Effects of Soybean Oligosaccharides and Their Functional Components on Skatole Production and Microbial Composition in Broiler Chicken Cecal Contents under In Vitro Conditions (Post-print)

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Abstract

This experiment aimed to investigate the effects of soybean oligosaccharides (SBO) and their functional components on skatole production and cecal microbiota composition in broiler chicken cecal content under in vitro conditions. A single-factor completely randomized design was adopted, using cecal content from 42-day-old broiler chickens as the inoculum, and the anaerobic culture medium was dispensed into sterile culture bottles, divided into 5 groups with 3 replicates per group. The control group was supplemented with 250 mol/L L-tryptophan, while the sucrose (SUC), stachyose (STA), raffinose (RAF), and SBO groups were supplemented with 1% SUC, STA, RAF, and SBO, respectively, on the basis of the control group. Additionally, a blank control without L-tryptophan but with all other components identical was set up for each group. Using the ANKOM RFS in vitro gas production system, anaerobic incubation was conducted at 39 °C for 24 h. The results showed: 1) The 24 h cumulative gas production of fermentation broth in SUC, STA, and SBO groups was extremely significantly higher than that in the control and RAF groups ($P<0.01$); indole concentrations in SBO, SUC, and STA groups were reduced by 98.15%, 97.72%, and 94.17% compared with the control group ($P<0.01$), respectively, and skatole concentrations were reduced by 79.04%, 71.88%, and 70.28% compared with the control group ($P<0.05$), respectively; lactic acid concentration in the SUC group was extremely significantly higher than that in all other groups ($P<0.01$); pH in the control group was extremely significantly higher than that in all other groups except the RAF group ($P<0.01$). 2) PCR-denaturing gradient gel electrophoresis (DGGE) technology was used to investigate differences in the microbial community composition of the fermentation broth. Microbial

community evenness in the SBO group was significantly lower than that in the control group (P<0.05); microbial community richness in SUC, STA, RAF, and SBO groups was significantly higher than that in the control group (P<0.05); microbial community similarity in SBO, STA, and RAF groups was higher than that in the control group; there were 3 specific bands in the experimental groups, with similar bacteria being Blautia producta, Parabacteroides distasonis, and Lactobacillus reuteri, respectively. In summary, under the conditions of this experiment, supplementation with 1% SUC, STA, and SBO significantly reduced the concentrations of indole and skatole produced from L-tryptophan metabolism in the in vitro cecal content culture medium of broiler chickens, increased microbial community richness, and promoted the proliferation of specific bacteria. The efficacy in reducing skatole followed the order: SBO > SUC > STA.

Full Text

Effects of Soybean Oligosaccharide and Its Functional Components on Skatole Production and Microbiota Composition in Broiler Cecal Contents Under In Vitro Conditions

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Abstract: This study investigated the effects of soybean oligosaccharide (SBO) and its functional components on skatole production and cecal microbiota composition in broiler cecal contents under in vitro conditions. A single-factor completely randomized design was employed, using cecal contents from 42-day-old broilers as the inoculum. The anaerobic culture medium was dispensed into sterile culture bottles and divided into five groups with three replicates each. The control group received 250 μmol/L L-tryptophan, while the sucrose (SUC), stachyose (STA), raffinose (RAF), and SBO groups were supplemented with 1% SUC, STA, RAF, and SBO, respectively, based on the control group. Additionally, each group included a blank control without L-tryptophan but with identical other components. Using the ANKOM RFS in vitro gas production system, microbial suspensions were anaerobically incubated at 39°C for 24 h. The results showed: (1) The 24 h cumulative gas production in the SUC, STA, and SBO groups was significantly higher than in the control and RAF groups (P<0.01). Indole concentrations in the SBO, SUC, and STA groups decreased by 98.15%, 97.72%, and 94.17% compared to the control group (P<0.01), while skatole concentrations decreased by 79.04%, 71.88%, and 70.28%, respectively (P<0.05). Lactate concentration in the SUC group was significantly higher than in all other groups (P<0.01), and pH in the control group was significantly higher than in all groups except the RAF group (P<0.01). (2) PCR-denaturing gradient gel electrophoresis (DGGE) analysis revealed that microbiota evenness

in the SBO group was significantly lower than in the control group (P<0.05), while microbiota richness in the SUC, STA, RAF, and SBO groups was significantly higher than in the control group (P<0.05). Microbiota similarity in the SBO, STA, and RAF groups was higher than in the control group. Three specific bands appeared only in the treatment groups, with similar bacteria identified as *Blautia producta*, *Parabacteroides distasonis*, and *Lactobacillus reuteri*. In conclusion, under the experimental conditions, supplementation with 1% SUC, STA, and SBO significantly reduced indole and skatole concentrations from L-tryptophan metabolism, increased microbiota richness, and promoted proliferation of specific bacteria in broiler cecal contents in vitro. The efficacy in reducing skatole followed the order: SBO > SUC > STA.

Keywords: skatole; soybean oligosaccharide; cecal microbiota; in vitro fermentation; broilers

Introduction

China' s broiler industry has developed rapidly with continuously expanding scale, while simultaneously, odor pollution from large quantities of manure discharged by chicken farms has intensified. Nutritional approaches to fundamentally reduce odor pollution in poultry production have attracted widespread attention and hold significant scientific importance for promoting sustainable, healthy development of the broiler industry and exploring environmentally friendly animal agriculture [1]. Skatole, a metabolite of microbial L-tryptophan degradation, is one of the major compounds responsible for the foul odor in pig and chicken excreta [2,3]. Research indicates that L-tryptophan availability and intestinal microbial composition and activity are primary factors affecting intestinal skatole concentrations in animals [4]. Skatole concentration in broiler ceca is significantly higher than in the ileum and rectum, with these differences primarily related to intestinal microbial diversity and richness [5]. Soybean oligosaccharide (SBO) is a product derived from soybeans and their processing byproducts, containing specific amounts of stachyose (STA), raffinose (RAF), and sucrose (SUC) (GB/T 22491–2008). Typically, 75% SBO contains 18% STA, 6% RAF, and 24% SUC [6]. Coon et al. [7] reported that the ileal digestibility of RAF and STA in turkeys was less than 1%, while fecal digestibility reached 84–90%, further demonstrating that SBO and its functional components are primarily utilized by microorganisms in the hindgut of poultry. Li et al. [8] investigated the effects of different fiber sources on L-tryptophan metabolism by pig rectal microbiota in vitro, showing that skatole production in the culture system was influenced by rectal microbial composition, which was closely related to the fiber source. Sheng et al. [9] demonstrated that exogenous L-tryptophan was the main factor affecting tryptophan and skatole concentrations in pig manure fermentation broth. Our previous research showed that dietary SBO supplementation significantly reduced indole and skatole concentrations in excreta of 42-day-old broilers

[10] and decreased skatole production from L-tryptophan metabolism under the action of broiler cecal and rectal microbiota [11]. However, which single component (monomer) of SBO plays the primary role remains unclear, and the underlying mechanism requires further elucidation.

1.1 Materials and Sample Collection

The SBO, SUC, RAF, and STA used in the experiment were commercial oligosaccharide products purchased from a biotechnology company in Henan Province. Using the anthrone colorimetric method [12], the measured total sugar contents of SBO, SUC, RAF, and STA were 76.10%, 64.53%, 68.21%, and 88.07%, respectively.

Thirty healthy Arbor Acres (AA) broilers (half male and half female) were raised to 42 days of age on a corn-soybean meal diet without antibiotics, with an average body weight of 2.75 kg. The birds were slaughtered, the abdominal cavity was opened, and the ceca were isolated, ligated with fine thread, excised, placed in prepared ziplock bags, weighed (approximately 6–7 g cecal contents per bird), and immediately stored at –80°C.

1.2 Experimental Design

A single-factor completely randomized design was employed with five groups: SUC, STA, RAF, SBO, and control, each with three replicates. The control group received 250 μmol/L L-tryptophan, while the SUC, STA, RAF, and SBO groups were supplemented with 3.10 g SUC, 2.27 g STA, 2.93 g RAF, and 2.63 g SBO, respectively (equivalent to 1% total sugar content) in a 200 mL in vitro culture volume. Additionally, considering differences in protein content among substrates, each group included a blank control without L-tryptophan but with identical other components.

1.3 In Vitro Culture Medium Preparation and Fermentation

The basal culture medium was prepared according to the method of Yokoyama et al. [13]. The pH was adjusted to 5.7 ± 0.3 (the pH of chicken cecal contents), and the medium was sterilized at 121°C for 15 min under 1 standard atmosphere [14]. Cecal contents were removed from the –80°C freezer, thawed in a laminar flow hood, and mixed at a 1:1 ratio from male and female broilers. Thirty grams of the mixed sample were suspended in 3 L sterile anaerobic culture medium, thoroughly stirred, and filtered through four layers of gauze to remove coarse particulate matter. The filtrate was dispensed into ANKOM RFS sterile culture bottles at 198 mL per bottle, with CO_2 continuously supplied throughout

the procedure to maintain microbial activity. Two milliliters of 5.1 mg/mL L-tryptophan solution were added to each group (final L-tryptophan concentration of 250 µmol/L), and the treatment groups received 1% SUC, STA, RAF, and SBO, respectively. Blank controls received 2 mL sterile distilled water, and all bottles were thoroughly shaken. The culture bottles were filled with CO_2, sealed with the gas production system caps, placed in a 39°C incubator, and anaerobically cultured for 24 h. Portions of fermentation broth were collected in capped centrifuge tubes and stored at -20°C and -80°C for later analysis.

1.4.1 Gas Production

The ANKOM RFS in vitro gas production system automatically detects gas produced in the bottles and records pressure information. Gas volume (mL) was calculated from pressure data using the formula:

$$V_{xt} = V_j \times \frac{P_{psi_t} - P_{psi_0}}{14.068}$$

where V_{xt} is the cumulative gas volume (mL) at time t (h), V_j is the headspace volume above the liquid in the module bottle (mL), P_{psi_t} is the cumulative pressure (psi) recorded by the GPM software for the sample module bottle at time t (h), P_{psi_0} is the cumulative pressure (psi) recorded by the GPM software for the blank module bottle at time t (h), and 1 psi = 6.895 kPa.

1.4.2 pH

After anaerobic incubation, the pH of fermentation broth was measured using a calibrated PHS-3C pH meter.

1.4.3 Indole and Skatole Concentrations

Indole and skatole concentrations in fermentation broth were determined by Agilent-1100 high-performance liquid chromatography according to the method described in reference [11].

1.4.4 Acetate, Propionate, Butyrate, and Lactate Concentrations

Acetate, propionate, butyrate, and lactate concentrations in fermentation broth were measured by Agilent-7890B gas chromatography according to the method described in reference [15].

1.5.1 Genomic DNA Extraction

Total bacterial genomic DNA was extracted from fermentation broth using a cetyltrimethylammonium bromide (CTAB) environmental microbial DNA extraction kit and purified with a DNA purification kit, then stored at -20°C.

1.5.2 PCR Amplification of Bacterial 16S rDNA Fragments

Universal bacterial primers were used as described in reference [10]. The PCR amplification system (50 µL) contained: 0.4 µL rTaq polymerase (5 U/µL), 5 µL 10×PCR buffer, 3.2 µL dNTPs (2.5 mmol/L), 1 µL GC-338F (20 µmol/L), 1 µL 518R (20 µmol/L), 50 ng template DNA, and ddH_2O to 50 µL. Reaction conditions were: 94°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 1 min; and final extension at 72°C for 10 min.

1.5.3 Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

DGGE analysis was performed using the Bio-Rad Dcode system. PCR products were separated on 7% polyacrylamide gels with a denaturing gradient of 35–55% at 150 V and 60°C for 5 h in 1×TAE buffer. Gels were stained with silver nitrate and photographed using a gel imaging system. Target bands were recovered using the Poly-Gel DNA extraction kit (OMEGA), cloned, and sequenced. Sequences were compared against the GenBank database to identify the closest bacterial relatives or clones.

1.5.4 PCR-DGGE Profile Analysis

Quantity One software was used for digital analysis of band numbers and densities. Diversity index (Shannon-Wiener index, H), richness (S), and evenness (E) were calculated as follows:

$$H = -\sum_{i=1}^{S} p_i \ln(p_i)$$

$$S = \sum_{i=1}^{N} \frac{N_i}{N}$$

$$E = \frac{H}{H_{max}}$$

where p_i is the proportion of the optical density of a single band to the total optical density of all bands in the sample, N is the abundance of bands in a single DGGE lane, N_i is the abundance of bands in lane i, S is the total number of bands across all samples, and H_{max} is the maximum value of H.

Pairwise comparison of microbiota differences between samples was performed by cluster analysis using MEGA 4.1 software based on Dice similarity coefficient (Cs), calculated as:

$$C_s = \frac{2j}{N_x + N_y}$$

where N_x and N_y are the numbers of bands in samples x and y, respectively, and j is the number of common bands between the two lanes.

1.6 Statistical Analysis

Data were analyzed by one-way ANOVA using IBM SPSS Statistics 22.0 software, with Duncan' s multiple comparison test. Bivariate correlation analysis was performed among skatole concentration, fermentation parameters, and microbiota structure. P<0.05 was considered significant and P<0.01 highly significant.

2.1 Cumulative Gas Production in Fermentation Broth

As shown in Table 1 , significant differences in cumulative gas production were observed among groups at 3 h (P<0.05), with highly significant differences at all subsequent time points (P<0.01). At 6 and 9 h, cumulative gas production in the RAF group was significantly lower than in the control group (P<0.01). Before 21 h, gas production in the RAF group remained lower than the control group, though not significantly (P>0.05). At 24 h, cumulative gas production in the SUC, STA, and SBO groups was significantly higher than in the control and RAF groups (P<0.01), with the control group showing the lowest production. No significant differences were observed among the SUC, STA, and SBO groups at any stage (P>0.05). Figure 1 [Figure 1: see original paper] visually illustrates the changes in cumulative gas production across time points.

Table 1 Effects of soybean oligosaccharide and its functional components on cumulative gas production in fermentation broth by cecal microbiota of broilers (mL)

Incubation time (h)	SUC	STA	RAF	SBO	Control
3	37.79$±3.10*ab*|37.89±1.22*ab*|33.10±1.31*b*|42.95±2.64*a*|44.60±1.55*a*||6|61.15±4.7				

In the same column, values with the same or no letter superscripts mean no significant difference (P>0.05), different lowercase letters indicate significant

difference (P<0.05), and different uppercase letters indicate highly significant difference (P<0.01). The same applies below.

Figure 1 Effects of soybean oligosaccharide and its functional components on cumulative gas production in fermentation broth by cecal microbiota of broilers

2.2 Indole, Skatole Concentrations and Fermentation Parameters

As shown in Table 2 , indole concentrations in the SBO, SUC, and STA groups decreased by 98.15%, 97.72%, and 94.17% compared to the control group (P<0.01), with no significant differences among these three groups (P>0.05). The RAF group showed no significant difference in indole concentration compared to the control (P>0.05). All treatment groups had significantly lower skatole concentrations than the control (P<0.05), with reductions of 79.04%, 71.88%, 70.28%, and 62.42% in the SBO, SUC, STA, and RAF groups, respectively (P<0.05), though no significant differences were observed among these four groups (P>0.05). No significant differences were found in acetate concentrations among groups (P>0.05). Lactate concentration in the SUC group was significantly higher than in all other groups (P<0.01), while the RAF and SBO groups showed no significant difference compared to the control (P>0.05). The pH in the control group was significantly higher than in all groups except RAF (P<0.01).

Table 2 Effects of soybean oligosaccharide and its functional components on indole, skatole concentrations and fermentation parameters in fermentation broth by cecal microbiota of broilers

Groups	Indole (ng/mL)	Skatole (ng/mL)	Acetate (mmol/L)	Lactate (mmol/L)	pH
SUC	5.21$±0.29$Bb$	8.05±0.73b	16.88±0.47	17.51±1.00Aa	5.65±0.49$BCbc$
STA	13.33±1.20Bb	8.51±0.81b			

No propionate and butyrate were detected in fermentation broth in all groups.

2.3.1 Microbial Diversity

As shown in Figure 2 [Figure 2: see original paper], bands 12, 20, 25, and 26 were present in all groups, while some bands appeared only in specific groups and others were absent from particular groups. Table 3 indicates that no significant differences were observed in microbiota diversity indices among groups (P>0.05). However, microbiota evenness in the SBO group was significantly lower than in the control group (P<0.05), and microbiota richness in the SUC, STA, RAF, and SBO groups was significantly higher than in the control group (P<0.05).

1-1, 1-2, 1-3 represent SUC groups; 2-1, 2-2, 2-3 represent STA groups; 3-1, 3-2, 3-3 represent RAF groups; 4-1, 4-2, 4-3 represent SBO groups; 5-1, 5-2, 5-3 represent control groups. The same applies to Figure 3.

Figure 2 PCR-DGGE fingerprints of microbiota in fermentation broth

Table 3 Effects of soybean oligosaccharide and its functional components on cecal microbiota diversity of broilers in vitro

Groups	Shannon-Wiener index	Evenness	Richness
SUC	3.03$±0.03|0.968±0.004ab|23.00±0.58a||STA|3.07±0.05|0.966±0.002ab|24.00±1.15a||RAF|3.13-		

2.3.2 Microbial Similarity

As shown in Figure 3 [Figure 3: see original paper], the DGGE profiles were clearly clustered into two groups with a similarity coefficient of 0.49. The upper cluster contained two control samples and one RAF sample with a similarity coefficient of 0.68, while the two control samples showed a similarity coefficient of 0.76. In the lower cluster, sample 1-2 from the SUC group had the lowest similarity coefficient (0.51) with other samples. Control sample 5-3 showed a similarity coefficient of 0.64 with other samples, which was markedly lower than the other three groups. Four clusters showed high similarity: SBO samples 4-1 and 4-3 (0.89), STA samples 2-2 and 2-3 (0.88), RAF samples 3-1 and 3-3 (0.84), and SUC sample 1-1 and SBO sample 4-2 (0.84). These samples were from groups supplemented with SBO, STA, and RAF, and all similarity coefficients were higher than those of the control group.

Figure 3 Unweighted pair group method clustering analysis of 15 fermentation broth samples

2.3.3 Sequence Analysis of Dominant DGGE Bands

Eleven common and specific bands from the PCR-DGGE fingerprints were excised, cloned, and sequenced. As shown in Table 4 , all eleven sequences showed 99–100% similarity to bacteria in the GenBank database. Bands 12, 20, 25, and 26 were common to all 15 fermentation samples, with similar bacteria identified as *Enterococcus gallinarum*, *Klebsiella pneumoniae*, *Dickeya chrysanthemi*, and *Shigella sonnei*, respectively. Band 4 appeared only in RAF group sample 3-1, with *Blautia producta* as the similar bacterium. Band 9 was present in SUC group samples 1-1 and 1-2, STA group samples 2-1 and 2-3, all RAF group samples, and SBO group samples 4-1 and 4-2, with *Parabacteroides distasonis* as the similar bacterium. Band 28 appeared in the SUC, STA, RAF, and SBO groups, with *Lactobacillus reuteri* as the similar bacterium. Thus, bands 4, 9, and 28 appeared only in the treatment groups.

Table 4 Analysis results of PCR-DGGE gel bands recovery sequence

Band No.	Similar strain	Accession No.	Similarity (%)	Classification	Note
4	*Blautia producta*	NR_{113270}	99	Firmicutes, Clostridia, *Blautia*	Unique to RAF group 3-1
9	*Parabacteroides distasonis*	NR_{041342}	99	Bacteroidetes, Bacteroidia, *Parabacteroides*	Present in SUC 1-1, 1-2; STA 2-1, 2-3; RAF; SBO 4-1, 4-2
12	*Enterococcus gallinarum*	NR_{104559}	100	Firmicutes, Bacilli, *Enterococcus*	Common to all groups
20	*Klebsiella pneumoniae*	NR_{041750}	100	Proteobacteria, Gammaproteobacteria, *Klebsiella*	Common to all groups
25	*Dickeya chrysanthemi*	NR_{074902}	100	Proteobacteria, Gammaproteobacteria, *Dickeya*	Common to all groups
26	*Shigella sonnei*	NR_{117738}	100	Proteobacteria, Gammaproteobacteria, *Shigella*	Common to all groups
28	*Lactobacillus reuteri*	NR_{075036}	100	Firmicutes, Bacilli, *Lactobacillus*	Present in SUC, STA, RAF, SBO groups

2.4 Correlations Among Skatole Concentration, Fermentation Parameters, and Microbiota Diversity

As shown in Table 5 , acetate concentration was positively correlated with microbiota diversity index and richness, with a highly significant positive correlation between acetate and diversity index (r=0.964, P<0.01) and a significant positive correlation between acetate and richness (r=0.954, P<0.05). pH was significantly positively correlated with microbiota evenness (r=0.909, P<0.05).

Table 5 Correlation of skatole concentration, fermentation parameters and diversity of cecal microbiota in fermentation broth of broilers

Items	Shannon-Wiener index	Evenness	Richness
Indole (ng/mL)	-0.228	-0.788	-0.477
Skatole (ng/mL)	-0.521	-0.619	-0.295
Acetate (mmol/L)	0.964**	0.909*	0.954*
Lactate (mmol/L)	-0.845	-0.564	-

** means extremely significant correlation (P<0.01), * means significant correlation (P<0.05).

3.1 Effects of Soybean Oligosaccharide and Its Functional Components on L-Tryptophan Metabolism and Skatole Production Under Broiler Cecal Microbiota

Fermentation substrate and microbial species influence gas production, gas production rate, and metabolites during fermentation [16]. Lan et al. [17] reported that STA showed greater gas production and rate compared to SBO and RAF in vitro fermentation using 81-day-old broiler cecal microbiota as inoculum. Our results showed that 24 h cumulative gas production in the SUC, STA, and SBO groups was significantly higher than in the control group, with no significant differences among these three groups, indicating that broiler cecal microorganisms can effectively utilize SBO and its functional components (except RAF) as carbon sources. Yi et al. [18] fermented RAF, STA, fructooligosaccharides, and mannan oligosaccharides using 42-day-old broiler cecal contents and found decreasing fermentability, with RAF showing the greatest gas production and rate. In contrast, our study showed the lowest gas production in the RAF group, possibly due to L-tryptophan addition, as the presence of protein substrate affected carbohydrate fermentation. Before 21 h, cumulative gas production in the RAF group was lower than the control, only exceeding the control at 24 h, suggesting slower RAF fermentation. This led to microorganisms initially utilizing L-tryptophan as substrate, increasing L-tryptophan degradation and

consequently indole and skatole production. Notably, indole production in the RAF group was 220.51, 212.39, and 221.50 ng/mL higher than in the SUC, STA, and SBO groups, respectively.

Li et al. [8] reported that beet pulp and fructooligosaccharide supplementation significantly reduced skatole concentration and relative production rate in L-tryptophan culture medium under pig rectal microbiota. Our study demonstrated that SBO and its functional components significantly reduced skatole concentration, with efficacy following the order: SBO > SUC > STA > RAF, while RAF showed no significant effect on indole concentration. These results indicate that both SBO and its functional components effectively reduce skatole production, with RAF being the least effective, primarily due to its slow fermentation rate, which caused microorganisms to preferentially utilize L-tryptophan, increasing its degradation products. Research shows that intestinal microbial growth and metabolism require both carbohydrates and proteins, with bacterial saccharolytic fermentation predominating in the distal broiler intestine, and putrefaction occurring only when carbohydrates are depleted [19]. The skatole-reducing effect of SBO and its components mainly stems from their preferential fermentation as microbial energy sources, thereby reducing bacterial fermentation of L-tryptophan and consequently decreasing skatole production.

Non-digestible carbohydrates (NDC) are fermented by colonic microorganisms to produce acetate, propionate, and butyrate [20]. Therefore, the content and proportion of these short-chain fatty acids (SCFA) in monogastric animals can indirectly reflect intestinal microbiota status [21]. Lan et al. [22] fermented four NDCs including SBO and water-soluble soybean polysaccharides, plus STA and RAF, using 81-day-old broiler cecal contents, reporting highest butyrate production and lowest pH and ammonia nitrogen concentration (199.3 mg/L) in the SBO group. In our study, SBO and its functional components showed no significant effect on acetate concentration, though the control group had the lowest acetate concentration (10.27 mmol/L). Macfarlane et al. [23] reported that fermentation substrate affects SCFA production, with protein-based substrates generally producing less SCFA than carbohydrate-based substrates. Our results showed that SBO supplementation significantly reduced lactate concentration and pH, with the control group showing the highest pH, which did not differ significantly from the RAF group. Low pH environments favor lactobacilli growth and reduce putrefactive bacteria, thereby decreasing skatole production.

Propionate and butyrate were not detected in our study using gas chromatography. Yi et al. [18] measured molar ratios of acetate, propionate, and butyrate in 24 h fermentation broth of STA and RAF as 5.6:2.5:1.9 and 5.6:2.3:2.1, respectively. This suggests that broiler intestinal microbiota primarily produce acetate and lactate from carbohydrate fermentation, with minimal propionate and butyrate production.

3.2 Effects of Soybean Oligosaccharide and Its Functional Components on Cecal Microbiota Composition In Vitro

In recent years, PCR-DGGE, qPCR, and metaproteomics have been widely applied in animal intestinal microbiota ecology research, becoming primary techniques for studying broiler intestinal microorganisms and explaining novel bacterial communities associated with malodorous compound formation [10,11]. Our study showed that SBO and its functional components had no significant effect on microbiota diversity index, consistent with Hou [11]. However, supplementation significantly increased microbiota richness, with SBO significantly reducing evenness. Differences in PCR-DGGE fingerprints and similarity coefficients between STA, RAF, SBO groups and the control may explain the skatole-reducing effects of SBO and its components. Additionally, three specific bands appeared only in treatment groups, corresponding to *Blautia producta*, *Parabacteroides distasonis*, and *Lactobacillus reuteri*, indicating that SBO and its functional components reduce indole and skatole production by promoting proliferation of these bacteria.

3.3 Correlation Analysis Among Skatole Concentration, Fermentation Parameters, and Cecal Microbiota Composition

Previous studies have focused on effects of SBO (or its components) on human or animal intestinal microbiota, with limited literature addressing skatole production, pH, and organic acids, and even fewer investigating their interrelationships [24,25]. Examining correlations among skatole, indole, and intestinal microbiota will help elucidate the microbial mechanisms by which SBO and its components reduce malodorous compound production, holding important scientific significance for exploring nutritional strategies to reduce skatole pollution. Hu et al. [26] fermented L-tryptophan using pig fecal bacteria and found that higher pH favored skatole production while lower pH favored indole production, indicating a relationship between environmental acidity and indole/skatole production. Wang et al. [25] reported a significant negative correlation between excreta pH and cecal lactate concentration in 42-day-old broilers (r=–0.927). Our study showed that SBO and its functional components significantly affected lactate concentration and pH, with SBO showing the lowest pH and the control the highest, consistent with the above reports. Higher gas production indicates better fermentation characteristics, which is less favorable for L-tryptophan degradation to indole. Zhang [5] reported that microbiota diversity index, richness, and total bacterial count were highly significantly positively correlated with skatole production in broilers (r=0.748, 0.783, and 0.700, respectively). Our study showed that acetate concentration was highly significantly positively correlated with diversity index and significantly positively correlated with richness, while pH was significantly positively correlated with evenness. However, indole and skatole concentrations showed no significant correlation with microbiota structure. Therefore, SBO and its functional components may reduce skatole

production by increasing or decreasing specific bacterial populations, which requires further investigation. Additionally, whether these results are consistent with changes in broiler intestinal microbiota needs verification through animal experiments.

Conclusions

1. Supplementation with 1% SBO, SUC, and STA significantly reduced indole concentration in broiler cecal fermentation broth by 98.15%, 97.72%, and 94.17%, respectively. Supplementation with 1% SBO, SUC, STA, and RAF significantly reduced skatole concentration by 79.04%, 71.88%, 70.28%, and 62.42%, respectively.
2. Supplementation with 1% SBO significantly reduced microbiota evenness, while SBO and its functional components significantly increased microbiota richness. SBO and its functional components promoted proliferation of *Blautia producta*, *Parabacteroides distasonis*, and *Lactobacillus reuteri*.

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