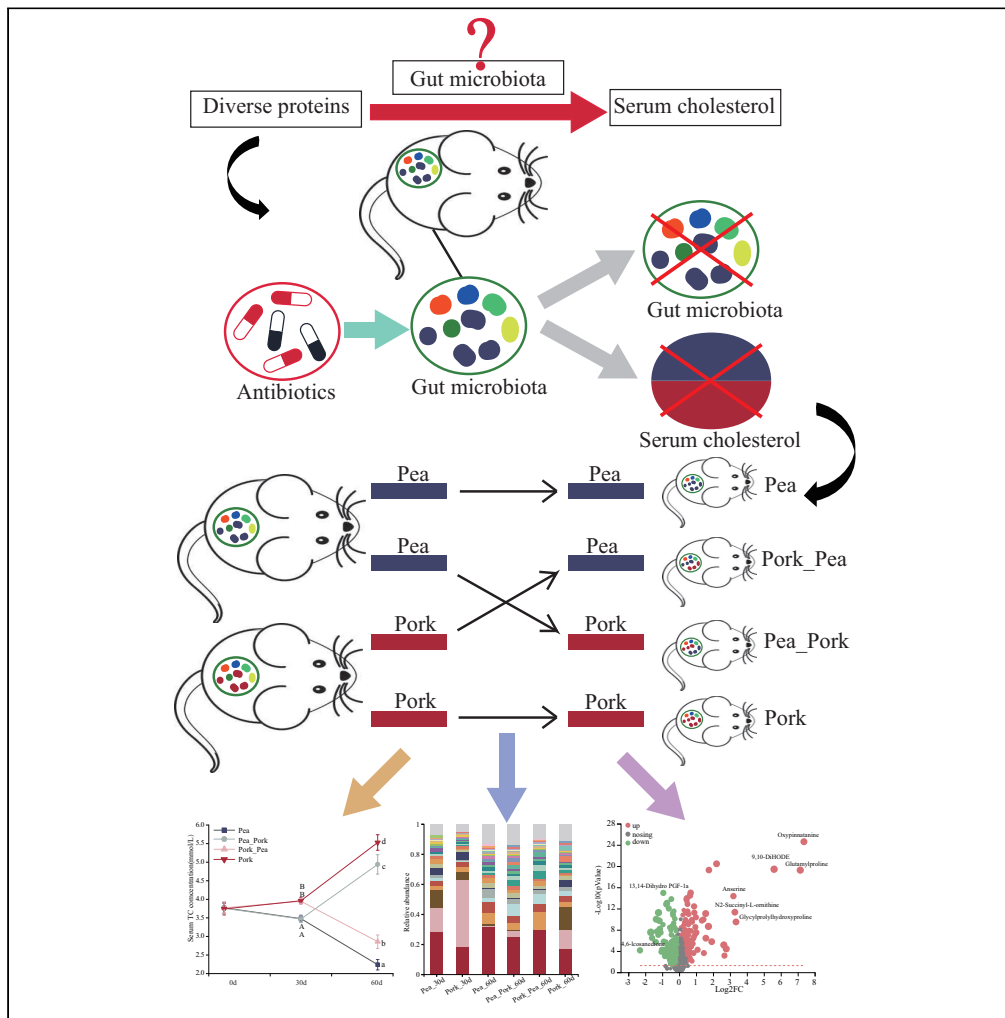


Article

Plant protein reduces serum cholesterol levels in hypercholesterolemia hamsters by modulating the compositions of gut microbiota and metabolites



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Highlights

Gut microbiota play a
decisive role in
modulating cholesterol
levels

Serum cholesterol levels
are reversed by protein
exchange correlating with
gut bacteria change

Pea protein alters cecal
metabolites involved in
amino acids and
cholesterol metabolism

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Article

Plant protein reduces serum cholesterol levels in hypercholesterolemia hamsters by modulating the compositions of gut microbiota and metabolites

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SUMMARY

Plant proteins exert effects of reducing cardio-cerebrovascular disease-related mortality partly via cholesterol-lowering, which was associated with gut microbiota. Here, we verify that there are significant differences in cholesterol levels among hamsters consuming different proteins. The decisive roles of gut microbiota in regulating host cholesterol are illustrated by the fact that the difference in serum cholesterol levels between hamsters feeding with pea protein and pork protein disappeared when treated with antibiotics. The results of cross-over intervention of pea and pork protein show that serum cholesterol levels are reversed with dietary exchange. The corresponding changes in microbiota suggest that Muribaculaceae are responsible for the inhibitory effect of pea protein on serum cholesterol level, whereas the opposite effect of pork protein is due to Erysipelotrichaceae. Moreover, pea protein supplement alters cecal metabolites including arginine/histidine pathway, primary bile acid biosynthesis, short-chain fatty acids, and other lipid-like molecules involved in cholesterol metabolism.

INTRODUCTION

Accumulating evidence from epidemiologic and intervention trials indicate that proteins from diverse sources exert distinct different healthy effects on humans. Consumption of excessive red meat tends to promote higher risks of mortality compared with other dietary ingredients such as whole grains, fish, poultry, nuts, beans, and low-fat dairy products (Micha et al., 2010; Pan et al., 2012; O'Connor et al., 2017). The latest epidemiological studies indicate that an increase of plant proteins in diets and substitution of red meat proteins or processed meat proteins with plant proteins are closely associated with the reduction of cardio-cerebrovascular disease (CVD)-related mortality (Naghshi et al., 2020; Budhathoki et al., 2019; Guasch-Ferré et al., 2019). Of note, lipid disorders characterized by hypercholesterolemia are the non-negligible risk factors for CVD (Ferdowsian and Barnard, 2009; Yokoyama et al., 2017). It has been well established that consumption of proteins from soybean, pea, rice, oat, and buckwheat could significantly reduce blood cholesterol concentration in human and animal models (Morita et al., 1997; Tomotake et al., 2000; Tong et al., 2012, 2016; Yang et al., 2013; Rigamonti et al., 2010). However, the underlying mechanisms of cholesterol regulation in response to protein from diverse sources like plant and animal remain inconclusive.

Protein from diverse sources, as an elemental but vital macronutrient in the diet, has huge influences on the composition and metabolism of the gut microbiota (Ijaz et al., 2018). The intake of soy protein promotes the growth of family Ruminococcaceae thereby producing more short-chain fatty acids (SCFAs) compared with meat proteins. Different proteins lower lipids at diverse degrees, which may be the consequence of reshaping the gut microbial community (Butteiger et al., 2016). Indeed, the first bacterium isolated from the rats long ago, which contains a group of cholesterol dehydrogenases encoded by the *ismA* gene, can convert intestinal cholesterol to coprostanol (Eysen et al., 1973; Kenny et al., 2020). Furthermore, the role of gut microbiota in cholesterol metabolism is supported by the fecal microbiota transplantation that the transplantation of microbiota from hypercholesterolemia human to normal mice induced elevated cholesterol levels which was associated with specific microbiota (Le Ray et al., 2019). These effects of gut microbiota on cholesterol metabolism are involved in complex metabolic pathway and host biology (Villette et al., 2020). It has been confirmed that gut microbial community structures can be changed by the short-term

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alterations of diets consisting fully of plant or animal products (David et al., 2014; Sonnenburg et al., 2016.). Apparently, the types of dietary protein have important effects on gut microbiota. Plant proteins have common characters in the mechanism of lowering serum cholesterol, that is, they can promote the excretion of bile acids in feces compared with casein and red meat protein, which leads to decreases in bile acid concentrations in enterohepatic circulation. However, there is still a lack of validated evidence for whether plant proteins lower cholesterol through gut microbiota and whether it determines the cholesterol metabolism for a long-term dietary intervention. In addition, the effects of different types of protein intake on gut metabolites, their reaction to gut microbiota, and the overall influence on cholesterol metabolisms are rarely reported.

With the increasing demand for high-quality protein worldwide, pea protein is being consumed all over the world because of its potential health benefits like lowering low-density lipoprotein cholesterol (LDL-C) and ameliorating gut microbiota balance, which has garnered enormous interest among scientists (Day, 2013; Dahl et al., 2012; Abeysekara et al., 2012). Meanwhile, one essential meat consumed in China and even around the world is pork, which is rich in protein and fat. Therefore, pea and pork proteins were studied to distinguish the cholesterol-modulating mechanism of diverse proteins by modulating the gut microbiota. Hamsters were treated with antibiotics (Abx) to investigate the decisive roles of gut microbiota in the effects of dietary proteins on cholesterol metabolism. The alteration of gut microbiota responsible for cholesterol metabolism induced by different proteins was further examined by the cross-over intervention of dietary protein either from pea to pork or from pork to pea in hamsters. Microbiota diversity analysis and metabolome were used to identify the dominant microbiota and metabolites acting as key modulators of cholesterol metabolism in response to diverse proteins.

RESULTS

Diverse proteins modulated serum and liver cholesterol levels to various degrees

A marked increase in body weight among all groups was observed between the initial weight and the final weight. There was no significant difference among each group except for the beef protein group exhibiting the most body weight gain of 46.5 ± 3.1 g (Table S1). There was no significant difference in the liver weight among all groups.

It was clearly shown that the serum and liver lipid profiles of plant groups were significantly different from those of animal groups after 30 days of feeding (Figure 1). Hamsters fed with plant proteins presented lower contents of total cholesterol (TC) (Figures 1A and 1F) and triglycerides (TG) (Figures 1D and 1I) in serum and liver than those fed with red meat proteins including pork and beef, similar to serum LDL-C levels (Figure 1B). Of note, one kind of animal protein, chicken protein, had significantly lower serum TC and LDL-C levels than red meat proteins, but not bean proteins (soybean and pea). Oat protein displayed the best cholesterol-lowering effect revealed by its lowest serum TC and LDL-C levels among all protein groups. Serum HDL-C content of the pea group was significantly higher than that of the control group. Intervention with plant proteins significantly reduced the atherosclerosis index (AI) and liver cholesterol ester (CE) compared with animal proteins. A similar trend of liver lipid profiles was observed, that is, the red meat groups presented the highest levels of liver TC, TG, and CE, whereas plant groups showed the lowest levels.

The effects of diverse proteins on lipid excretion (Figures S1A–S1D) and serum levels of apolipoproteins (Apo) (Figures S1E–S1H) were also detected. Fecal weights of hamsters fed with oat, pea, and beef proteins were significantly higher than those of the other groups. Plant and chicken proteins significantly increased fecal TC compared with red meat proteins. Moreover, plant proteins significantly promoted the fecal total lipids and bile acid than animal proteins. The significantly higher ApoE contents were found in all groups compared with control, and the beef group showed the highest content. The ApoA1 levels of the plant groups were significantly higher than those of the animal groups, and the ApoB content was significantly decreased only induced by pork protein. There was no significant difference in ApoB/ApoA1 between control, oat, rice, and soybean groups, which was significantly lower than that in animal groups.

The enzymes related to liver lipid metabolism also showed significant difference between plant and meat protein groups (Figure S2). The levels of liver hydroxymethyl glutarate monoacyl CoA (HMG-CoA) reductase in plant groups were lower than those of animal groups, among which pork group showed much higher level than the other groups (Figure S2A). The concentrations of liver cholesterol 7- α -hydroxylase

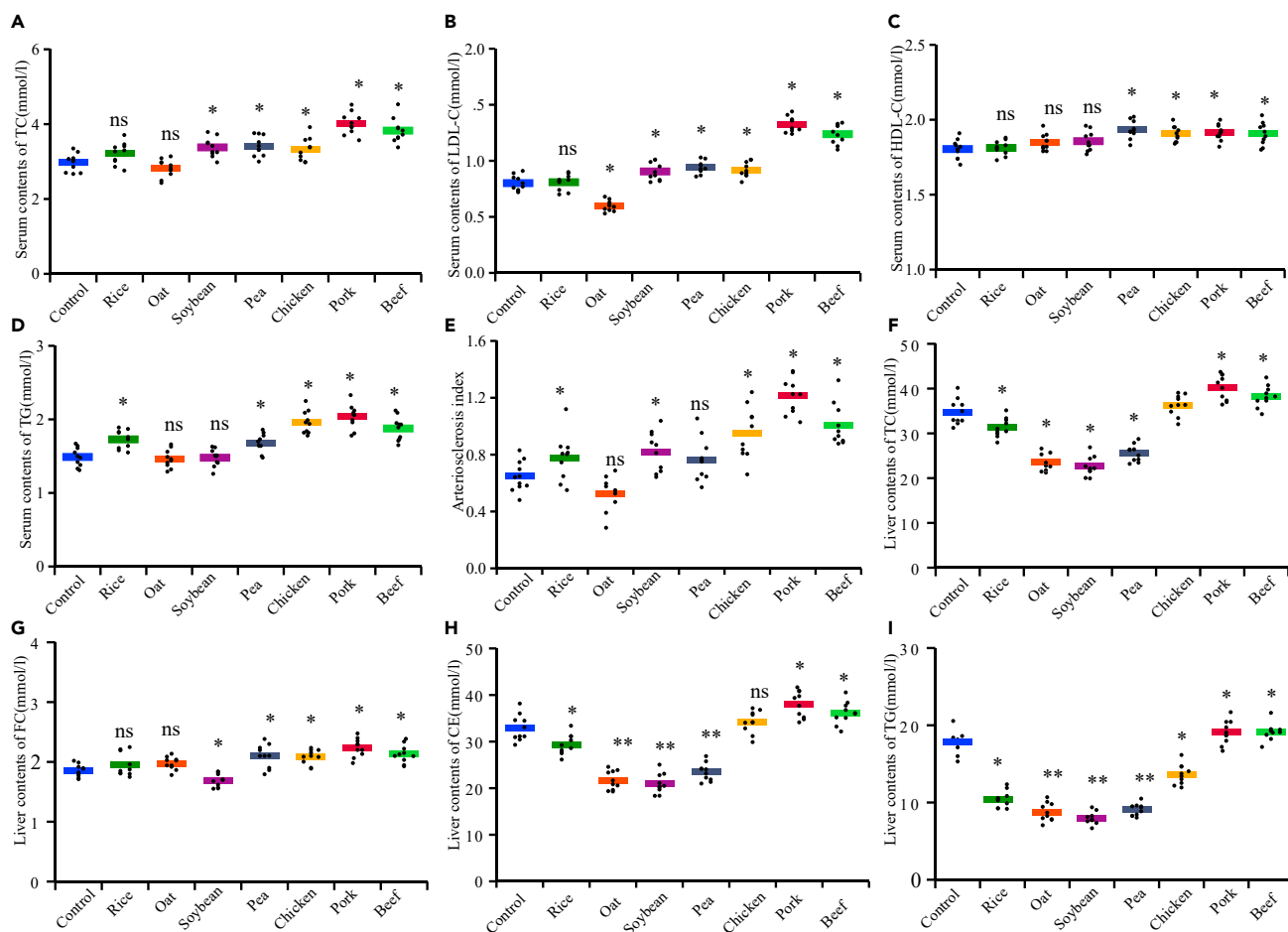


Figure 1. Effects of diverse proteins on serum and liver lipid profiles

(A–D) Serum levels of TC: total cholesterol (A); LDL-C: low-density lipoprotein cholesterol (B); HDL-C: high-density lipoprotein cholesterol (C); TG: triglycerides (D)

(E) Arteriosclerosis index, which was calculated by TC content minus HDL-C content and divided by HDL-C content.

(F–I) Liver levels of TC (F); FC: free cholesterol (G); CE: cholesterol ester (H); TG (I).

Statistical significance was calculated by Student's t test (* $p < 0.05$; ns, not significant).

(CYP7A1) were significantly enhanced by plant proteins, whereas they were reduced by pork protein (Figure S2B). No significant difference in fatty acid synthase concentration was found among control, soybean, and chicken groups, whereas a considerable decline in pea and a slight increase in red meat groups was observed (Figure S2C). Interestingly, among plant groups, the content of acyltransferase was the highest in soybean group, and the contents of low-density lipoprotein receptor (LDLR) and lipoprotein lipase (LPL) were the highest in oat group (Figures S2D–S2F).

Diverse proteins differentially altered gut microbiota

The total number of reads obtained in the sequencing were 3,866,650. There are 117 taxa at the genus and 55 taxa at the family level detected in this experiment. According to the values obtained from Shannon and Simpson indices (Figures S3A and S3B), oat and pea groups displayed the highest diversity, followed by rice and soybean, which also showed significant higher diversity compared with meat groups at significance of $p < 0.05$ and $p < 0.01$, respectively. As for Chao and Ace indices implying the evenness of microbial community (Figures S3C and S3D), there were significantly lower values in beef group compared with other groups among which no statistical difference was found. The distance of principal-component analysis (PCA) and principal co-ordinates analysis (PCoA) based on weighted unifracs plots displaying partitions by group was visually representative of the similarity among all samples (Figures S3E and S3F). Pea and

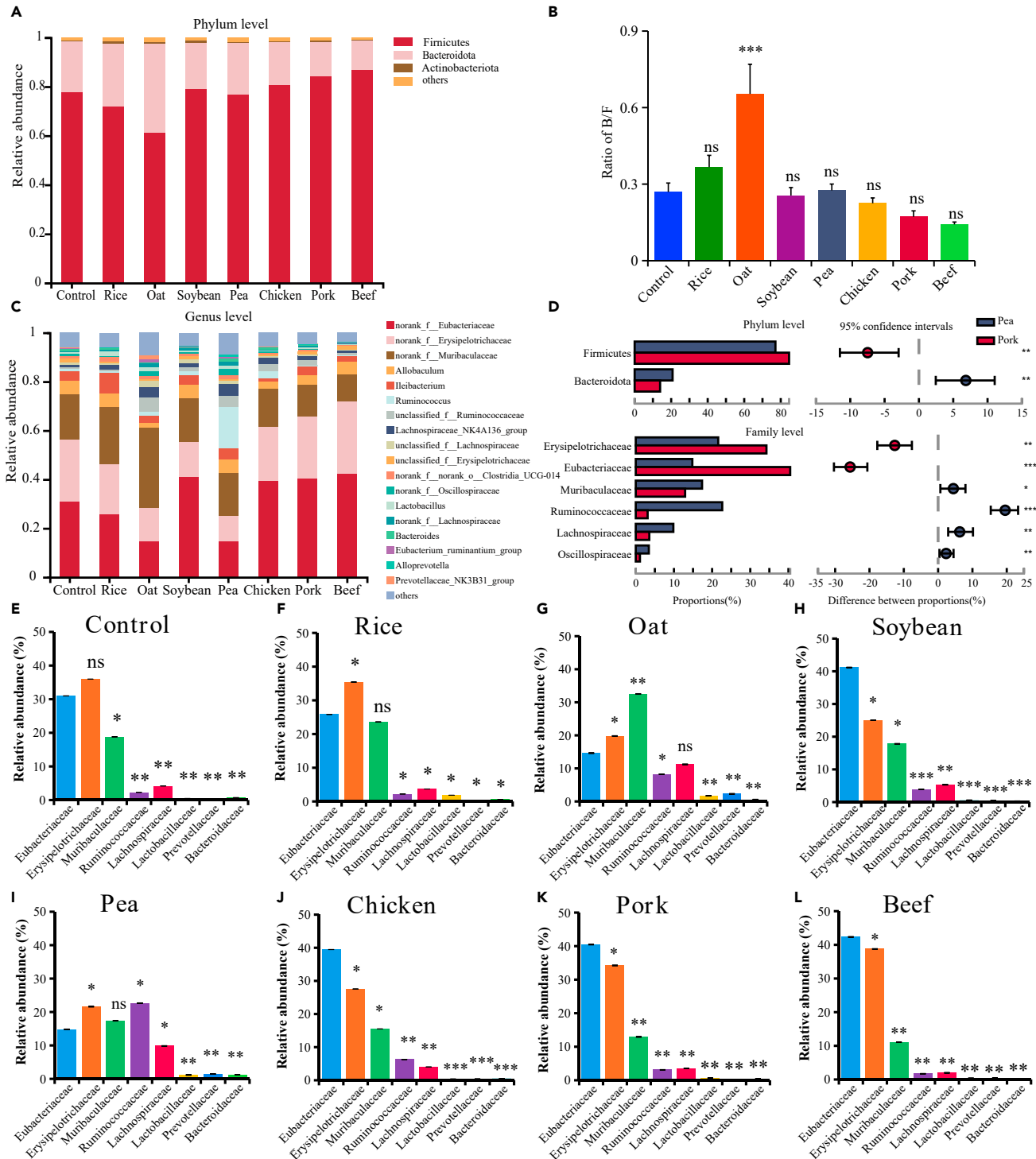


Figure 2. Changes of gut microbiota composition in response to diverse dietary proteins

(A) The composition of gut microbiota in fecal samples from hamsters after 30 days' administration of diverse proteins at phylum level.

(B) The ratio of Bacteroidota and Firmicutes in different groups.

(C) The composition of gut microbiota in fecal samples from hamsters after 30 days' administration of diverse proteins at genus level; taxa with abundance below 1% were presented as others.

(D) Gut microbiota significantly different between pea and pork protein groups at phylum and family levels. The bar plots show the abundance of diverse bacteria. Positive differences in mean of relative abundance indicate bacteria with higher abundance in the pea protein group, whereas negative differences

Figure 2. Continued

indicate bacteria with higher abundance in pork protein group. Statistical analysis was performed by the Wilcoxon test coupling with false discovery rate (FDR) correction.

(E–L) Discrepant bacteria at family level in control (E), rice (F), oat (G), soybean (H), pea (I), chicken (J), pork (K), and beef (L).

Statistical significance was calculated by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant). All error bars indicate SE.

oat groups were clearly separated from the other groups, whereas meat groups were clustered closely, which suggested that hamsters fed with meat proteins had similar microbiota community structures. Similar trend was displayed in the hierarchical clustering tree at operational taxonomic unit (OTU) level showing that oat and pea as one subgroup was such different from the others; control and rice groups, soybean group as two different subgroups both were clustered away from meat subgroup (Figure S3G). The hierarchical clustering analysis disclosed apparent separation of plant from meat groups, which was in agreement with the results of PCA and PCoA. These findings demonstrated that the composition of gut bacteria exhibited profoundly diverse responses to diverse proteins.

At the phylum level, Firmicutes and Bacteroidetes were the two predominant phyla in all groups, accounting for 61.33%–86.84% and 12.27%–36.32%, respectively. Hamsters fed with animal proteins had higher relative abundance of Firmicutes but lower Bacteroidetes than those fed with plant proteins. The most accumulated relative abundance of Firmicutes was observed in beef group, whereas the lowest one was present in oat group (Figure 2A). Correspondingly, oat group had the highest ratio of Bacteroidetes to Firmicutes, followed by rice group without significant difference from control, soybean, and pea groups, whereas pork and beef groups showed the lowest ratio (Figure 2B). The microbiota composition and structures were distinctly different among different protein groups revealed by the plot at the genus level (Figure 2C). Pea group presented even microbiota composition revealed by four genera *norank_f_Erysipelotrichaceae*, *norank_f_Eubacteriaceae*, *Ruminococcus*, and *norank_f_Muribaculaceae*, respectively, accounting for 10.43%, 14.81%, 16.78%, and 17.35% of relative abundance, followed by the four genera *Ileibacterium*, *unclassified_f_Ruminococcaceae*, *Lachnospiraceae_NK4A136_group*, and *Allobaculum*, respectively, accounting for 4.57%, 4.67%, 4.77%, and 5.75%. These data were in agreement with the results presented in alpha diversity (Figure S3). The animal groups shared great similarity in microbial community structures dominated by *norank_f_Eubacteriaceae* (40.75% \pm 0.84%), *norank_f_Erysipelotrichaceae* (25.85% \pm 2.89%), and *norank_f_Muribaculaceae* (13.16% \pm 1.28%). Comparisons of the gut microbiota composition in pea and pork showed that significant difference was not only present in the dominant phyla Firmicutes and Bacteroidetes but also in the top 6 families including Eubacteriaceae, Erysipelotrichaceae, Muribaculaceae, Ruminococcaceae, Lachnospiraceae, and Oscillospiraceae (Figure 2D). The relative abundance of different microbiota at family level was subsequently characterized among all groups (Figures 2E–2L). Apart from the pea group, which was evenly enriched in Erysipelotrichaceae, Ruminococcaceae, Eubacteriaceae, and Muribaculaceae, all groups were dominated by Eubacteriaceae, Erysipelotrichaceae, and Muribaculaceae (Figure 2I). The relative abundance of Erysipelotrichaceae was the highest in control group (35.96%) (Figure 2E), as well as in rice group (35.42%) (Figure 2F), but that of Muribaculaceae was the highest in oat group (Figure 2G). Compared with the control group, both oat and pea groups led to an increase in Muribaculaceae and decreases in Eubacteriaceae and Erysipelotrichaceae. Soybean and meat groups presented similar relative abundance of Eubacteriaceae but different ratio of Erysipelotrichaceae/Muribaculaceae, in which soybean (1.40) and chicken (1.78) groups showed much lower ratio than red meat groups (2.65 for pork; 3.50 for beef) (Figures 2H and 2J–2L).

The important bacterial taxa contributing to the discrepancies produced by diverse proteins were depicted in the linear discriminant analysis (LDA) effect size (LEfSe) plots (Figure 3A). Most taxa selected by LEfSe analysis were enriched in oat and pea groups, whereas none was detected in control, soybean, and pork groups. Among the 4 taxa in meat groups and 23 taxa in plant groups, Bacteroidota and Firmicutes were significantly enriched in oat and beef groups, respectively. These data were consistent with the above-mentioned findings (Figure 2A). The Muribaculaceae family predominated by genera *norank_f_Muribaculaceae* was enriched in the oat group, which was of great importance revealed by LDA value >4.5 . Within Bacteroidota phylum, three genera *Prevotellaceae_UCG_001*, *Prevotellaceae_NK3B31_group*, and *norank_f_Muribaculaceae* were enriched in oat group and two genera *Bacteroides* and *Alloprevotella* were enriched in pea group (Figure 3B). Within Firmicutes phylum, the genus *norank_f_Erysipelotrichaceae* was enriched in beef group; two orders Oscillospirales and Clostridia_UCG_014 were enriched in pea groups, two genera *Eubacterium_ruminantium_group* and *Family_XIII_UCG-014* were enriched in oat

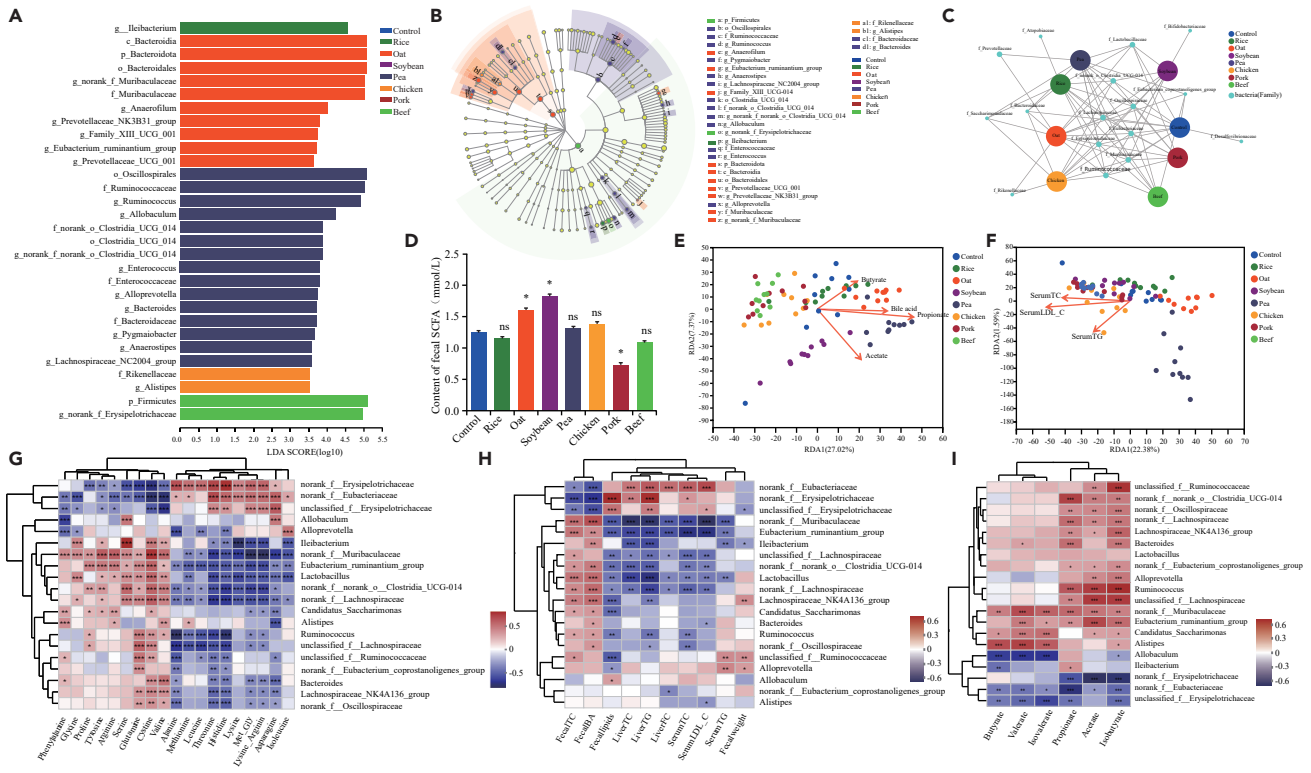


Figure 3. Discrepant bacteria and correlation analysis

- (A) Taxa with linear discriminant analysis (LDA) score 3 or greater from phylum to genus levels in gut microbiota communities of diverse protein groups.
 (B) Taxa with linear discriminant analysis effect size (LEfSe) representing bacteria from genus to phylum level in different groups.
 (C) The interconnection networks of gut microbiota at family level in different groups. Species node sizes represent relative abundance at family levels, and edges represent the association patterns of individual family with the dietary protein types.
 (D) Contents of short-chain fatty acids (SCFAs) in different groups.
 (E) Redundancy analysis (RDA) of samples from different groups based on OTU data and gut microbial metabolites.
 (F) Serum lipid profile.
 (G) Correction analysis of top 20 genera and amino acid composition of diverse proteins.
 (H) Lipid profiles.
 (I) Gut microbial metabolites.

Statistical significance was calculated by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant). All error bars indicate SE.

group, and one genus *Ileibacterium* was enriched in rice group. The interconnection networks illustrated the co-occurrence patterns of bacterial community at family level in diverse groups (Figure 3C). Desulforibroniaceae, Atobobiaceae, and Bifidobacteriaceae were unique in control, rice, and soybean group, respectively. Prevotellaceae was unique in both pea and oat groups, and Rikenellaceae, in oat and chicken groups.

As the main metabolites of gut microbiota and the direct effects on lipid profiles, fecal SCFA content of the pork group was the lowest, but those of soybean and oat groups were the highest, followed by pea and chicken groups, without significant difference from control, rice, and beef groups (Figure 3D). Redundancy analysis (RDA) was conducted to clarify the correlation between diverse proteins and SCFAs or serum lipid profiles (Figures 3E and 3F). Propionate contributed the differences generated by diverse proteins much more than acetate, bile acid, and butyrate (Figure 3E). Rice and pea groups were positively correlated with acetate, propionate, and butyrate, whereas the meat groups displayed the converse trend. Positive association of soybean group with acetate and rice group with butyrate was observed, but not with meat groups. Pea and oat groups exhibited negative association with serum TC and LDL-C, whereas meat groups presented the opposite correlation (Figure 3F). Overall, plant proteins had better effects on cholesterol-lowering with gut microbiota alteration, which promoted the generation of SCFAs compared with meat proteins.

The relationship between serum lipids, amino acid compositions (Table S2), and gut microbiota was analyzed by Spearman to explore the underlying mechanism of gut microbiota affecting host cholesterol with diverse proteins (Figures 3G–3I). *Norank_f_Eubacteriaceae*, *norank_f_Erysipelotrichaceae* and *unclassified_f_Erysipelotrichaceae* were clustered in Branch 1, which showed inverse trend compared with the other genera (Branch 2). Likewise, the amino acid compositions were divided into two clusters based on the converse correlation with the gut microbiota. The left set of amino acids including phenylalanine (Phe), glycine (Gly), proline (Pro), tyrosine (Tyr), arginine (Arg), serine (Ser), glutamine (Glu), cystine (Cys), and valine (Val) displayed negative correlation with Branch 1; the right set including alanine (Ala), methionine (Met), leucine (Leu), threonine (Thr), histidine (His), lysine (Lys) asparagine (Asp), and isoleucine (Ile) showed negative correlation with Branch 2. Val and Cys displayed negative association with *norank_f_Eubacteriaceae* ($R = -0.67, -0.68$), whereas Ser and His showed positive association with *norank_f_Erysipelotrichaceae* and *Ileibacterium* ($R = 0.79, 0.76$), respectively (Figure 3G). The results of correlation revealed that host lipid profiles were extensively associated with the gut microbiota (Figure 3H). Branch 1 was negatively associated with fecal TC and bile acid, whereas it was positively correlated with liver lipids and serum TC and LDL-C. *Eubacterium_ruminantium_group* displayed negative association with serum and liver lipid profiles, especially serum LDL-C ($R = -0.62$). SCFAs are important metabolites of gut microbiota including acetate, butyrate, and propionate (Figure 3I). Butyrate, valerate, and isovalerate were clustered into the first branch, whereas propionate, acetate, and isobutyrate were clustered into the second branch. The first branch was positively correlated with *Alistipes*, whereas it was negatively correlated with *Allobaculum*. *Ruminococcus* and *unclassified_f_Lachnospiraceae* were positively associated with acetate ($R = 0.56, 0.57$) and isobutyrate ($R = 0.62, 0.57$). Branch 1 was negatively associated with all SCFAs, which showed positive correlation with *norank_f_Muribaculaceae* and *Eubacterium_ruminantium_group*.

Abx treatment abolished the effects of proteins on serum cholesterol modulation.

To investigate the decisive roles of gut microbiota in regulating cholesterol by proteins, the hamsters were treated with cocktail of vancomycin and bacitracin (Figure 4A). No statistical difference was detected in Shannon, Simpson, and Chao indices between pea and pork groups before the Abx treatments (Figures 4B and 4C). At the end of the experiment, huge declines were observed in both richness and evenness of microbiota, revealed by the decrease in Chao index observed in Pea_Abx from 594.02 to 58.78 and Prok_Abx from 610.66 to 51.92, and decrease in Ace index from 636.77 to 75.52 of Pea_Abx and from 648.31 to 68.5 of Prok_Abx (Figures 4D and 4E). The hierarchical clustering tree at OTU level showed that all samples could be divided into two subgroups containing the Abx- and the none Abx-, respectively (Figure 4F). Similar clusters were found in the PCA and PCoA plots, which indicated a distinct separation between the genera of hamsters before Abx and after Abx treatment (Figures 4G and 4H). There were overlaps between pea and pork groups both before (Pea_Abx_0d versus Pork_Abx_0d) and after (Pea_Abx_30d versus Pork_Abx_30d) Abx treatment at PC1 levels accounting for the largest part in PCA and PCoA plots, which demonstrated that Abx increased the similarity of gut microbiota between Pea_Abx and Pork_Abx. These results indicated that Abx treatment abolished the differences in gut microbiota induced by pea and pork proteins.

As anticipated, the relative abundance of Firmicutes and Bacteroidetes at phylum level was significantly decreased in the presence of continuous Abx administration based on the decreased alpha diversity (Figure 4I). After the treatment with Abx, Proteobacteria became the dominant bacteria at the phylum level. There were considerable diminutions of specific phyla, such as Firmicutes and Bacteroidetes, affected by pea and pork protein supplement as elucidated earlier (Figure 2D). Particularly, the difference in abundance of microbiota between pea and pork groups disappeared in the presence of Abx, apart from Lactobacillaceae and Bacteroidaceae, which accounted for limited abundance in the discrepant bacteria (Figure 4J). These results illustrated that Abx treatment largely eliminated the difference in gut microbiota composition.

Concomitantly, the Abx treatment resulted in an increase in the contents of serum TC, LDL-C, and HDL-C of hamsters compared with the hamsters without Abx treatment feeding with pea or pork proteins, but there was no significant difference in cholesterol levels between Pea_Abx and Pork_Abx groups (Figure 4K). It indicated that the inhibitory effects of pea and pork proteins on cholesterol disappeared with the elimination of gut microbiota induced by Abx. There were significant differences in lipid profiles of hamsters before and after Abx treatment. After Abx treatment, the liver FC, TG, and CYP7A1 concentrations were not significantly different between pea and pork groups, but liver TC, CE, HMG-CoA reductase, LDLR,

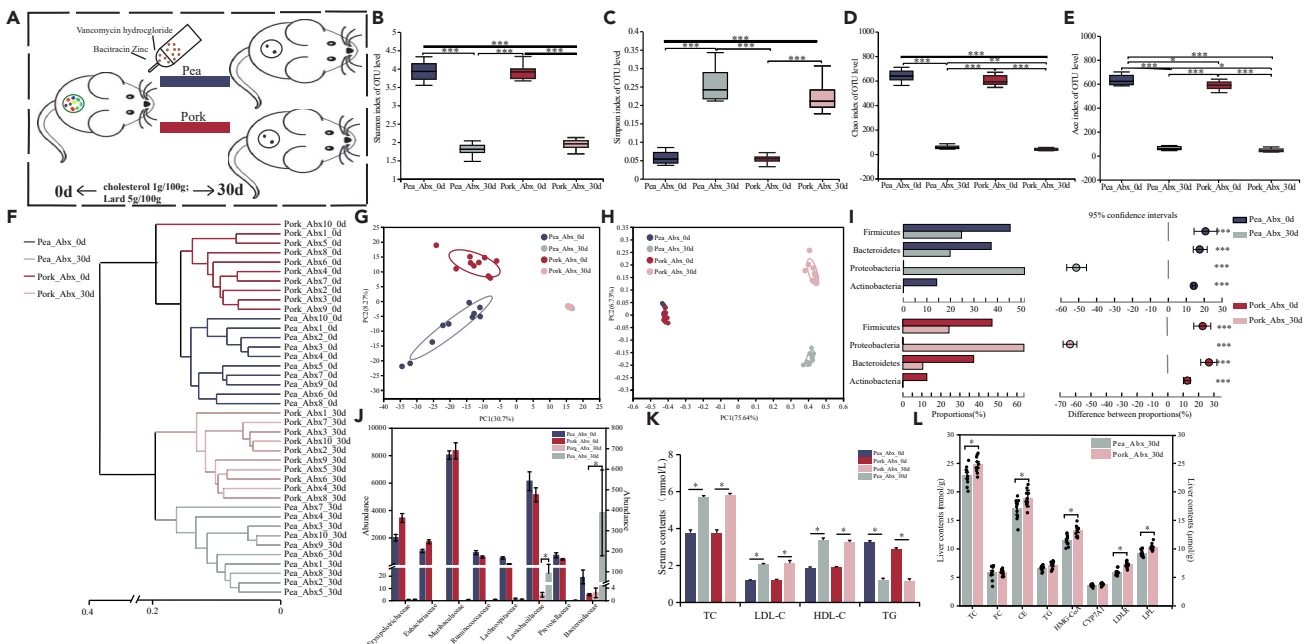


Figure 4. Effects of pea and pork protein with antibiotics treatment on gut microbiota composition and structure

(A) The overview of the antibiotics study design.

(B–E) Boxplot of alpha diversity indices at OTU level expressed as Shannon index (B), Simpson index (C), Chao index (D), and Ace index (E).

(F) Hierarchical clustering of gut bacteria at OTU level.

(G) Principal-component analysis (PCA).

(H) Principal co-ordinates analysis (PCoA) based on unweighted UniFrac of all groups at 0 and 30 days.

(I) Mean proportions of discrepant species at phylum level in different groups by two-group comparisons using Welch's t test: Pea_Abx_0d versus Pea_Abx_30d; Pork_Abx_0d versus Pork_Abx_30d.

(J) Abundance of the discrepant gut microbiota at family level.

(K) Serum cholesterol content of hamsters treated with Abx.

(L) Liver contents of TC, FC, CE, TG, HMG-CoA, CYP7A1, LDLR, and LPL. Statistical significance was calculated by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All error bars indicate SE.

and LPL levels of pea group were significantly lower than those of pork group (Figure 4L). Notably, the significance of decreases in liver TC induced by pea compared with pork protein was not so much apparent in hamster after Abx treatment compared with that without Abx treatment (Figure 1F). It implied that the effects of pea and pork protein supplementation on modulating serum cholesterol level were abolished in the absence of gut microbiota in hamster model.

Cross-over intervention of pea and pork proteins reversed cholesterol levels

Hamsters fed with pea and pork protein in the first month were further divided into 2 groups for the cross-over intervention (Figure 5A). The corresponding cross-over effects of Pea_Pork and Pork_Pea on the levels of serum TC, LDL-C, and TG were observed clearly (Figures 5B–5E). The serum TC and LDL-C levels significantly decreased in pea group, whereas they increased in pork group during the first month compared with the original levels before intervention (Figures 5B and 5C). Pea protein exhibited obviously higher serum HDL-C and lower TG contents than pork protein (Figures 5D and 5E). However, these changes were reversed by the subsequent cross-over intervention of proteins after 60 days of feeding. Pork protein intervention led to the highest level of serum TC and LDL-C, which reached the same level of Pea_Pork group. The similar reversed trend was found in pea group, which showed the lowest level of serum TC and LDL-C with the comparable level of Pork_Pea group, as well as the reversed changes in HDL-C and TG. Moreover, the most body weight gain was presented in Pork_Pea, much higher than the other groups (Table S1). Pea protein intake significantly reduced liver TC and FC contents in contrast to pork protein, and no significant difference was found between Pea_Pork and Pork_Pea in the contents of TC, FC, and TG (Figure 5F). HMG-CoA reductase and LDLR contents were significantly enhanced in pea group compared with pork after 60 days. Notably, there was no significant difference in the contents of HMG-CoA reductase, CYP7A1,

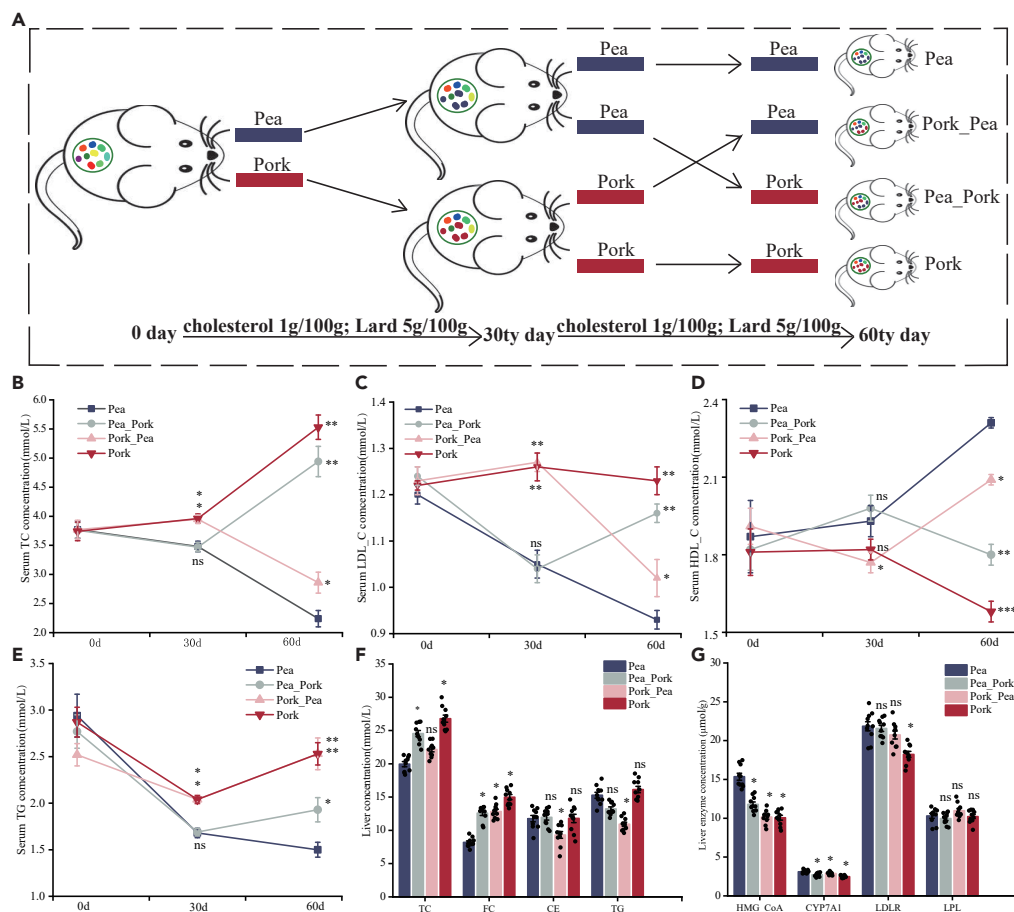


Figure 5. Effects of pea and pork protein supplement on cholesterol level in hamsters

(A) The overview of the cross-over intervention of pea and pork protein study design.

(B–E) Serum levels of TC (B); LDL-C (C); HDL-C (D); and TG (E).

(F) Liver concentrations of TC, FC, CE, and TG.

(G) Liver concentrations of HMG-CoA, CYP7A1, LDLR, and LPL.

Statistical significance was calculated by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant). All error bars indicate SE.

LDLR, and LPL between Pea_Pork and Pork_Pea groups (Figure 5G). These findings confirmed the capacity of pea protein to lower cholesterol, which was strong enough to reverse the side effects of pork protein by dietary shifts.

Cross-over intervention of pea and pork proteins reshaped gut microbiota

The alterations of dietary protein from pea to pork and pork to pea both greatly changed hamsters' gut microbiota composition. Pork protein reduced microbiota diversity and evenness significantly based on its lower Shannon, Chao, and Ace values and higher Simpson value than pea protein in the first period of 30 days (Figures S4A–S4D). The same trend can be observed at the cross-over period when the microbial diversity and evenness of Pork_Pea group were elevated. Notably, no significant difference was found between Pea_Pork and Pork_Pea of which microbial richness and evenness were lower than pea but higher than pork. PCA and PCoA plots for the beta diversity disclosed the significant distinction of the microbial composition structures between pea and pork groups after the first feeding period, whereas the individual meat groups displayed similar gut microbial structures as reflected by the close clustering within each group (Figures S4E and S4F). It is worthy to notice that the farthest distance from pea to pork was just equal to that from Pork_Pea to pork and from Pea_Pork to pea as shown in PCA and PCoA plots (Figures S4G and S4H). According to the cluster tree, the two dietary exchange groups came together into one, which subsequently joined in pea group for 60 days, and finally formed one cluster with Pork_30d, Pork_60d, and

Pea_30d (Figure S4I). There was a favorable similarity within each group, which was consistent with the results of PCA and PCoA.

The dominant phyla were Firmicutes ($64\% \pm 8\%$) and Bacteroidetes ($30\% \pm 8\%$) in all groups, which was identical to the previous results (Figure 6A). There was a significant difference in the proportions of Firmicutes and Bacteroidetes between pea and pork groups after the first 30 days of feeding. This discrepancy was enhanced during the following cross-over feeding period revealed by the significant increase in Bacteroidetes of pea group, whereas decrease in pork group. Pea_60d showed no significant difference in the relative abundance of Firmicutes and Bacteroidetes from Pea_Pork_60d or Pork_Pea_60d. The gut microbiota of hamsters fed with pork protein was characterized by high *norank_f_Eubacteriaceae* at the first month, which was almost depleted with conversion to pea protein at the second month (Figure 6B). Pea protein replacing pork also decreased the relative abundance of *norank_Erysipelotrichaceae*, whereas it increased *norank_f_Muribaculaceae* and *Lachnospiraceae_NK4A136_group*. Conversely, *norank_f_Eubacteriaceae*, *norank_f_Muribaculaceae*, and *Lachnospiraceae_NK4A136_group* were greatly decreased and *Ruminococcus* was elevated by substitution of pea protein with pork. Two-group comparisons were used to select the different microbiota between pea and pork groups at the first feeding period (Figure 6C). Consumption of pork protein significantly enhanced the relative abundance of Eubacteriaceae, whereas it decreased Muribaculaceae, Erysipelotrichaceae, and *Ruminococcus* compared with pea protein at family level. LEfSe analysis illustrated the important roles of *Clostridia* contributing to the enrichment of Firmicutes in pork group, whereas Muribaculaceae contributing to the enrichment of Bacteroidetes in pea group according to the LDA values (Figure 6D). Comparisons among these four groups showed significant difference that total proportions of Erysipelotrichaceae and Eubacteriaceae were higher in Pork group, whereas the total proportions of Muribaculaceae and Ruminococcaceae were higher in pea, Pea_Pork, and Pork_Pea groups (Figure 6E). Likewise, the vital microbiota contributing to the discrepancy induced by diverse proteins were selected by LEfSe analysis (Figure 6F). The Erysipelotrichia including Erysipelotrichaceae family was of great importance in pork group, whereas the Muribaculaceae family consisting of *norank_Muribaculaceae* was enriched in pea group.

The microbiota affected by dietary proteins was identified by two-group comparisons (Figure 7). Ruminococcaceae, Muribaculaceae, and Lachnospiraceae were identified as the most primary bacteria among all groups, followed by Erysipelotrichaceae. The comparison between pea and pork showed that the most different microbiota with great abundance were Muribaculaceae, followed by Ruminococcaceae, both of which were increased in the presence of pea protein, whereas pork group was characterized by Erysipelotrichaceae and Eubacteriaceae, which were barely found in the pea group (Figure 7A). In Pea_Pork and Pork_Pea groups with the same amount of pea and pork protein intake, there were significant differences in Saccharimonadaceae and Eubacteriaceae accounting for less than 5% of relative abundance (Figure 7B). The ability of pea and pork proteins to reshape gut microbiota was next examined by the comparison of cross-over intervention. The significant decreases in proportions of Lachnospiraceae, Erysipelotrichaceae, Rikenellaceae, and Eubacteriaceae were observed after changing dietary protein from pea to pork, as well as increases in relative abundance of Ruminococcaceae, Muribaculaceae, and Bacteroidaceae (Figure 7C). Substitution of pork protein with pea significantly increased the relative abundance of Ruminococcaceae, Muribaculaceae, and Bacteroidaceae, and almost diminished Erysipelotrichaceae and Eubacteriaceae (Figure 7D). The gut microbiota composition was similar between pork and Pea_Pork groups and pea and Pork_Pea groups (Figures 7E and 7F). These results suggested that dietary exchange of diverse proteins definitely reversed the gut microbiota.

Cross-over intervention of pea and pork proteins altered cecal metabolites

SCFAs as the key metabolites generated by gut microbiota were beneficial for the lipid homeostasis and cholesterol metabolism and thus were detected, as well as its further indirect effects on cecal metabolites. Pea group showed the highest content of total SCFAs with the primary constituents of acetate, propionate, and butyrate, whereas isobutyrate, isovalerate, and isohexanoate as branched-chain fatty acids were not statistically different among the 4 groups (Figure 8A). Heatmap on the basis of metabolite levels showed the highest similarity to the abundance and composition between Pea_Pork and pork, whereas the lowest correlation between pea and Pea_Pork, suggesting that substitution of pea protein with pork led to the most apparent changes in cecal metabolites. Likewise, cross-over diet profoundly altered the cecal metabolites, resulting in the distinct separations that Pea_Pork were close to pork and Pork_Pea were close to pea in the PCA plot. It implied that the differences caused by dietary exchange of protein were similar to the

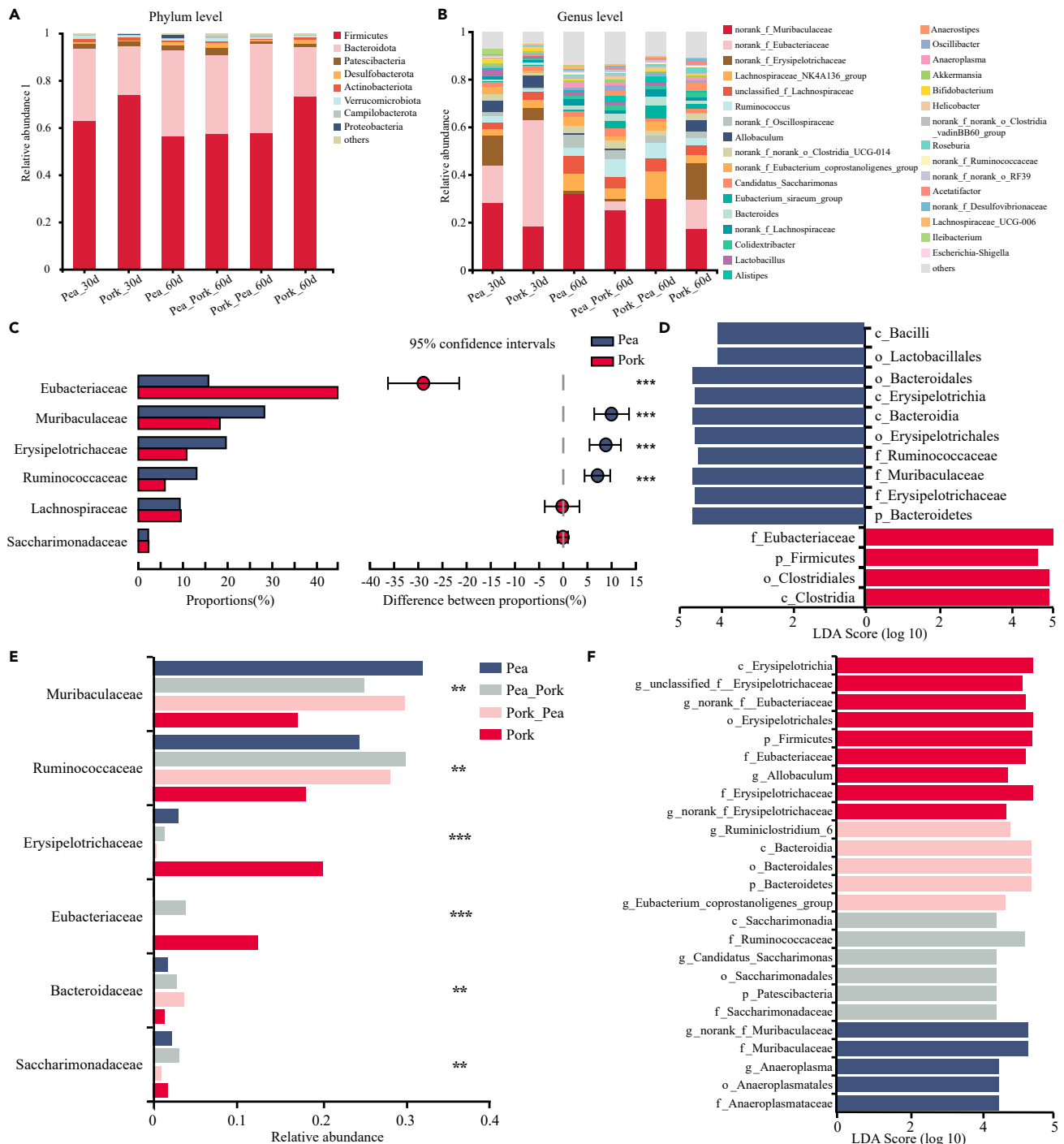


Figure 6. Changes of gut microbiota composition in response to pea protein and pork protein

(A and B) The composition of gut microbiota at phylum level (A) or genus level (B) in different groups; taxa with abundance below 1% were presented as others. (C) Mean proportions of discrepant species on phylum level in different groups at 30 days by two-group comparisons using Welch's t test: Pea versus Pork at 30 days.

(D) Taxa with LDA score 3 or greater from phylum to genus levels in gut microbiota communities of pea and pork protein groups at 30 days.

(E) Proportions of gut microbiota on family level with significant difference among different groups at 60 days. Kruskal-Wallis H test coupling with FDR correction was applied to evaluate the significant difference among the four groups at 60 days.

(F) Taxa with LDA score 3 or greater from phylum to genus levels in gut microbiota communities of different groups at 60 days.

p < 0.01, *p < 0.001. All error bars indicate SE.

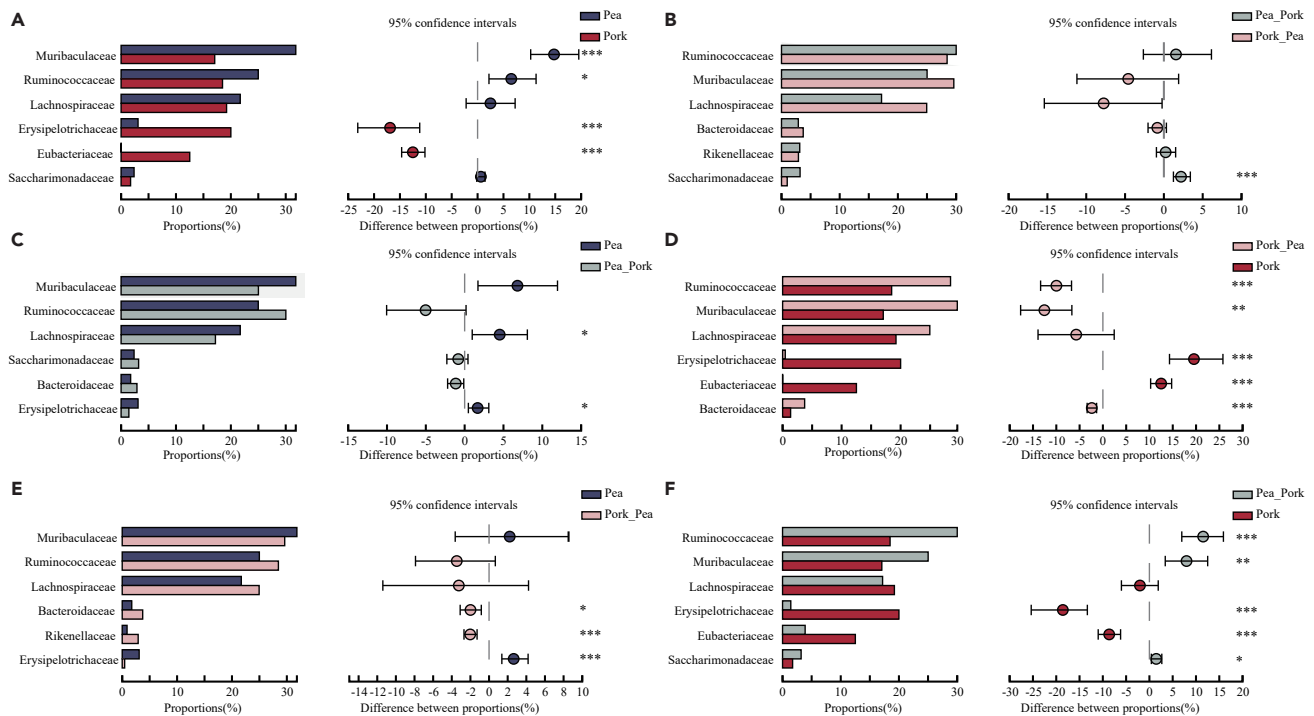


Figure 7. ■ ■ ■

Mean proportions of discrepant species at phylum level in different groups at 60 days by two-group comparisons using Welch's t test: (A–F) Pea versus Pork (A); Pea_Pork versus Pork_Pea (B); Pea versus Pea_Pork (C); Pork_Pea versus Pork (D); Pea versus Pork_Pea (E); Pea_Pork versus Pork (F).

* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. All error bars indicate SE.

whole protein itself (Figure 8B). Among 846 metabolites detected, 497 metabolites were identified with significant different expression quantities by pairwise comparisons with the orthogonal partial least squares discriminant analysis model (Figures 8C and S5). Based on the HMDB and KEGG databases, the identified metabolites were classified as lipids and lipid-like molecules, acids and derivatives, and organoheterocyclic and organic oxygen compounds. Among the 7 categories in KEGG metabolic pathway, most abundant metabolites (228 kinds of metabolites) were annotated in Metabolism type, with 51 kinds of metabolites in lipid metabolism and 40 in amino acid metabolism, followed by Organismal Systems (72 kinds of metabolites) and Human Disease (41 kinds of metabolites) (Figures 8D and 8E).

Pea and pork protein intervention significantly altered fecal metabolites of hamsters with 138 up-regulated and 126 down-regulated metabolites, some of which exhibited differences between pork and pea groups (Figure 9). First, comparison between pea and pork groups showed that pea protein consumption resulted in significant decreases in oxypinnatanine and glutamylproline concentrations and increases in 13,14-dihydro PGF-1a and 4,6-icosanedione (Figures 9A and 9B). Among the top 30 metabolites with high variable importance in projection (VIP) scores, glutamylproline, oxypinnatanine, and glycylylprolylhydroxyproline belonging to oligopeptide were decreased much more by pea protein. Moreover, the expression of N2-succinyl-L-ornithine, N-succinyl-L,L-2,6-diaminopimelate, and anserine involved in amino acid metabolism were also lower in pea group. Conversely, the sterol lipid compounds like 1 α ,25-dihydroxy-11 α -(1R)-oxiranyl]vitaminD3 and 22-dehydroclerosterol were enhanced by pea protein (Figure 9C). Second, comparison between the cross-over diet groups and pea or pork groups revealed 102 up-regulated and 130 down-regulated metabolites in Pea_Pork group compared with pea group (Figures 9D–9F), and 104 up-regulated and 141 down-regulated ones in Pork_Pea group compared with pork group (Figures 9G–9I). It is worthy to notice that the discrepant features were similar between Pea_Pork versus pea, Pork_Pea versus pork, and pork versus pea, especially the relative expression of oxypinnatanine, glutamylproline, 9,10-DiHODE, N2-succinyl-L-ornithine, and anserine with high fold change values. Similarly, these metabolites in the volcano plot displayed high VIP scores (Figures 9D–9I). In terms of specific metabolic features, dietary exchange led to the direct change of the metabolites away from those in the original

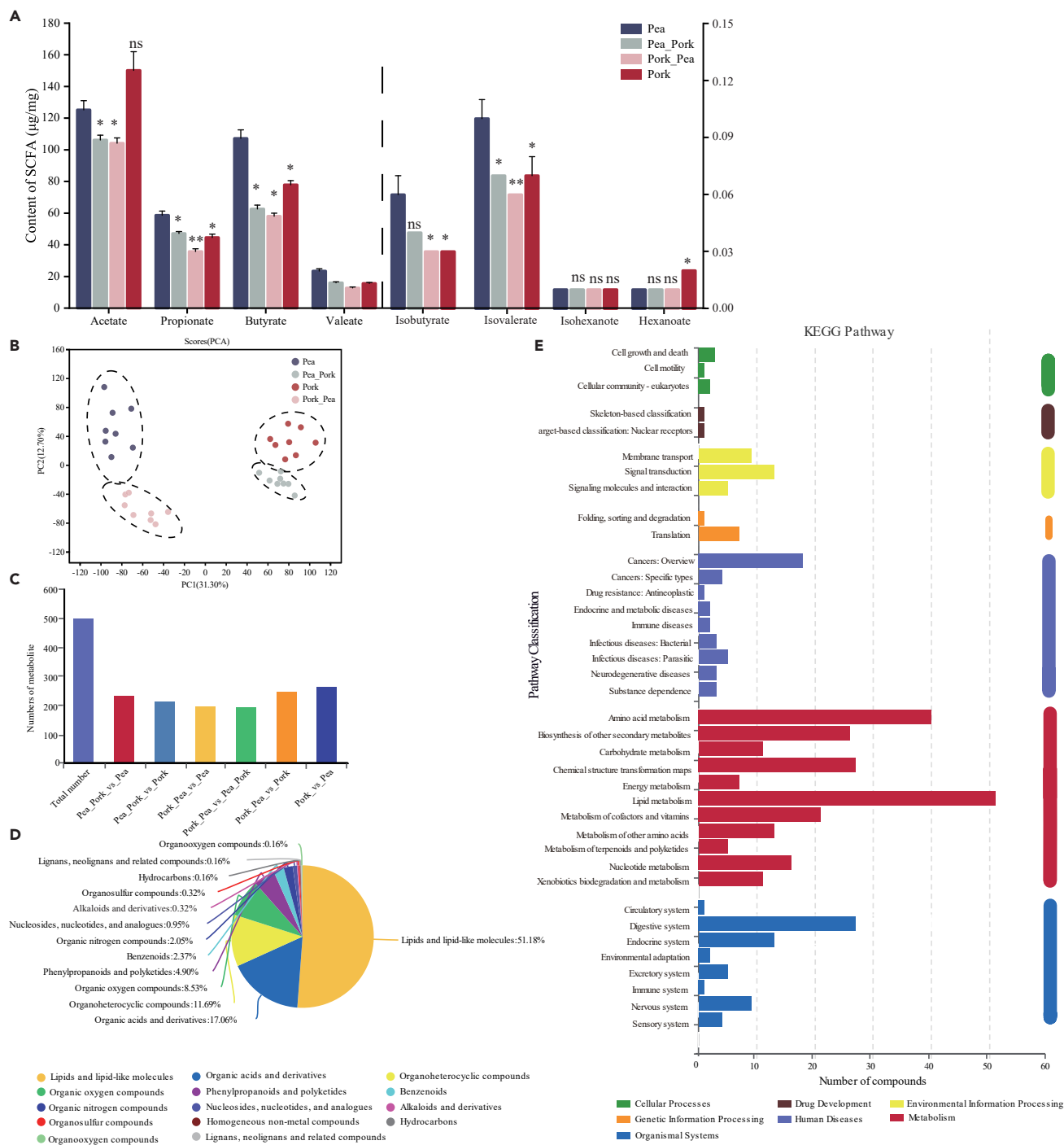


Figure 8. Overview of metabolic signatures among all groups

(A) Cecal SCFA contents of hamsters with cross-over intervention of pea and pork.

(B) PCA of metabolic features among all groups.

(C) Numbers of discrepant metabolites in comparison of the two groups.

(D) Pie chart based on counts of HMDB chemical taxonomy (superclass) for all metabolites detected in this study class.

(E) KEGG pathway classification of metabolites detected and annotated.

Statistical significance was calculated by Student's t test (*p < 0.05, **p < 0.01; ns, not significant). All error bars indicate SE.

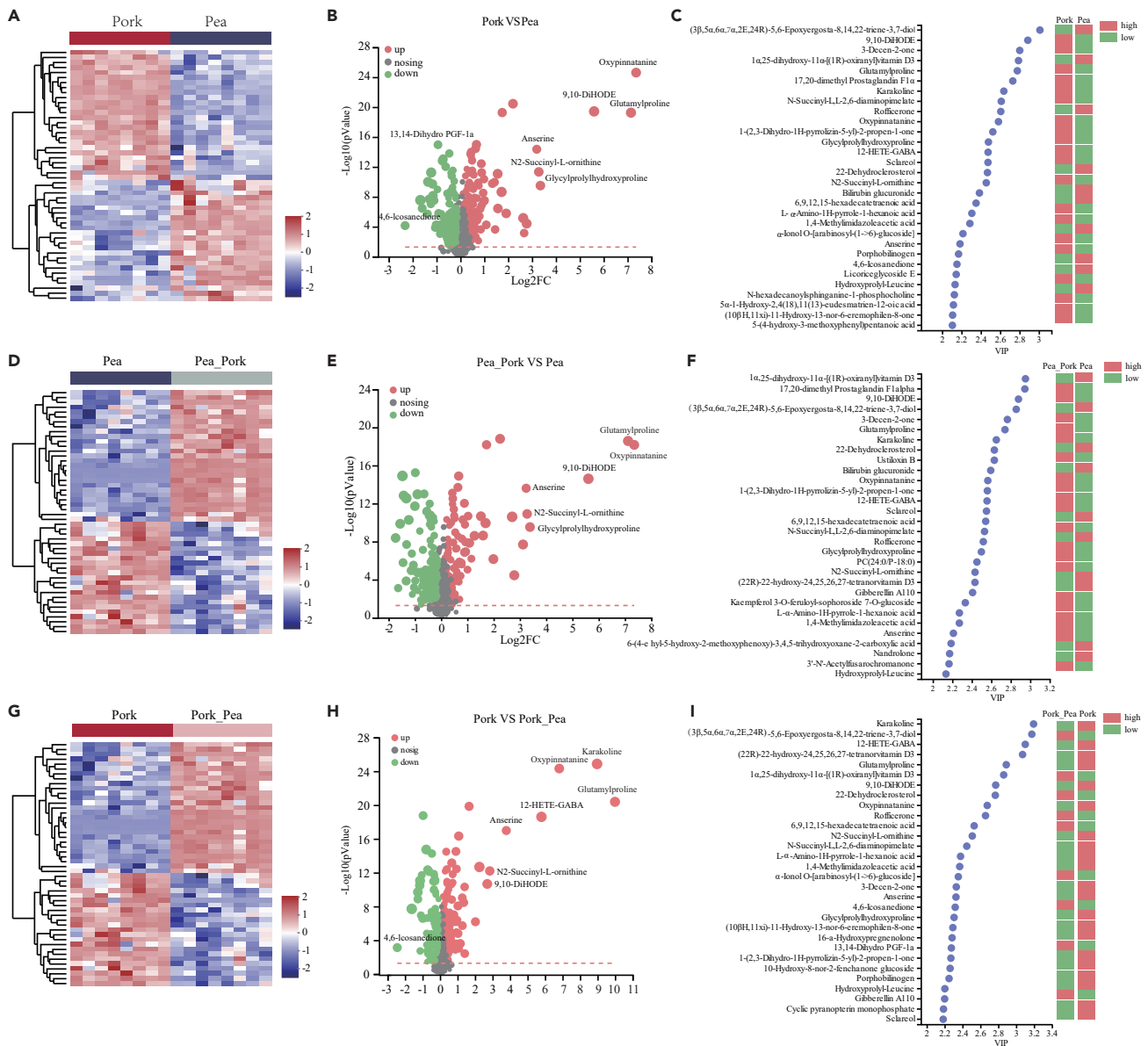


Figure 9. Cecal metabolome alterations

(A–C) Comparison between pork and pea groups by heatmap of discrepant metabolites (A); volcano plot of discrepant metabolites (B); discrepant metabolic signatures ranked by variable importance in projection (VIP) score (C).

(D–F) Comparison between Pea_Pork and pea groups by heatmap of discrepant metabolites (D); volcano plot of discrepant metabolites (E); discrepant metabolic signatures ranked by VIP score (F).

(G–I) Comparison between Pork_Pea and pork groups by heatmap of discrepant metabolites (G); volcano plot of discrepant metabolites (H); discrepant metabolic signatures ranked by VIP score (I). The red spots or pieces indicate up-regulation, and the green ones indicate down-regulation.

diet pattern. The expression of metabolites significantly differed neither between pea and Pork_Pea nor between pork and Pea_Pork. Third, the most effective metabolites with the highest VIP scores were analyzed as shown in Figures 10A–10C. The metabolites derived from amino acid metabolism showed that L-arginine was up-regulated in pea and Pork_Pea compared with pork group, but N2-succinyl-L-ornithine, anserine, hercynine, oxypinnatanine, and glutamylproline were down-regulated (Figures 10a1–a6). The bile acid metabolites involved in cholesterol metabolism showed that glycocholate was down-regulated in pea group, whereas taurine, 27-hydroxycholesterol, and 3 β ,7 α -dihydroxy-5-cholestenoate were up-regulated (Figures 10b1–10b4). Other metabolites involved in lipid metabolism showed increases in

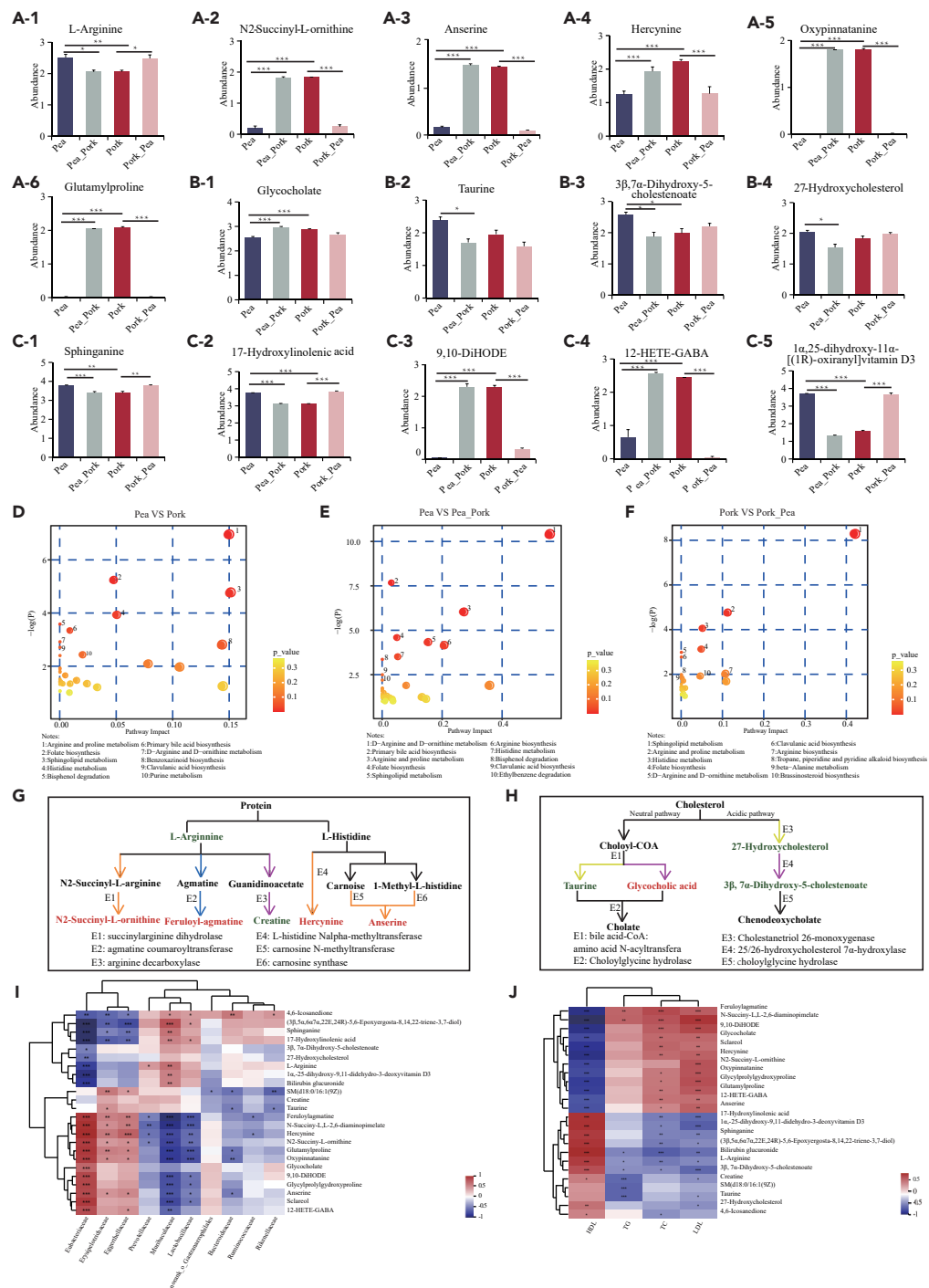


Figure 10. Alterations of specific cecal metabolites and pathways

(A–C) Relative expression of discrepant metabolites: L-arginine (a-1), N2-succinyl-L-ornithine (a-2), anserine (a-3), hercynine (a-4), oxypinnatanine (a-5), glutamylproline (a-6), glycocholate (b-1), taurine (b-2), 3 β ,7 α -dihydroxy-5-cholestenoate (b-3), 27-hydroxycholesterol (b-4), sphinganine (c-1), 17-hydroxylinolenic acid (c-2), 9,10-DiHODE (c-3), 12-HETE-GABA (c-4), 1 α ,25-dihydroxy-11 α -[(1R)-oxiranyl]vitamin D3 (c-5).

(D–F) The main metabolic pathways according to integrative analysis of the pathway impact and p value of metabolic signatures in comparison of pork and pea (D), Pea_Pork and pea (E), pork and Pork_Pea (F).

(G) A diagram of major relevant pathway of arginine and histidine metabolism. Green color of metabolites represents the up-regulation and red color represents the down-regulation in the presence of pea protein. Arrow with different color

Figure 10. Continued

represents significantly different pathway observed in comparison of different groups: orange represents pork versus pea, Pea_Pork versus pea, pork versus Pork_Pea; purple represents pork versus pea, Pea_Pork versus pea; blue represents pork versus pea, pork versus pork_Pea.

(H) A diagram of major relevant pathway of primary bile acid biosynthesis. Green and red metabolites represent up-regulation and down-regulation in the presence of pea protein. Arrow with different color represents significantly different pathways observed in comparison of different groups: purple represents pork versus pea, Pea_Pork versus pea; yellow represents Pea_Pork versus pea, respectively.

(I) Correlation analysis of specific metabolites with gut microbiota at family level.

(J) Correlation analysis of specific metabolites with serum lipid profiles.

Statistical significance was calculated by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant). All error bars indicate SE.

sphinganine and 17-hydroxylinolenic acid in the presence of pea protein, but decrease in 9,10-DiHODE. 12-HETE-GABA belonging to fatty amides was decreased in pea and Pork_Pea groups, whereas another fatty amide of 1 α ,25-dihydroxy-11 α -[(1R)-oxiranyl]vitaminD3 was increased (Figures 10c1–10c5).

The functions of these altered metabolites were identified by KEGG pathway analysis. The significantly different pathways common in the pairwise comparisons (pork versus pea; Pea_Pork versus pea; pork versus Pork_Pea) were mainly involved with amino acid metabolism, including arginine and proline metabolism, D-arginine and D-ornithine metabolism, and histidine metabolism (Figures 10D–10F). The primary bile acid biosynthesis as one of the most important pathways also contributed to the differences of Pea_Pork from pea. Accordingly, the pathways of amino acid metabolites converted from protein and the pathways of bile acid metabolites converted from cholesterol were identified as shown in Figures 10G and 10H, respectively. Spearman correlation analysis disclosed the relationship of cecal metabolites with gut microbiota or serum lipid profiles (Figures 10I and 10J). The gut microbiota were divided into three clusters, including left, middle, and right clusters, and the cecal metabolites were divided into two clusters, including up and down clusters (Figure 10I). Metabolites in the up cluster exhibited significantly negative correlation with the microbiota in the left cluster, whereas the metabolites in the down cluster displayed positive correlation with these microbiota in which Erysipelotrichaceae was identified as the “harmful” bacteria. On the contrary, the microbiotas in the middle cluster identified as the “beneficial” bacteria, such as Muribaculaceae and Lactobacillus, were positively correlated with metabolites in the up cluster but negatively correlated with metabolites in the down cluster. Correspondingly, metabolites in the up cluster were negatively correlated with serum HDL-C, whereas positively correlated with serum LDL-C and TC (Figure 10J). The negative correlation of serum HDL-C was the tightest with N-succinyl-L,L-2,6-diaminopimelate ($R = -0.89$), followed by feruloyl-*agmatine* ($R = -0.79$). The closest correlation was positively observed between N-succinyl-L,L-2,6-diaminopimelate and LDL-C ($R = 0.74$), as well as feruloyl-*agmatine* and TC ($R = 0.65$). In addition, the closest correlation between microbiotas and metabolites were presented by Eubacteriaceae, followed by Muribaculaceae.

DISCUSSION

Our present study revealed that the effects of proteins from diverse sources on cholesterol metabolism were closely dependent on gut microbiota susceptible to diet. It was consistent with the previous reports that consumption of plant-based and animal-based diet could alter microbial community structure differently (Butteiger et al., 2016; David et al., 2014; Devkota et al., 2012; Raman et al., 2019). Moreover, gut microbiota was indeed associated with various circulating metabolites like amino acid and lipid (Vojinovic et al., 2019; Fu et al., 2015; Rothschild et al., 2018). It has been identified that Bacteroides were related to metabolism of aromatic and branched-chain amino acid in a cohort of obese individuals (Liu et al., 2017a, 2017b). Our study initially demonstrated that the different effects of diverse protein on cholesterol regulation were resulted from the alteration of gut microbiota, which was confirmed by the Abx treatment experiment. Furthermore, the cross-over proteins of pea and pork could reverse gut microbiota composition and cecal metabolites thus affecting serum cholesterol level.

The plant- and meat-based proteins elicited variation of cholesterol levels in multiple ways, which have aroused great attention from researchers. In the early decades, the mechanism of cholesterol lowering by proteins mainly focused on the hepatic enzymes related to lipid metabolism, such as HMG-CoA reductase and CYP7A1 in the liver of hamster. In recent years, the composition and structure of gut microbiota have been well evidenced to be associated with cholesterol homeostasis (Villette et al., 2020).

Muribaculaceae, cultured and identified from the bacterial family S24-7, was negatively correlated with serum TC and TG and was strongly associated with SCFA secretion (Martinez et al., 2009; Lagkouvardos et al., 2019; Smith et al., 2019). The enhanced diversity of gut microbiota is crucial for protection of intestinal function, and reduced diversity was always accompanied by obesity and a diet with much sugar and fat (Sommer et al., 2017; Turnbaugh et al., 2008; Yatsunenkov et al., 2012). Our study elucidated that plant proteins induced higher bacterial diversity and Muribaculaceae proportion than meat proteins. Conversely, the red meat groups showed significantly higher proportions of Eubacteriaceae and Erysipelotrichaceae than plant groups. It is reported that the abundance of Erysipelotrichaceae was affected by cholesterol excretion in hamsters. Accumulating abundance of Erysipelotrichaceae in gut lumina of the mammal model of colon cancer was associated with inflammatory bowel diseases, and its reduction was observed in hamster model of hypercholesterolemia (Oliphant and Allen-Vercoe, 2019; Schaubeck et al., 2016). The present study demonstrated that the key role of “beneficial” microbe Muribaculaceae is responsible for the cholesterol regulation of plant proteins, especially pea and oat proteins, as well as the role of “harmful” microbe Erysipelotrichaceae for the cholesterol regulation of meat proteins.

The *norank_f_Muribaculaceae*, *Eubacterium_ruminantium_group*, and *Lactobacillus* showed negative association with serum cholesterol concentrations and positive association with SCFAs, which confirmed the beneficial effects of these bacteria on cholesterol regulation. Further correlation analysis also supported this finding that the increase in abundance of these three bacteria were positively correlated with Glu, Cys, Val, and Ser, and thus suggested the key role of amino acid compositions in lowering lipid and cholesterol levels. These results indicated that the discrepancy in the gut microbial community between plant and red meat groups was profoundly due to the different AA compositions of different proteins. Essential amino acids as a source of nitrogen and sulfur are irreplaceable for their enormous physiological importance (Wu, 2016). Glutamine supplementation can mitigate the waist circumference and blood lipopolysaccharide in obese individuals by modulating gut microbiota (Abboud et al., 2019). Val is an essential substrate involved in protein metabolism because it contributes to gut bacterial improvement of piglets (Yin et al., 2020). In the present study, the proportions of Glu and Val are higher in plant groups than in meat groups, and corresponded with three main genera enrichment, which were not characterized or cultured until now (Kenny et al., 2020). Therefore, diverse proteins have effects on the cholesterol metabolism not only by the absorption as amino acid or peptides in small intestine but also probably in the form of metabolites from amino acid in large intestine.

The Abx treatment eliminated specific microbiome and abolished discrepancies in gut microbiota in response to diverse protein interventions, which also deleted the difference in serum and liver cholesterol contents between pea and pork groups. These results agreed with the previous findings of Scott et al. who observed that Abx treatment eliminated the effects of tryptophan generated by gut microbiota catabolism (Scott et al., 2020). The Abx treatment was also reported to deplete most microbiota and abolish the beneficial effects of proanthocyanidin from grape seed on high-fat-diet-fed mice (Liu et al., 2017a, 2017b). The difference in two predominant gut microbiota, Firmicutes and Bacteroidetes, between pea and pork groups disappeared in the presence of Abx administration. As the abundance of Firmicutes and Bacteroidetes diminished largely, the Proteobacteria elevated correspondingly (Hwang et al., 2015). Firmicutes and Bacteroidetes are the two dominant phyla collectively accounting for 94% of total taxa at the end of experiment which is in line with the findings on gut microbiota at the aforementioned phylum level. Proteobacteria, as one of the most abundant bacteria, have the potential to drive chronic colitis in mice involved in cholesterol metabolism (Carvalho et al., 2012). In the present study, the elimination of differences in cholesterol levels between pea and pork groups supported the decisive roles of gut microbiota in cholesterol regulation.

Given gut microbiota was so important for the cholesterol-lowering effects of diverse proteins, whether immediate changes in diet could reshape the gut microbiota was necessary to be investigated. Accordingly, the dynamic reversal of gut microbial community was observed clearly when the diet converted from both pea to pork and pork to pea. The close correlation of dynamic reversal by diet with gut microbiota and further lipid profiles reconfirmed that diet alterations of appropriate protein could have beneficial effects on lipid metabolism by modulating the gut microbiota structure. The cross-over intervention from pork to pea protein significantly increased the proportions of Muribaculaceae, Ruminococcaceae, and Lachnospiraceae with correspondingly decreased Eubacteriaceae and Erysipelotrichaceae, which was coincident with the effects of pea protein. The replacement of pea protein with pork exhibited no profound

changes in the beneficial gut microbiota such as Muribaculaceae and Ruminococcaceae, which could be attributed to the strong resilience of gut microbiota induced by pea protein. The gut microbiota has been reported to restore the equilibrium of gut ecosystem to the external challenges that is resilience (Fasarella et al., 2020). In contrast, the replacement of pork protein with pea significantly reserved the gut microbiota compositions according to the diminution in probiotics like Muribaculaceae, Ruminococcaceae (Shang et al., 2016) and accumulation in the potential pathogenic bacteria Erysipelotrichaceae (Kaakoush, 2015). These evidences of “beneficial” microbe Muribaculaceae mainly responsible for pea protein effects and “harmful” microbe Erysipelotrichaceae for pork protein was consistent with the aforementioned results of gut microbiota compositions. The contrary alterations of gut microbiota found in the diet exchange possibly resulted from the alterations of multiple metabolites induced by pea protein supplement in complex metabolic pathways, which is illustrated next.

The significant changes in cecal metabolites in response to diverse proteins were mainly classified as the amino acid derivatives derived from protein metabolism, bile acids biosynthesis converted from cholesterol metabolism, SCFA secretion produced from gut microbiota, or lipid and lipid-like molecules involved in lipid metabolism. First, the differences in amino acids metabolites between pea and pork protein were mainly attributed to the arginine metabolism pathway, and histidine metabolism pathway. The effect of pea protein was not only related to arginine but also closely involved with its luminal metabolites, based on the results of down-regulated N2-succinyl-L-ornithine and feruloyl-*agmatine*, but up-regulated creatine. L-arginine supplementation has been reported to enhance whole-body insulin sensitivity and reduce plasma lipid and cholesterol levels (Moon et al., 2017; Dashtabi et al., 2015). In addition to the direct benefits, accumulating gut luminal arginine was also closely associated with the circulating amino acid metabolism by interacting with gut microbiotas to produce relevant metabolites (Liu et al., 2017a, 2017b; Claeysens et al., 2007). However, the correlation between L-arginine metabolites and gut microbiota relevant to cholesterol metabolism was not as clear as the function of L-arginine itself based on the plasma absorption. Our study disclosed that the metabolism of arginine/creatine pathway was beneficial for cholesterol-lowering, rather than arginine/N2-succinyl-L-ornithine or/feruloyl-*agmatine* pathways. Although the content of arginine in pork protein was higher than that in pea protein, the gut luminal arginine of hamster feeding with pea protein was much more than that of hamster feeding pork protein, this showing that more arginine promoted more growth of “beneficial” bacteria, such as Muribaculaceae and *Lactobacillus*, and their interaction produced more “beneficial” metabolites, such as creatine which is beneficial for cholesterol regulation. Conversely, less gut luminal arginine of hamster feeding with pork protein led to more growth of “harmful” bacteria, such as Erysipelotrichaceae, and further produced more “harmful” metabolites, such as N2-succinyl-L-ornithine and feruloyl-*agmatine*, which have negative effect on the cholesterol regulation of pork protein. As the histidine metabolism, the pathways of anserine or *hercynine* were inhibited in the presence of pea protein so as to promote probiotic growth and suppress cholesterol levels. Furthermore, oxypinnatanine and glutamylproline as unknown metabolites of amino acid might identify to be unfavorable for lowering-cholesterol regulation.

Second, primary bile acid biosynthesis involved in cholesterol metabolism was also affected by the dietary exchange from pork to pea protein. A key intermediate product from cholesterol to cholate, glycocholate, was observed to positively correlate with TC and LDL-C, whereas negatively correlate with HDL-C, which was also evidenced by Gu et al. (2017) who reported the potential roles of gut microbiota and bile acids in antidiabetic treatment. Primary bile acids are synthesized in the liver and discharged into intestine, where the conversion of cholesterol into bile acids occurs, and then subject to extensive metabolism by gut microbes, namely, deconjugation of glycine or taurine and biotransformation of the unconjugated primary bile acids to secondary bile acids (Heinken et al., 2019; Wahlström et al., 2016). This complex transformation of primary and secondary bile acids is crucial for their biological function contributing to absorption of dietary lipids (Derrien and van Hylckama Vlieg, 2015). In the present study, pork protein resulted in the significant inhibition of acidic pathway and the stimulation of neutral pathway through which cholesterol was converted to cholate by glycocholate pathway. However, pea protein promoted the production of cholate by taurine pathway, and its effect on chenodeoxycholate synthesis by 27-hydroxycholesterol/3 β ,7 α -dihydroxy-5-cholestenoate pathway was more significant, indicating the close relationship between bile acid biosynthesis and the changes in gut microbiota and cholesterol regulation.

Third, the differences between pea and pork protein were also associated with lipid-like metabolism, such as sphingomyelin/ceramide pathway activation plays a crucial role in oxidized LDL-induced atherosclerotic

lesions (Augé et al., 2004); 17-Hydroxylinolenic acid or 9,10-DiHODE as linolenic metabolites indeed regulate the lipid profiles by inhibition of HMG-CoA activity (Schuchardt et al., 2013); two fatty amides, 12-HETE-GABA and 1 α ,25-dihydroxy-11 α -[(1R)-oxiranyl]vitaminD3, also contribute to the diverse effects of protein. Consistently, as productions of gut microbiota, the SCFA contents in the caecum, including acetate, propionate, and butyrate, were significantly increased by pea protein intervention together with the increase in relative abundance of Muribaculaceae, which supported their positive association as reported by previous studies (Lagkouvardos et al., 2019; Smith et al., 2019; Shang et al., 2016). These metabolite alterations resulting from gut microbiota corresponded to the changes in lipid and cholesterol levels induced by the diverse proteins. Gut microbiota can utilize digestion products of food ingredients that diverse proteins produce different nutrients for gut microbiota to generate various metabolites, in turn, to involve in host metabolism (Alexander and Turnbaugh, 2020). Therefore, the cholesterol-lowering effects of pea protein can be attributed to its promotion of beneficial gut microbiota like Muribaculaceae and Lachnospiraceae, which resulted in the corresponding regulation of cecal metabolites involved in amino acid, bile acid, and lipid metabolism.

In conclusion, this is the first time that the different cholesterol-regulating effects of proteins from diverse sources have been identified and these differences have been correlated with changes in gut microbiota so as to clarify the effective mechanism of cholesterol metabolism. Plant proteins such as pea protein showed significantly lower serum or liver cholesterol levels than meat proteins such as pork protein, which corresponded to the changes in gut microbiota including the increased abundances of Muribaculaceae by pea protein and Erysipelotrichaceae by pork protein. The decisive roles of gut microbiota were confirmed by the findings that no more difference in serum or liver cholesterol was observed in the presence of Abx with the elimination of gut microbiota. The effects of gut microbiota on cholesterol metabolism depended on the dietary pattern, which was reversible by shifting protein types. The relevant SCFA levels responsible for Muribaculaceae and Erysipelotrichaceae were also distinguished between pea and pork proteins. As the cecal metabolites induced by diverse proteins supplement, cross-over intervention either from pork to pea or from pea to pork led to reversed changes in arginine, taurine, and proline metabolism and sphingomyelin/ceramide pathway. Overall, our study demonstrated that the difference in cholesterol regulation between pea and pork proteins could be attributed to the improvement of amino acid metabolism, bile acid biosynthesis, and SCFAs secretion, which depended on gut microbiota, and, in comparison, consumption of pea protein was beneficial for cholesterol regulation by stimulating the growth of beneficial bacteria of Muribaculaceae.

Limitations of study

In this study, we studied metabolite changes caused by different proteins by untargeted metabolomics. However, to further identify how proteins modulate host cholesterol, targeted metabolomics should be used in further studies. Moreover, amino acids may also have a huge impact on cholesterol metabolism, which should be tested in the future.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.103435>.

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AUTHOR CONTRIBUTIONS

L.-T.T. and F.W. designed and supervised this study. T.X. and A.W. prepared the samples and conducted the animal experiments. T.X., W.Q., and X.Z. performed the data analysis. L.W., C.L., and L.L. assisted in statistical analysis of the metadata. L.-T.T. and T.X. wrote the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibiotics		
Vancomycin hydrochloride	Shanghai yuanye Bio-Technology Co., Ltd.	CAS# 1404-93-9
Bacitracin zinc	Shanghai yuanye Bio-Technology Co., Ltd.	CAS# 1405-89-6
Critical commercial assays		
E.Z.NA® stool DNA kit	Omega Bio-tek	
AxyPrep DNA gel extraction kit	Axygen Biosciences	
Deposited data		
GitHub	This paper	82,101,182,149
BioProject	This paper	PRJNA689329
Software and algorithms		
FLASH	1.2.11	https://ccb.jhu.edu/software/FLASH
Fastp	0.19.6	https://github.com/OpenGene/fastp
Uparse	7.0.1090	http://www.drive5.com/uparse/
Qiime	1.9.1	http://qiime.org/install/index.html

RESOURCE AVAILABILITY

Lead contact

Furthermore information and requests for resources and reagents should be directed to and will be filled by the lead contact, Fengzhong Wang (wangfengzhong@sina.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The biochemical data in this work are available at GitHub: 82101182149_Protein. The raw data were deposited to NCBI SRA database and are available for download under BioProject accession code PRJNA689329. The raw data of untargeted metabolome analysis are freely accessible at GitHub: 82101182149_Metabolites.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal experiment

All animal experimental protocols were approved by the Ethical Committee of Experimental Animal Center of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College. All the male hamsters (91.67 ± 0.29 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in a specific pathogen-free (SPF) animal center. The hamsters were acclimatized at $20.0 \pm 0.5^\circ\text{C}$ and $50 \pm 10\%$ humidity with a 12 h light-dark cycle and given free access to water and food. The experimental diets were prepared by Beijing Nuokangyuan Biotechnology Co., Ltd. (Beijing, China) according to the diet recommended by the American Institute of Nutrition (AIN)-93G formula with few modifications (Table S3).

METHOD DETAILS

Preparation of diverse proteins

Rice, soybean and pea proteins were provided by Anhui Lurong Co., Ltd. (Anhui, China) and Shandong Yantai Dongfang Protein Co., Ltd. (Shandong, China), respectively. Oat powder was provided by Hebei Shijiazhuang Lingfeng Co., Ltd. (Hebei, China), and oat protein was prepared by the alkali-solution and acid-

isolation method. Chicken, beef, and pork powder were provided by Shandong Qingdao Derunlong Food Co., Ltd. (Shandong, China), and the animal proteins were prepared by defatting the meat powder with petroleum ether. Briefly, meat powder was stirred with petroleum ether (m/v 1:4) for 1 h and then the upper organic solvent was removed. The extraction was repeated 3 times to achieve maximum defatting.

Animals and diets

All animal experimental protocols were approved by the Ethical Committee of Experimental Animal Center of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College. All the male hamsters (91.67 ± 0.29 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in a specific pathogen-free (SPF) animal center. The hamsters were acclimatized at $20.0 \pm 0.5^\circ\text{C}$ and $50 \pm 10\%$ humidity with a 12 h light-dark cycle and given free access to water and food. The experimental diets were prepared by Beijing Nuokangyuan Biotechnology Co., Ltd. (Beijing, China) according to the diet recommended by the American Institute of Nutrition (AIN)-93G formula with few modifications (Table S3). Proteins in these experimental diets were substituted with the obtained proteins prepared previously, and the protein in the control group was casein.

In the protein intervention study, four-week-old hamsters were divided into eight groups ($n = 10$) based on serum total cholesterol content after 7-day acclimatization and then fed experimental diets for 30 days. The control group was conventionally raised with standard diet including no extra cholesterol, and the experimental groups were fed with high cholesterol diets containing proteins from rice, oat, soybean, pea, chicken, pork, and beef, respectively (Table S3).

In the antibiotic (Abx) treatment experiment, twenty hamsters were divided into two groups based on the serum total cholesterol (TC) content and fed with pea or pork protein after 7-day acclimatization. Two phyla *Firmicutes* and *Bacteroidetes* belong to *Bacteria* were manifested to be the majority of mammal's gut microbiota (Ley et al., 2008). Vancomycin hydrochloride and Bacitracin Zinc have strong sterile effects on *Firmicutes* and *Bacteroidetes* while were hardly absorbed by the host gastrointestinal tract (Hwang et al., 2015). The hamsters were administered drinking water containing Vancomycin hydrochloride (0.5 g/L; Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China) and Bacitracin Zinc (1.0 g/L; Shanghai yuanye Bio-Technology Co., Ltd.) for 30 days. The lipid profiles and the gut microbiota were analyzed before (Pea_Abx_0d, Pork_Abx_0d) and after (Pea_Abx_30d, Pork_Abx_30d) the feeding with Abx treatment.

In the cross-over dietary protein experiment, there were two groups each containing 20 hamsters respectively feeding pea and pork protein during the first month. After 30 days of feeding, fasting blood was collected and the pea group was then divided into two groups based on their serum TC concentration for further feeding pea (pea) and pork protein (Pea_Pork), respectively. Likewise, the pork group was divided into two groups for further feeding pea (Pork_Pea) and pork protein (pork), respectively.

The daily food intake of hamsters was monitored to ensure no difference in the contents of protein, starch and lipid among all experiments, and the body weight was recorded once a week. Hamsters were fasted overnight before being sacrificed at the end of these experiments. Blood was collected from the orbital plexus by diethyl ether anesthetization, and centrifuged at 4°C , 3500 rpm for 10 min. The liver, caecum, colon and fresh feces were immediately collected and placed on liquid nitrogen. All the tissues were stored at -80°C for further analysis.

Biochemical analysis

The lipid profiles, including serum TC, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol LDL-C, and triglyceride (TG), were detected by Fully Automatic Biochemical Analyzer (Hitachi, Tokyo, Japan). The apolipoprotein A, B, E contents in serum; the TC, TG and free cholesterol (FC) contents in liver; the TC and total bile acid contents in feces were measured using Elisa kits from Appligen Technologies Co., Ltd. (Beijing, China). The liver cholesterol ester (CE) was calculated by liver TC minus liver FC. The concentrations of 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG-CoA reductase), cholesterol 7α -hydroxylase (CYP7A1), fatty acid synthase (FAS), cholesterol acyltransferase (ACAT), lipoprotein lipase (LPL), and low-density lipoprotein receptor (LDLR) in liver were measured with Elisa Kits (Shanghai Enzyme-linked Biotechnology Co., Ltd. Shanghai, China) following manufacturer's instructions.

SCFA analysis

Total SCFAs were extracted from fecal samples (10 mg) supplemented in 10 μ L of internal standards (0.0125 μ L/ μ L 2-ethylbutyric acid, Sigma-Aldrich) and 500 μ L of methanol (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China). The extracts were detected using a gas chromatographic coupled with a mass spectrometer (Agilent Technologies Inc, CA, USA), and quantified by the Masshunter quantitative software. SCFAs standards were mixtures of acetate, propionate, butyrate, isobutyrate, valerate, hexanoate, isohexanoate (Darmstadt, Germany), and isovalerate (Sigma-Aldrich).

Gut microbiota analysis

Genomic DNA was extracted from fecal samples using the E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The quality of extracted DNA was checked by 1% agarose gel, and DNA concentration and purity were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA).

The V3–V4 hypervariable regions of the 16S rRNA gene were subjected to high-throughput sequencing by Beijing Allwegene Tech, Ltd (Beijing, China) with the Illumina Miseq PE300 sequencing platform (Illumina, Inc., CA, USA). The V3-V4 region of the bacteria 16S rRNA gene was amplified with the universal primers of the forward 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse 806 R (5'-GACTAC HVGGGTWCTAAT-3') by an ABI GeneAmp 9700 PCR thermocycler (ABI, CA, USA). These primers contained a set of 8-nucleotide barcodes sequences unique to each sample. The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, single extension at 72°C for 10 min, and end at 10°C. The PCR mixtures contain 5 \times TransStart FastPfu buffer 4 μ L, 2.5 mM dNTPs 2 μ L, forward primer (5 μ M) 0.8 μ L, reverse primer (5 μ M) 0.8 μ L, TransStart FastPfu DNA Polymerase 0.4 μ L, template DNA 10 ng, and finally ddH₂O up to 20 μ L. Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and quantified using QuantiFluor-ST (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 \times 300) on an Illumina MiSeq platform (Illumina, San Diego, USA).

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH. In brief, quality control criteria mainly included removal of low-quality sequences and barcode sequence: the reads at any site receiving an average quality score of <20 within the 50 bp sliding window set to reduce sequencing error, the truncated reads shorter than 50 bp, reads containing ambiguous characters. Moreover, only overlapping sequences longer than 10 bp were allowed to assemble and the maximum mismatch ratio of the overlap region is 0.2. Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted. There was 2 nucleotide mismatch allowed in primer matching while no allowance in barcode mismatching. The acquired high-quality sequences were classified into operational taxonomic units (OTU) at 97% identity using UPARSE version 7.1. Chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA Silva v138 database using a confidence threshold of 0.7. After these progresses, all sequences were picked randomly for further analyses according to the least number to eliminate the differences caused by sequencing depth among diverse samples.

Untargeted LC-MS metabolomics

The metabolites were extracted from caecum contents using a 400 methanol: water (4:1,v/v) solution. The mixture was allowed to settle at –20°C and treated by High throughput tissue crusher Wonbio-96c (Shanghai wanbo biotechnology co., LTD) at 50 Hz for 6 min, then followed by vortex for 30s and ultrasonication at 40 kHz for 30 min at 5°C. The samples were placed at –20°C for 30 min to precipitate proteins. After centrifugation at 13000 g at 4°C for 15 min, the supernatant was carefully transferred to sample vials for LC-MS/MS analysis. Equal volumes of all samples were mixed for preparing pooled quality control (QC) samples.

Chromatographic separation of the metabolites was performed on an ExionLCTMAD system (AB Sciex, USA) equipped with an ACQUITY UPLC system with UPLC HSS T3 column (100 mm \times 2.1 mm i.d., 1.8 μ m; Waters, Milford, USA), and a quadrupole time-of-flight mass spectrometer (Triple TOFTM5600+, AB Sciex, USA) with an electrospray ionization (ESI) source. The raw data was imported into the Progenesis QI 2.3 (Nonlinear Dynamics, Waters, USA) for peak detection and alignment. Metabolic features detected

at least 80% in any set of samples were retained. Statistical analysis was performed on log transformed data for normalization and imputation to identify significant differences in metabolite levels between comparable groups. All of the metabolite variables were scaled to pareto Scaling prior to orthogonal partial least squares discriminant analysis (OPLS-DA). The OPLS-DA model validity was evaluated from model parameters R^2 and Q^2 , which provided information for the interpretability and predictability, respectively, to avoid the risk of over-fitting of the model. Variable importance in the projection (VIP) was calculated in OPLS-DA model. *p* values were estimated with paired Student's *t*-test on Single dimensional statistical analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

The differences in the concentrations of lipids in serum, liver, fecal, the contents of enzymes related to lipid metabolism in liver, the contents of SCFAs and bile acid in fecal, and relative abundance of bacteria in the protein groups and control; in pea_pork, pork_pea, pork and pea groups were evaluated using Student's *t*-test. *p* value less than 0.05 was declared significantly. Community richness was evaluated by Simpson, Ace, Chao and Shannon. Bray Curtis similarity clustering analysis was performed by R package (version R 3.0.2). Multiple group comparisons were conducted by Kruskal-Wallis H test and two-group comparisons were performed by Wilcoxon ran-sum test. LDA coupled with effect size measurements (LEfSe) was performed to discover highly-dimensional gut bacteria and characterize the differences between two or more biological conditions.