

# Incomplete degradation products of galactomannan from *Sesbania cannabina* modulated the cecal microbial community of laying hens

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## Abstract

Galactomannan and its degradation products have been gaining attention based on their possible means for improving the natural defense of the host through modulation of the bacterial population in the gut. Herein, incomplete degradation products of galactomannan (IDPG) was supplemented into the diet of aged laying hens to investigate the efficacy of IDPG on the gut microbiome. Four treatments with six replicates of twelve 68-wk-old laying hens (Hy-Line variety brown) each were fed a basal diet supplemented with 0%, 0.01%, 0.025%, and 0.05% IDPG for 8 wk. Results showed that the propionate concentration significantly increased in laying hens fed a diet supplemented with 0.025% or 0.05% IDPG relative to the control diet ( $P < 0.05$ ). Moreover, the results of 16S rRNA gene sequencing revealed that there was a notable elevation of microbiome species diversity due to the addition of IDPG, with a noted enrichment to phyla *Bacteroidetes* at the expense of *Firmicutes* and *Proteobacteria*. Metabolic prediction of the cecal microbiome suggested significant improvements to carbohydrate and lipid metabolism and a significant depletion for energy metabolism and infectious diseases. More importantly, a strong positive correlation between levels of genera *Bacteroides*, *Rikenellaceae\_RC9\_gut\_group*, and *Prevotellaceae\_UCG-001* with high production of propionate was found using multivariate analysis. Our study demonstrated that IDPG acted by mainly enriching the phyla *Bacteroidetes* in the cecum, increasing species diversity, and cecal propionate concentrations. It seems that IDPG can be used as feed additives in laying hen farming due to its capacity to positively modulate the cecal microbiome and aid improve overall health.

## Lay Summary

The health and nutritional status of poultry are largely interconnected with the gut microbiome, which directly or indirectly affects gut morphology, nutrition, and immune responses. Dietary fiber is resistant to digestion in the small intestines of monogastric animals but is completely or partially fermented in the distal gut, thus it is understood that they could stimulate gut health. Incomplete degradation products of galactomannan (IDPG) is an important member of the dietary fiber family of molecules, however, there exists scant research on their beneficial effects on human or animal health. Our study demonstrated that IDPG acted by mainly enriching the phyla *Bacteroidetes* in the cecum, which are common bacteria in the gut that are involved in the fermentation of carbohydrates. Thereafter, the enriched phyla *Bacteroidetes* produced propionate and reduced the abundance of phyla *Firmicutes* and *Proteobacteria* by competitive inhibition. IDPG has also increased species diversity and enhanced the stability of intestinal flora, thereby exhibiting excellent prebiotic activity.

**Key words:** *Bacteroidetes*, cecal, incomplete degradation products of galactomannan, laying hen, propionate

**Abbreviations:** CCA, canonical correspondence analysis; HPLC, high-performance liquid chromatography; IDPG, incomplete degradation products of galactomannan; KEGG, Kyoto Encyclopedia of Genes and Genomes; MOS, manno-oligosaccharides; OTUs, operational taxonomic units; PCA, principal component analysis; PCR, polymerase chain reaction; PUL, polysaccharide utilization loci; QIIME, quantitative insights into microbial ecology; RDP, ribosomal database project; SCFA, short-chain fatty acids; TCA cycle, tricarboxylic acid cycle

## Introduction

Microbial communities in the gastrointestinal tract greatly influence various biological functions of a given host. Examples of such functions include maintaining host health, improving performance, and ensuring food and animal products' safety (Chung et al., 2012; Mohd Shaufi et al., 2015; Sekirov et al., 2018). In the gastrointestinal tract, there are more microbes

in the cecum than in the small intestine and colon, although all three have similar roles in digestion and absorption of nutrients (Mohd Shaufi et al., 2015). Each family of microbes is functionally interdependent, with an example being how certain indigestible fibers pass un-degraded from the small intestine into the ceca those microorganisms produce short-chain fatty acids (SCFA: mainly acetic acid, propionic acid,

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and butyric acid) and ammonia. Each of these ceca fermentation products is then absorbed by the intestinal epithelium to the host, a mechanism similar to the rumen (Li et al., 2017). As a result, analysis of the cecal microbiome represents a pivotal area of poultry nutrition research, which will lead to a better understanding of cecal microbial biodiversity and their interactions with the host. To our knowledge, some additives, including insect (Borrelli et al., 2017), probiotic (Brzóška et al., 2012), and dietary fiber (Cetin et al., 2005), may have regulating effects on gut microbiomes of grown livestock. Among these examples, dietary fibers can beneficially affect the host by selectively stimulating the growth or activity of certain bacterial species inhabiting the digestive tract without being digested (Patterson and Burkholder, 2003). Therefore, they were thought to be ideal feed additives.

Recently, as an important member of dietary fiber, galactomannan (GM) has garnered interest not only due to its outstanding immuno-enhancing activity but also due to its ability to modify the gut microbiota population profile in a positive sense (Gamal-Eldeen et al., 2006; Hernandez et al., 2011; Shtriker et al., 2018). Based on the consensus that a well-balanced cecal microflora is important for physical health (Anderson et al., 2000), galactomannan maintains this balance by reducing the load of pathogenic bacteria and enhancing the growth of beneficial bacteria through different mechanisms. It has been reported that galactomannan reduces attachment of Gram-negative bacteria such as *Escherichia coli* with intestinal mucosa through a mechanism involving binding bacterial FimH of type-1 fimbriae. The cause of this binding was found to be a high bacterial affinity for mannose residues on intestinal mucosa (Fernandez et al., 2002; Hooge, 2004). Moreover, studies have shown that galactomannan enhances the number of beneficial bacteria, such as *Lactobacillus* and *Bifidobacteria spp.* (McLaughlin et al., 2015). Notably, galactomannan and its degradation products induced the abundance of one specific identified species, *Bacteroides ovatus*, a well-known mannan fermenter (Bagenholm et al., 2017; Park et al., 2018). More importantly, the increased production of SCFA caused by probiotics induced by galactomannan and its degradation products, in which SCFA are involved in cell proliferation in the intestinal mucosa, reduced the pH of the brush border microenvironment and block the adhesion of pathogens (Lan et al., 2004; Reichardt et al., 2018). Furthermore, Zartl et al. (2018) found that the low molecular weight galactomannan has a better effect on inducing probiotics growth than high molecular weight galactomannan. This suggests that it may be necessary to shorten long-chain galactomannan prior to the investigation of galactomannan effect on gut microbial composition.

In nature, galactomannan is diffusely found in many plants or plant derivatives, such as locust bean (Pinheiro et al., 2011), *Sesbania* species (Pollard et al., 2011), and coffee grounds (Gu et al., 2020). Herein, we put our attention on the galactomannan from *Sesbania cannabina* seed and obtained incomplete degradation products of galactomannan (IDPG) through  $\beta$ -endo-mannanase hydrolysis (Tao et al., 2020b). After hydrolysis, the average molecular weight of IDPG was approximately one-tenth of the original galactomannan (342 to 20,000 Da). Previous results showed that IDPG supplementation significantly increases egg production and decreases feed conversion ratio ( $P < 0.05$ ; Tao et al., 2021). Hassanein and Soliman (2010) suggested that the establishment of useful bacterial colonies could improve the nutrient digestibility in

the intestine, thereby decreasing the value of feed conversion ratio. Herein, we aimed to identify the shifts in microbiota composition as well as SCFA production induced by IDPG to make certain beneficial effects on gastrointestinal health. It is our hope to fill the gap in galactomannan and its degradation products in poultry farming and provide a practical basis for further research on the relationship between galactomannan and microbial community in other animals or humans.

## Materials and Methods

The experimental protocols used in this experiment, including animal care and use, were reviewed and approved by the Animal Care and Use Ethics Committee of Nanjing Forestry University (Nanjing, China).

### Preparation of IDPG

IDPG was prepared from enzymatic hydrolysis of *S. cannabina* seeds. The *S. cannabina* seeds of 50 kg used in this experiment were purchased from a local farm in Yancheng city, Jiangsu province of China in 2020. At first, ground (Mini plant shredder F2102, Taisite instrument Co., Ltd., Tianjin, China) *S. cannabina* seeds were suspended into distilled water to reach a galactomannan concentration of 40 g/L, the pH of the solution was adjusted to 4.8 with 0.05 M citric acid buffer and then treated with  $\beta$ -mannanase (20 U/g galactomannan, 72 h, 50 °C). Endo- $\beta$ -mannanase (EC 3.2.1.78) was prepared from *Trichoderma reesei* Rut C-30 using avicel as a substrate. Enzymes were deactivated (10 min, 100 °C) after the incubation period, and then the suspensions were centrifuged (16,465  $\times$  g, 10 min) with the resultant supernatants being referred to as IDPG solution. Finally, solid IDPG was prepared using a spray dryer (BUCHI, Flawil, Switzerland). The content of IDPG in the solid was 45.96% determined by a sulfuric acid hydrolysis method and high-performance anion-exchange chromatography with pulsed amperometric detection using mannose and galactose as an external standard. The content of protein and ash was 12.05% and 6.72%, respectively. The protein content of GM was monitored using the Bio-Rad protein assay with bovine serum albumin as a standard (Bradford, 1976). The ash content of GM was analyzed according to AACC-Methods 08-01.01 (Anonymous, 2000). These compositional analysis methods of the solid were in line with the work of Tao et al. (2020a).

### Animal care and experimental design

A total of 288 laying hens (68-wk-old, Hy-Line variety brown) were randomly distributed into 4 dietary treatments consisting of 6 replicates (cages) with 12 birds per replicate and fed a basal diet supplemented with 0% (control group), 0.01%, 0.025%, and 0.05% IDPG for 8 wk, respectively. Ingredient composition and nutrient content of the basal diet are given in Table 1. In an environmentally controlled house, birds were allowed free access to water and mash feed in 3-level cages (120 cm  $\times$  60 cm  $\times$  50 cm; 0.09 m<sup>2</sup> per chick) with controlled ventilation and lighting (16L:8D). The laying hens were fed twice per day (6:00 a.m. and 3:00 p.m.) and feed was mixed in the trough to ensure total consumption by the laying hens. According to the feeding situation of the previous day, the feeding amount for each new day was appropriately adjusted to ensure that there was no remaining feed in the trough each night. After 2 wk of preliminary testing, a formal experiment was carried out over 8 wk.

**Table 1.** Composition and nutrient levels of basal diets (as-fed basis)

Ingredients	Content, %	Nutrient levels <sup>1</sup>	
Corn	63.50	Apparent metabolizable energy, MJ/kg	11.16
Soybean meal	18.80	Crude protein, %	15.37
Fish meal	1.50	Calcium, %	3.79
Rapeseed meal	2.00	Total phosphorus, %	0.64
Corn gluten meal	1.20		
Soybean phospholipid	1.00		
Limestone	9.17		
Dicalcium phosphate	1.40		
DL-Methionine	0.10		
Sodium chloride	0.33		
1% Premix <sup>2</sup>	1.00		
Total	100		

<sup>1</sup>Nutrient levels were the calculated values.

<sup>2</sup>1% Premix was provided by Huamu Institute of Animal Science and Technology, and provided per kilogram of diet: vitamin A (transretinyl acetate), 1.08 × 10<sup>4</sup> IU; vitamin D3 (cholecalciferol), 2.7 × 10<sup>3</sup> IU; vitamin E (all-rac- $\alpha$ -tocopherol), 27 mg; menadione, 0.84 mg; thiamin, 0.72 mg; riboflavin, 5.4 mg; nicotinamide, 9 mg; calcium pantothenate, 36 mg; pyridoxine-HCl, 2.7 mg; biotin, 0.09 mg; folic acid, 0.24 mg; vitamin B12 (cobalamin), 0.009 mg; Fe (from ferrous sulfate), 100 mg; Cu (from copper sulfate), 8.0 mg; Mn (from manganese sulfate), 100 mg; Zn (from zinc oxide), 100 mg; I (from calcium iodate), 0.9 mg; Se (from sodium selenite), 0.3 mg.

### Sample collection

At 76 wk of age, a total of 24 birds from all treatments were randomly selected (1 bird per replicate) and euthanized by cervical dislocation and then necropsied. Their entire gastrointestinal tracts were rapidly removed and placed on a chilled stainless steel tray. Cecal samples were quickly removed aseptically, the content of which was collected and divided into two portions. Each portion was then rapidly frozen in liquid nitrogen and stored at -80 °C until further analysis.

### Determination of the concentration of SCFA and lactate in the cecum

The concentration of SCFA and lactate in cecal content samples of laying hens was measured by high-performance liquid chromatography (HPLC). Sample (0.10 ± 0.01 g) was mixed with 2 mL deionized water by a vortex meter (Vortex 1, IKA-Werke GmbH & CO. KG, Staufen, Germany) for 10 min. The resultant mixture was centrifuged at 16,465 × g for 30 min, and the obtained supernatant was diluted to be in the range of calibration, filtered through a 0.22 µm nylon filter, and then 10 µL of which was injected into the HPLC. The HPLC was equipped with a Bio-Rad Aminex HPX-87H column (7.8 mm × 300 mm) in combination with a Bio-Rad (Cat. No. 125-0139) guard column. The mobile phase of 5 mmol/L H<sub>2</sub>SO<sub>4</sub> ran at 0.6 mL/min under 55 °C. External standards such as lactic acid, acetic acid, propionic acid, and butyric acid used for instrument calibration were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Quantification data and chromatograms were collected and analyzed with Agilent analysis system. The concentrations of cecal SCFA were calculated as follows:

$$\text{SCFA concentration (mg/g cecum)} = \frac{\text{SCFA concentration (mg/L)} \times V_{\text{cecal mixture}} \text{ (L)}}{\text{dry cecum (g)}} \times 100 \% .$$

### Total bacterial genomic DNA extraction and 16S rDNA sequencing

For 16S rRNA gene sequencing, the total genomic DNA of each sample was extracted using the HiPure Stool DNA Kits (Magen, Guangzhou, China) according to the manufacturer's protocols. Thereafter, the 16S rDNA V3 to V4 hypervariable regions of ribosomal RNA gene were amplified using polymerase chain reaction (PCR) containing primers (341F: 5'-CCTACGGGNGGCWGCAG-3'; 806R: 5'-GGACTACHVGGGTATCTAAT-3'; Guo et al., 2017), where the barcode was an eight-base sequence unique to each sample. The applied reaction conditions were as follows: 95 °C (2 min), followed by 27 cycles of 98 °C (10 s), 62 °C (30 s), and 68 °C (30 s), with a final extension at 68 °C (10 min). PCR reactions were performed in triplicate 50 µL mixture containing 5 µL of 10 × KOD buffer, 5 µL of 2.5 mM dNTPs, 1.5 µL of each primer (5 µM), 1 µL of KOD polymerase, and 100 ng of template DNA.

PCR products were purified from 2% agarose gels using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. The DNA amplicon concentration of each sample was quantified using the ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, CA, USA). Finally, the purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina platform according to the standard protocols.

### Tag and operational taxonomic unit assessment

To get high-quality and clean reads, FASTP was employed to further filter the raw reads according to the following rules removing reads containing more than 10% of unknown nucleotides (N) and less than 60% of bases with quality (Q-value) > 20. Next, the paired-end clean reads were merged as raw tags using FLASH (Magoč and Salzberg, 2011; version 1.2.11) with a minimum overlap of 10 bp and mismatch error rates of 2%. Subsequently, noisy sequences of the obtained raw tags were filtered by Quantitative Insights Into

Microbial Ecology (QIIME; version 1.9.1) pipeline under specific filtering conditions to obtain high-quality clean tags (Caporaso et al., 2010; Bokulich et al., 2013). Clean tags were next evaluated for the presence of chimeric amplification using the UCHIME algorithm, and the determined chimeric tags were removed to ultimately unlock effective tags. Effective tags were clustered into operational taxonomic units (OTUs) of  $\geq 97\%$  similarity using UPARSE (Edgar, 2013) pipeline. In addition, the tag sequences with the highest abundances were selected as representative sequences within each cluster. Based on that, between groups, Venn analysis was performed using R project (version 3.4.1) to identify unique and common OTUs. Moreover, the alpha-diversity index of Chao1, Simpson, and Shannon indices were calculated in QIIME, and the beta diversity was estimated using weighted and unweighted UniFrac distance matrix generated by GUniFrac package (version 1.0) in R project. Statistical analysis of ANOSIM test was calculated using R project.

### Taxonomic abundance profiling

A naive Bayesian model was used to classify the representative sequences into organisms using Ribosomal Database Project classifier (Wang et al., 2007; version 2.2) based on SILVA database (Pruesse et al., 2007) with the confidence threshold values ranging from 0.8 to 1.

### Molecular functional enrichment prediction

The Kyoto Encyclopedia of Genes and Genomes pathway analysis of the OTUs was inferred using Tax4Fun (Aßhauer et al., 2015; version 1.0). Heat maps for the differentiated pathways were generated.

### Multivariate analysis

Multivariate analyses were conducted to study the correlation between host cecal SCFA and lactate concentrations and microbial composition at the genus level by canonical correspondence analysis (CCA) using CANOCO 4.5 software (ter Braak, 1988). A total of 4 environmental variables (lactate, acetate, propionate, and butyrate) and 24 OTUs corresponding to the genus level were included in the CCA. A heat map based on the Pearson correlation test was used to assess the eventual association between the amount of key bacterial species and SCFA levels.

### Statistical analysis

Data were analyzed by ANOVA using SPSS (2008) statistical software (Ver.16.0 for windows, SPSS Inc., Chicago, IL, USA). Differences in means among treatment groups were separated

using Tukey's multiple range test. *P*-values less than 0.05 were considered indicative of statistical significance.

## Results

### Effect of IDPG on cecal SCFA and lactate contents

Until the end of the experiment, 288 chickens survived healthily, and then the cecal SCFA and lactate were measured. As seen from Table 2, acetate is the main component in the cecum of laying hens, followed by butyrate, and finally propionate. Laying hens fed diets containing IDPG showed lower cecal lactate and acetate contents than those fed no IDPG. However, this difference was not statistically significant. In contrast, compared with the control group, the chickens fed a diet supplemented with IDPG (0.025% and 0.05%) showed a significant increase in cecal propionate ( $P < 0.05$ ). Nevertheless, there was no obvious difference in the cecal butyrate contents between the IDPG-supplemented group and the control group. Concerning total SCFA, there was no significant difference between the varied IDPG dosages (0.025% and 0.05%) and the control group.

### Tag and OTU-based analysis

The cecal microbiota from groups supplemented with 0.025% and 0.05% IDPG were divided into two intersecting clusters that separated from the control group and group supplemented with 0.01% IDPG and occupied distinct positions in the principal component analysis (PCA) plot (Figure 1a). Furthermore, there did not appear to be much difference in microbial communities between the 0.025% IDPG-supplemented group and the 0.05% IDPG-supplemented group. In agreement with the PCA results, the numbers of OTUs in the groups supplemented with 0.025% and 0.05% IDPG increased significantly compared with the control group (Table 3). In addition, a Venn diagram was generated based on the OTUs distributed among four groups (Figure 1b). In total, 733 OTUs were common to four groups of cecal samples. More importantly, compared with the control group, 191, 305, and 242 OTUs were unique in the groups supplemented with 0.01%, 0.025%, and 0.05% IDPG, respectively, which was consistent with PCA results.

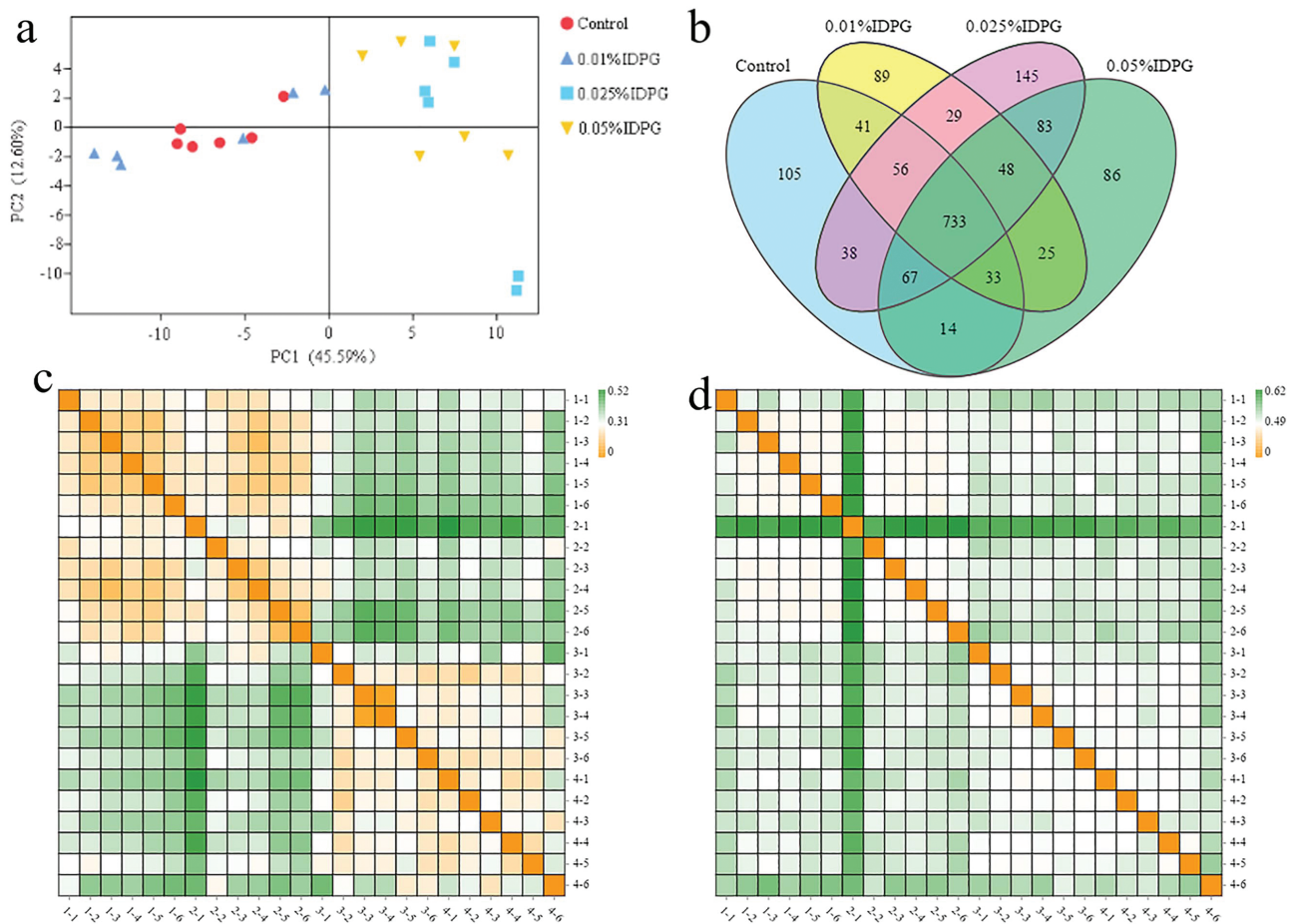
To analyze alpha diversities of the samples, we calculated several indices including Chao1, Shannon, and Simpson values (Table 3). No difference in the Shannon index was found among treatments. However, the Chao1 index was higher ( $P < 0.05$ ) in groups supplemented with 0.025% or 0.05% IDPG than in the control group. The Simpson index showed a slight increase in the IDPG-based group compared with the control group, yet the difference was not significant.

**Table 2.** Contents of cecal short-chain fatty acids and lactate in laying hens fed diets supplemented with different doses of incomplete degradation products of galactomannan

Organic acid	IDPG level, %				SEM	<i>P</i> -value
	0	0.1	0.025	0.05		
Lactate, mg/g cecum	2.56	0.68	2.10	1.26	0.40	0.114
Acetate, mg/g cecum	22.09	17.49	20.17	21.09	1.17	0.172
Propionate, mg/g cecum	1.92 <sup>b</sup>	2.18 <sup>b</sup>	3.59 <sup>a</sup>	3.99 <sup>a</sup>	0.43	0.001
Butyrate, mg/g cecum	6.46	6.20	6.91	7.75	0.36	0.276
Total SCFA, mg/g cecum	30.47 <sup>a</sup>	25.88 <sup>b</sup>	30.67 <sup>a</sup>	32.84 <sup>a</sup>	1.69	0.006

<sup>a,b,c</sup>Different superscript letters in the same row differ significantly ( $P < 0.05$ ).





**Figure 1.** Analysis of OTUs and diversity of cecal microbiome of four groups supplemented with different doses of incomplete degradation products of galactomannan (IDPG). PCA plot (a); Venn diagram of the OTUs in the four treatments (b); Heat maps based, respectively, on weighted and unweighted UniFrac distances of gut microbial communities of four treatments (c, d). Control group (1-1 to 1-6), 0.01% IDPG group (2-1 to 2-6), 0.025% IDPG group (3-1 to 3-6), and 0.05% IDPG group (4-1 to 4-6).

**Table 3.** Alpha diversities and OTUs of the cecal samples in four groups supplemented with different doses of incomplete degradation products of galactomannan

Items	IDPG level, %				SEM	P-value
	0	0.01	0.025	0.05		
OTUs	1,585 <sup>b</sup>	1,562 <sup>b</sup>	1,884 <sup>a</sup>	1,798 <sup>a</sup>	34.09	<0.001
Chao1	2,516 <sup>c</sup>	2,568 <sup>bc</sup>	2,894 <sup>a</sup>	2,715 <sup>b</sup>	43.70	<0.001
Shannon	6.63	6.45	6.75	6.61	0.06	0.465
Simpson	0.96	0.95	0.97	0.97	0.00	0.07

<sup>a,b,c</sup>Different superscript letters in the same row differ significantly ( $P < 0.05$ ).

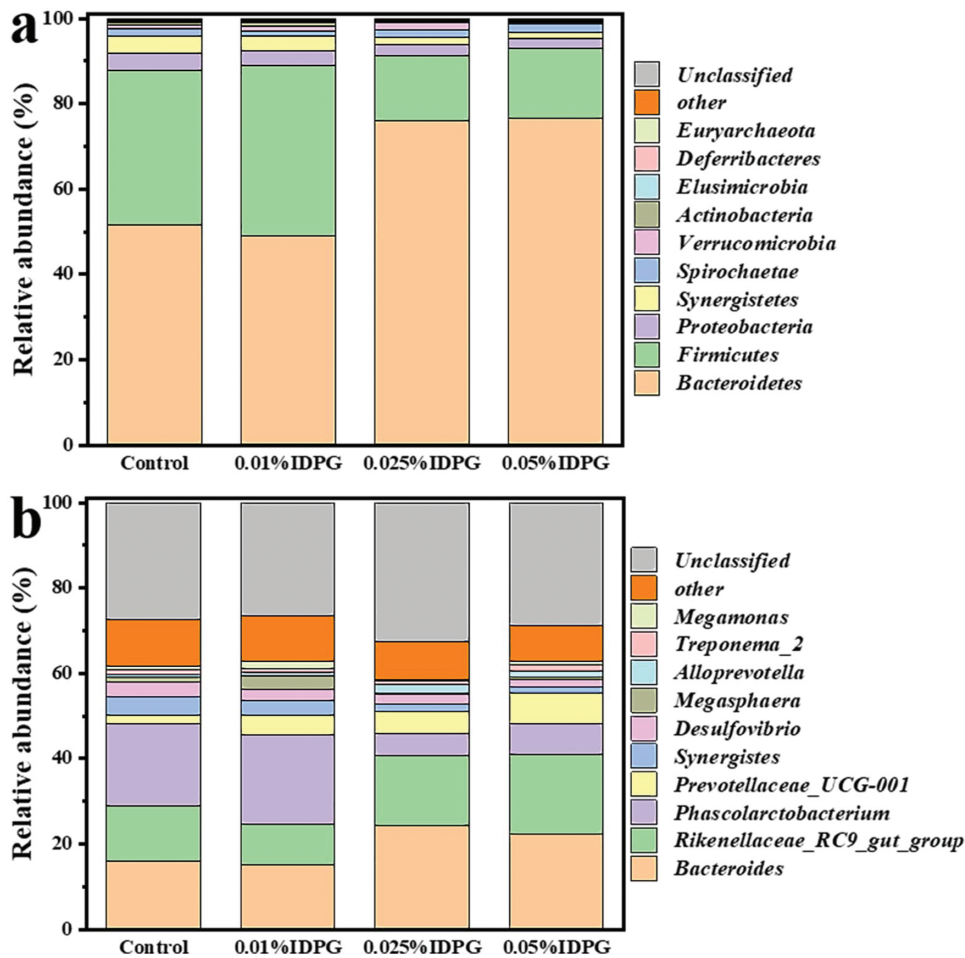
Furthermore, the beta-diversity analysis showed a strong difference in the relative abundance of microbial species (weighted UniFrac) between IDPG-supplemented groups and the control group, but not in the type of microbe (unweighted UniFrac; Figure 1c and d). Moreover, R statistic ANOSIM computed on phylogenetic distances among samples revealed that IDPG administration of 0.025% or 0.05% promoted a shift in the bacterial community (Table 4), yet no difference was observed between the 0.01% IDPG-supplemented group and the control group.

**Table 4.** ANOSIM analysis between the incomplete degradation products of galactomannan-supplemented group (0.01%, 0.025%, and 0.05%) and the control group

Comparison	R value	P-value
Control vs. 0.01% IDPG	-0.04	0.641
Control vs. 0.025% IDPG	0.91	0.003
Control vs. 0.05% IDPG	0.92	0.001

### Change of gut microbiota composition after IDPG-based diet

As shown in Figure 2, samples highlighted the predominance of *Bacteroidetes* followed by *Firmicutes*, *Proteobacteria*, and *Synergistetes* at the phylum level, together accounting for more than 95% of the total sequences in all groups. Moreover, a noticeable increase in relative abundance ( $P < 0.05$ ) of phyla *Bacteroidetes* was seen in the IDPG treatment groups compared with control (Figure 2a). Specifically, the values increased from 51.71% in the control birds to 76.09% in the 0.025% IDPG-fed laying hens, and further to 76.71% in the 0.05% IDPG-fed laying hens of the total cecal microbiome. We also observed a drastic decrease in *Firmicutes*, *Proteobacteria*, and *Synergistetes* with exposure to 0.025% or 0.05% IDPG compared with the control group ( $P < 0.05$ ). At a finer level, the majority



**Figure 2.** The cecal microbiome of laying hens fed diets supplemented with different doses of incomplete degradation products of galactomannan. Compositions of gut bacteria in at the phylum level (a). Composition of gut bacteria in ceca at the genus level (b).

of classifiable sequences belonged to genera *Bacteroides* (16.06%), *Rikenellaceae\_RC9\_gut\_group* (12.83%), *Phascolarctobacterium* (19.16%), *Prevotellaceae\_UCG-001* (2.19%), *Synergistes* (4.16%), and *Desulfovibrio* (3.48%) in the control group, whereas the values for the 0.025% IDPG-supplemented group were 24.24%, 16.34%, 5.31%, 5.27%, 1.66%, and 2.28%, respectively (Figure 2b). Within the phyla *Bacteroidetes*, the relative abundance of genera *Bacteroides*, *Rikenellaceae\_RC9\_gut\_group*, *Prevotellaceae\_UCG-001*, and *Alloprevotella* significantly increased in 0.025% and 0.05% IDPG-supplemented group ( $P < 0.05$ ) relative to the control group. However, there was a decrease in the relative abundance of genera *Phascolarctobacterium* (19.16% to 5.31% and 7.04%) and *Megasphaera* (1.31% to 0.34% and 0.70%) within the phyla *Firmicutes* in 0.025% and 0.05% IDPG-supplemented group ( $P < 0.05$ ) relative to the control group. We also found a noticeable reduction of genera *Synergistes* in 0.025% and 0.05% IDPG-supplemented group compared with the control group ( $P < 0.05$ ). With respect to the genus *Desulfovibrio*, its relative abundance in the cecal microbiome of the 0.05% IDPG-supplemented group was significantly reduced ( $P < 0.05$ ) compared with the control group, while that in the 0.025% IDPG group was reduced but not significantly.

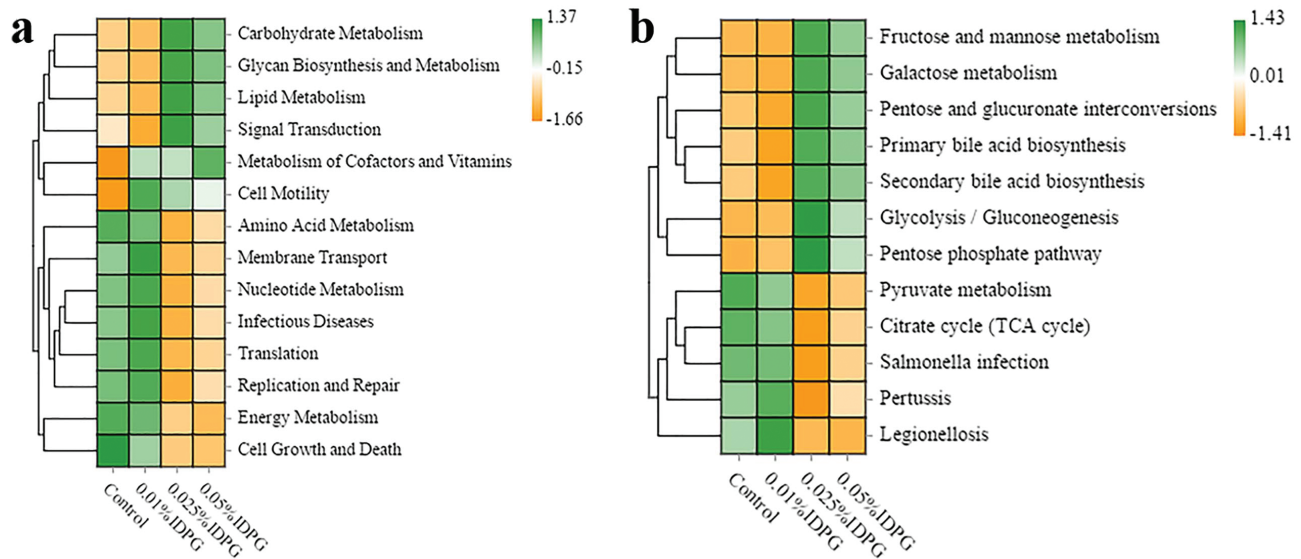
#### Molecular functional enrichment predictions

Metabolic functions, as predicted by Tax4Fun, showed that the IDPG-supplemented group had the highest number of

enriched functions compared with the control group. Within carbohydrate metabolism, pathways such as fructose and mannose metabolism, galactose metabolism, pentose phosphate pathway, and glycolysis/glycogenesis were each specifically enriched in the (0.025% and 0.05%) IDPG-fed laying hens ( $P < 0.05$ ; Figure 3b). However, the pathway of tricarboxylic acid (TCA) cycle within the carbohydrate metabolism depleted in the (0.025% and 0.05%) IDPG-supplemented groups relative to the control ( $P < 0.05$ ). In addition, significant depletions on amino acid metabolism and energy metabolism accompanied by enrichment on the lipid metabolism were observed in the laying hens fed a diet supplemented with 0.025% or 0.05% IDPG ( $P < 0.05$ ; Figure 3a). More importantly, on subclass level 3 metabolic prediction, *Salmonella* infections in the 0.025% or 0.05% IDPG-fed group were lower than that in the control group ( $P < 0.05$ ).

#### Cecal SCFA and lactate concentration and correlation with key bacterial species

The CCA results showed that the concentration of butyrate, propionate, acetate, and lactate had a consistent correlation with the bacterial composition at the genus level. The effect of propionate was the most significant, followed by butyrate, acetate, and finally lactate. Moreover, high levels of *Bacteroides*, *Rikenellaceae\_RC9\_gut\_group*, and *Prevotellaceae\_UCG-001* strongly correlated with elevated production of propionate. In contrast,



**Figure 3.** Heat maps of the comparison of cecal microbial functionality in the laying hens supplemented with different doses of incomplete degradation products of galactomannan. Tukey's honestly significant difference showed significant differences between the groups ( $P < 0.05$ ). Metabolic prediction based on subclass level 2 and 3, respectively (a, b).

*Phascolarctobacterium* and *Synergistes* correlated negatively with propionate (Figure 4b).

## Discussion

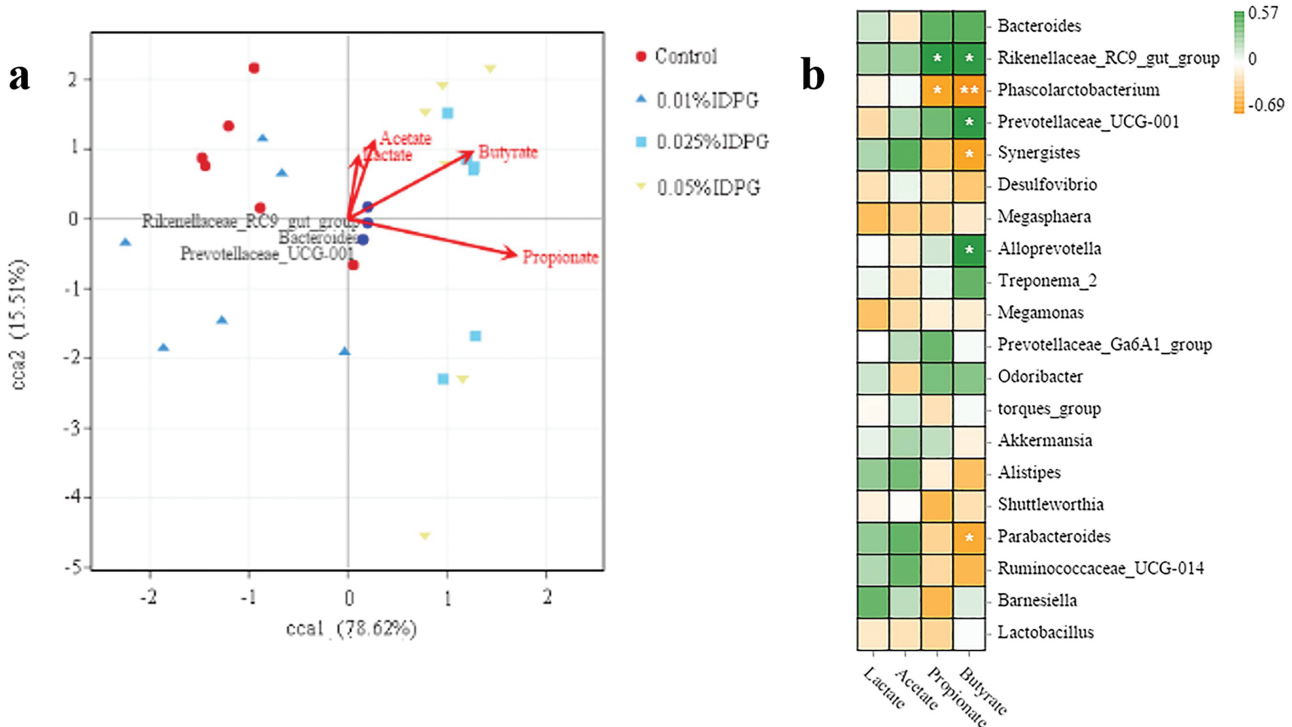
In the present study, propionate concentration increased markedly in the cecum, with higher doses (0.025% and 0.05%) of IDPG driving the presence of more propionate. This result is consistent with a report that galactomannan serves as a strong catalyst for propionate production by intestinal flora (Reichardt et al., 2009). Moreover, 0.025% or 0.05% IDPG supplementation resulted in a slight increase in butyrate concentration. In addition, a decrease in lactate concentration was noted. Regarding both butyrate and lactate, these changes were not found to be statistically significant. This could be explained by the fact that lactate is an intermediate of the fermentation reactions that ultimately produce acetate, butyrate, and propionate. Therefore, decreased lactate may actually be utilized to produce propionate and butyrate (Seeliger et al., 2002; Duncan et al., 2004). Another interesting finding is the dramatic increase in propionate concentration because propionate is an inhibitor of cholesterol synthesis. This leads to a decrease in plasma cholesterol levels alongside increased cecal propionate, a finding which was revealed in previous studies (Demigné et al., 1995; Chen et al., 2013). In addition, propionate is an important microbial metabolite fermented from complex carbohydrates, which correlate to advantageous effects on host health (Bergman, 1990). Thereby, we have deduced that the elevated propionate could be essential for providing appropriate intestinal environments and alterations to the intestinal microbiota.

A wealth of data indicate that gut microbiota is strong determinant of host physiology and general health. In particular, gut microbiota play a critical role in maintaining normal physiological functions of the intestine (Wu et al., 2018). To investigate the effect of IDPG on the gut microbiome, we analyzed 16S rRNA sequences from the cecal contents of aged laying hens after 8 wk of feeding with IDPG. The PCA and

a Venn diagram of the observed OTUs (Figure 1a and b) revealed a significant difference in the cecal microbial community between the 0.025% and 0.05% IDPG-supplemented group and the control group as well as the 0.01% IDPG-supplemented group. Moreover, IDPG supplementation significantly increased alpha-diversity levels compared with the control group, as determined by estimations such as Chao1 and Shannon index. Chao1 index reflects the species richness of the intestinal microorganisms and is sensitive to rare species. The elevated Chao1 indices herein imply the effect of IDPG as a driver of increased numbers of rare microorganism species. Interestingly, it has been reported that the level of diversity in each microbiome was significantly linked to the relative abundance of the *Bacteroidetes*, and microbiomes enriched for *Firmicutes* or *Actinobacteria* had a lower level of diversity (Turnbaugh et al., 2009). Therefore, the elevation of alpha diversity in the cecal microbiome of hens fed diets supplemented with IDPG suggests a shift to the proportions of *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Additionally, the slight increase in the Simpson index induced by the IDPG addition reveals that the addition of IDPG makes the original dominant species more prominent. Similarly, beta-diversity analysis, based on weighted and unweighted UniFrac distance, indicates that IDPG mainly brought about a main change to the abundance of shared species, followed by species richness. Based on these results, this change in shared species abundance may have occurred mainly in the phyla *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. To confirm this, we then classified these representative phyla.

*Bacteroidetes* and *Firmicutes* are the two most abundant bacterial phyla in the cecum of laying hens, where *Bacteroidetes* account for more than 50% of the total cecal microbiome of laying hens over 26 wk of age (Videnska et al., 2014). Feeding an IDPG-based diet induced a significant increase in the phyla *Bacteroidetes* from 51.71% to a maximum level representing 76.71% of the total cecal microbiome. The increase in the abundance of the phyla *Bacteroidetes* could be explained by an increase in the genera *Bacteroides*, *Rikenellaceae\_RC9\_gut\_group*, and *Prevotellaceae\_UCG-001*. Other phyla, such





**Figure 4.** The correlation between short-chain fatty acids concentration and key microbes at the genus level. Canonical correspondence analysis plot (a); Heat map (b). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

as *Firmicutes*, *Proteobacteria*, and *Synergistetes* tended to correspondingly decrease in abundance. The decreased abundance of the phyla *Firmicutes* can be explained by the decrease in the genera *Phascolarctobacterium* and *Megasphaera*. Consistent with our findings, Hoving et al. (2018) demonstrated that manno-oligosaccharides (MOS) increased the abundance of *Bacteroidetes* and decreased the abundance of *Firmicutes* in the cecal microbiome of the female mice.

*Bacteroidetes* are common bacteria in the gut that are involved in many important metabolic activities, including fermentation of carbohydrates, synthesis of propionate via the succinate pathway, biotransformation of bile acids, and prevention of pathogen colonization (Flint et al., 2008). Comparative and functional genomic studies predict that the ability of gut *Bacteroides* species to utilize diverse glycans depends on a series of gene clusters that are referred to as polysaccharide utilization loci (PUL; Bjursell et al., 2006). Recently, a detailed genetic, biochemical, and enzyme structural characterization of a galactomannan-specific PUL from *B. ovatus* revealed an interplay of two mannan-specific surface glycan-binding proteins, two GH26 endo- $\beta$ -mannanases and a GH36 exo- $\alpha$ -galactosidase in the deconstruction of this plant cell wall polysaccharide (Reddy et al., 2016; Bagenholm et al., 2017). Moreover, in the genera *Bacteroides*, enzymes involved in carbohydrate depolymerization are attached to the cell surface, which may be an advantage in nutrition uptake (Senoura et al., 2011). A new mannan catabolic pathway in genera *Bacteroides* was also revealed involving 1,4- $\beta$ -mannanase, a mannobiose and/or sugar transporter, mannobiose 2-epimerase, and mannosylglucose phosphorylase before finally progressing to glycolysis (Senoura et al., 2011). Accordingly, the energy-efficient strategy for mannan metabolism, in which a unique mannosylglucose phosphorylase can directly produce phosphorylated mannose without

ATP consumption, would provide intestinal anaerobic bacteria with evolutionary advantages. These outstanding characteristics constitute the major nutrient acquisition strategy deployed by *Bacteroidetes* bacteria and thus are intrinsically linked to the colonization of nutritional niches and the establishment of microbial ecosystems. Unlike *Bacteroidetes*, which are enriched for many carbohydrate metabolism pathways, *Firmicutes* are enriched for transport systems (Turnbaugh et al., 2009). In our present study, consumption of IDPG induced a relative increase in certain *Bacteroidetes* at the expense of *Firmicutes* (mainly *Phascolarctobacterium* and *Megasphaera*), which is similar to what was observed for mice-fed MOS-enriched diets (Hoving et al., 2018). Notably, the use of an IDPG-based diet resulted in lower *Firmicutes* to *Bacteroidetes* ratios. This result is important considering that an increased ratio of *Firmicutes* to *Bacteroidetes* has been shown to be associated with obesity in humans and mice due to the increased energy harvesting capacity of bacterial species in the phyla *Firmicutes* (Turnbaugh et al., 2006, Turnbaugh et al., 2009). The phylum *Proteobacteria*, known for containing numerous pathogenic bacteria such as *Salmonella* and *Helicobacter*, is related to dysbiosis and the progression of several diseases (Shin et al., 2015). The observed reduction of the abundance of phylum *Proteobacteria* therefore may be ascribed to the ability of mannose in IDPG to bind to mannose-binding lectins of Gram-negative bacteria expressing type 1 fimbriae and as a consequence reduced bacterial attachment to the intestinal epithelial cells (Ganner and Schatzmayr, 2012). In all, the reduction of the abundance of *Proteobacteria* in laying hens fed the diet supplemented with IDPG was shown to help with maintaining favorable intestinal environments and overall host health.

In response to the dramatic change in the cecal microbial community induced by IDPG, the metabolic function of the



cecal microbiome was also altered. Metabolic prediction of the cecal microbiome revealed significant enrichment for a number of expected functional categories, including carbohydrate and lipid metabolism (Figure 3). Moreover, metabolic profile-based clustering indicated that the noted alteration of metabolic function was highly consistent with changes to species abundance. This results in changes to several pathways related to the metabolism of IDPG, specifically carbohydrate metabolism (e.g., fructose and mannose metabolism, galactose metabolism, pentose phosphate pathway, and glycolysis/glycogenesis). Several other pathways were markedly depleted alongside modified carbohydrate metabolism, including amino acid metabolism, energy metabolism, and infectious diseases. This dramatic shift of energy metabolism may be ascribed to the depletion of the TCA cycle at level 3 metabolic prediction and the unique energy-efficient pathway for IDPG metabolism in genera *Bacteroides*, as previously mentioned. Another point that cannot be ignored was that inclusion of IDPG in the diet of laying hens notably depleted *Salmonella* infection based on subclass level 3 metabolic prediction. This again serves as a strong indicator that IDPG supplementation can greatly assist a laying hen's ability to maintain overall health.

Interestingly, we found a strong positive correlation between levels of genera *Bacteroides*, *Rikenellaceae\_RC9\_gut\_group*, and *Prevotellaceae\_UCG-001* and production of propionate. In accordance with this result, the proportion of propionate that is present in total SCFA correlated with the relative abundance of phyla *Bacteroidetes*. This was congruent with a report on the impact of diet on intestinal microbiota composition and fermentation products in obese men as reported by Salonen et al. (2014). In addition, genera *Phascolarctobacterium* and *Synergistes* correlated negatively with propionate concentrations, as predicted based on the dramatic change in the microbiome community. As a consequence, we can gain valuable insight through gut microbe-metabolite associations that the significant enrichment of phyla *Bacteroidetes* induced by IDPG produce propionate and competitively restrain the proliferation of other phyla such as *Firmicutes*, *Proteobacteria*, and *Synergistete*.

In this study, changes in the gut microbiota as a result of implemented IDPG into the diets of laying hens were found to both be statistically significant and provide highly unique observations. Specifically, the phyla *Bacteroidetes* were shown to play a key role in the coordination of IDPG degradation and an ensuing increase to cecal propionate concentrations. However, the pathway by which gut microbiota exert their positive effects on the intestinal tract as well as throughout the rest of the body remains a critical and unanswered question. We believe that the beneficial effects on hens of IDPG dietary supplementation merit further investigation considering the current lack of knowledge regarding IDPG utilization and the growing interest in gastrointestinal health in both animals and humans.

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## Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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