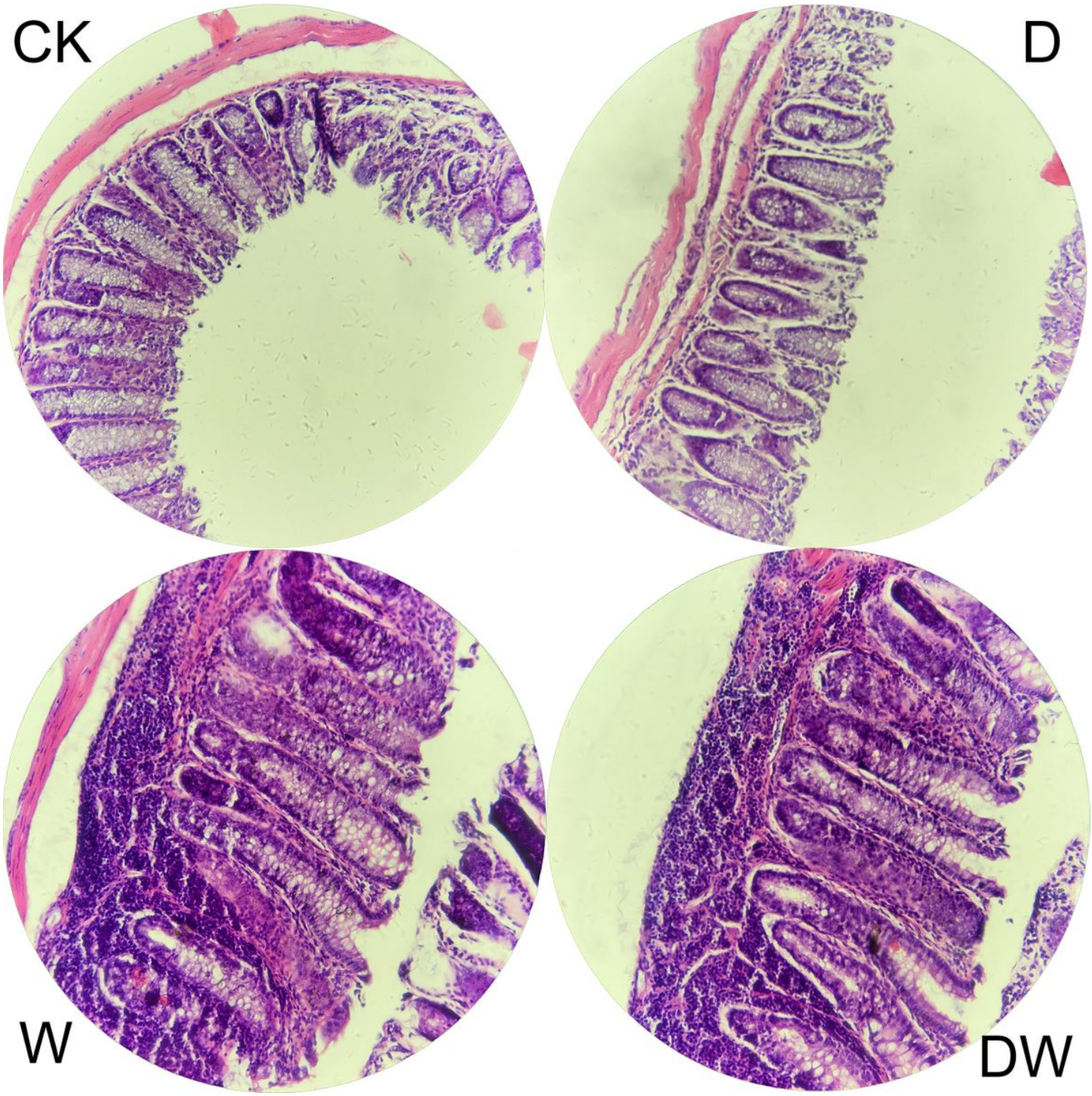

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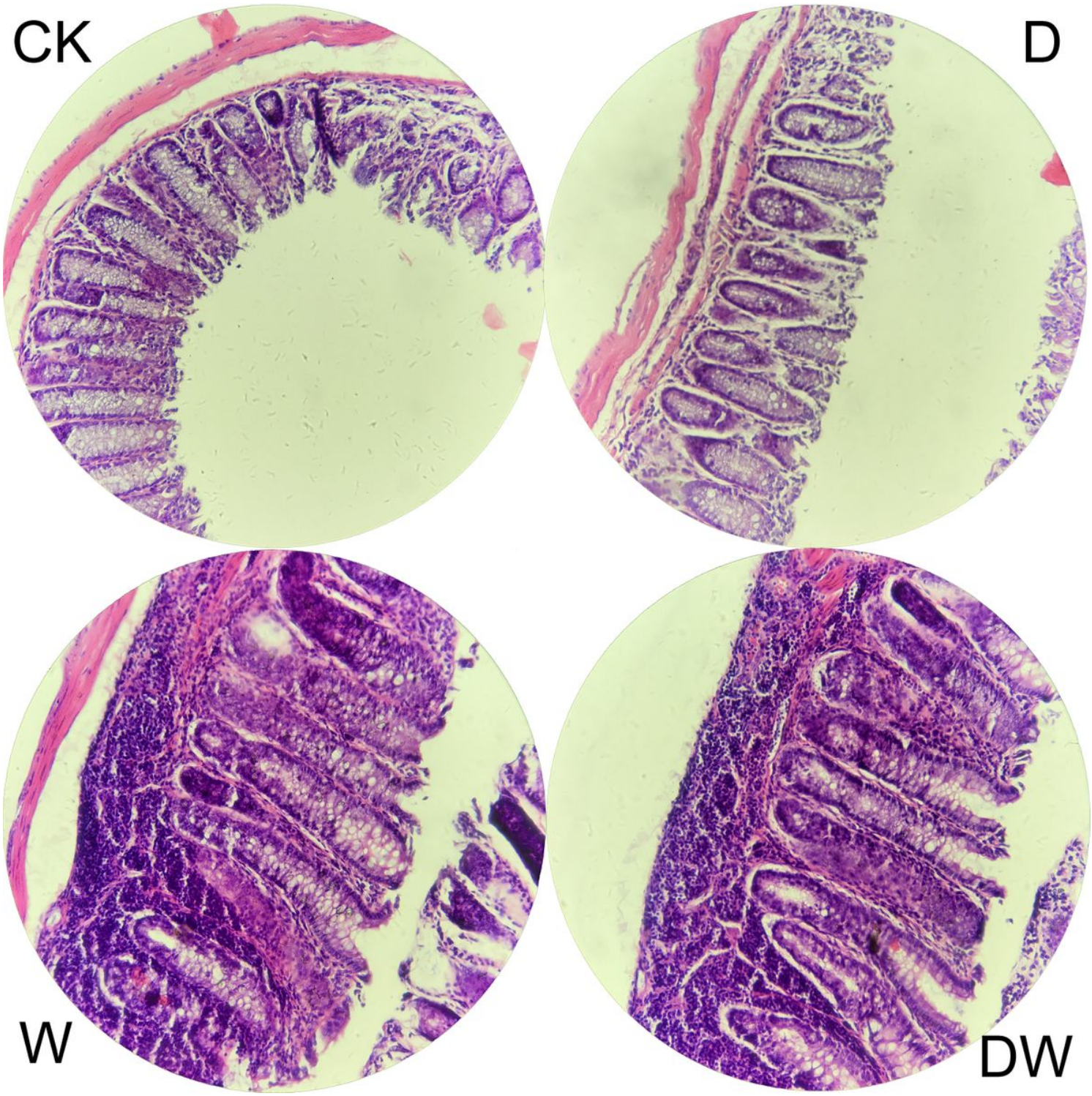

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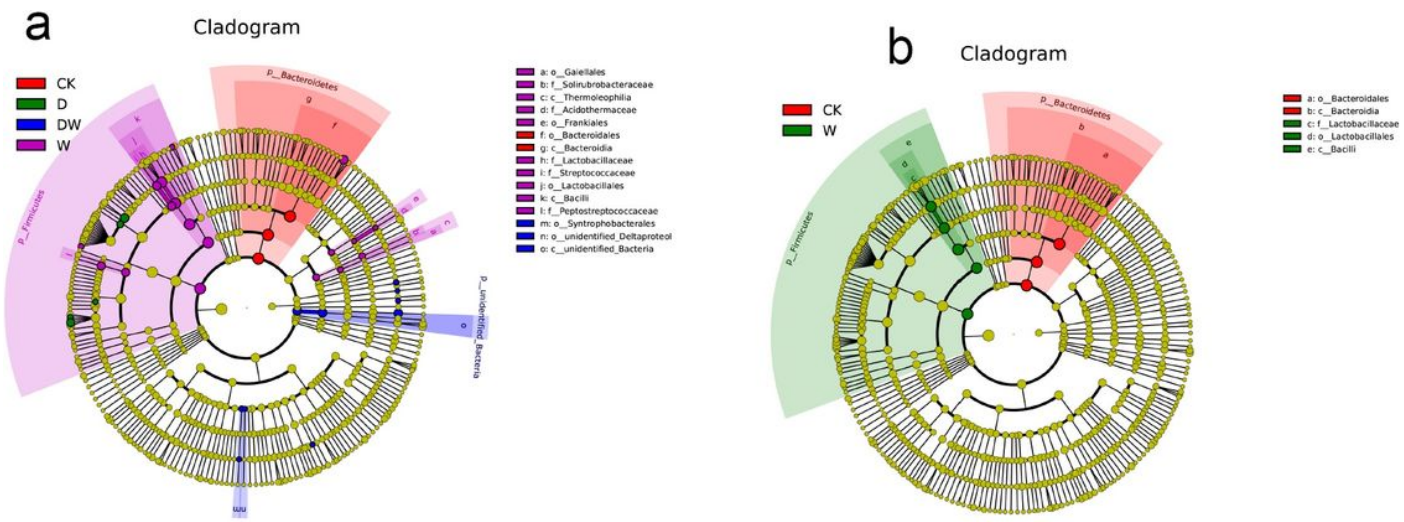
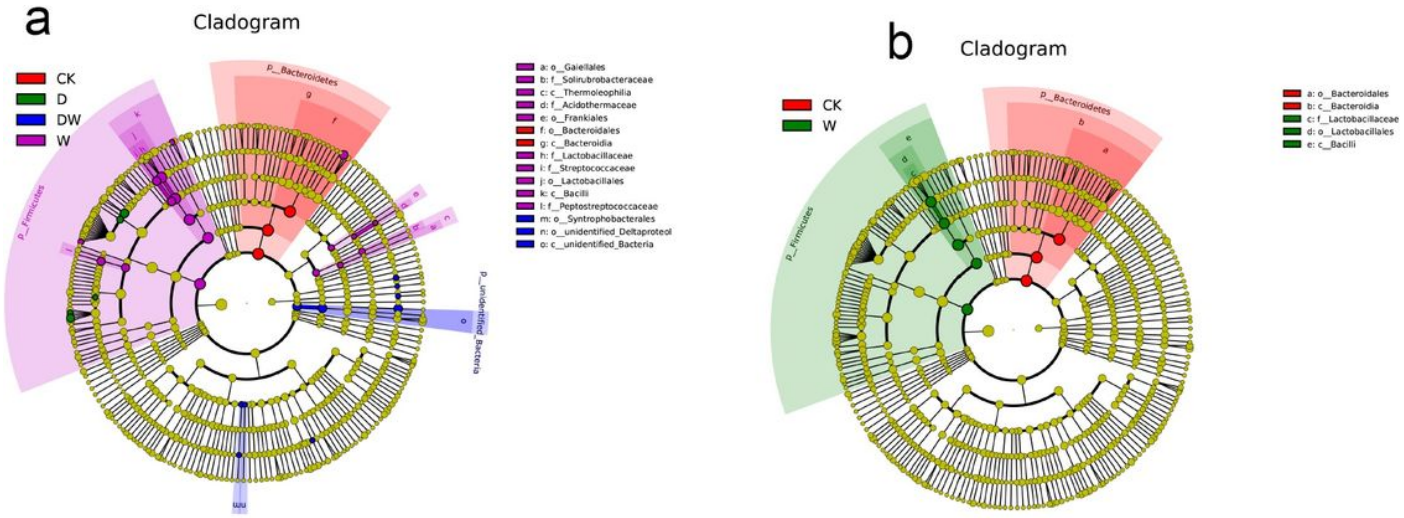


Figure 5

Evolutionary branches in LDA Effect Size (LEfSe) analysis to mice gut flora (a: LDA score is 3, b: LDA score is 4, n=4).



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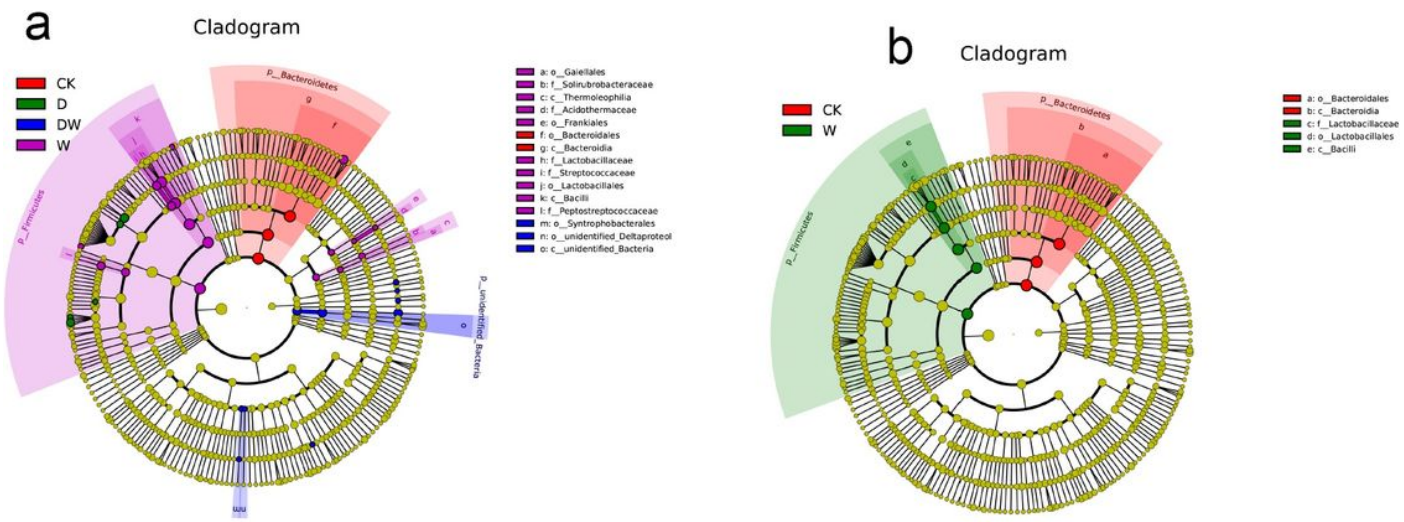

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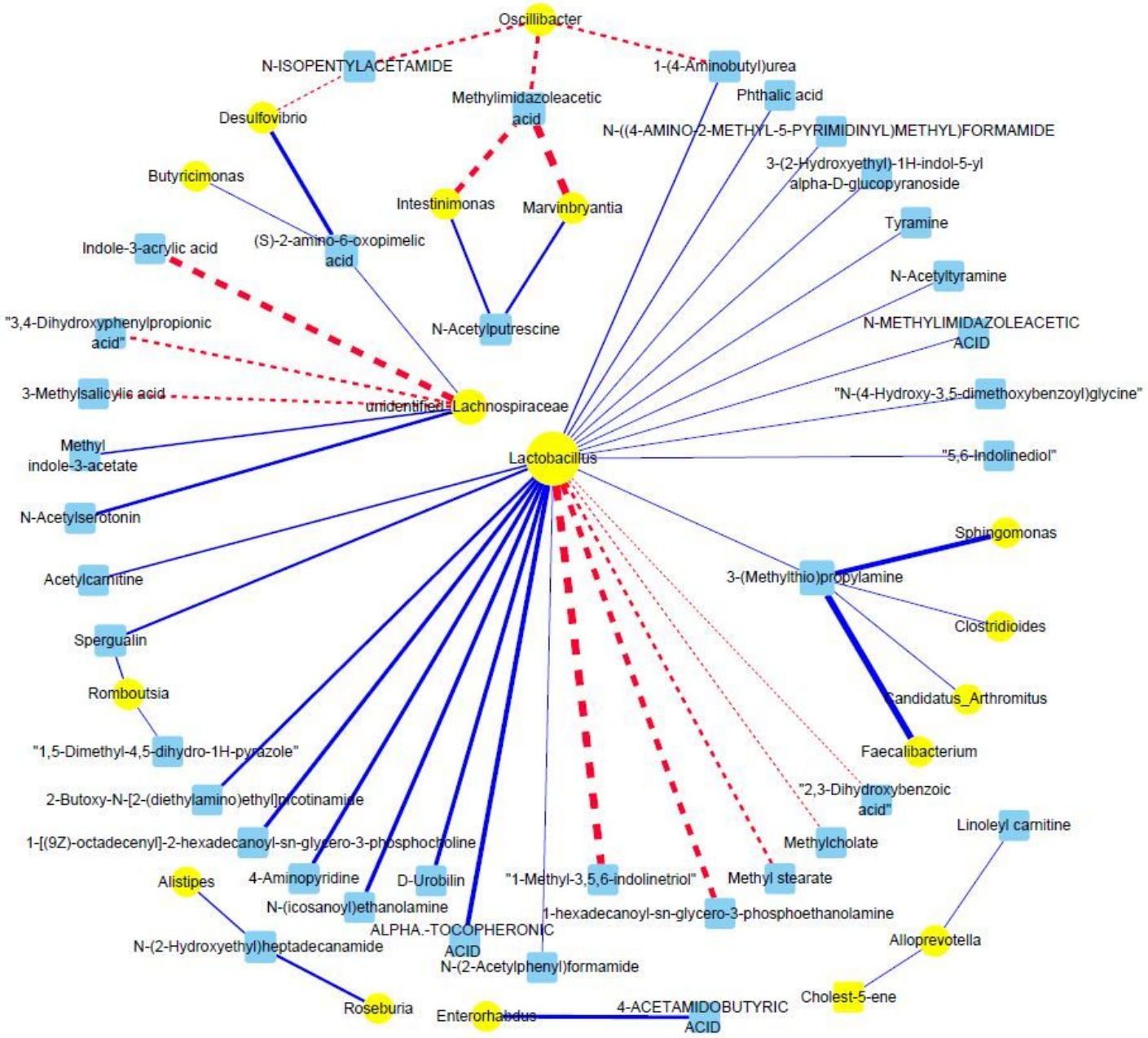
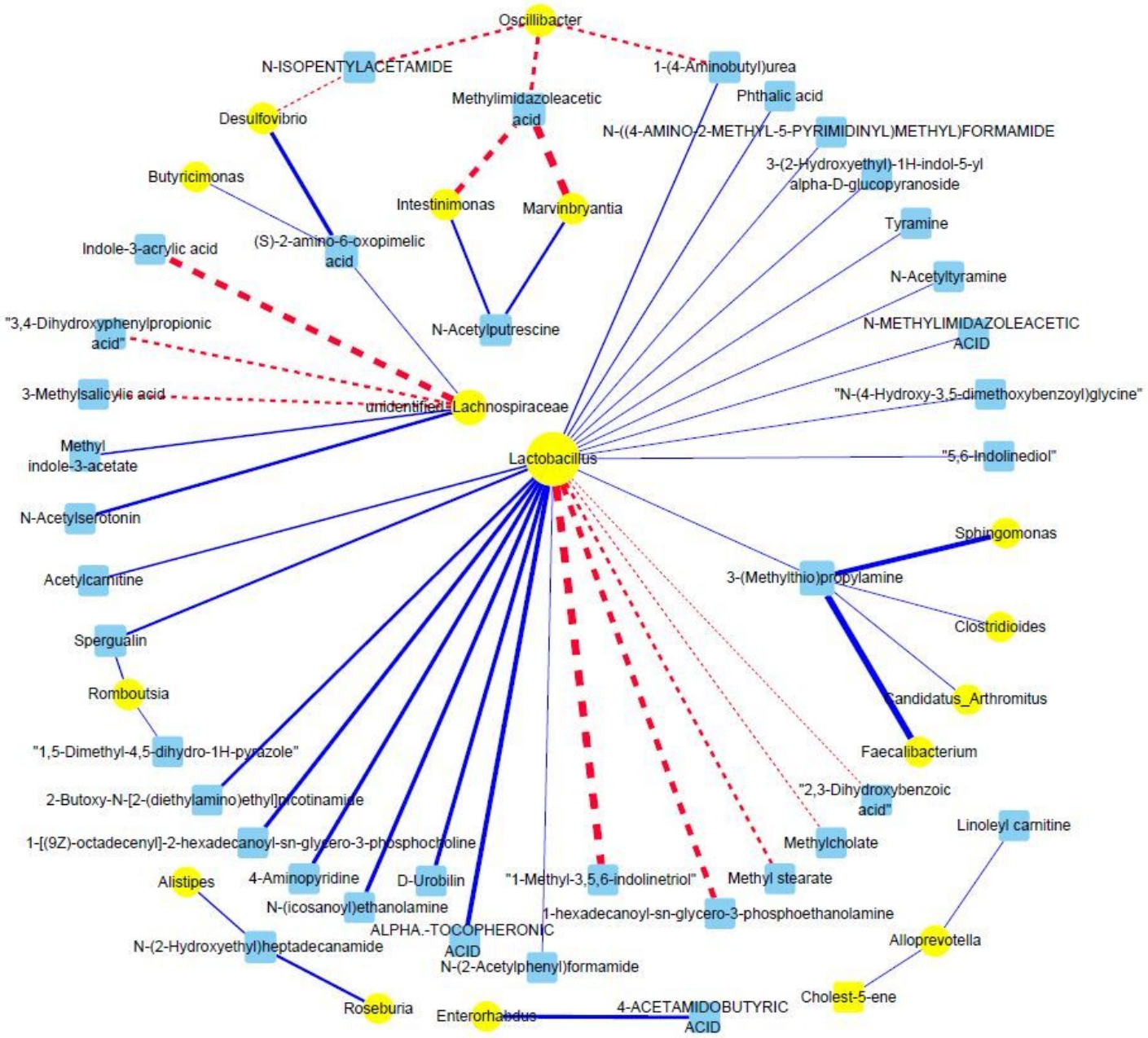


Figure 6

Associations of gut microbial species with their metabolites. yellow round nodes: different genus of the intestinal microbiota, blue round nodes: metabolites of the microbiota, red dashed lines: negative correlation, blue solid lines: positive correlation. The width of lines indicated the magnitude of correlations, from -0.7 to -1.0 or from 0.7 to 1.0 (Spearman). Size of nodes represents how many correspondences of this element involved with another type of element.



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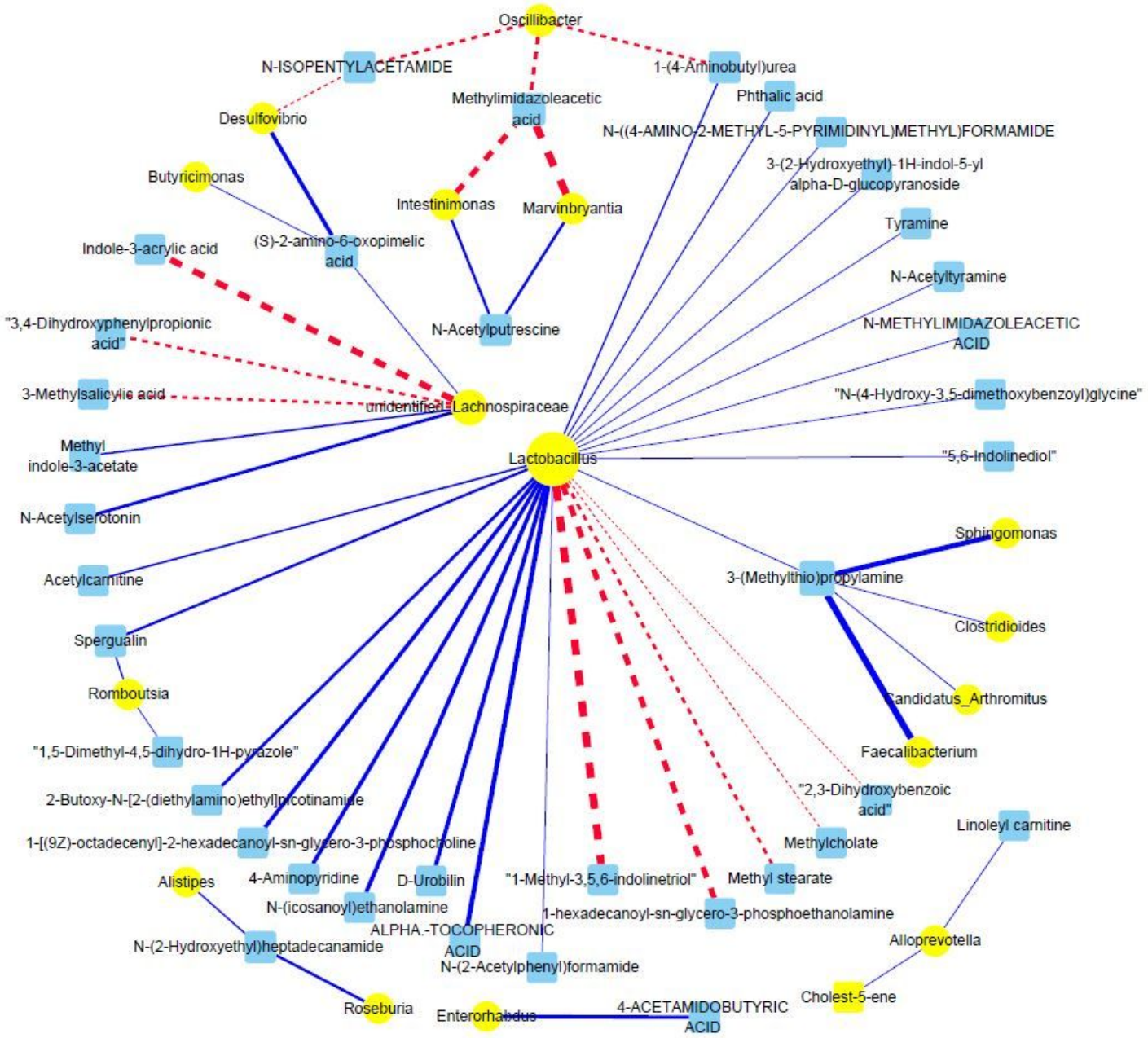

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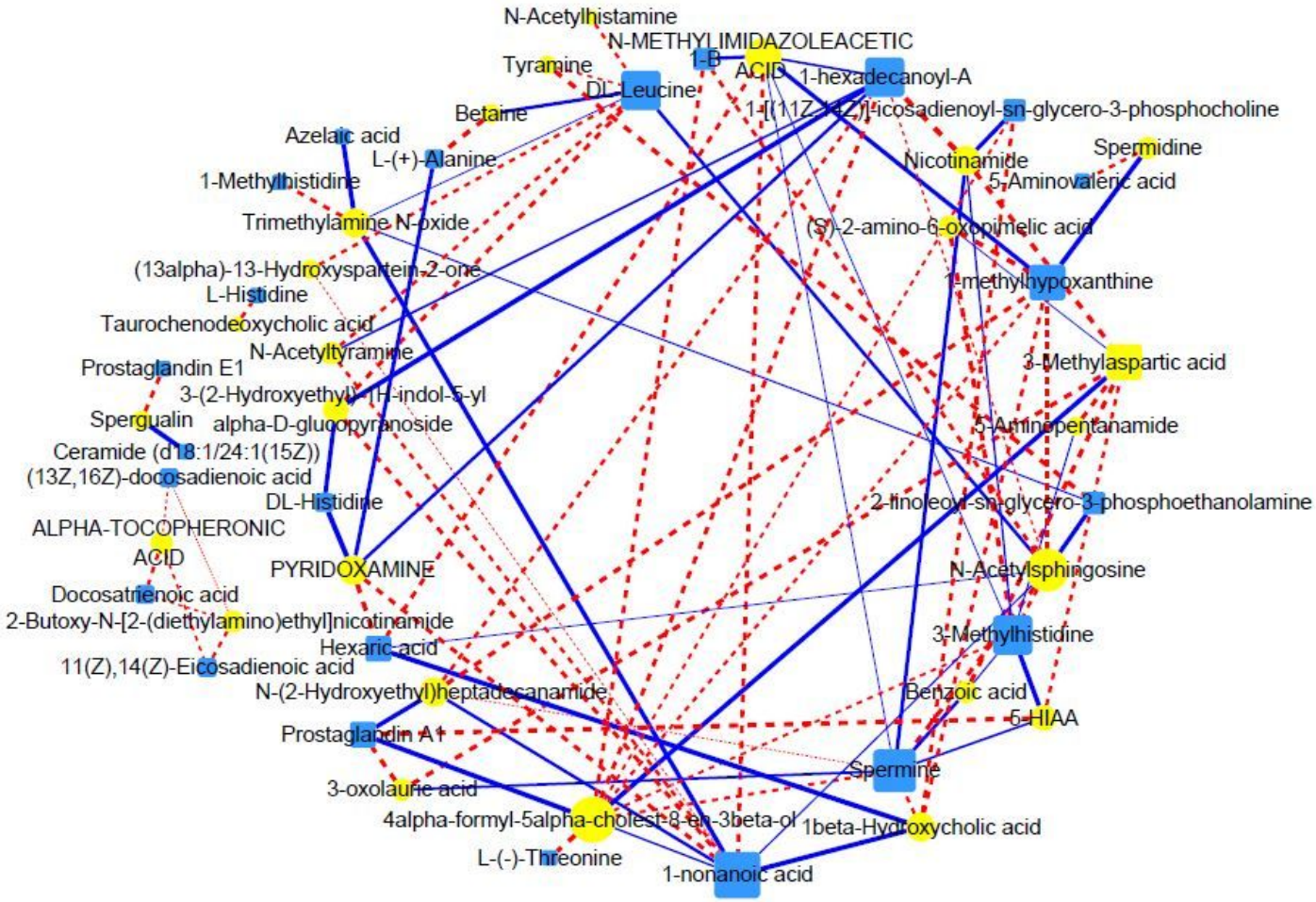

Figure 7

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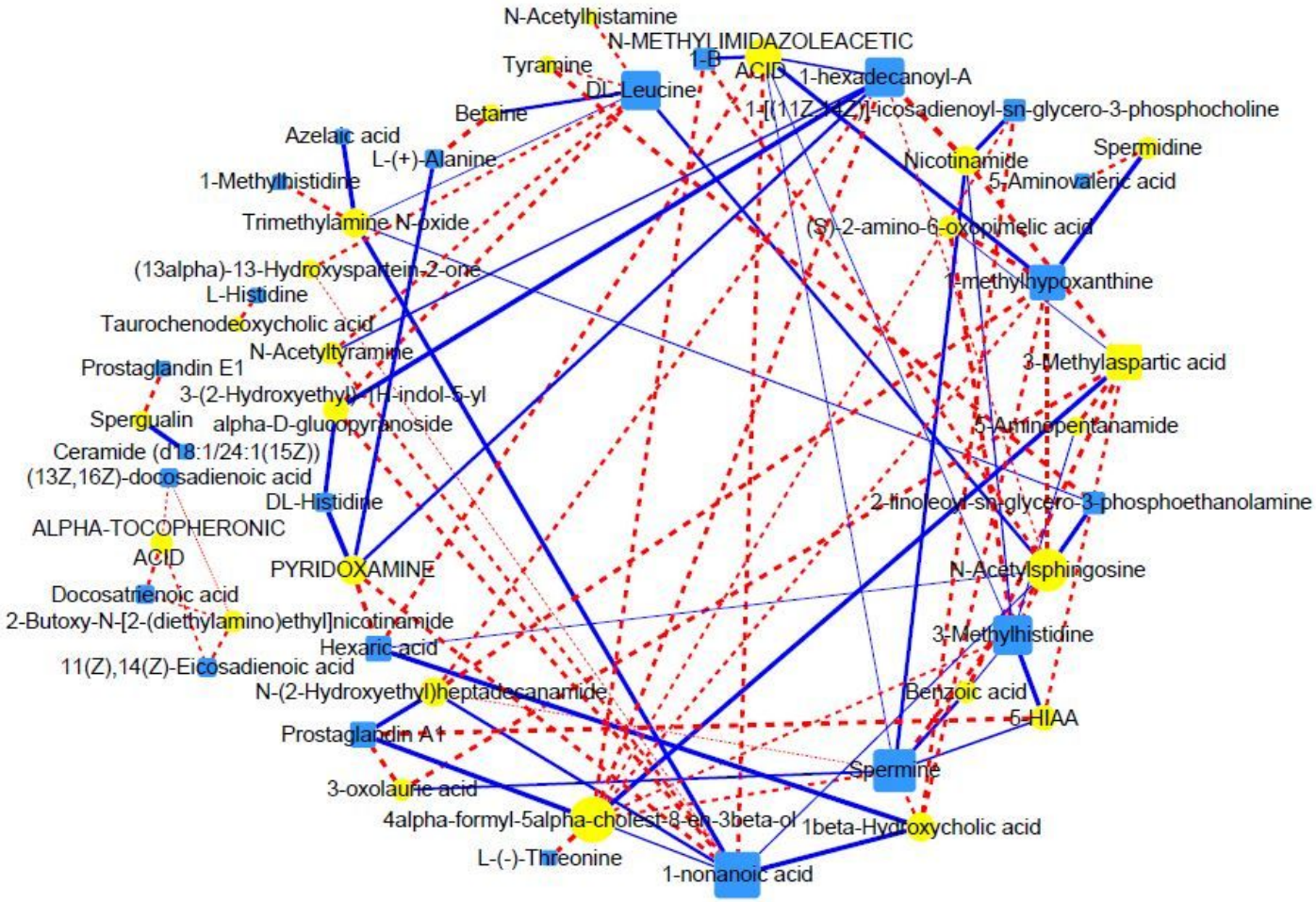

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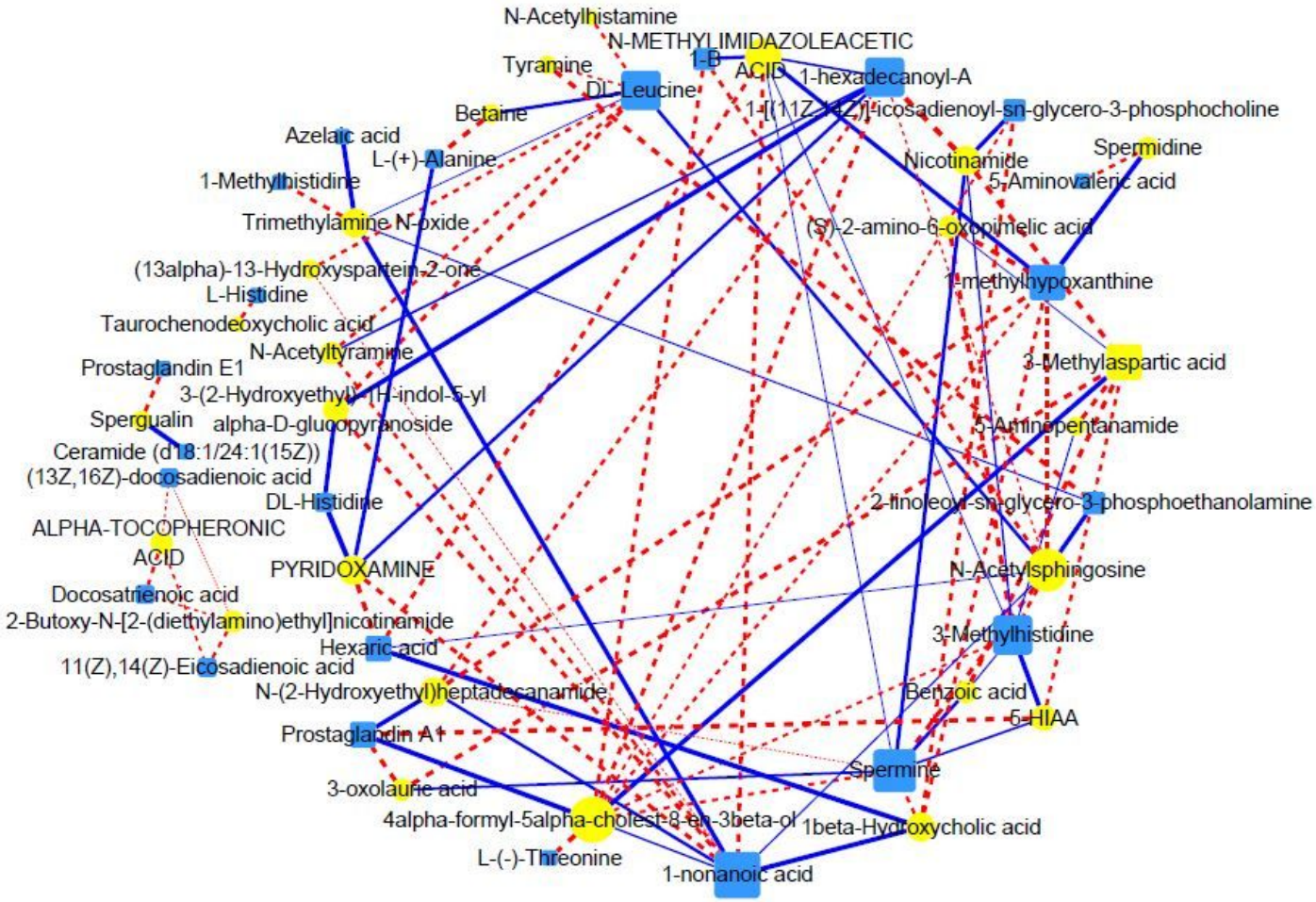

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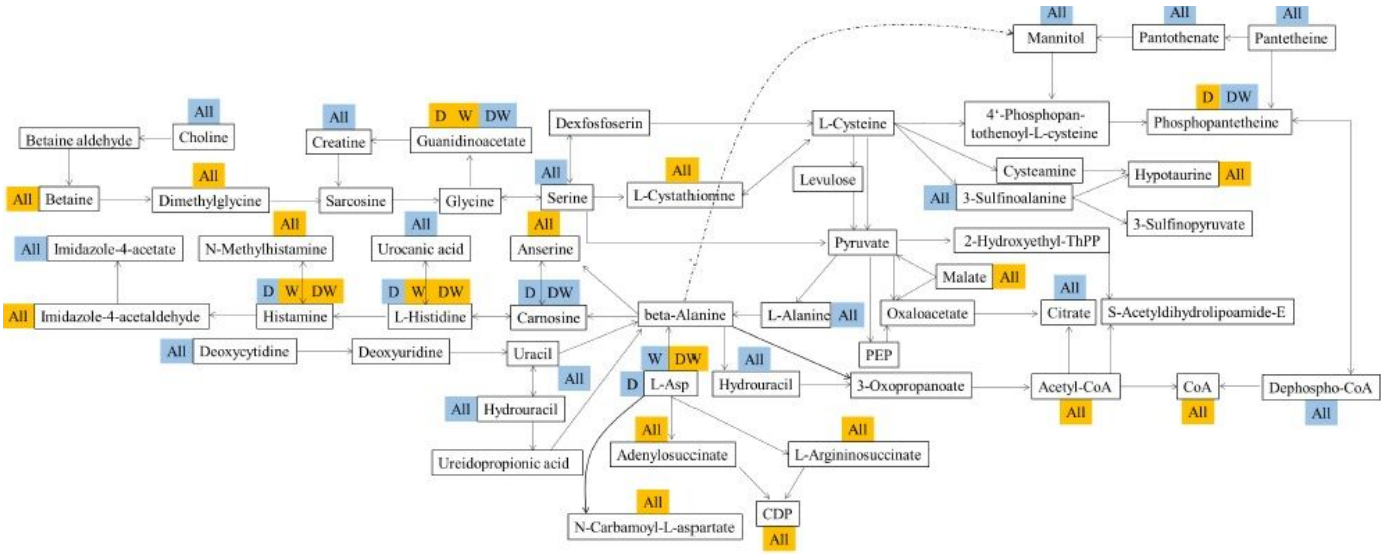
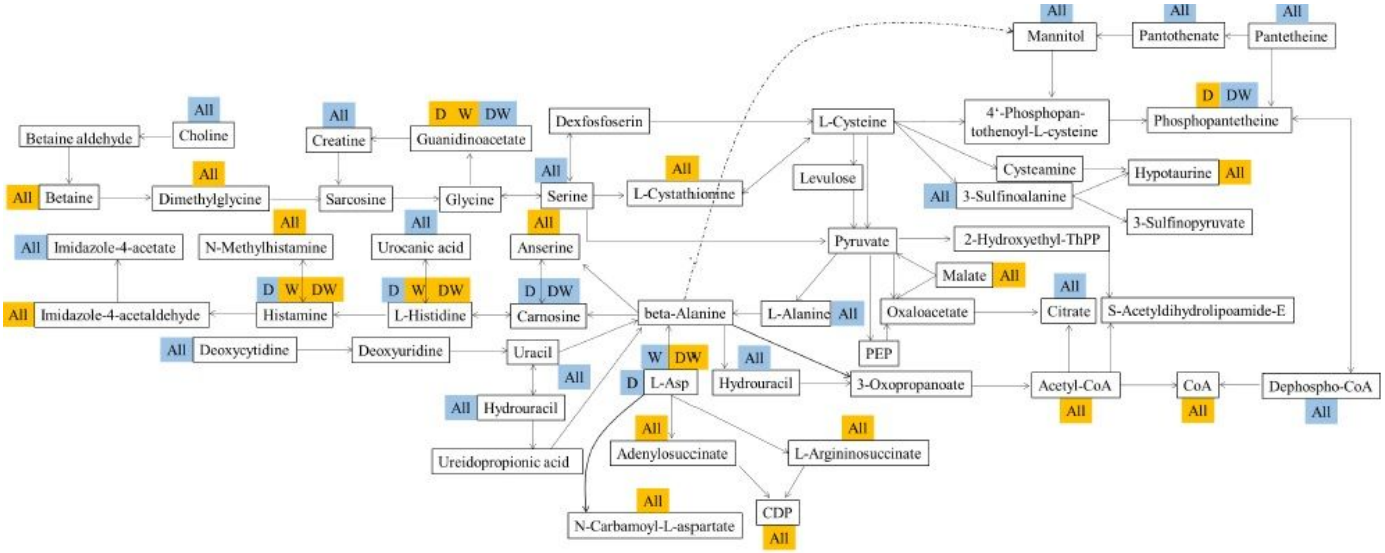


Figure 8

Enriched metabolic pathways in mice liver according to the announced metabolites. orange: upregulation, blue: downregulation, all: D, W, and DW



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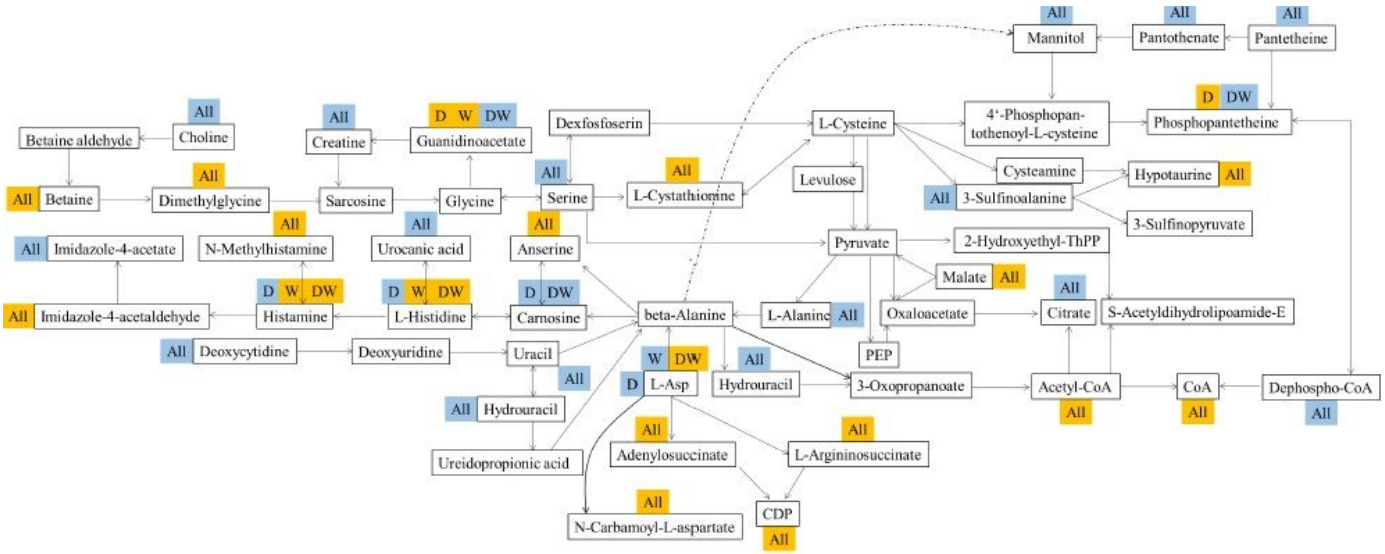

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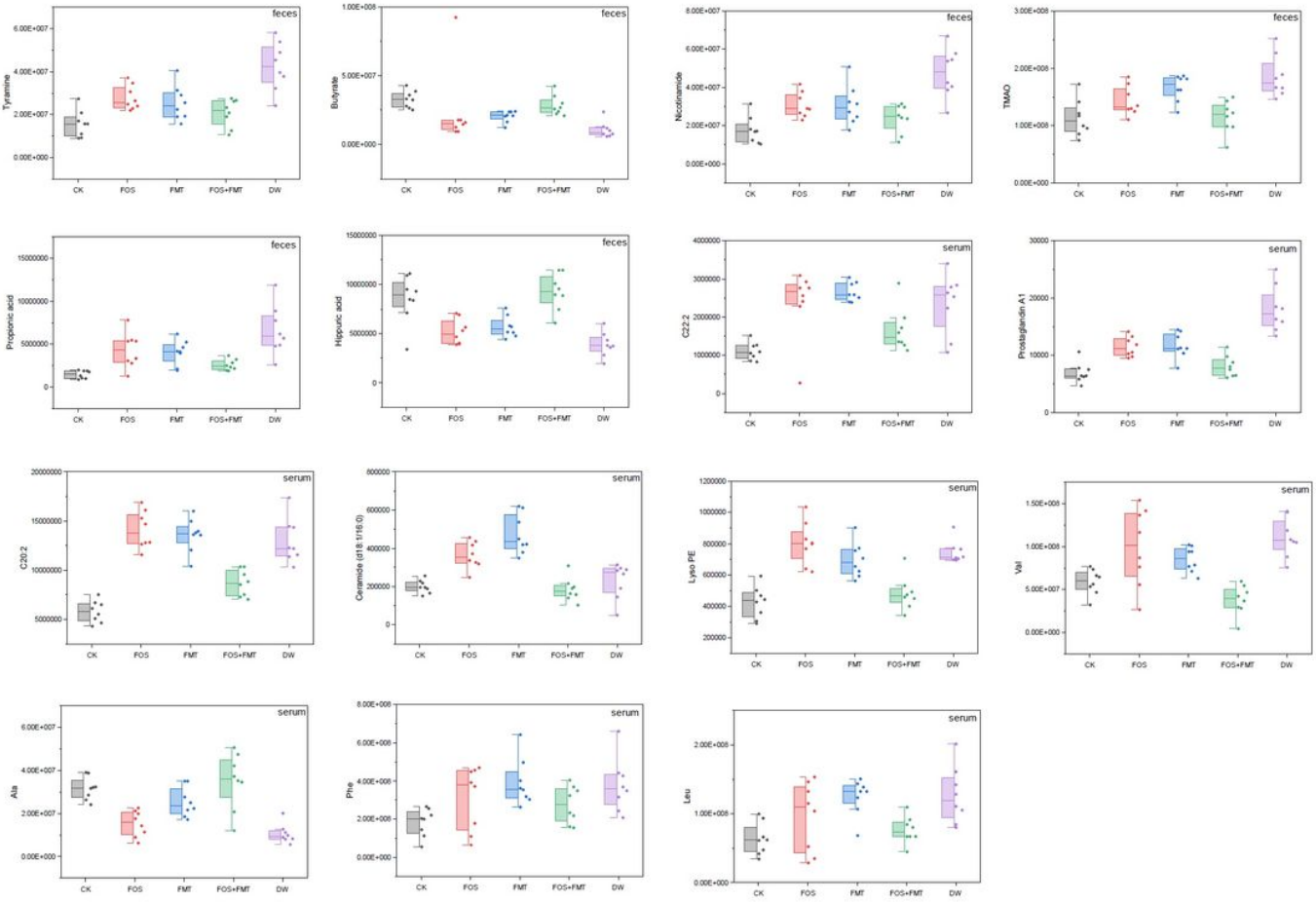
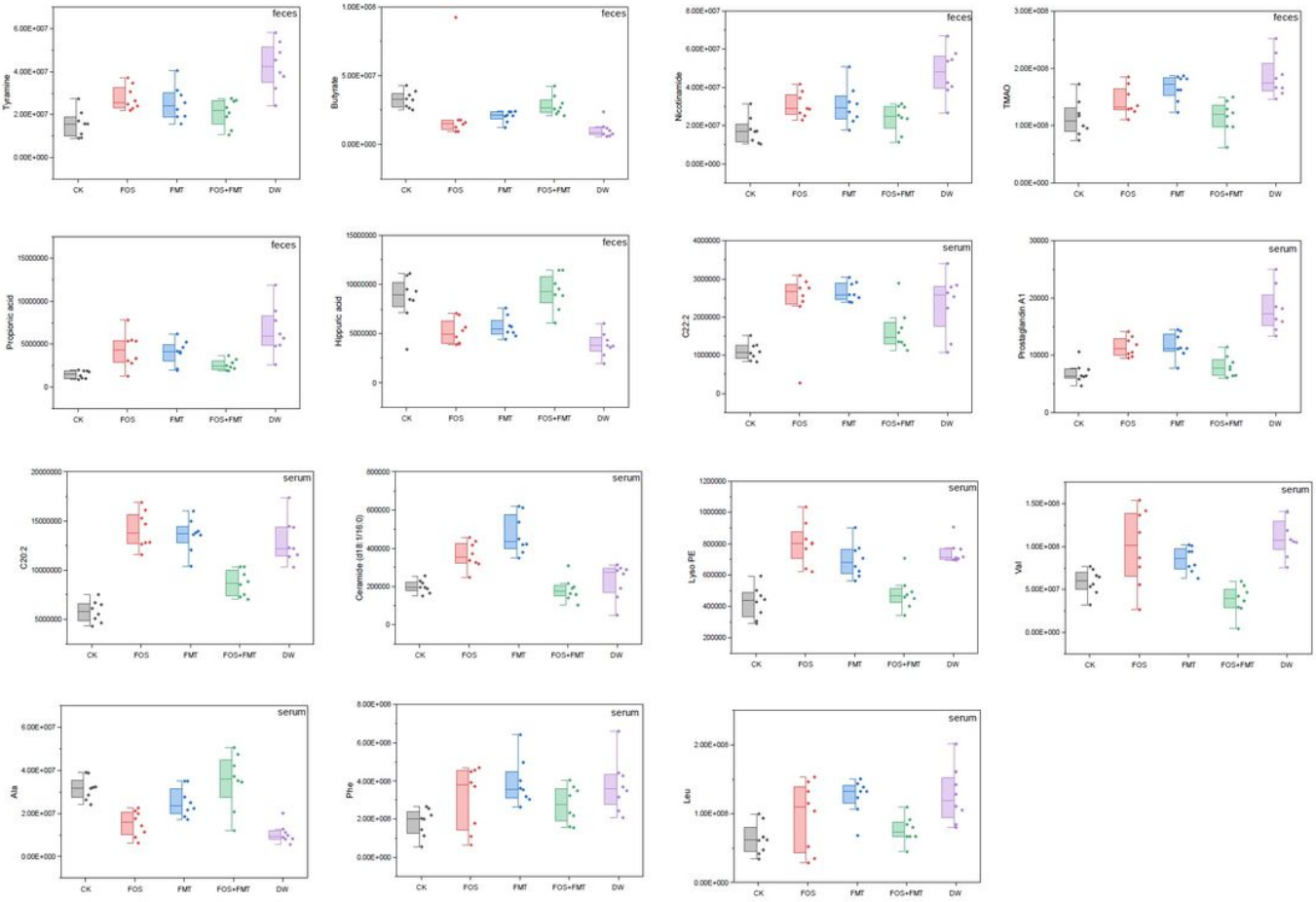


Figure 9

Alterations to metabolites of gut flora and serum after intervention to mice circulation (n=8).



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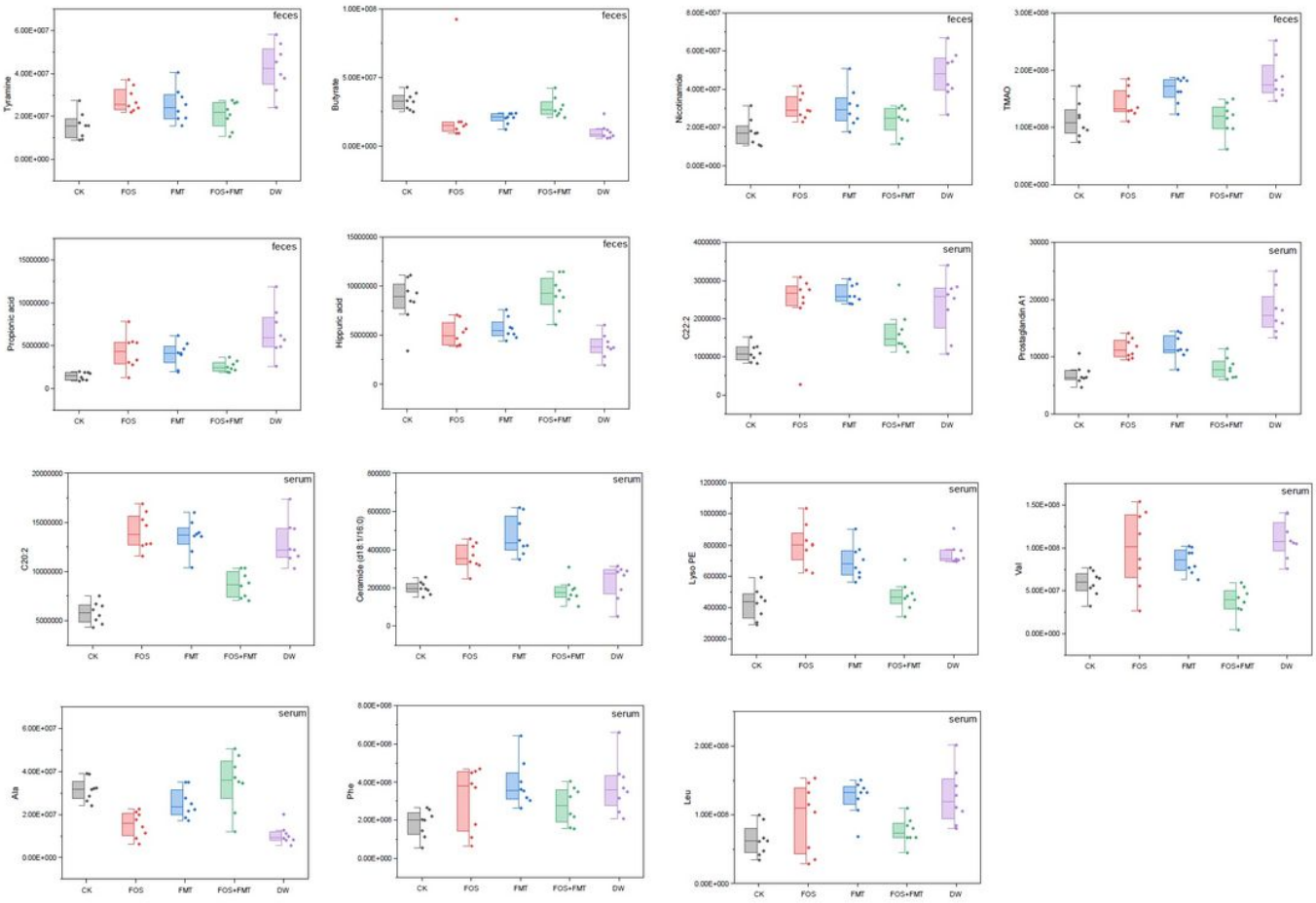

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