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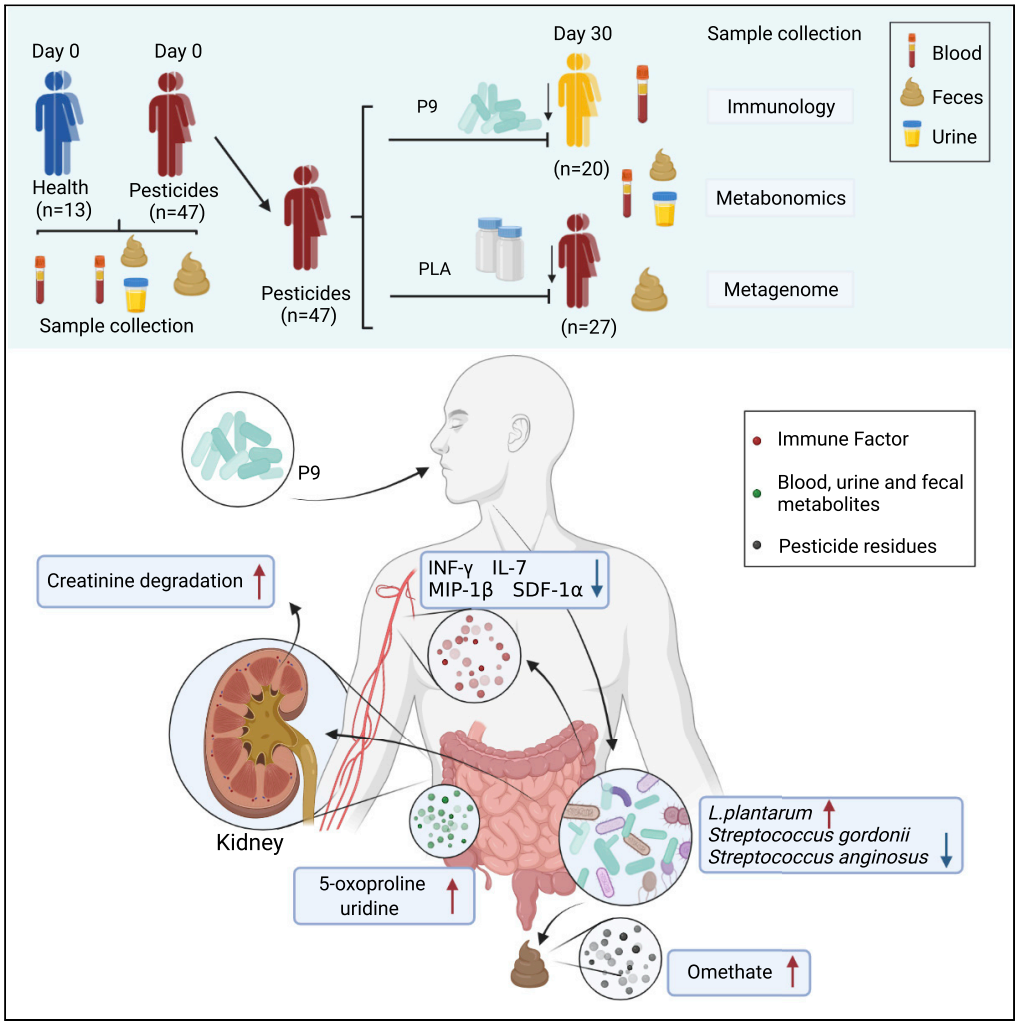
Lactiplantibacillus plantarum P9 improved gut microbial metabolites and alleviated inflammatory response in pesticide exposure cohorts



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Highlights

High-frequency pesticide exposure induced inflammatory responses to occur

P9 maintained gut microbiota homeostasis in subjects with high pesticide exposure

P9 significantly increased the level of beneficial metabolites in the subjects

P9 reduced inflammatory response and promoted excretion of pesticide residues

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Lactiplantibacillus plantarum P9 improved gut microbial metabolites and alleviated inflammatory response in pesticide exposure cohorts

Wenjun Liu,[1,2,4] Changkun Li,[1,4] Bohai Li,[1,4] Qingxiang Shang,[1] Zhe Han,[3] Yuan Zhang,[1] Xiufang Liu,[1] Hui Fan,[1] Jiachao Zhang,[3] Yongfu Chen,[1,*] and Heping Zhang[1,5,*]

SUMMARY

Multiple pesticide residue accumulations increase the probability of chronic metabolic diseases in humans. Thus, we applied multi-omics techniques to reveal how the gut microbiome responded to pesticide exposure. Then, we explored how probiotic *Lactiplantibacillus plantarum* P9 (P9) consumption impacted the gut microbiota and immune factors after high pesticide exposure. Multi-omics results indicated frequent exposure to pesticides did not alter the composition of the intestinal microbiota, but it did increase the abundance of Lipopolysaccharide in the gut, which might contribute to chronic inflammation. Supplementation with P9 maintained the homeostasis of the gut microbiota and reduced the abundance of pathogens in the high pesticide-exposed subjects. By detecting metabolites, we observed uridine and 5-oxoproline concentrations increased significantly after P9 consumption. Furthermore, P9 alleviated immune factors disorder and promoted pesticide residue excretion. Our findings provide new insights into the application of probiotics for pesticide detoxification, and suggest probiotics as daily supplements for pesticide exposure prevention.

INTRODUCTION

Pesticides are considered essential components in maintaining high agricultural productivity in modern farming. A wide variety of pesticides are currently used worldwide, including organophosphates, pyrethroids, and others (Moser et al., 2015). China's agricultural production has experienced the most rapid increase in pesticide use. It now uses 1.5 to 4 times more pesticides than the world average (Zhang et al., 2015), consuming approximately 300,000 tons of pesticides every year (Chu et al., 2018). Accordingly, concomitant pesticide use increases environmental and human health concerns. Dialkyl phosphate metabolites (DAPs), as important metabolites of organophosphorus pesticides *in vivo*, were measured in three populations in Greece. The former had significantly higher median DAP levels than the latter two controls (Koureas et al., 2014). Eight pesticides were found in people living in the village of Burkina Faso located in Africa (Lehmann et al., 2018). Multiple pesticide residue accumulations increase the probability of many chronic metabolic diseases in humans. Therefore, we should pay more attention to the harm of various pesticides synergism on the populations with high-frequency exposure to pesticides.

Acetylcholinesterase (AChE) activity was considered an essential symbol of pesticide poisoning in the human body in previous studies. However, AChE activity changed only when people were exposed to organophosphate and carbamate insecticides. Not all pesticide exposure could lead to inhibition of the activity of AChE. In the general population, biomonitoring of AChE has much higher interindividual variability than the intraindividual variability of cholinesterase (Brock and Brock, 1993). Therefore, it is urgent to develop and validate a new biomarker that is representative and more practical for assessing the damage of multiple pesticides exposure (Barrón Cuenca et al., 2019; Godoy et al., 2019; Lozano-Paniagua et al., 2018). There is evidence from experimental and epidemiological studies to indicate that pesticide exposure may disturb the immune system, as shown by the fluctuation of cytokines. In general, pesticides damage immune cells by inducing oxidative stress, mitochondrial dysfunction, ER stress, disruption of the ubiquitin protease system or autophagy, and inhibition of enzymes with esterase activity (Fukuyama and Tajiki-Nishino, 2020; Mokarizadeh et al., 2015). Therefore, an altered immune system, including inflammatory cytokines and metabolites, may be a susceptibility marker for pesticide-induced immunotoxicity, which

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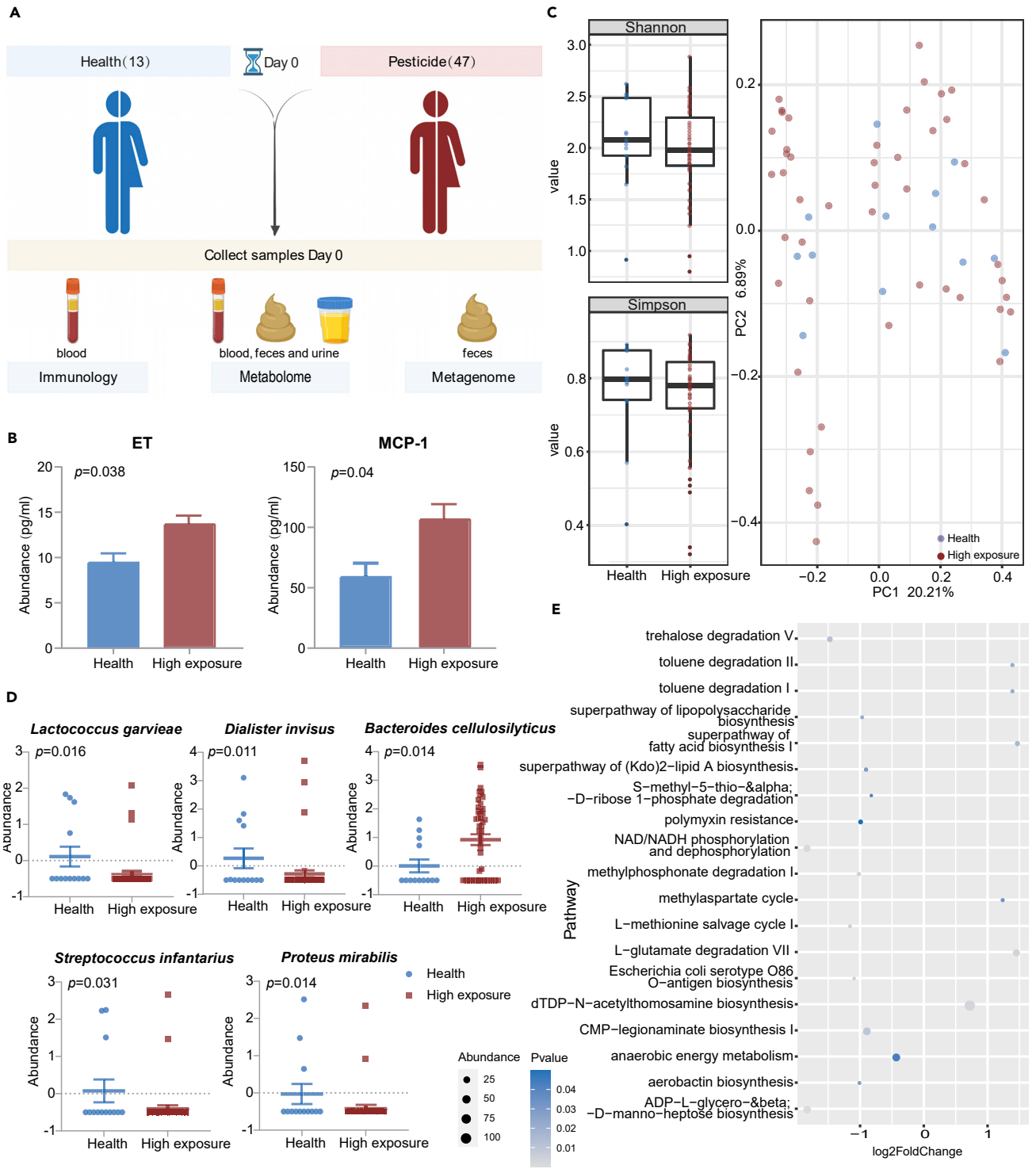


Figure 1. Experimental design and comparative analysis of immune index and intestinal microbiota after high exposure to pesticides

(A) The population was divided into two groups: healthy group (n = 13) and high-pesticide exposure group (n = 47). Samples were collected and the data were analyzed.

(B) Significant difference in immune factors between the healthy group and the high exposure to pesticides ($p < 0.05$), the average value of error line means ± SEM.

Figure 1. *Continued*

(C) Shannon index, Simpson index in healthy group and high-exposure group, and based on the principal coordinate analysis of Bray-curtis, the different color points in the (PCoA) between healthy group and the high-exposure group represented the samples of different groups.

(D) The species with significant changes between the healthy group and the high-exposure group ($p < 0.05$), the average error line means ± SEM.

(E) Significant difference in metabolic pathway between the healthy group and the pesticide high-exposure group, and Log2 Fold Change represented a logarithmic change. The value <0 represented the related metabolic pathway of enrichment in the healthy group, and the value >0 represented the related metabolic pathway of enrichment in the high-pesticide exposure group.

ultimately influences the development of immune-mediated diseases (Corsini et al., 2013; Fukuyama and Tajiki-Nishino, 2020).

Numerous studies have demonstrated that the gut microbiota is involved in basic physiological functions such as immune regulation, nutrient metabolism, and hormone secretion in the host (Valdes et al., 2018). Intestinal microbial consortia can also enhance host protection against environmental contaminants and xenobiotics. The toxicity of xenobiotics can reduced be detoxification or total elimination after bacterial metabolism. A total of 1369 environmental pollutants were shown to be transformed by microorganisms through biocatalytic reactions, which included reduction, hydrolysis, functional group removal, N-oxide cleavage, proteolysis, and denitrification (Claus et al., 2016; Haiser and Turnbaugh, 2013). For pesticide pollutants *in vitro* or *in vivo*, some bacteria have shown excellent degradation and relieve the organ damage induced by pesticides (Li et al., 2018; Zhan et al., 2018). On the other hand, exposure to multiple pesticides could also lead to metabolic disorders by interfering with the structure and function of the intestinal microbiota (Jin et al., 2015; Liang et al., 2019, 2020). Glyphosate, an organophosphorus pesticide, may alter the balance of the honey bee gut microbiota and the early colonization of the intestinal microbiota, increasing the susceptibility of honeybees to pathogens (Motta et al., 2018). Accordingly, the gut microbiota plays a crucial role in the degradation and relief from the harm of multiple pesticides, which may solve the problem of pesticides *in vivo* (Meng et al., 2020). However, to date, little is known about the potential mechanism of pesticide degradation by gut microbiota.

To bridge this gap, the traditional food-sourced probiotic *Lactiplantibacillus plantarum* P9 (P9) was applied to elucidate the regulatory effect of P9 on gut microbiota and metabolites in pesticide-exposed populations by metagenomics and metabolomics techniques. The probiotic P9 was found to possess a high capacity to degrade three commonly used OPPs, namely, dimethoate, phorate, and omethoate, in an *in vitro* study (Li et al., 2018). P9 also has shown degradation activity of phorate and relieved the toxicity of phorate in rats (unpublished data). The present study will lay a foundation for the application of probiotics in the field of pesticide detoxification. Our study divided the population into two groups, first to study how high-frequency exposure to pesticides in healthy people can harm health and then to apply probiotics to patients with high exposure to pesticides. The therapeutic effect of the probiotics was evaluated from part to whole by the multigroup method. Given the widespread pesticide pollution and its harm to human health, it is of great significance to explore the interaction mechanism between probiotics and pesticide exposure and the host intestinal microbiota. This is of great significance for the future application of probiotics to relieve the toxicity of pesticides on human health.

RESULTS

Here, we recruited 60 subjects to participate in this experiment and divided them into two groups based on their degree of pesticide exposure. First, we explored how the gut microbiota responded to pesticide exposure (Figure 1A). Then, we further divided the population into two groups to reveal how probiotic intervention impacted the gut microbiota and immune factors during high pesticide exposure (Figure 3A).

Pesticide exposure triggered inflammatory responses in humans and altered the human intestinal microbiome

To explore how the human intestinal microbiome and immune system responded to high-frequency pesticide exposure, we detected various indicators of the tested population. First, in terms of immune markers, we found a significant increase in the concentration of two inflammatory markers (ET endothelin, $p = 0.038$; MCP-1 monocyte chemotactic protein-1, $p = 0.04$) (Figure 1B). Both ET and MCP-1 are associated with kidney disease, meaning that large amounts of exposure to pesticides may indirectly damage the kidneys.

Second, we explored intestinal microbiota changes in healthy people by evaluating the diversity of the intestinal microbiome among the groups by their degree of pesticide exposure. There was no significant difference between the high-pesticide exposure group and the healthy group (Figure 1C). Interestingly, we found significant variations in species abundance between the healthy and high-exposure groups (Figure 1D). In addition, we found that the abundance of *Bacteroides cellulosilyticus* increased significantly in the high-exposure group, while the abundance of *Lactococcus garvieae* and *Proteus mirabilis* decreased significantly. Finally, we also found that changes in the metabolic pathways of some intestinal microbiota were caused by alterations in the abundance of species. High pesticide exposure caused significant changes in 19 pathways (Figure 1E). The abundance of L-glutamate degradation VII (synthesis of butyric acid) and the DTDP-N-acetylthyroxine biosynthesis pathway in the high-exposure group was significantly higher than that in the healthy group.

High exposure to pesticides induced disruption of hormone metabolism

From the above results, we observed that high pesticide exposure would affect the metabolic pathways of microbes, so we further verified the changes in metabolites. There was a clear trend of separation of fecal metabolites between the healthy and highly exposed groups, and we looked for some differential metabolites (Figure 2A). The hormone metabolites (androstenedione and 2,3-dinor-8-iso-PGF2alpha) were significantly increased in the high-exposure group (Figure 2B). These metabolites were related to the steroid hormone biosynthesis and arachidonic acid metabolism pathways. These results indicated that exposure to pesticides disturbed hormone metabolism *in vivo*. Hormone biosynthesis disturbance was also found in another pesticide exposure cohort study (Freire et al., 2021). In addition, the lipid metabolites PE (20:0/0:0) and PC (12:0/0:0) were also increased significantly in the high-exposure group, indicating that pesticides could promote biosynthesis in the intestine or increase the synthetic ability of the intestinal microbiota. Based on the metabolites in serum and urine, the intestine was the most vulnerable organ to pesticide exposure.

The potential relationship between the inflammatory response and high pesticide exposure

Based on the above analysis, association analysis was performed to explore the relationship between pesticide exposure frequency, intestinal microbiota, metabolic pathways and products, and immune factors (Figure 2C). The results showed that the metabolic pathway of lipopolysaccharide biosynthesis exhibited a positive correlation with *Dialister invisus* in healthy populations after high-frequency exposure to pesticides ($r = 0.97$). MCP-1 was positively correlated with *D. invisus* ($r = 0.45$). The metabolic pathway of lipopolysaccharide synthesis is the main pathway of lipopolysaccharide synthesis in Gram-negative bacteria. Lipopolysaccharide affects the normal function of the host immune system, which mediates the increase in related metabolic pathways and metabolites. It then affects immune factors, leading to the occurrence of inflammatory reactions.

Consumption of the probiotic *L. plantarum* P9 promoted the excretion of pesticide residues and alleviated inflammation due to pesticide exposure

The volunteers showed significant changes in immune factors in their bodies after P9 supplementation. We compared the immune indicators between 0 and 30 days of P9 ingestion, and we found that the levels of proinflammatory factors such as INF-γ, SDF-1α, and IL-7 decreased significantly (Figure 3B). The expression level of macrophage inflammatory protein-1β (MIP-1β) was also significantly decreased in the highly exposed population after P9 administration. There was no significant change in the placebo group. In addition, significant changes in the levels of VC and VD were observed in the P9 group (Figure S1A). We also measured the urinary albumin (ALB) levels in the subjects, and unfortunately, while P9 significantly affected the expression of ALB it did not restore it to normal (Figure S1B). All of these results suggest that P9 intake alleviated the inflammatory response in people highly exposed to pesticides.

We also compared the changes in fecal levels of pesticide residues and showed that fecal levels of omethoate were significantly higher after P9 intake, indicating that P9 aided the excretion of pesticide residues in patients with high pesticide exposure (Figure 3C).

L. plantarum P9 maintained host intestinal microbial homeostasis

The above results indicated that probiotics are effective in reducing inflammation; therefore, the changes in the gut microbiota produced by P9 intervention are of particular importance. There were no significant

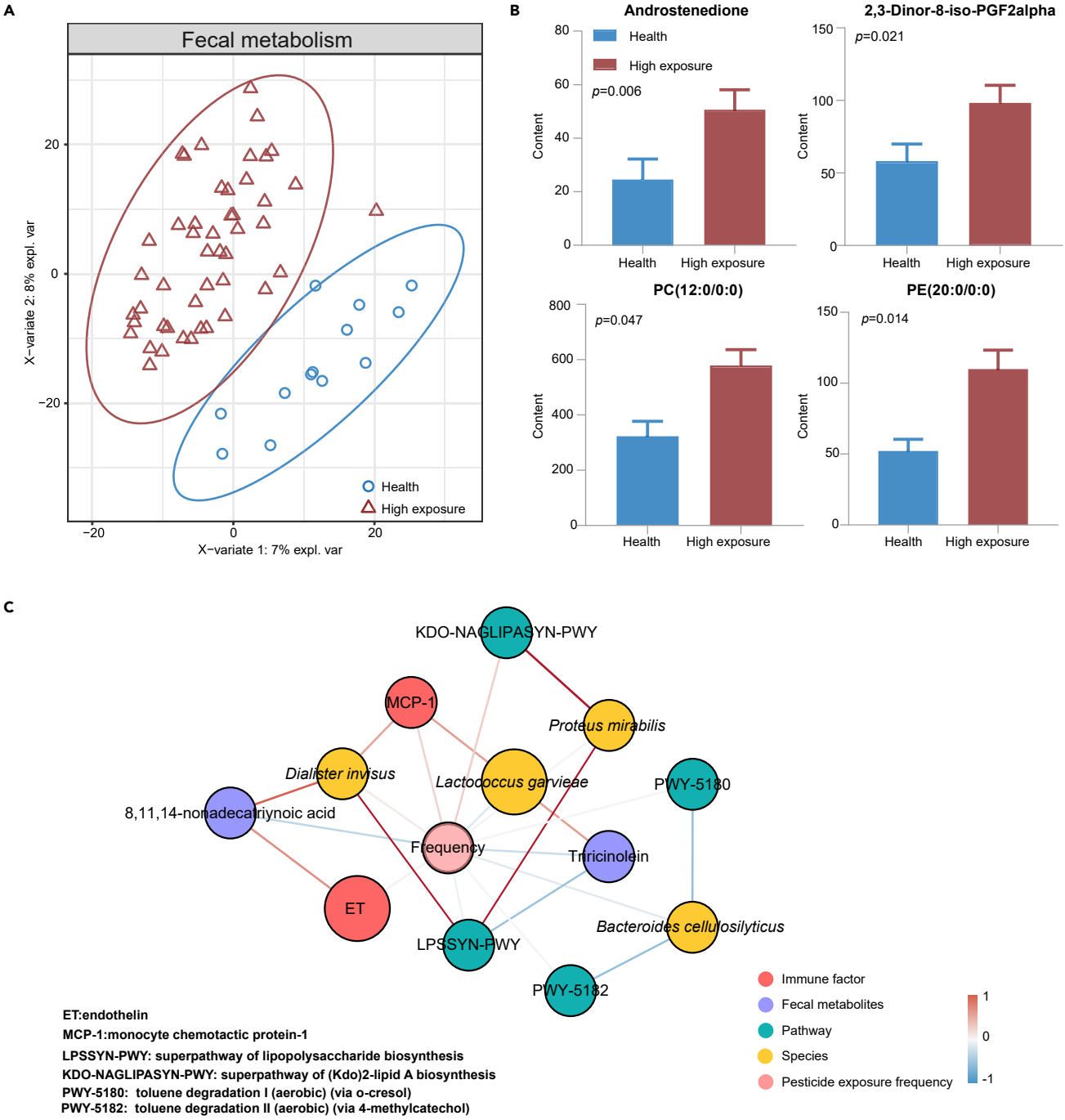


Figure 2. Analysis of fecal metabolites and creation of network diagrams in the healthy and highly exposed groups

(A) Partial Least Squares Discriminant Analysis of stool samples in the healthy group vs the high-exposure group.

(B) The metabolite with significant changes between the healthy group and the high-exposure group ($p < 0.05$), the average error line means ± SEM.

(C) Correlation network between species, metabolic pathways, metabolites, pesticide exposure frequency, and immune factors that underwent differential changes after high pesticide exposure. Edge widths and colors (red represents positive correlation, blue represents negative correlation.) were proportional to the correlation strength of the Pearson rank.

differences between subjects in the P9 groups in terms of gut microbial structure on days 0 and 30 ($p > 0.05$). However, the P9 group was closer to normal. The intake of P9 maintained the stability of the intestinal microbiota of the subjects. Interestingly, the abundance of *L. plantarum* increased significantly

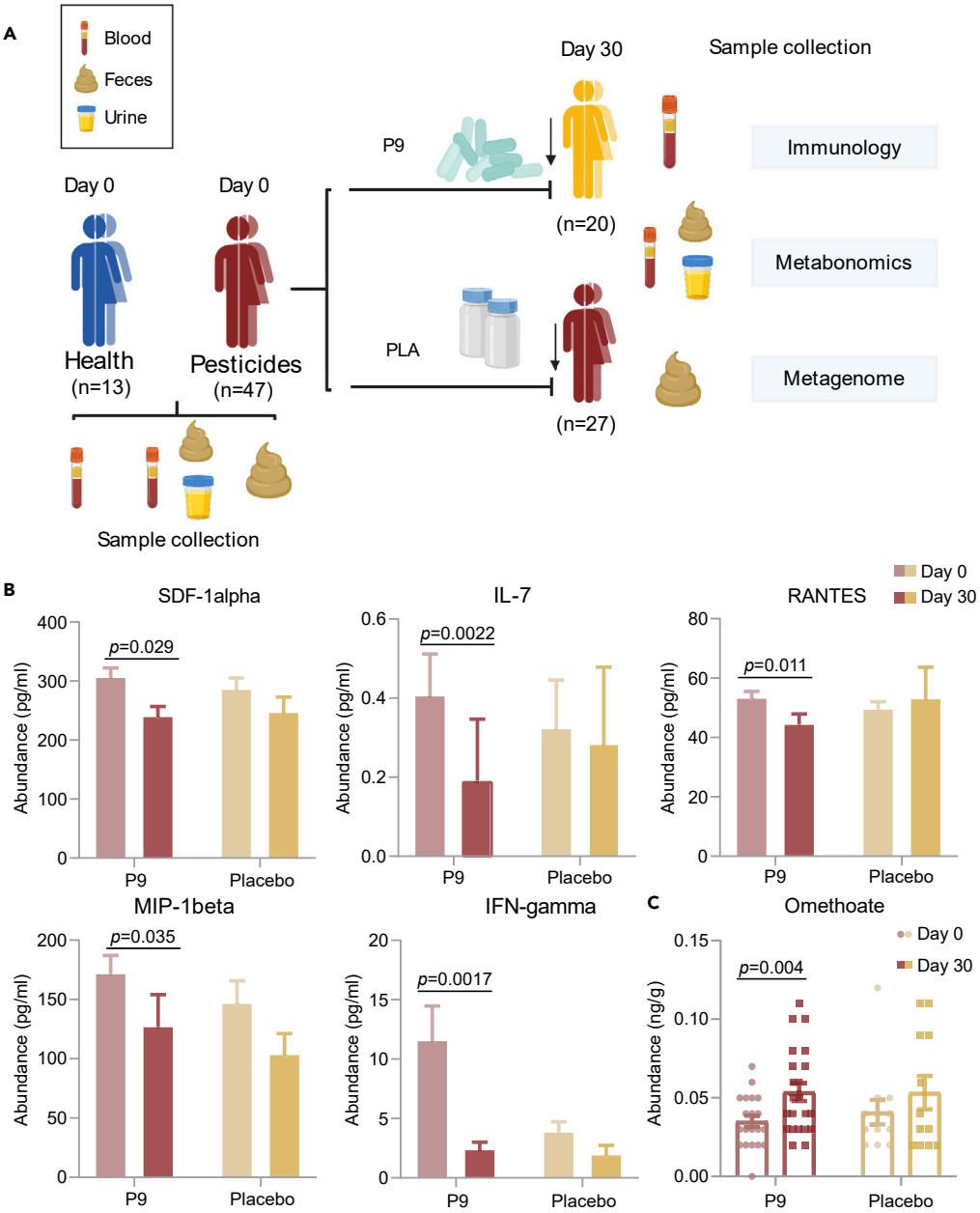


Figure 3. Experimental design and effect of P9 intake on immune factors and fecal pesticide residues in the P9 group and placebo group.

(A) The high-exposure population was divided into two groups: P9 treatment group (n = 20) and placebo group (n = 27). Serum, urine, and stool samples were collected and analyzed after continuous administration of placebo and P9 for 30 days. P9 bacterial powder was supplemented from the beginning of the experiment (i.e. 0 days) and continued for 30 days.

(B) Immunity factors with significant changes at the end of the P9 intervention compared to day 0 ($p < 0.05$), error line is mean ± SEM.

(C) Expression of fecal pesticide residues omethoate in different groups (See also Figure S1).

after 30 days of P9 ingestion, followed by a significant decrease in the abundance of pathogens such as *Streptococcus gordonii* and *Streptococcus anginosus* (Figure 4B). *L. plantarum* P9 can influence the intestinal microbiota of people highly exposed to pesticides to some extent, increasing the abundance of beneficial species while decreasing the level of pathogenic species (Figure S1C).

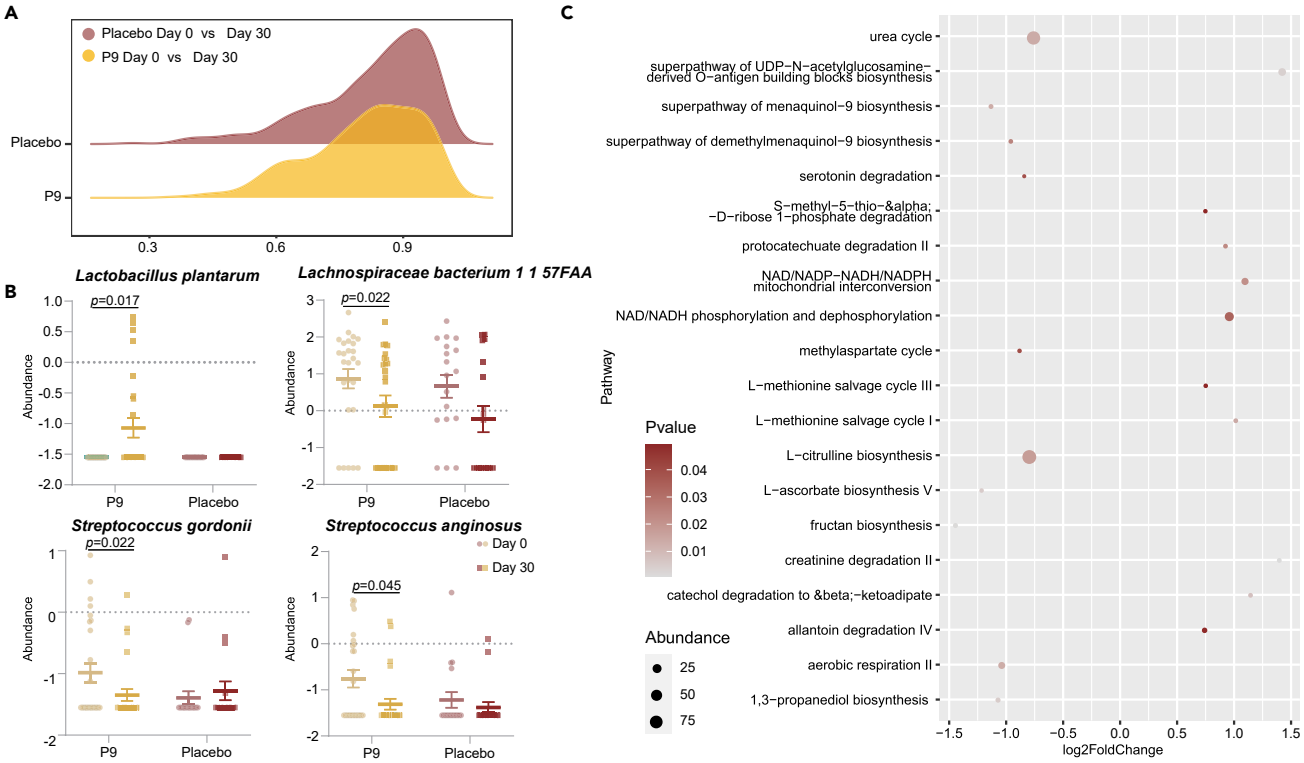


Figure 4. Experimental design and intestinal microbiota after P9 intervention

(A) Kernel density estimates based on Bray-Curtis distances, with different color representing different groups of samples at different times.

(B) Significant difference species between healthy and high-exposure groups of changes in ($p < 0.05$), error line means ± SEM.

(C) Significant differences in metabolic pathways before and after P9 intervention, and Log2 Fold Change represented a logarithmic change. Values < 0 represent enrichment of relevant metabolic pathways at day 0 and values > 0 represent enrichment of relevant metabolic pathways at day 30 (See also Figure S1).

On this basis, we then investigated the effect of P9 intervention on metabolic pathways in the gut microbiota. P9 intervention significantly upregulated pathways such as creatinine degradation II and allantoin degradation IV while significantly decreasing metabolic pathways such as L-citrulline biosynthesis and serotonin degradation (Figure 4C).

Ingestion of *L. plantarum* P9 led to significant changes in metabolism in people with high pesticide exposure

After understanding the effects of P9 on the gut microbiota of the subjects, we investigated the changes in the metabolome induced by P9 intervention, including the serum, urine, and fecal metabolomes. We compared the metabolites of the three fractions at two time points before and after the intervention and screened out some metabolites with differences. The results showed that after 30 days of P9 administration, fecal levels of chenodeoxycholic acid sulfate were significantly lower, while triprolidine levels were significantly higher (Table S3). Urine metabolite levels of methylpyrazine, retinoic acid, and vanillactic acid were significantly reduced after 30 days of P9 intervention (Table S4). In addition, serum metabolites such as 4-3-pyridyl-butanoate, 11-deoxycorticosterone, uridine, and 5-oxoproline were significantly increased, while 4-alpha-hydroxymethyl-5, alpha-cholesta-8,24-dien-3, and beta-ol were significantly decreased (Table 1). In summary, under the influence of P9, the metabolites of the human body changed significantly.

The potential mechanism of the interaction between probiotics and intestinal microbes to reduce inflammation

The above results suggest that P9 intake in a population with high pesticide exposure can stabilize intestinal microecological stability and alleviate inflammation, so we explored in depth the potential relationship between probiotics and inflammatory factors. We performed a correlation analysis ($r > 0.4$) of strains,

Table 1. The biomarker metabolites in serum after P9 intake

Metabolites	Fold change[a]	p value[b]	Pathway
Uridine	10.27	0.0040	Pyrimidine ribonucleosides salvage I/UTP and CTP dephosphorylation I
3-methoxy-4-hydroxyphenylglycol	6.00	0.0002	Noradrenaline and adrenaline degradation
S-formylglutathione	4.47	0.0191	formaldehyde oxidation
3,4-dihydroxy-5-all-trans-nonaprenylbenzoate	2.98	0.0000	
5-oxoproline	2.90	0.0054	
3,4-dihydroxy-5-all-trans-decaprenylbenzoate	2.44	0.0030	ubiquinol-10 biosynthesis
2-oxoglutarate	2.43	0.0328	Citrate cycle (TCA cycle)/Glyoxylate and dicarboxylate metabolism/ Biosynthesis of terpenoids and steroids/Biosynthesis of alkaloids derived from shikimate pathway
β-carotene 15,15′epoxide	2.42	0.0007	
Reduced riboflavin	2.39	0.0001	
4-hydroxy-2-nonenal-N-acetyl-L-cysteine	2.17	0.0009	4-Hydroxy-2-nonenal detoxification
Norspermine	0.50	0.0013	
Alpha-D-ribose 1,5-bisphosphate	0.47	0.0000	
4alpha-hydroxymethyl-5alpha-cholesta-8,24-dien-3beta-ol	0.47	0.0000	
Hyodeoxycholate	0.46	0.0020	
Resolvin E2	0.45	0.0007	Aspirin-triggered resolvin E biosynthesis
SII-trans-10′-apo-beta-carotenal	0.44	0.0003	
(E,Z)-2,6-Farnesal	0.44	0.0000	
Simvastatin acid	0.43	0.0143	
Simvastatin lactone	0.41	0.0000	
11-desoxycortisol	0.41	0.0001	Glucocorticoid biosynthesis
Estrone-sulfate	0.37	0.0027	
3-acetamidopropanal	0.19	0.0032	Spermine and spermidine degradation I
PPP_i	0.07	0.0000	Tetrahydrobiopterin *de novo* biosynthesis
11-desoxycortisol	0.03	0.0000	Glucocorticoid biosynthesis

See also Tables S3 and S4.

[a]Fold change were the ratio of metabolites relative concentration in day 30 to day 0 of P9 group.

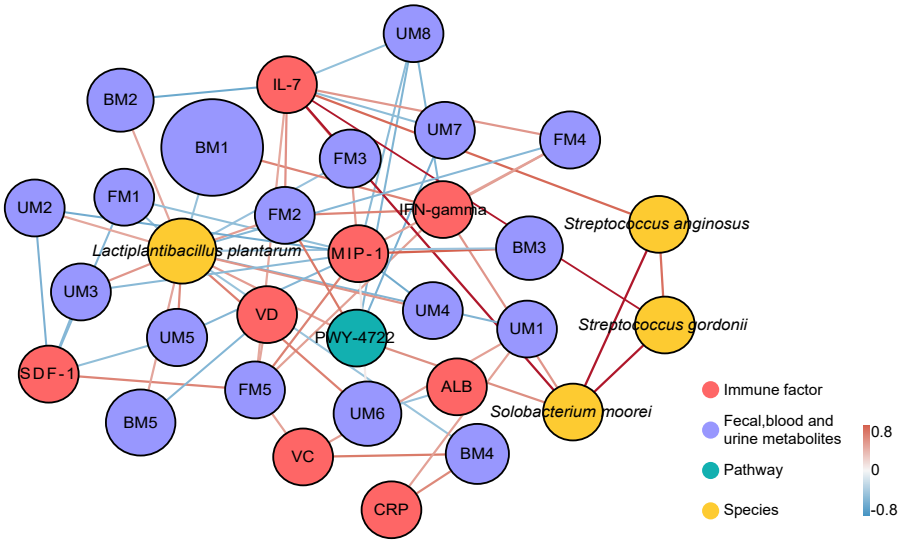
[b]p represents the adjusted p value ($p < 0.05$ and fold change >2).

immune factors, metabolic pathways, and metabolites that produced significant changes before and after P9 consumption (Figure 5A). The results showed a positive correlation between P9 and the degradation of creatinine II and a strong correlation between related metabolites and immune factors guided by this metabolic pathway. In addition, metabolites associated with *L. plantarum* were also positively correlated with immune factors, among others. Therefore, we drew a mechanism map based on the whole network diagram to roughly describe the potential mechanisms by which *L. plantarum* alleviates the inflammatory response by modulating the intestinal microbiota (Figure 5B). All of these results suggest that *L. plantarum* P9 plays a key role in alleviating the onset of the inflammatory response triggered by pesticide exposure.

DISCUSSION

Here, the subjects underwent significant changes in some species of the intestine, such as *D. invisus*, during frequent exposure to pesticides. *D. invisus* was previously reported to induce an inflammatory response in

A



FM1: Dehydroxyzyleuton
FM2: Hexylresorcinol
FM3: PA(22:0/18:3(9Z,12Z,15Z))
FM4: 4alpha-carboxy-5alpha-cholesta-8-en-3beta-ol
FM5: 17Z-octadecenoic acid
PWY-4722 : creatinine degradation II

BM1: 2-Phosphoglycolate
BM2: 5-Formiminotetrahydrofolate
BM3: Sulfate
BM4: Dolichyl diphosphate
BM5: trans-2-Enoyl-OPC6-CoA
BM6: 9-cis-Retinoic acid

UM1: Vecuronium
UM2: Riboflavin reduced
UM3: Epicatechin 3-O-(3-O-methylgallate)
UM4: 2,5-Dihydro-4,5-dimethyl-2-thiazole
UM5: N-methyl arachidonoyl amine
UM6: Cer(d18:0/15:0)
UM7: Epicatechin 3-O-(4-methylgallate)
UM8: 13'-carboxy-alpha-tocopherol

B

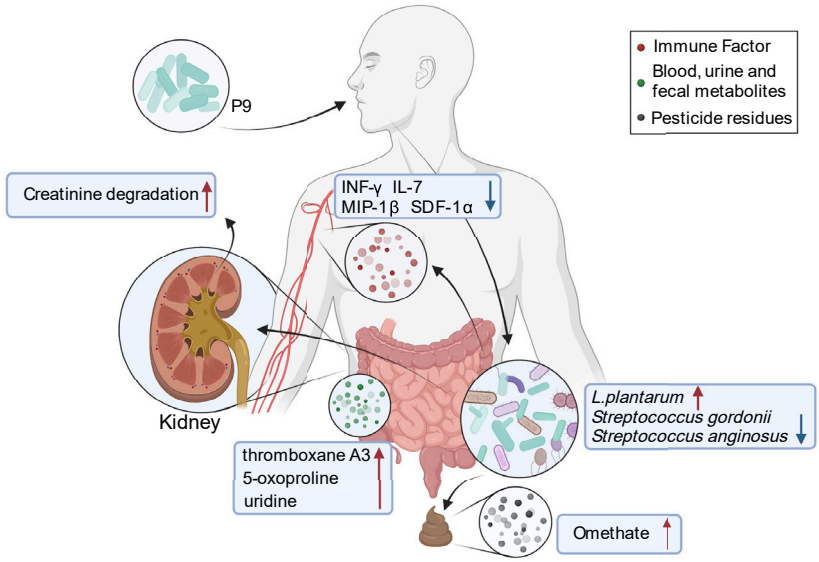


Figure 5. The network of bacteria, metabolic pathways, metabolites, and immune factors that changed before and after the intake of P9 in people with high pesticide exposure

(A) Edge width and color (red represents positive correlation, blue represents negative correlation) is proportional to the correlation intensity of Pearson rank correlation coefficient.

(B) Potential mechanism of *Lactiplantibacillus plantarum* P9 regulating intestinal microbiota and alleviating inflammation in patients with high pesticide exposure.

the host during a variety of mixed infections by producing endotoxin (Domann et al., 2003; Kaakoush et al., 2015). Supporting these analyses, *D. invisus* was positively correlated with the superpathway of lipopolysaccharide biosynthesis (r = 0.97). A large number of studies have shown that pesticides could perturb the gut microbiota and increase the level of endotoxin in the intestinal tract, which activates the NF-κB pathway and induces inflammatory responses (Chmelar et al., 2019; Zhang et al., 2016). *L. plantarum* P9 (P9) administration showed protective effects against pesticide-induced intestinal microbiota disorder, as it significantly decreased the abundance of pathogenic bacteria such as *S. gordonii* and *S. anginosus* and significantly increased the abundance of *L. plantarum*. P9 administration decreased the abundances of endotoxin-producing intestinal microbes such as *D. invisus*, which may reduce inflammatory responses caused by endotoxin.

The abundance of *L. plantarum* was also positively correlated with the metabolic pathway creatinine degradation II (r = 0.47). The increased activity of the metabolic pathway creatinine degradation II accelerated the degradation of creatinine, which alleviated the pressure brought by the pesticides on the kidney (Jerez-Morales et al., 2021). This may be a protective mode of P9 against pesticide-induced harmful effects in humans.

As an exogenous toxic compound, pesticide-induced tissue inflammation has been verified in many *in vivo* and *in vitro* experiments (Corsini et al., 2013; Jacobsen-Pereira et al., 2020). In a farmers cohort study, proinflammatory IL-6 in the subjects with long-term exposure to mixtures of pesticides (fungicides, insecticides, and herbicides) was significantly elevated (p = 0.04) in the plasma compared to that of controls. Circulating inflammatory biomarkers such as IL-6, TNF-α, monocyte chemotactic protein 1 (MCP-1), and E-selectin are associated with a variety of metabolic disorders (Goldberg, 2009).

In our study, the inflammatory factors ET and MCP-1 were also significantly increased in the plasma of the high-exposure group. ET and MCP-1 are two types of immune factors closely related to kidney disease that are highly expressed in many kinds of nephropathy and can cause different degrees of damage to the affected kidney in a variety of ways. Therefore, ET and MCP-1 have important significance as clinical diagnostic indicators of kidney disease (Ciarambino et al., 2021; Giraldi et al., 2020; Puthumana et al., 2021; Srivastava et al., 2021). In conclusion, inflammation caused by pesticides may be an important inducer of other secondary diseases, and ET and MCP-1 were the key factors by which pesticides interfered with the immune system in this study.

The intake of *L. plantarum* P9 improved these inflammatory factors. Downstream metabolites of creatinine metabolic pathway II, such as 2-phosphate and PA (22:0/18:3 (9Z, 12Z, 15Z)), were positively correlated with P9 and negatively correlated with proinflammatory factors such as MCP-1 and IL-7. 2-Phosphoglycolate salvage metabolism was previously identified in the genomes of *Lactobacillus delbrueckii* and *Lactobacillus johnsonii* (Ortman et al., 2020). Since the intake of P9 increased the abundance of *L. plantarum*, creatinine metabolic pathway II was significantly upregulated and thus the content of proinflammatory factors IL-7 and MCP-1 decreased, alleviating the inflammatory response, all of which proved the therapeutic effect of P9 on high-pesticide exposure people.

In the serum, uridine, one of four components that comprise RNA, has been reported to have an anti-inflammatory effect in many inflammatory animal models; for example, in acute lung injury, bronchial asthma (Cicko et al., 2015), and dextran sulfate sodium (DSS)-induced colitis in mice (Jeengar et al., 2017), 5-oxoproline has been found to be significantly related to a lower risk of incident chronic kidney disease (Yu et al., 2014), and it was a significantly increased biomarker in the serum after intake of P9 by the high-pesticide exposure group. This indicates that 5-oxoproline may be a beneficial metabolite to the kidney due to the moderating effect of P9. Moreover, in previous studies, compared with the control group, *L. plantarum* administration attenuated the inflammatory response and improved the intestinal microbiota richness in the organophosphorus pesticide rat model, which is consistent with the results of the present study. These findings suggest that treatment with *L. plantarum* P9 could regulate the disturbed intestinal microbiota and prevent further inflammation in people suffering from high pesticide exposure.

Overall, we have drawn three conclusions from the present study. First, frequent exposure to pesticides can increase the expression of proinflammatory factors in the body and trigger the onset

of chronic inflammation. *L. plantarum* P9 could moderate the inflammatory response induced by high-frequency pesticide exposure and promote the excretion of pesticide residues by increasing the abundance of the probiotic *L. plantarum* in the pesticide-exposed population. Finally, metabolites produced by P9 itself or by the host under the regulation of P9 also play an important role in the anti-inflammatory induction of pesticides. Our findings provide new insights into the application of probiotics in the field of pesticide detoxification and demonstrate objective and detailed data supporting the application of *L. plantarum* P9 as a supplement for the prevention of pesticide exposure in daily life.

Limitations of the study

Our study elucidates that high frequency of pesticide exposure can lead to increased synthesis of lipopolysaccharides and induce inflammatory responses in healthy populations. The volunteers included in this study were exposed to a combination of pesticides including organophosphorus and organochlorine, focusing on the hazards of the combination of pesticides on humans while the hazards of single pesticide exposure on humans are not clear; therefore, we expect that in future studies the hazards of single pesticides on the organism can be clarified based on the gut microbiota.

STAR★METHODS

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  - Data and code availability
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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

H.Z. and Y.C. designed the project; Z.H., C.L., and J.Z. wrote the manuscript; B.L., C.L., W.L., Q.S., Y.Z., X.L., and H.F. collected samples and performed experiments; Z.H., C.L., B.L., and J.Z. analyzed the data and generated graphics. All authors read and approved the final paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Lactiplantibacillus plantarum P9	Inner Mongolia Agricultural University	N/A
Biological samples		
Human Stool Samples	This study	N/A
Human Serum Samples	This study	N/A
Human Urine Samples	This study	N/A
Chemicals, peptides, and recombinant proteins		
ready-to-use QuEChERS salt powder	Paya et al. (2007)	N/A
Critical commercial assays		
NEBNext® Ultra™DNA Library Preparation Kit	NEB	N/A
Albumin (ALB) Elisa kit	Jiuqiang corporation	N/A
InvitrogenTM cytokine & phemokine convenience 34-plex human procartaplex panel 1A	Thermo Fisher Scientific	N/A
QIAamp stool kit	QIAGEN	cat# 51604
Deposited data		
Macrogenomic data	This paper	BioProject: PRJNA744284
Software and algorithms		
R	The R Foundation for Statistical Computing	https://www.r-project.org/
Cytoscape (v 3.7.1)	Killcoyne et al. (2009)	https://cytoscape.org
MetaPhlan2	Franzosa et al., 2018	https://github.com/biobakery/biobakery/wiki/metaphlan2
HUMANN2	Franzosa et al., 2018	https://huttenhower.sph.harvard.edu/humann2
GraphPad Prism (v 8.0)	GraphPad Prism Software, Inc.	https://www.graphpad.com
Human Metabolome Database	Wishart et al. (2007)	http://www.hmdb.ca/
METLIN	Smith et al. (2005)	https://metlin.scripps.edu/
MASSBANK	Horai et al. (2010)	https://massbank.eu/
Progenesis QI	Nonlinear Dynamics	http://www.nonlinear.com/progenesis/qi/
Other		
Hitachi Automatic Physiological and Biochemical instrument	Hitachi	N/A
QuEChERS	Bristol	N/A
Eppendorf™ Refrigerated Centrifuge 5424 R	Eppendorf	N/A
Kinetex® 1.7 μm EVO C18	Phenomenex	N/A
ACQUITY UPLC® HSS T3	Waters	N/A
Q-TOF	Waters	N/A
Illumina HiSeq 2500 sequencer	Biolabs	N/A
Genevac evaporator	SP Scientific	N/A
Qubit® 2.0 Flurometer	Life Technologies	N/A
QTRAP® 6500+ UPLC–MS/MS System and analytical standards	Sigma–Aldrich	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and data should be directed to and will be fulfilled by the lead contact, Heping Zhang (hepingdd@vip.sina.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The raw macrogenomic data have been placed in the National Center for Biotechnology Information and have been available to the public since the date of publication. The log number is listed in the Key resources table.
- This paper does not report original code.
- Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In this study, a total of 60 farmers were recruited from four agricultural villages in the rural areas of Qi County town in Henan Province, China. The cohort for our study was comprised of 30 women (50%) and 30 men (50%), aged 40–65 years, all volunteers from three adjacent villages, who had essentially the same dietary patterns and working hours. These participants were healthy individuals with no major medical conditions. Our study was conducted between July and September, which is the busiest season in the agricultural areas of Henan Province. Prior to conducting the study, we surveyed the participants on their frequency of pesticide use. There were 17 most frequently used pesticides, including plant growth regulators (mepiquat chloride and pyraclostrobin), pyridine pesticides (imidacloprid), pyrethroid pesticides and their metabolites (β-cypermethrin, cypermethrin, 4-fluoro-3-phenoxybenzoic acid, and 3-phenoxybenzoic acid), antibiotic insecticides (emamectin benzoate), organophosphorus insecticides and their metabolites (glyphosate, dichlorvos, methomyl, leuconazole, diethyl phosphate, and dimethyl phosphorothioate), sulfonylurea insecticides (tribenuron methyl), and amide insecticides (alachlor). These results suggest that the farmers in our study were exposed to multiple pesticides and that any changes in physical indicators may be due to a combination of pesticides. During the experiment, these volunteers worked in the field for at least 4 h a day and frequently sprayed the crops with pesticides. The details of these volunteers are shown in Table S1.

This study was examined and permitted by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO. KY2020006), and all volunteers gave consent prior to participating in this study.

METHOD DETAILS

Experimental design

In our experiment, according to the frequency of pesticide exposure, volunteers were divided into two groups: a healthy group (spraying times per week $\leq$2) and a high-exposure group (spraying times per week >2). Our experimental design is shown in Figures 1A and 3A. The whole experiment is divided into two stages. In the first stage, the population was divided into two groups: a healthy group (n = 13) and a high pesticide exposure group (n = 47). In the second stage, two interventions were used in the high-exposure population: placebo group (n = 20) and *L. plantarum* P9 (P9) group (n = 27). The P9 group was supplemented with P9 (6×10^{10} cfu/day), while the placebo group only ate the same powder without P9. The powder is composed of maltodextrin, galactose oligosaccharide, strawberry powder and xylitol, which have no effect on the experimental results. The participants consumed these products for 30 days. After 30 days, the indices in the serum, urine and feces were measured. All 60 subjects persisted until the end of the study and did not take any antibiotics one month before or during the study. In addition, all participants were asked not to take any other drugs or supplements.

The physiology and biochemistry of serum and urine were assessed

The physiology and biochemistry of serum and urine were detected by a Hitachi Automatic Physiological and Biochemical instrument using a chemical colorimetry method. The concentrations of albumin (ALB) was detected using kits under the guidance of the instruction manual.

The serum immunoassays

The serum cytokine levels (Th1/Th2 cell related: IFN-γ; inflammatory cytokines: C-reaction protein, IL-7; chemokines: eotaxin, MCP-1, MIP-1β, RANTES, SDF-1α) were detected using the InvitrogenTM cytokine & phemokine convenience 34-plex human procartaplex panel 1A under the guidance of the instruction manual.

Targeted quantification of 13 kinds of pesticides

In this study, 13 pesticides with higher use frequency were selected based on the questionnaire. These pesticides were detected in serum, urine, and fecal samples using a QTRAP® 6500^{+} UPLC–MS/MS System and analytical standards (purchased from Sigma–Aldrich). Pesticide extraction, sample preparation and determination methods were performed according to Shin et al. with some modifications (Shin et al., 2018). Briefly, 2 mL of urine sample or 1 g of freeze-dried fecal sample was added to a 5-mL microcentrifuge tube, and then 0.4 mL acetic acid sodium acetate buffer and β-glucuronidase solution (1%, v/v) were added. The mixed solution was diluted with 3 mL of acetonitrile and shaken for 1 min. Then, 100 μL of human serum was transferred to a 2-mL microcentrifuge tube and mixed with 50 μL acetic acid sodium acetate buffer. This mixed solution was extracted with 400 μL of acetonitrile and shaken for 1 min. A 1% ratio (w/v) of ready-to-use QuEChERS salt powder was added to the mixed solution of urine, serum and fecal samples for the effective extraction of the pesticides (Paya et al., 2007). The tube was centrifuged at 4°C and 16,800×g for 5 min. The supernatant was transferred into a new microcentrifuge tube, the remaining precipitate was extracted again with 400 μL of acetonitrile, and the above operation was repeated. The supernatant was transferred to the tube containing the first supernatant. Finally, 200 μL of the extracted solution was added to a brown sample bottle, and 4 μL of the final extraction sample was injected into a QTRAP® 6500^{+} system for pesticide quantification. The whole process above was carried out on ice.

Conditions of UPLC-QTRAP-MS/MS

The UPLC conditions were as follows: chromatography column: Kinetex® 1.7 μm EVO C18; mobile phase A: ultrapure water containing 0.1% formic acid; mobile phase B: methanol containing 0.1% formic acid; column temperature: 35°C; mobile phase flow rate: 0.3 mL/min; gradient elution: 0.0–0.5 min 5% B, 0.5–5.0 min 5–55% B, 5.0–7.0 min 55–95% B, 7.0–10.0 min 95–95% B, 10.0–13.5 min 95–5% B, 13.5–15.0 min 5–5% B.

The parameters of the QTRAP 6500^{+} mass spectrometry were set as follows: curtain gas: 20 psi; collision gas (CAD): medium; ion source gas 1 (GS1): 50 psi; ion source gas 2 (GS2): 50 psi; temperature (TEM): 550°C; ion spray voltage of positive mode: 5500 V; ion spray voltage of negative mode: 4500 V. Multiple reaction monitoring (MRM) was used for the detection and quantitation of pesticides. Information on the MRM ion-pair channel and the corresponding declustering potential (DP) and collision energy (CE) are listed in Table S2.

Untargeted metabolomics analysis

Extraction of serum metabolites

Serum sample preparation was performed according to Dunn et al. (2011) with some modifications. A total of 100 μL of human serum was placed in a 2-mL microcentrifuge tube, mixed with 900 μL of 50:50 (v/v) methanol/ultrapure water solution and shaken for 1 min. After standing at 4°C for 60 min, we centrifuged the mixed solution at 4°C, 13000×g for 15 min. Then, 900 μL of the supernatant was concentrated using a Genevac evaporator. The sediment was resuspended in 200 μL methanol/ultrapure water (1:1) solution and vortexed for 60 s. The reconstituted solution was centrifuged at 4°C and 13000×g for 15 min and filtered with a 0.22 μm pore size membrane. Ten microliters of the final solution was injected into the UPLC-Q-TOF system for analysis.

Extraction of fecal metabolites

Fecal sample preparation was performed following previously reported methods (Vanden Bussche et al., 2015). Briefly, 1 g of freeze-dried fecal sample was diluted with 9 mL of 4:1 (v/v) methanol/solution and shaken for 5 min. After centrifugation at 4°C and 12,000×g for 15 min, the supernatant was concentrated using a Genevac evaporator and dissolved in 2 mL 4:1 (v/v) methanol/ultrapure water solution. The mixed solution was filtered through a 0.22 μm microporous membrane, and 10 μL of ultrapure water from the filtered sample was injected into UPLC-Q-TOF for analysis.

Extraction of urine metabolites

The extraction method of urine was similar to that of feces. Briefly, 5 mL of urine sample was transferred to a 50 mL centrifuge tube and diluted with 20 mL of methanol. After shaking and centrifugation at 4°C and 12,000×g for 15 min, the supernatant was concentrated and resuspended in 500 μL 4:1 (v/v) methanol/ultrapure water solution and vortexed for 60 s. The reconstituted solution was centrifuged at 4°C and 13,000×g for 15 min and passed through a 0.22 μm pore size membrane. Ten microliters of filtered sample was injected into the UPLC-Q-TOF system.

Conditions of the UPLC-Q-TOF system

The UPLC conditions were as follows: chromatography column: ACQUITY UPLC® HSS T3 (1.8 μm, 2.1 × 100 mm). Mobile phase composition: using ultrapure water containing 0.1% formic acid as mobile phase A and methanol containing 0.1% formic acid as mobile phase B; Column temperature: 45°C; Mobile phase flow rate: 0.40 mL/min; The gradient elution: 0.0–1.5 min 5% B, 1.5–7.0 min 5–25% B, 7.0–8.0 min 25–60% B, 8.0–12.0 min 60–95% B, 12.0–14.0 min 95–95% B, 14.5–16.0 min 95–5% B, 16.0–18.0 min 5–5% B.

The parameters of Q-TOF mass spectrometry were set as follows: Source temperature: 120°C; Desolvation temperature: 500°C; Capillary voltage: 3000 V; Cone voltage: 4000 V; Desolvation gas flow rate: 800 L/h; Cone gas flow rate: 50 L/h; Scan time: 0.2 s; Mass range: 50–1200 *m/z*; Data acquisition methods: MS^E mode (collision energy changed from 10 to 60 eV); The reference ion for positive ion mode: 556.2771 *m/z*; The reference ion for negative ion mode: 554.2615 *m/z*.

Metabolomics data analysis

The raw UPLC-Q-TOF data were first imported into Progenesis QI software for peak extraction, alignment, adduct annotation and retention time correction. The dataset was mean-centred and Pareto-scaled. The significant differential metabolites were identified by comparing their MS/MS mass spectra with the local standards library and online mass spectrum databases, including Human Metabolome Database (HMDB) (Wishart et al., 2007), METLIN database (Smith et al., 2005) and MASSBANK database (Horai et al., 2010).

DNA extraction and shotgun metagenomic sequencing

Using the QIAamp stool kit to extract total DNA from stool samples, we measured the purity and completion of DNA using 1% agarose gel electrophoresis (Zhang et al., 2016). The composition of the DNA was determined using a Qubit® 2.0 Flurometer. We decided to use an amount of DNA with an OD between 1.8 and 2.0 and above 1ug to construct the library and used a total of 1 μg of DNA per sample as the sample for the preparation of input material. We generated sequencing libraries using the NEBNext® Ultra™DNA Library Preparation Kit as recommended by the manufacturer and added index codes attributed to each sample to match sequences to their source. In short, DNA samples are split into 350bp files by sonication, the ends of individual DNA fragments were polished, A-tailed, and ligated to full-length aptamers, making them available for Illumina sequencing and further PCR amplification. In the end, the PCR products were purified by the AMPure XP system, the size distribution of the library was analyzed by the Agilent 2100 Bioanalyzer, and then the DNA was quantified using real-time PCR.

Illumina HiSeq 2500 sequencer was used for the shotgun metagenomic sequencing. Bioinformatics is used to control the quality of the original data obtained by sequencing. The double-terminal gene fragment of about 150 bp was obtained by sequencing, on the basis of which a sequencing library with the length of 300 bp was constructed. MetaPhlan2 software was used to annotate and classify the metagenomic species. Based on the UniRef90 database, HUMANN2 was performed to annotate the functional characteristics and metabolic pathways of the metagenome (Franzosa et al., 2018).

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were obtained from two non-interfering experiments and are expressed as mean ± SE(SEM). Statistically significant differences between groups were assessed using the Student's t-test because of false discovery rate (FDR). The overall statistical analysis was completed based on the R. Wilcoxon rank-sum test and Man-Whitney test were used to analysis the different abundances of strains, immune factors, metabolites, pesticide residues and Serum biochemical indexes. It was found that $p < 0.05$ was significantly different. GraphPad Prism (v 8.0) was used to produce bar charts and heatmap data represented as mean ± SE(SEM). The software package "ade4" in R is used for principal coordinate analysis (PCOA); The package "ggplot2" was used to generate box diagram, scatter diagram; The heatmap was built using the "pheatmap" package. In addition, the p value in the bubble graph iwascorrected using the "DESeq2" packet filter. Finally, the mechanism network was inferred from metagenomic sequencing data using Pearson rank correlation coefficient and visualized in Cytoscape (v 3.7.1) (Killcoyne et al., 2009).