Dietary protein sources differentially affect the growth of *Akkermansia muciniphila* and maintenance of the gut mucus barrier in mice

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Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, KEGG orthology; AB-PAS, Alcian blue-periodic acid Schiff; CPD, chicken protein-based diet; SPD, soy protein-based diet; GF, germ-free; SPF, specific-pathogen-free.

Keywords: *Akkermansia muciniphila*, chicken protein, germ free mice, oxidative phosphorylation, soy protein

Abstract

Scope

The gut microbiota plays an essential role in linking diet to host health. The specific role of different dietary proteins on the gut microbiota and health is less understood. Here we investigated the impact of proteins derived from chicken and soy on the gut microbiota and host gut barrier in C57BL/6 mice.

Methods and results

Specific-pathogen-free and germ-free mice were assigned to either a chicken or a soy protein-based diet for 4 weeks. Compared with a chicken protein-based diet, intake of a soy protein-based diet reduced the abundance of *A. muciniphila* and the number of goblet cells, lowered the level of *Muc2* mRNA, and decreased the thickness of the mucus layer in the colon of specific-pathogen-free mice. In germ-free mice colonization with *A. muciniphila* combined with intake of a chicken protein-based diet resulted in a higher expression of the *Muc2* mRNA in colon, and surprisingly, an increased potential for oxidative phosphorylation in *A. muciniphila* compared with colonized mice fed a soy protein-based diet.

Conclusion

These findings suggest possible mutually beneficial interactions between the growth and function of *A. muciniphila* and host mucus barrier in response to intake of a chicken protein-based diet contrasting the intake of a soy protein-based diet.

1. Introduction

Meat accounts for an increasing proportion in human diets, but this increase has been associated with many metabolic disorders, and continued high intake of animal protein is not sustainable. [1, 2] Furthermore, problems associated with high meat intake may also potentially be related to a concomitant decrease in plant-derived foods rich in dietary fibers and other bioactive components. Research has in particular focused on associations between intake of red meat, the gut microbiota, and disorders such as cardiovascular disease. [3, 4] The gut microbiota is now recognized as a crucial factor in human physiology and nutrition, linking diets to host health. [5, 6, 7] However, little attention has been paid to the effect of different dietary proteins including chicken meat, a widely consumed food, on the gut microbial composition and host physiological responses.

In an *in vitro* study, incubation of human fecal samples with digested freeze dried boiled chicken meat increased the abundance of *Bifidobacterium* spp., *Bacteroides, Coriobacterium,* and *Atopobium* compared with beef or fish meat. [8] In rat studies, intake of a chicken protein-based diet with normal protein content resulted in a significantly higher abundance of *A. muciniphila* in the gut microbiota compared with a soy protein-based diet, [9] implicating possible association between a chicken protein-based diet and *A. muciniphila*, which could further play a critical role in host health.

A. muciniphila is a mucin degrader most abundantly present in the colon able to colonize the mucus layer of the human intestinal tract and reported to play an important role in

maintaining the gut barrier. [10, 11, 12] *A. muciniphila* is capable of using mucin as a carbon, nitrogen, and energy source, and releasing sulfate in a free form. [13] The heavily glycosylated gel-forming mucin *Muc2* encoded by *Muc2* gene is the main component of mucin.[14] which forms a physical barrier and defense system along the intestine with varying local properties. If there is no mucus layer, as in the case of *Muc2*$^{-/-}$ mice, colonization of enteric pathogens occurs to a greater extent and more easily than in wild type animals. [15]

As a nutritional source, mucins may serve as even more important modulators of the gut microbiota composition and *A. muciniphila* growth than colon pH. [16] *A. muciniphila* has been proposed as a beneficial gut microbe playing a critical role in alleviating metabolic disorders such as obesity, [17, 18, 19] diabetes and colitis [20, 21, 22, 23, 24]. Recent studies have also shown that supplementation with *A. muciniphila* decreased metabolic endotoxemia and induced a more favorable plasma metabolite profile. [25, 26] The colonization of this bacterium in the gut depends, to a high degree, upon dietary components. [27, 28] However, little is known about the underlying mechanisms.

In the present study, we explored the impact of chicken or soy protein-based diets on the abundance and functional potential of *A. muciniphila* in the gut microbiota, and further used germ-free mice to examine how dietary proteins affected colonization and growth of *A. muciniphila*.

2. Experimental Section

2.1. Diets

Chicken and soy protein diets were prepared with reference to the AIN-93G formula by replacing casein with chicken protein or soy protein. [29] Briefly, soy protein isolates were obtained from a commercial company (Lin Biological Products, Linyi, Shandong, China) and isoflavones were removed by extraction with 80% methanol. Chicken protein was prepared as described by Zhao et al. [9] Chicken *pectoralis major* muscle was cooked in a pouch till the core temperature reached 70ºC. Then, the cooked meat was minced, and fat was removed with a mixture of dichloromethane and methanol (2:1, v:v). Meat powder was passed through a 25 mesh screen. The resulting powder contained greater than 90% proteins. The proteins were incorporated at a percentage of 20% (% weight) into the diets, which contained 39.75% cornstarch, 13.20% sucrose, 7.00% soybean oil, 5.00% fiber, 3.50% mineral mix (AIN-93-G-MX), 1.00% vitamin mix (AIN-93-VX), 0.3% L-cystine, 0.25% choline bitartrate and 0.0014% tert-butylhydroquinone. The acclimatization diet, purchased from Jiangsu Xietong Pharmaceutical Bio-engineering Co., Ltd., was identical to the diet offered to the mice after weaning. The nutrients and amino composition of the diets are listed in Supporting Information Table S1.

2.2. Feeding and sampling

Eighteen 5-weeks-old conventional male C57BL/6 mice were single housed in an SPF animal facility (SYXK(Su)2011-0037) with 12h-light-dark cycles. The mice were allowed to access the acclimatization diet and water *ad libitum*. After a 7-days adaption (6-weeks-old), the mice were randomly divided into two groups and fed either SPD or CPD (n=9, each group). Before changing the diets, fecal samples were collected. After 4-weeks feeding, mice were sacrificed by cervical dislocation. Cecal and descending colonic contents and colon tissue were collected in sterile tubes for microbial and RT-PCR analysis. Colonic content samples from two animals in the SPD group and one animal in the CPD group were unavailable because no stools were present in the descending colon. The middle part of the colon was fixed in methanol-Carnoy's solution for measuring the mucus layer and goblet cells.

A total of 20 6-weeks-old male C57BL/6 germ-free mice were single housed in an isolator facility. The mice were kept in the same isolator after weaning (4-week-old), and no adaption time was applied. Mice were randomly divided into four groups (n=5) and subjected to one of the four treatments, namely, either solely being fed CPD or SPD that was irradiated at 25kGy, or colonization with *A. muciniphila* on day 14 of the feeding period. The colonization was performed by oral gavage administrating of 5×10^8 cfu of *A. muciniphila* in 200 μL of phosphate-buffered saline. *A. muciniphila* (strain DSM 22959, Germany) was cultured under anaerobic conditions in brain heart infusion broth (BD Bioscience, San Jose, CA) supplemented with 0.2% porcine mucin (Sigma-Aldrich, St Louis, MO). The concentration was monitored by measuring the absorbance at 600 nm. The total

period of feeding was 4 weeks. Mice were sacrificed by cervical dislocation. Cecal and colonic contents were collected for microbial and metatranscriptomic analyses. Colon tissue was collected for RT-PCR. The colon tissue was also fixed in methanol-Carnoy's fixative for measuring mucus layer and goblet cells.

2.3 Dosage information

Chicken and soy protein diets were prepared with reference to the AIN-93G formula by replacing casein with chicken protein or soy protein. [29] The protein content (20%) and fat content (7%) of the diets were at the normal dose. The colonization of *A. muciniphila* in germ-free mice was performed by oral gavage administrating of 5×10^8 cfu of *A. muciniphila* in 200 µL of phosphate-buffered saline according to the previous studies. [22, 30]

2.3. 16S rRNA gene sequencing

Total microbial DNA was extracted from cecal contents, colonic contents, or feces using a commercial stool DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The V4 hypervariable region of the 16S ribosomal RNA gene was selected for amplification. The universal primers used were F515 (5'-GTGCCAGCMGCCGCGG-3') and R806 (5'-GGACTACHVGGGTWTCTA AT-3') that carried an eight-base unique barcode sequence for each sample. [31] PCR reactions were run and amplicons were sequenced as described previously. [32] Detailed methods for all analyses can be found in Supporting Information Detailed Methods.

2.4. In vitro cultivation of *A. muciniphila*

Soy protein and chicken protein were in vitro digested with pepsin and trypsin as previously described. [33] Briefly, the protein powder (1 g, each) were mixed in 9 mL of 1×PBS buffer (pH= 7.4). The mixture was adjusted to pH 2.0 with 1 mol/L HCl, and pepsin (≥400 units/mg of protein, from porcine gastric mucosa, product number P7125, Sigma-Aldrich, St. Louis, MO) was added at a ratio of 1:31.25 of the substrate. The mixture was incubated at 37 °C for 2 h with continuous shaking, and then the enzyme was inactivated by adjusting the pH to 7.5 with 1 mol/L NaOH on ice. Then, trypsin (1645 units/mg of protein, from porcine pancreas, lyophilized powder, type II-S, product number T7409, Sigma-Aldrich, St. Louis, MO) was added at a ratio of 1:50 substrate. The reaction mixture was maintained under the same conditions as above. After 2 h of trypsin digestion, enzyme activity was terminated by heating at 95 °C for 5 min. The digestion products were freeze-dried. *A. muciniphila* strain DSM 22959 (provided by Dr. Li Liu at Nanjing Agricultural University) was grown anaerobically in BHI media supplemented with 0.2% mucin, freeze-dried digestion products of soy protein or chicken protein. The growth profile was evaluated by intermittently measuring absorbance at 600 nm.

2.5. Histological observations

Colon tissue was fixed in methanol-Carnoy's fixative overnight. The 5-mm-thick sections were embedded in paraffin. Serial transversal sections (4μm thick) were stained with periodic acid-Schiff (PAS) and Alcian blue in order to visualize mucin-producing cells (goblet

cells) and the mucus layer. [20] The number of PAS-positive cells was counted on ten random crypts per section (3 sections per sample). The thickness of mucus layer was measured on ten random visual fields per section under a light microscope (ZEISS Axio Imager 2, Oberkochen, Germany). The histological observations were done on 33 biological samples for all treatments.

2.6. RNA isolation and real-time quantitative PCR analysis

Total RNA was extracted from colon tissue by using Takara MiniBEST universal RNA extraction kit (Takara, Kusatsu, Japan) followed by reverse transcription into cDNA using PrimeScript™ RT Master Mix (Takara, Kusatsu, Japan). Quantitative real-time PCR was performed with an Applied Biosystems QuantStudio 6 flex real-time PCR system (Life Technologies, Waltham, Massachusetts). Data analysis was performed according to $2^{-\triangle\triangle Ct}$ method. [34] The SPD group was set as calibrator when performing the statistical analysis. The primers for each specific gene are listed in Supporting Information Table S2.

2.7. Metatranscriptomic analysis

Total RNA was extracted from cecal contents of germ-free mice colonized with *A. mucniphila* to explore the transcriptome profile of this bacterium in response to intake of different protein-based diets. Detailed methods for all analyses can be found in Supporting Information Detailed Methods.

2.8. Statistical analysis

The effects of diet on the measured variables were evaluated by one-way analysis of variance in which protein diet was set as the independent, and the measured variables were set as the dependent. The means were compared by Duncan's multiple comparison using the SAS system (version 9.2), and a *P* value lower than 0.05 was considered significant. When wild-type and germ free mice were compared, multiple analysis of variance was applied. The diet effect on the frequency of *A. muciniphila* was evaluated by Kruskal-Wallis test, and *P* value lower than 0.05 was considered significant.

3. Results and discussion

3.1. A chicken protein-based diet maintains whereas a soy protein-based diet impairs the growth of *A. muciniphila*

To investigate how different protein sources affected the gut microbiota, male C57BL/6 mice kept under standard SPF conditions were fed soy (SPF-SPD) or chicken (SPF-CPD) protein-based diets for 28 days. We observed no significant differences between the two diet groups in weight gain or feed intake over time during this relatively short feeding period (Figure 1A and B). We collected fecal samples on day 0, and cecal and colonic content samples on day 28. DNA was extracted for 16S rRNA gene amplicon sequencing. A total of 2,117,748 reads were obtained from 48 samples with an average of 44,119 $\pm$ 9,531 reads per sample, and an average total read length of 255 bp (Supporting Information Table S3).

Diversity analyses indicated that most species had been captured by this sequencing depth (Supporting Information Figure S1).

Although no differences were observed in body weight gain or feed intake between CPD- and SPD-fed mice, diet had a substantial impact on the gut microbiota composition (Figure 2A). As the gut microbiota composition in fecal samples has been reported to largely reflect that of the colonic content, [35, 36] we compared the fecal samples collected at day 0 with the colonic content sampled on day 28. This comparison revealed that intake of the SPD and the CPD altered the gut microbiota composition with a good separation between the day 28 colonic and the day 0 fecal samples. However, importantly we also observed a clear separation between the colonic samples from mice fed the SPD or the CPD (Figure 2A). Similarly, cecal samples from SPD and CPD fed mice also clearly separated from each other (Figure 2A), similarly to the reported comparison between cecal content samples from rats fed chicken or soy protein-based diets. [9] We noticed that the microbial composition of the colonic and cecal samples exhibited a high degree of similarity in the mice fed either CPD or SPD based on unweighted unifrac distance analysis (Figure 2B), whereas the microbial composition exhibited higher similarity in colonic or cecal samples independent of diet when based on weighted unifrac distance analysis (Figure 2C).

At the phylum level, Firmicutes and Bacteroidetes were as expected the two predominant phyla in all the samples (Figure 2D), accounting for 54.29% to 98.20% of the total OTUs. Before the experimental protein diets were provided (day 0), Bacteroidetes was the dominant phylum in fecal samples, and the shift to the experimental diets increased the

abundance of Firmicutes. Of note, in mice receiving the SPD we observed a decreased in the relative abundance of Verrucomicrobia, while we did not observe a similar decrease in the abundance of Verrucomicrobia in mice receiving the CPD. A linear discriminant analysis of effect size (LEfSe) revealed that the abundances of 180 OTUs were significantly changed by the diets (Supporting Information Table S4). CPD increased the abundance of 54 and 36 OTUs in cecal and colonic samples, respectively, whereas SPD increased the abundance of 23 OTUs in both cecal and colonic samples. Among these OTUs, some uncommon bacteria were observed, including *Rhizobiales*, *Facklamia* and *Achromobacter*. This could result from contamination from commercial DNA extraction kits or laboratory prepared reagents. [37, 38] Although this could affect the results of bioinformatics analysis, their abundances were too low to affect the general results. Of note, higher abundances of the phyla Actinobacteria and Verrucomicrobia were observed in cecal and colonic samples from CPD-fed SPF mice, compared with an increased abundance of the phyla Firmicutes and Proteobacteria in SPD-fed SPF mice (Figure 2D).

At the genus level, intake of both diets increased the abundance of *Lactobacillus*, but decreased the abundance of the *S24-7* family in the *Bacteroidales* order (Figure 3). Importantly, the soy protein-based diet significantly decreased the prevalence of *A. muciniphila* while chicken protein-based diet maintained the prevalence and abundance of this bacterium (Figure 4, Table 1, $P < 0.05$). This suggested that SPD might impair the growth of *A. muciniphila* which may translate into effects on host health. Thus, recent studies have indicated that *A. muciniphila* may reduce insulin resistance, protect against atherosclerosis,

and increase the number of goblet cells in the colon of obese mice, [22, 23, 39] and supplementation with polysaccharide has been reported to promote the proliferation of *A. muciniphila*. [28] Thus the digested products of soy protein in the intestine may not efficiently support colonization and/or growth of *A. muciniphila*. On the other hand, the digested products of chicken protein allowed growth of *A. muciniphila*. To examine the underlying mechanisms, in vitro cultivation and germ-free mice feeding experiments were implemented.

3.2. A chicken protein digest maintains whereas a soy protein digest impairs the in vitro growth of *A. muciniphila*

A. muciniphila (strain DSM 22959) was cultured in the brain heart infusion (BHI) media supplemented with mucin, pepsin-trypsin-digested chicken or soy proteins. Supplementation with mucin substantially promoted the growth of *A. muciniphila*. Supplementation of digested chicken protein also increased the growth of *A. muciniphila* compared with BHI medium alone, while the growth of the bacterium was suppressed in the medium supplemented with the digested soy protein (Figure 5, $P<0.05$).

3.3. Intake of the chicken protein-based diet maintains mucus barrier functions and intestinal homeostasis by regulating the proliferation of goblet cells and the growth of *A. muciniphila*

The growth of *A. muciniphila* in the gut depends on mucin excretion from goblet cells.[12] Mucin production is dependent on the threonine level in the diet, as threonine constitutes a large fraction of the amino acids in the protein backbone of mucin.[40, 41] Soy protein contains of a much lower level of threonine than chicken protein (Figure 6A, $P < 0.01$), and interestingly, the mice in the SPF-SPD group exhibited a smaller number of PAS-positive goblet cells and a thinner mucus layer in the colon compared with the SPF-CPD group (Figure 6B-D, $P < 0.05$). This was accompanied by a lower level of *Muc2* gene expression in the colon tissue of SPD-fed SPF mice suggesting the threonine content of the protein source in the feed may affect mucin production (Figure 6E, $P < 0.05$). To further investigate how the dietary protein source might affect gut function and homeostasis in the absence of gut bacteria or in the presence of *A. muciniphila*, one group of germ-free mice were fed CPD (GF-CPD) or SPD (GF-SPD) for 4 weeks. Another group was similarly fed CPD (GF-CPD-AKK) or SPD (GF-SPD-AKK) for 2 weeks and then gavaged with live *A. muciniphila*. We observed no significant differences between these groups in weight gain, but a slight difference in feed intake at the beginning of the feeding period (Supporting Information Figure S2). The germ free mice not gavaged with *A. muciniphila* exhibited a lower level of *Muc2* gene expression, reduced mucus thickness, and a smaller number of goblet cells compared with the SPF mice. Of note, the number of neutral goblet cells was higher in CPD-fed germ-free mice (GF-CPD)

compared with the SPD-fed counterparts (GF-SPD) ($P < 0.05$, Figure 5B&C), indicating the protein source per se could affect goblet cell numbers. No significant difference was observed between the two groups in the mucus thickness and *Muc2* mRNA level ($P>0.05$, Figure 6B, D and E). Colonization with *A. muciniphila* caused a slight insignificant increase in the number of goblet cells independent of diets (P=0.34). However, *Muc2* mRNA level was higher for GF-CPD-AKK group than the GF-SPD-AKK group ($P<0.05$, Figure 6E), suggesting an interplay between protein source and *A. muciniphila*. However, the thickness of the mucus layer did not differ significantly between the two diet groups ($P > 0.05$, Figure 6D). The lack of effect on mucus thickness may reflect the fact that it takes up to 8 weeks for germ-free mice to attain homeostatic conditions of the mucus system when the intestinal microenvironment is altered. [42, 43, 44] In the present study, the feeding period was only two weeks after colonization with *A. muciniphila*, and thus, a homeostatic balance was probably not reached. Taken together, our results still indicate that intake of chicken protein may help maintaining mucus barrier functions and intestinal homeostasis by regulating the proliferation of goblet cells and the growth of *A. muciniphila*, contrasting the effect of intake of a soy protein-based diet.

3.4. Effects of chicken protein-based and soy protein-based diets on *A. muciniphila* genes involved in energy metabolism

To examine how dietary chicken and soy proteins might affect the growth and function of *A. muciniphila*, the cecal contents from GF-SPD-AKK and GF-CPD-AKK mice were subjected to a metatranscriptome analysis. A total of 157.74 Gb high quality reads were obtained from 10

samples (Supporting Information Table S5). A total of 611 genes were differentially expressed, of which 215 genes were more highly expressed in the CPD group than in the SPD group (Figure 7A, Supporting Information Table S6). KEGG analysis indicated that genes involved in ribosome function and oxidative phosphorylation were more highly expressed in the GF-CPD-AKK group than in the GF-SPD-AKK group (Figure 7B, Supporting Information Tables S7&8). These genes include genes encoding ribosomal proteins, NADH dehydrogenase associated genes (Amuc_1614/K00333, Amuc_1613/K00333, Amuc_1612/K00335, Amuc_1604/K00343, Amuc_1610/K00337), and succinate dehydrogenase associated genes (Amuc_0985/K00239, Amuc_0986/K00241) (Figure 7C, Supporting Information Figure S3). The enzymes involved in the TCA cycle and oxidative phosphorylation pathways normally play a critical role in adapting energy usage or conservation to the requirements of cells, [45] promoting the growth of aerobic bacteria. Although *A. muciniphila* was initially described as strictly anaerobic, it has been found to be oxygen-tolerant, and may even benefit from the presence of nanomolar concentrations of oxygen through a complex electron transport chain. [46] *A. muciniphila* colonizes the mucus layer close to epithelial cells, where low level of oxygen is present. [47] This might explain an association between the enhanced potential for oxidative metabolism and the higher abundance of *A. muciniphila* in the SPF-CPD group compared to the SPF-SPD group. A recent mouse study indicated that intake of a fiber free diet dramatically increased the abundance of *A. muciniphila* along with increased levels mRNAs encoding mucin-degrading genes such as alpha-N-acetylglucosaminidase, and a thinning of the mucus layer. [48] In the present

study where the experimental diets actually had a slightly higher fiber content, we observed a striking difference comparing mice fed the SPD with mice fed the CPD with a lower abundance of *A. muciniphila* and thinner mucus layer in the SPD group than in the CPD group. In addition, mucin-degrading alpha-N-acetylglucosaminidase (K01205) was expressed to a higher level (P_{adj} = 0.04, Supporting Information Table S7) in *A. muciniphila* from the GF-SPD-AKK group compared with the GF-CPD-AKK group, pointing to distinct differences in the effects of chicken protein and soy protein on not only the host mucin production, but also on functions of *A. muciniphila* in the gut.

4. Concluding remarks

In this study we report on the striking differences in the responses of the gut microbiota to intake of a chicken protein- and a soy protein-based diet. Both diets induced significant changes in the composition of the gut microbiota. Intake of a chicken protein-based diet maintained levels of *A. muciniphila* in the gut microbiota, whilst a soy protein-based diet reduced the abundance of this bacterium. This conclusion was supported by in vitro culturing of *A. muciniphila* showing that supplementation with a soy protein digest reduced the growth of *A. muciniphila* whereas supplementation with a chicken protein digest did not. In SPF-CPD mice, the number of goblet cells and *Muc2* mRNA level were higher, and the thickness of the mucus layer was greater than in SPF-SPD mice. The use of germ-free mice corroborated the effect of protein diets on proliferation of goblet cells and also pointed to a combined effect of intake of chicken protein and colonization by *A. muciniphila*. Finally, comparing the transcriptional profiles of *A. muciniphila* in the mice fed the two diets, we

noticed higher levels of expression of genes involving ribosome synthesis and function, and surprisingly higher oxidative energy metabolism in *A. muciniphila* in the mice fed the chicken protein-based diet than in the mice fed the soy protein-based diet. Taken together, our results point to a complex interplay between intake of different protein sources and the gut microbiota, clearly distinguishing soy protein from chicken protein, with a noticeable effect on the abundance and the functional potential of *A. muciniphila*.

Ethics approval

All animal experimental protocols were approved by the Animal Care Committee of Nanjing Agricultural University. SPF animals were housed in an SPF animal facility (reference number SYXK(Su)2011-0037), and germ free mice were housed in an SPF animal facility with germ free isolators (reference number SCXY(Jing)2014-11).

Availability of sequencing data

The sequencing data are available on NCBI. The 16S rRNA gene sequencing accession ID is SRP154879. The metatranscriptome sequencing data accession ID is SRP132485.

Author contributions

C.L., G.Z. and X.X designed the study. F.Z., X.L. and S.S. carried out the animal study and collected samples. F.Z. carried out the histological and gene expression measurement. F.Z. and X.L. carried out the in vitro cultivation. F.Z. performed statistical analyses and produced the figures, guided by G.H. and C.L. L.L. provided the bacterium *Akkermansia muciniphila*. F.Z. and C.L. wrote the first version of the manuscript, and K.K. contributed to the interpretation, restructured and revised the manuscript. M.M. contributed to the interpretation and writing.

Conflict of interest statement

The authors declare no conflict of interests.

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Figure legends

Figure 1. Body weight gain and feed intake in SPF mice fed the chicken protein or the soy protein-based diet.

A) body weight gain. B) feed intake. One-way ANOVA with repeated measures was applied to compare the time and feed effects on body weight gain and feed intake of mice fed soy or chicken protein-based diets.

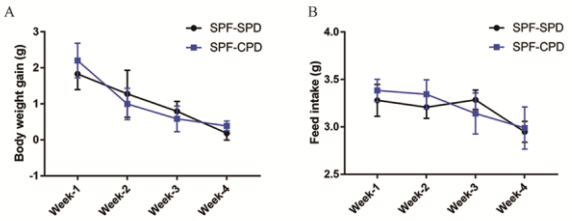

Figure 2. The composition of the gut microbiota in SPF mice fed the chicken protein-based or the soy protein-based diets. A) PCoA plot of OTU data. B) Cluster analysis based on unweighted UniFrac distance. C) Cluster analysis based on weighted UniFrac distance. D) Taxon-based analysis at the phylum level. Each column represents one sample. CPD-cecum and SPD-cecum: cecal content samples from CPD-fed and SPD-fed SPF mice, respectively (n=9 each); CPD-colon and SPD-colon: colonic content samples from CPD-fed SPF mice (n=8) and SPD-fed SPF mice (n=7); CPD-feces and SPD-feces: fecal samples from CPD-fed (n=8) and SPD-fed (n=7) SPF mice before diet change, respectively.

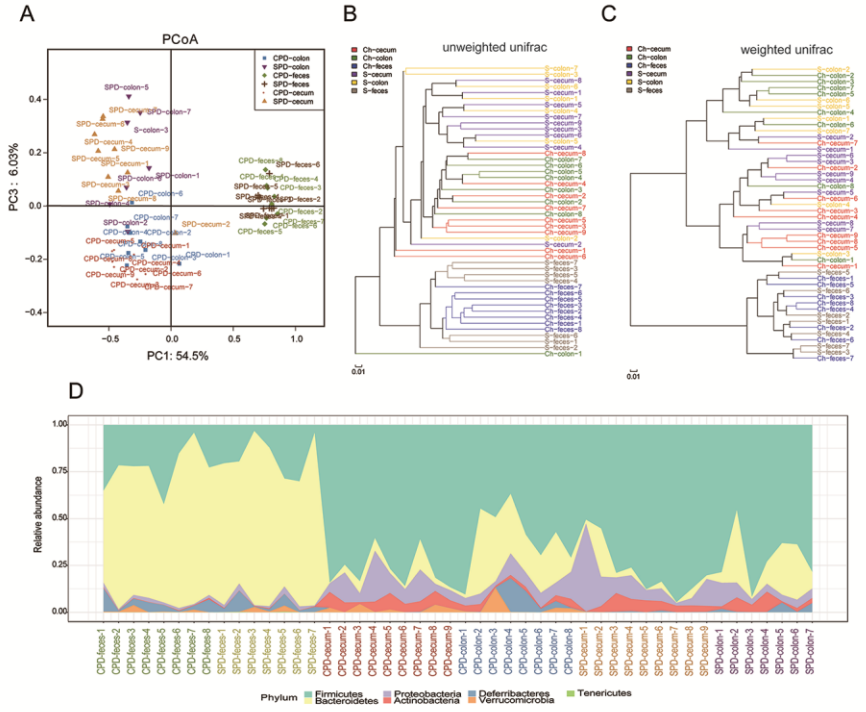

Figure 3. Heatmap of identified OTUs (CLR transformed) at the genus level. Each column represents one sample and each row represents an OTU.

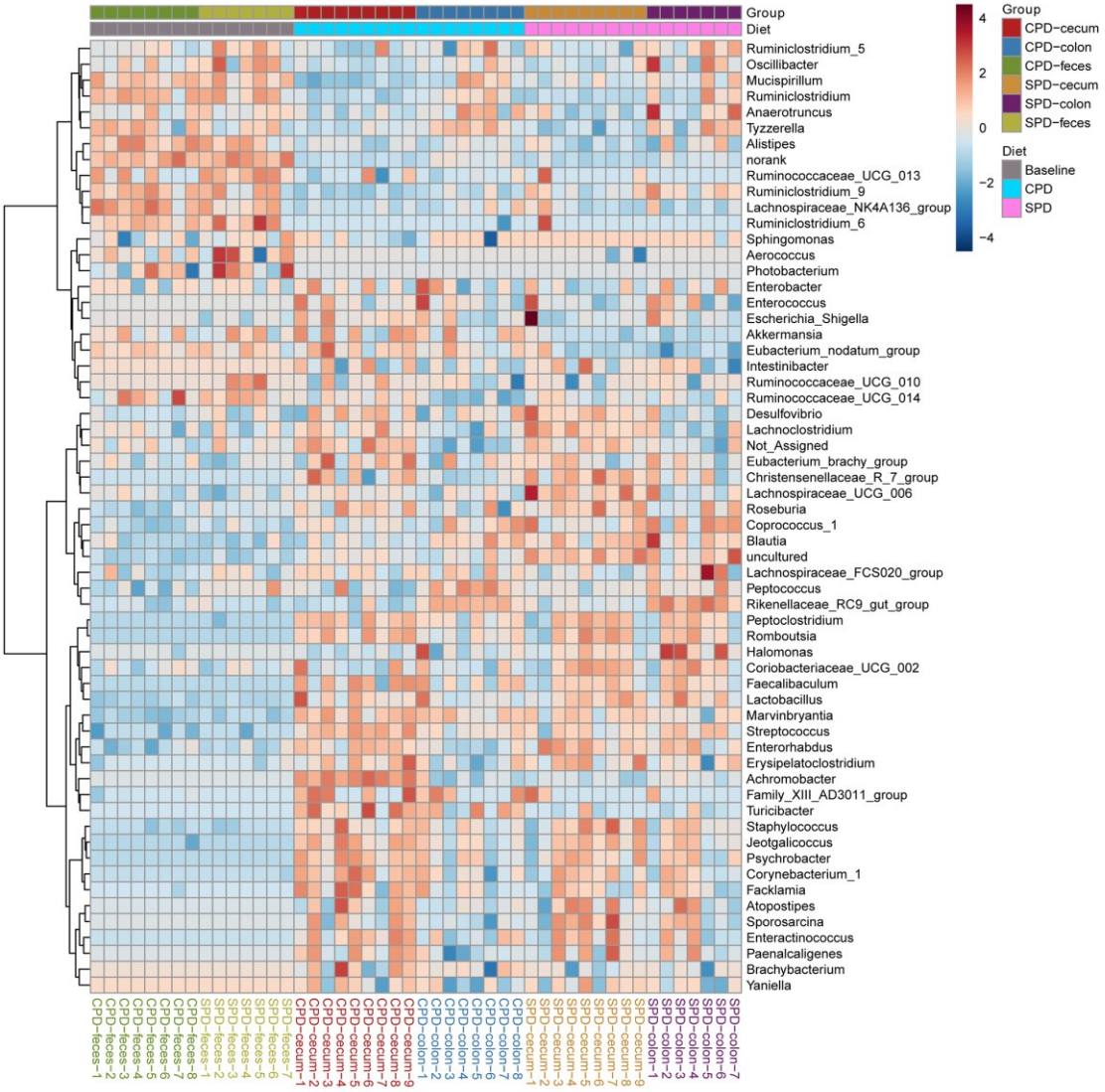

Figure 4. Relative abundance (CLR transformed) and frequency of *A. muciniphila* in mice fed the chicken protein- or the soy protein-based diets. SPF-CPD and SPF-SPD, chicken and soy protein-based diet SPF groups; feces-day 0, fecal samples before diet change; colon/cecum, colonic and cecal content samples on day 28.

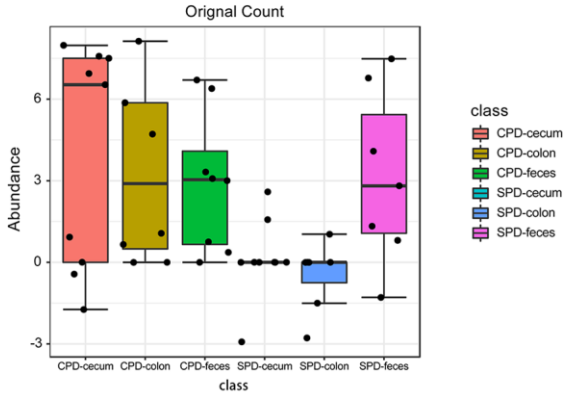

Figure 5. Effects of chicken protein digest and soy protein digest on the growth of *A. muciniphila*.

A. muciniphila was grown in BHI medium supplemented or not with mucin, chicken protein digest or soy protein digest. The absorbance at 600 nm was measured every 6 h using a nanodrop spectrophotometer. The data of each time point were analyzed by one-way analysis of variance and means were compared by the Duncan's multiple comparisons. The letters "a,b,c,d" denote significant differences among treatments at a given time point ($P < 0.05$).

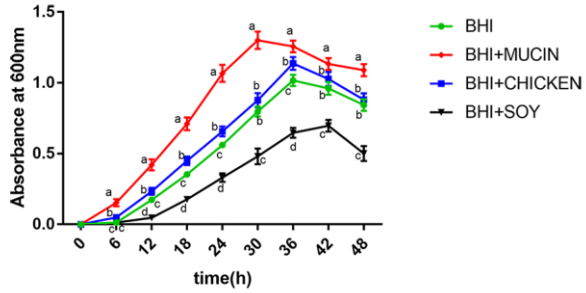

Figure 6. Goblet cells and mucus thickness in colon. A) The threonine content in chicken and soy proteins. The means were compared by t-test. *** *P*<0.001. B) Representative alcian blue PAS micrographs (scale bars, 50μm). C) Number of goblet cells per crypt. In each group, samples from 3-5 GF mice and 7-8 SPF mice were selected. A 2×3 factorial ANOVA was applied in which diet was an independent variable, whereas SPF/GF and AKK were considered a combined independent variable. A,B,C,D, denote significant differences in the number of neutral goblet cells (P<0.05). a,b, denote significant differences in the number of acidic goblet cells (P<0.05). D) Mucus thickness. A,B denote significant differences (P<0.05). E) *Muc2* mRNA expression level (n=9 each for the SPF mouse groups, and n=5 each for the GF mouse groups). Two outliers in the SPF-SPD group and one outlier in the SPF-CPD group were excluded. A,B,C,D, denote significant differences (P<0.05).

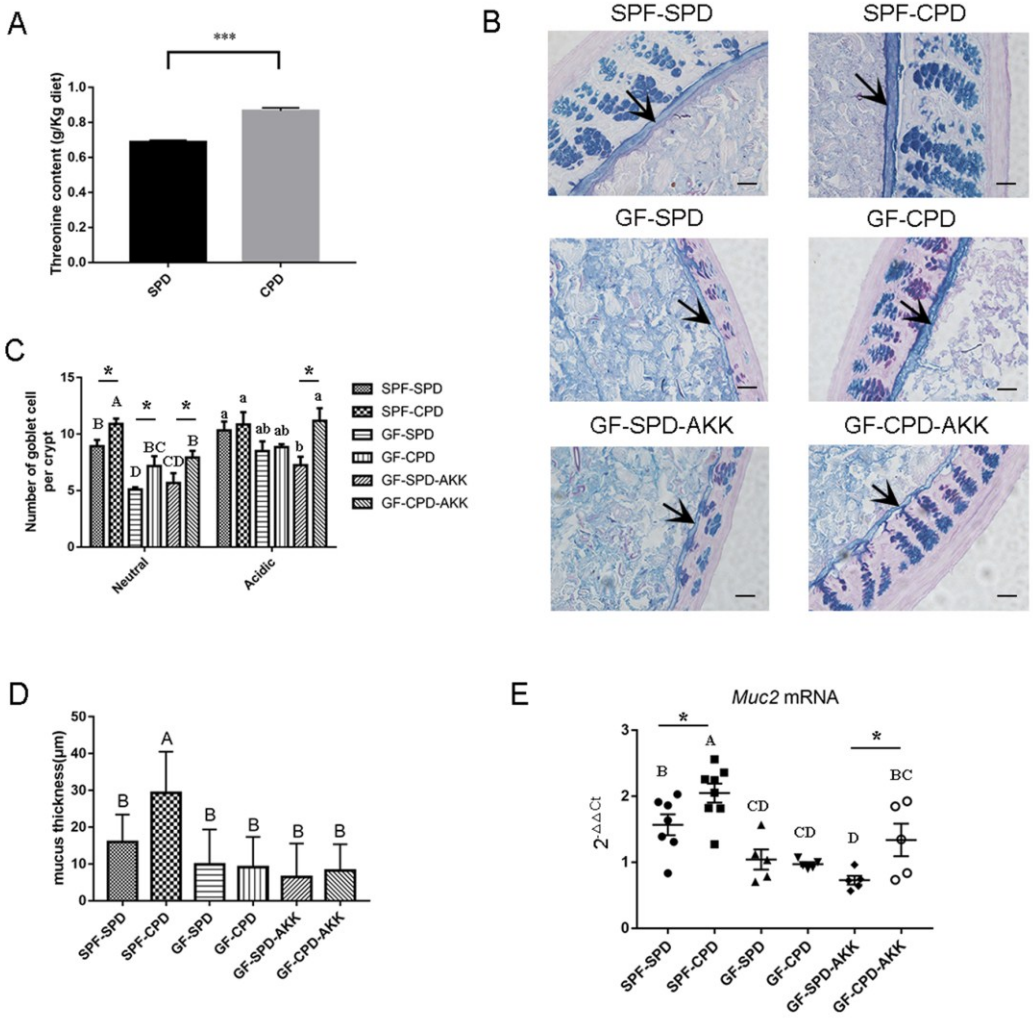

Figure 7. Gene expression in *A. muciniphila* in cecal samples. A) Volcano plot for upregulated (red dots) and downregulated (green dots) genes in the GF-CPD-AKK group. B) KEGG enrichment analysis for downregulated (left) and upregulated (right) pathways in the GF-CPD-AKK group. C) Differentially expressed KOs in the GF-SPD-AKK and the GF-CPD-AKK groups (n=5 each).

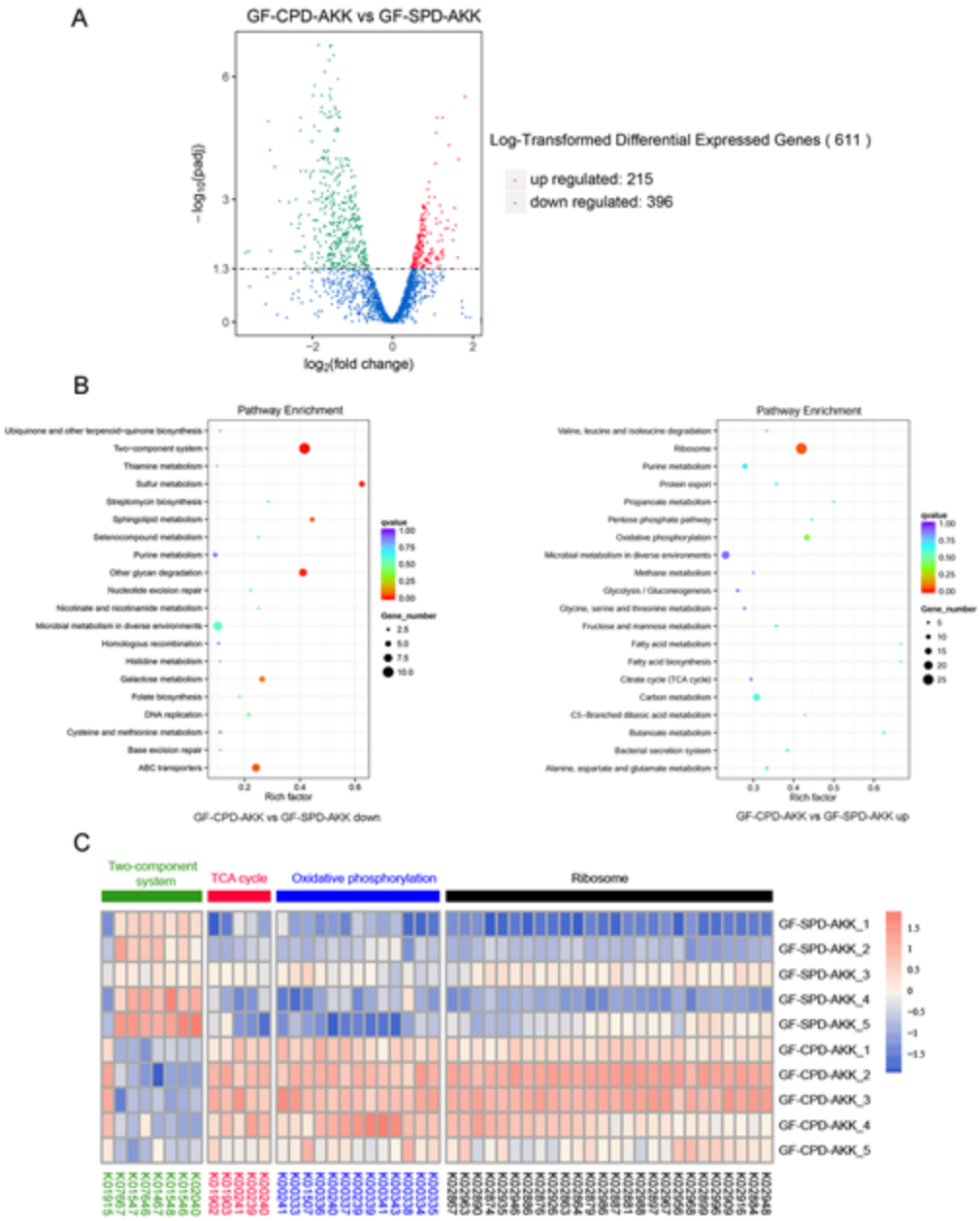

Table 1 Wilcoxon scores for occurrence of *Akkermansia muciniphila*

Time	Diets	Samples sources		
		feces	**colon**	**cecum**
0d	CPD	30.00(7/8)a		
	SPD	33.00(7/7)a		
28d	CPD		27.00(6/8)a	30.33(8/9)a
	SPD		12.43(1/7)b	14.33(2/9)b

Kruskal-Wallis test was used for comparisons between each two groups. The numbers in parentheses are the number of samples in which *A. muciniphila* were detected and the total number of samples in each group. The figures outside the parentheses are average Wilcoxon scores. "a,b" denote significant differences between two diet groups ($P < 0.05$).

Graphical text:

A chicken protein-based diet maintains, whilst a soy protein-based diet reduces the abundance of *A. muciniphila* and the number of goblet cells, lowered the level of *Muc2* mRNA, and decreases the thickness of the mucus layer in the colon. These effects were associated with differences in the functional repertoire of *A. muciniphila* in mice fed the two dietary proteins.