Journal of Animal Science and Research
ISSN 2576-6457 | Open Access

RESEARCH ARTICLE

Volume 6 - Issue 1

Effects of Cashew Nut Shell Liquid on Hindgut Fermentation and Microbiota of Chickens

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Received: 20 Jun, 2022 | **Accepted:** 01 Jul, 2022 | **Published:** 08 Jul, 2022

Citation: Hosokawa M, Hikita C, Suzuki Y, Koike S, Kobayashi Y (2022) Effects of Cashew Nut Shell Liquid on Hindgut Fermentation and Microbiota of Chickens. J Anim Sci Res 6(1): dx.doi.org/10.16966/2576-6457.159

Abstract

Cashew nut shell liquid (CNSL), a feed additive for chickens, was evaluated for its influence on the hindgut environment by determining fermentation and bacterial profiles using cecal contents, fecal slurry and pure cultures. A farm-scale study showed that CNSL feeding to broiler chickens from 0 to 42 days of age improved body weight gain and lowered mortality. Cecal levels of short chain fatty acids increased with CNSL feeding, while ammonia and indole levels decreased. Feeding CNSL decreased the relative abundance of cecal bacteria belonging to some clostridial groups and Ruminococcaceae. In particular, *Clostridium perfringens* was undetectable following CNSL feeding. A pure culture study showed that CNSL (≥ 6.25 μg/ml) inhibited *C. perfringens* growth, accompanied by cell surface disruption based on electron microscopic observation. Fermentation profiles of slurry cultures prepared from feces of chickens were altered by CNSL supplementation toward less ammonia production. The lowered ammonia was correlated positively with the abundance of unclassified Lachnospiraceae and unclassified Ruminococcaceae, and negatively with unclassified Enterobacteriaceae and *Sutterella*. These results suggest that CNSL could exert its beneficial influence on the hindgut health of chickens through impacting the microbiota.

Keywords: Additive; Cashew; Chicken; Fermentation; Hindgut microbe

Introduction

The gastrointestinal (GI) microbiota plays a pivotal role in health maintenance of chickens; in particular, growth of epithelial cells, regulation of mucin and antibody production, and inhibition of pathogenic microbes in the GI tract [1-4]. As the global demand for animal protein sources is increasing, a feeding regimen that ensures higher efficiency and weight gain, especially in the broiler industry, has been required. Therefore, the maintenance of healthy animal GI microbiota is essential. However, broiler chickens are exposed to various factors that hamper the maintenance of GI health. Chickens raised in highly dense feeding systems are exposed to foreign bacteria from contaminated bedding and oxidative stress, leading to changes in GI microbiota [5]. Temporal disruption of GI microbiota can also occur following vaccination and dietary changes during growth, which may negatively impact animal health. Reduction of feed intake and weight gain, lowered growth performance and increased occurrence of digestive disorders cause huge economic losses in the chicken industry. The GI tract of chickens harbors non-beneficial/pathogenic microbes such as *Campylobacter*, *Salmonella*, *Escherichia coli*, and *Clostridium perfringens* as common constituents [6], which can be risk factors for host animal health upon abnormal growth. This also represents a serious problem for consumers in relation to food-poisoning. Thus, the maintenance of healthy GI microbiota is vital in terms of safer animal production and product consumption.

To maintain a healthy GI status, dietary management is the most important factor. Antibiotics as feed additives have been the main approach for stable maintenance of the GI microbiota, prevention of disorders and growth promotion for many years [7]. However, the use of antibiotics for growth promotion has been banned or reconsidered due to human health concerns, including antibiotic resistance and its transmission to humans *via* the food chain, bodies of water and others [8]. Therefore, there has been active exploration of alternatives to antibiotics throughout the world. As a result, probiotics, prebiotics, and other natural materials have been proposed as alternatives [9-13].

One such additive is cashew nut shell liquid (CNSL), a by-product of the cashew nut industry, formerly utilized as a raw material for producing paints, lacquers, coatings and others [14]. CNSL contains alkyl-phenols represented by anacardic acid, cardanol and caldol [15], which possess selective antibacterial activity mostly against Gram-positive bacteria via surfactant action [16,17]. Although CNSL was initially targeted for modulating rumen microbiota and fermentation in ruminant animals [14,18-21], its functionality has not been assessed for mono-gastric farm animals.

Among the segments of the GI tract in chickens, the cecum possesses the most dense and diverse microbiota, in which Gram-positive Firmicutes occupy the majority (50-90%) of microbes [1,2,22-24]. Since CNSL acts against Gram-positive bacteria,

feeding CNSL to chickens might alter the cecal microbiota toward a more favorable direction, possibly promoting animal health and, consequently, improving growth and production performance. The present study aimed to clarify the effect of CNSL on the hindgut microbiota and fermentation profiles in relation to animal health and growth performance. The functionality of CNSL was evaluated using a farm-scale feeding study, followed by pure culture and fecal slurry culture studies.

Materials and Methods

For animal sampling, we followed the Guidelines for Animal Experiments, Hokkaido University (2007) and the Act on Welfare and Management of Animal (2005).

Farm-scale feeding study (experiment 1)

A total of 143,170 one-day-old chicks (UK Chunky) at 2 blocks in the same commercial farm in Miyagi Prefecture, Japan were used. One block was assigned to the control group (n=54,384) and the other to the treatment group (n=88,786), with a stocking density of 18.4 and 16.9 birds/m^2, respectively. Growth performance of the birds in these 2 blocks had been reported almost equal with 1.830 *vs.* 1.885 in feed conversion ratio, which might warrant possible comparison between the following 2 treatments even not allocated to the exactly same block. Birds in the control group were fed a normal diet, while those in the treatment group were fed the same diet supplemented with CNSL (100 μg/g diet) from 19 to 40 days of age. For the supplementation, commercial CNSL additive (Clostop®, Idemitsu Co., Ltd., Tokyo, Japan) in powdered form was used. The feeding program was according to the protocol of Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan, in which 4 diets were used, *i.e.,* pre-starter (day 1 to 10), starter (day 11 to 25), grower (day 26 to 40) and finisher (day 41 and thereafter), all of which were anti-biotic free. The pre-starter and starter diets contained 24.0 and 23.0% crude protein (CP), respectively, with 3000 kcal/kg metabolizable energy (ME). Meanwhile, the grower and finisher diets contained 22.0 and 21.0% CP, respectively, with 3150 kcal/kg ME. Nutritional composition was basically adjusted by changing proportion of soybean to corn.

Feed intake, body weight, and mortality (including mortality due to inflammation) were recorded during the period from day 1 to 42. For inflammation, detailed identification of the cause for occurrence was not carried out. On day 42, at slaughtering in bird processing plant, 10 birds from each group were randomly selected for sampling cecal contents. Then, 0.1 g of cecal content was suspended in 0.5 ml of 0.85% saline and centrifuged (4°C, 16,000 × g, 5 min). The supernatant and precipitated samples were kept at -30°C until chemical and microbial analyses, respectively.

Pure culture study (experiment 2)

C. perfringens JCM 3817 obtained from Japan Collection of Microorganisms, RIKEN, Tsukuba, Japan was used. The minimum inhibitory concentration (MIC) of CNSL for *C. perfringens* was determined by spectrophotometrically monitoring bacterial growth at 660 nm (Miniphoto; Taitec, Saitama, Japan). GAM broth (Nissui Pharmaceutical, Tokyo, Japan) was prepared anaerobically and supplemented with CNSL at 7 levels ranging from 0 to 12.5 μg/ml final concentration. MIC was defined as the minimum concentration of CNSL required for growth inhibition.

The morphology of *C. perfringens* exposed to CNSL was observed using scanning electron microscopy (SEM). *C. perfringens* was grown in GAM broth to the exponential phase, and then 99.5% ethanol (control) or ethanol-dissolved CNSL (200 μg/ml final concentration) was added. The sample was incubated for another 5 h and the culture was employed for SEM observation as follows. Bacteria harvested by centrifugation (4°C, 16,000 × g, 5 min) was washed with 20 mM K phosphate buffer (pH 7.2), soaked in 2.5% glutaraldehyde in K phosphate buffer and then fixed with 1% osmic acid in K phosphate buffer. The sample was then dehydrated using different ethanol concentrations of 50, 70, 90, and 99.5%. The next dehydration employed a critical point drier (EM CPD300; Hitachi, Tokyo, Japan). The sample was then coated with gold-palladium by ion spatter (E101; Hitachi) and observed using a high resolution scanning electron microscope (JSM-6301F; Japan Electron, Tokyo, Japan) at an acceleration voltage of 5 kV.

Fecal culture study (experiment 3)

Feces was collected from 63- or 117-week-old layers (n=11) at the experimental farm of Field Science Center, Hokkaido University, Sapporo, Japan, which were confirmed to be PCR-positive for *C. perfringens*. The layers received a diet (Hokuren-Kumiaishiryo, Sapporo, Japan) containing 16% crude protein, 3.5% crude fat, 6% crude fiber, 14% crude ash, 3% calcium, 0.4% phosphorus, and 2,840 kcal ME/kg. Fecal samples were used to evaluate the ability of CNSL to modulate fecal fermentation and the microbiota in a batch culture experiment. Feces from each bird were diluted with McDougall's buffer [25] to a 1:2 ratio and strained through 2 layers of surgical gauze to obtain the fecal slurry inoculum. The culture tube containing CNSL dissolved in 99.5% ethanol and left over night to allow the ethanol to evaporate was filled with the abovementioned inoculum prepared from each bird's feces. The CNSL level was set at 100 μg/ml final concentration. For the control, the same amount of ethanol was added and treated as above. After addition of the inoculum, the head space of the culture tube was flushed with N_2 gas, sealed with a butyl rubber stopper and a plastic screw cap, and then anaerobically incubated at 39°C for 24 h. After incubation, the culture was centrifuged (4°C, 16,000 × g, 5 min) to separate the supernatant and precipitate, both of which were kept at -30°C for chemical and microbial analyses, respectively.

Analyses

Chemical analysis was performed as follows. Short chain fatty acids (SCFA) were analyzed by a gas chromatograph (GC-14B; Shimazu, Kyoto, Japan) fitted with a flame-ionization detector using a fused silica capillary column (ULBON HR-20 M, 0.53 mm × 30 m; Shinwa Chemical Industries, Kyoto, Japan). Gas chromatography was conducted as described in Oh et al. [26]. Ammonia was spectrophotometrically assayed using a phenol-hypochlorite assay [27]. Indole and skatole were determined by a colorimetric method [28].

DNA from cecal contents and fecal cultures was extracted and purified by the repeated beads beading plus column (RBB+C) method [29] and quantified by absorbance using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR assay using DNA from cecal contents in the feeding study was conducted to quantify specific bacterial groups including total bacteria, total lactobacilli, total bifidobacteria, *Escherichia coli*, *Eubacteriumhallii*, *Faecalibacteriumprausnitzii*, *Clostridium coccoides-Eubacteriumrectale* group, *C. perfringens*, *Clostridium* cluster XIVab, Ruminococcaceae, *Bacteroides-Prevotella-Porphyromonas*. All the quantification procedures, including primers, standards, PCR conditions and the calculations, were according to Koike, et al. [30], Myint, et al. [31] and Yamada, et al. [32]. In brief, real-time PCR was performed with a Light Cycler system and a First Start DNA Master SYBR Green I reaction

kit (both from Roche, Penzberg, Germany). A 10-fold dilution series of the standard plasmid for the respective target (16S rDNA sequence specific to each bacterial group) was run along with the samples. Quantification was achieved using standard curves obtained from the amplification profile of known concentrations of the standard plasmid for the respective target. Specificity of each PCR amplification was confirmed by melting curve analysis of the PCR products, in which the temperature was increased from 70 to 95°C at a rate of 0.1°C/s. The relative abundance of each bacterial group was calculated as a percentage of the total level of bacteria.

To comprehensively analyze the microbial community in fecal cultures with and without CNSL, DNA samples from the cultures were applied to MiSeq analysis (Illumina, San Diego, CA, USA). Sequencing was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). The V3 to V4 regions were amplified using two primer sets, Amplicon PCR Forward Primer (5'-CCTACGGGNGGCWGCAG-3') and Amplicon PCR Reverse Primer (5'-GACTACHVGGGTATCTAATCC-3') for the bacterial rRNA gene. The first PCR was carried out in a total volume of 25 µl, consisting of 12.5 µl 2x KAPA HiFi HotStart ReadyMix, 5 µl each primer (1 µM) and 2.5 µl template DNA (5 ng/µl). The following PCR condition was used: 25 cycles, consisting of denaturation at 95°C (30 s), annealing at 55°C (30 s) and extension at 72°C (30 s). In order to attach the index, the 2nd PCR was carried out in a total volume of 50 µl, consisting of 10 µl Nextera XT Index Primer mix (N7xx/S5xx), 10 µl PCR grade water, 25 µl 2xKAPA HiFi HotStart ReadyMix and 5 µl 1st PCR product. The following PCR condition was used: 8 cycles consisting of denaturation at 95°C (30 s), annealing at 55°C (30 s) and extension at 72°C (30 s). Amplicon sequencing was carried out using MiSeq as described by Caporaso et al. [33]. Data quality control and analyses were performed using the QIIME2 (ver. 2020.8). Operational taxonomical unit (OTU) was generated from sequences clustered at a 97% similarity threshold using the UCLUST algorithm [34]. Chimeric sequences were removed from the analysis using the Chimera Slayer algorithm. Taxonomy was assigned using the Green genes database (ver. 13.8) at a 99% similarity threshold. Differences in biodiversity between samples in the control and CNSL supplemented cultures were compared by alpha diversity metrics: Chao1, Shannon index, observed OTUs and Good's coverage. The sequences obtained were deposited in the DNA Data Bank of Japan nucleotide sequence database under the accession number DRA011527.

The data for growth performance, and chemical and microbial analyses were subjected to Student's t-test to detect the effect of CNSL in feeding and fecal culture studies. Statistical significance and tendency were defined at $P< 0.05$ and $P< 0.10$, respectively.

Results

Farm-scale feeding study

Parameters of growth performance during the period from day 1 to 42 are shown in table 1. Body weight gain in CNSL-fed birds was significantly higher than that in control birds. Total mortality and mortality caused by inflammation were numerically lower in the CNSL group than in the control, though no statistical significance was detected.

Table 2 shows cecal fermentation parameters with or without CNSL dietary supplementation. Feeding CNSL increased the concentration of total SCFAs and the molar proportion of acetate, whereas the molar proportion of propionate was decreased. Concentrations of ammonia and indole decreased with CNSL feeding. There was no effect of CNSL feeding on butyrate and skatole production.

Table 1: Effect of cashew nut shell liquid (CNSL) feeding on growth performance (day 1 to 42) of broiler chickens.

	Control	CNSL
The number of chicken (birds)	54,384	88,786
Feed conversion ratio	1.95 ± 0.10	1.91 ± 0.05
Body weight gain (g/day)	54.31 ± 1.43	57.47 ± 3.01*
Total mortality (%)	3.16 ± 0.01	2.25 ± 0.01
Mortality by inflammation (%)[a]	1.36 ± 0.01	0.86 ± 0.00

*, significantly different from control (P< 0.05).
a, Detailed identification of the cause for occurrence was not carried out (shown as % of birds having inflammation in total birds processed).

Table 2: Effect of cashew nut shell liquid (CNSL) feeding on cecal fermentation products in broiler chickens.

Fermentation product	Control	CNSL	P value
Total short chain fatty acid (µmol/g cecal content)[a]	67.5 ± 17.6	100.0 ± 31.2*	0.014
Acetate (molar %)	60.5 ± 4.6	68.1 ± 3.2	0.001
Propionate (molar %)	23.5 ± 1.5	16.3 ± 3.2*	0.000
Butyrate (molar %)	12.2 ± 3.8	12.4 ± 4.0	0.906
Ammonia (µgN/g cecal content)	284.6 ± 80.9	191.3 ± 42.6*	0.007
Indole (µg/g cecal content)	32.3 ± 11.5	10.0 ± 3.1*	0.000
Skatole (µg/g cecal content)	149.5 ± 60.1	149.8 ± 85.2	0.994

*, significantly different from control (P< 0.05).
a, sum of acetate, propionate, and butyrate (iso-butyrate, iso-valerate and valerate were negligibly detected).

The relative abundance of cecal bacteria is shown in figure 1. *C. perfringens* was detected in the control group (4 of 10 birds showed $10^{6\text{-}10}$ /g in quantitation), but were unquantifiable in all birds of the CNSL-fed group. The relative abundance of *C. coccoides-E. rectale* group, *Clostridium* cluster XIVab, Ruminococcaceae and *Bacteroides-Prevotella-Porphyromonas* decreased by CNSL feeding. Cecal abundance of other bacterial groups was not influenced by CNSL feeding. Since the exact abundance of *C. perfringens* with CNSL feeding was not clear due to lower than quantifiable level, statistical analysis was not available. However, we further examined this species, assuming that CNSL potentially decreases *C. perfringns*.

Pure culture study

Growth curves of *C. perfringens* JCM 3817 in GAM broth with different concentrations of CNSL are shown in figure 2. The growth of this bacterium was inhibited by CNSL in a dose-dependent manner. At 12 h, the control culture had reached the stationary phase, whereas growth of the CNSL-supplemented culture (6.25 and 12.5 µg/ml) was not observed. Thus, the MIC of CNSL was defined as 6.25 µg/ml. SEM observation of the morphology of *C. perfringens* (Figure 2) revealed extensive damage to the cell surface by CNSL exposure, in which bubble-like bumps were formed.

Fecal culture study

We screened 11 *C. perfringens*-PCR-positive birds (10^5-10^6 copies 16S rDNA/g of feces) from a total of 21 birds before starting the fecal culture study. Fermentation products as influenced by CNSL in fecal cultures are shown in table 3. CNSL supplementation decreased the molar proportion of acetate, while increasing that of propionate. The

Table 3: Effect of cashew nut shell liquid (CNSL) supplementation on fermentation products in fecal culture of laying hens.

	Control	CNSL	P value
Total short chain fatty acid (mM in culture)[a]	43.9 ± 7.7	39.4 ± 12.0	0.432
Acetate (molar %)	73.6 ± 3.8	66.3 ± 5.4*	0.011
Propionate (molar %)	14.9 ± 2.2	20.5 ± 5.3*	0.022
Iso-butyrate (molar %)	1.9 ± 0.8	2.4 ± 1.1	0.366
Butyrate (molar %)	6.3 ± 2.0	6.9 ± 2.0	0.552
Iso-valerate (molar %)	1.6 ± 1.0	2.3 ± 1.1	0.224
Valerate (molar %)	1.7 ± 1.1	1.6 ± 1.5	0.905
Ammonia (μgN/ml culture)	230.2 ± 40.6	170.5 ± 55.2*	0.037
Indole (μg/ml culture)	14.0 ± 4.2	10.9 ± 5.2	0.240
Skatole (μg/ml culture)	54.9 ± 25.2	58.0 ± 22.2	0.813

*, significantly different from control (P< 0.05).
a, sum of acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate.

concentration of ammonia decreased with CNSL supplementation. Other fermentation products were not influenced by CNSL.

To obtain detailed information on hindgut microbial responses to CNSL, MiSeqanalysis was performed using DNA from fecal cultures. The number of total reads ranged from 20,081 to 24,046, with a mean value of 21,670 ± 992. Good's coverage (%) was 100, indicating that sufficient sequences to discuss bacterial composition were obtained. There was no effect of CNSL supplementation on diversity indices of fecal microbiota having similar Chao 1 (282 and 232 for control and CNSL, respectively), Shannon (6.06 and 6.54) and observed OTU (232 and 282). The bacterial community structure is shown in figure 3. At a phylum level, CNSL supplementation decreased the detection frequency of Firmicutes, while increasing that of Proteobacteria. At a genus level, CNSL supplementation decreased the detection frequency of unclassified Lachnospiraceae, unclassified Ruminococcaceae and *Coprococcus*, while increasing that of unclassified Enterobacteriaceae and *Sutterella*.

Correlations between relative abundance of specific bacterial groups from MiSeq analysis and fermentation parameters are shown in figure 4. The relative abundance of unclassified Lachnospiraceae and unclassified Ruminococcaceae was positively correlated with ammonia concentration and the molar proportion of acetate, and negatively correlated with the molar proportion of propionate. The relative abundance of *Coprococcus* was positively correlated with ammonia concentration, while that of unclassified Enterobacteriaceae was negatively correlated. The relative abundance of *Sutterella* was negatively correlated with acetate proportion and positively correlated with propionate proportion.

Discussion and Conclusion

The farm-scale study using broiler chickens shows that CNSL feeding directs cecal fermentation toward more SCFA production and less ammonia production (Table 2). SCFAs lower hindgut pH and inhibit the growth of Enterobacteriaceae including pathogens [35]. Besides, SCFAs act as signaling molecules *via* G-protein-coupled receptors and regulate the host immune system. Therefore, SCFAs are beneficial products for animal health. CNSL-fed birds showed higher acetate and lower propionate proportions in their ceca, although the quantified bacteria did not clearly explain these changes, i.e.,bifidobacteria and *E. hallii* involved in acetate production did not change with CNSL feeding (Figure 1).

On the other hand, in the fecal culture study, CNSL supplementation decreased the molar proportion of acetate, while increasing that of propionate (Table 3). These shifts were supported by fecal bacterial changes; acetate and propionate proportions correlated with unclassified Lachnospiraceae, unclassified Ruminococcaceae and *Sutterella* (Figure 4). In fact, unclassified Lachnospiraceae remarkably decreased with CNSL feeding (13.3 to 3.6%). Such fermentation shifts (decreased acetate and increased propionate) have been recognized as typical when CNSL is fed to ruminants [18-20]. Therefore, CNSL is also considered to alter the microbiota and fermentation in chickens, even though the pattern of shifts is different between fecal and cecal samples and/or between broilers and layers. Acetate maintains gut health thorough establishment of the immune system [36], while propionate contributes to the improvement of animal energy status [37]. Promotion of these SCFAs by CNSL might act favourably for animal health and nutrition.

Decreased ammonia production was consistently observed in the cecal contents of birds fed CNSL and in fecal cultures supplemented with CNSL (Tables 2 and 3). Ammonia is associated with intestinal disorders [3,38], and high ammonia concentrations in poultry barns reduce the productivity of chickens by inducing inflammation in the trachea and intestine [39]. Based on the present study results, CNSL might be a beneficial additive to suppress ammonia production in the intestine and feces, possibly improving the health and productivity of chickens. The fecal culture study indicated that ammonia concentrations were positively correlated with unclassified Lachnospiraceae, unclassified Ruminococcaceae and *Coprococcus*, and negatively correlated with unclassified Enterobacteriaceae (Figure 4). Since unclassified Lachnospiraceae, unclassified Ruminococcaceae and *Coprococcus* belong to Clostridiales, many of which are known to be involved in ammonia production [40], the decrease of these bacteria by CNSL (Figure 3) might lead to the decrease in ammonia concentration in the fecal culture. Clostridia in broiler ceca were also suppressed by CNSL feeding (Figure 1), presumably leading to decreased ammonia, as well as indole (Table 2) as another putrefactive and carcinogenic product in the GI tract [41].

Necrotic enteritis (NE) is a disease caused by *C. perfringens* that impacts economic, welfare and food safety aspects in broiler chicken production [42,43]. Although *C. perfringens* is a commensal bacterium, it produces NetB toxin with intestinal overgrowth under a state of dysbiosis, resulting from microbial infection, feed switching, vaccination and high-protein feed [43]. Previous studies have attempted to suppress the growth of *C. perfringens* using probiotics such as *Bacillus subtilis* [44] and *Bacillus coagulans* [11] in NE-induced chickens. In the present study, the cecal level of *C. perfringens* was unquantifiable in all birds of the CNSL-fed group (Figure 1). CNSL addition to *C. perfringens*-positive fecal cultures also decreased the abundance of this species (experiment 3, data not shown).Thus, CNSL could be a growth inhibitor of *C. perfringens*, and its potency could be equal to or greater than other probiotics. In fact, the pure cultures study clearly revealed that CNSL inhibits the growth of *C. perfringens* at a supplementation level of at least 6.25 μg/ml, and this inhibition occurs by physical disruption of the bacterial cell surface (Figure 2) through its surfactant action [16]. CNSL has selective antibacterial activity, especially against Gram-positive bacteria [14,45]. These bacteria lack an outer membrane, which acts as a barrier against the penetration of alkyl-phenols (main antibacterial compounds of CNSL) into the cell wall [17]. Thus, it is reasonable that Gram-positive *C. perfringens* is suppressed by CNSL.

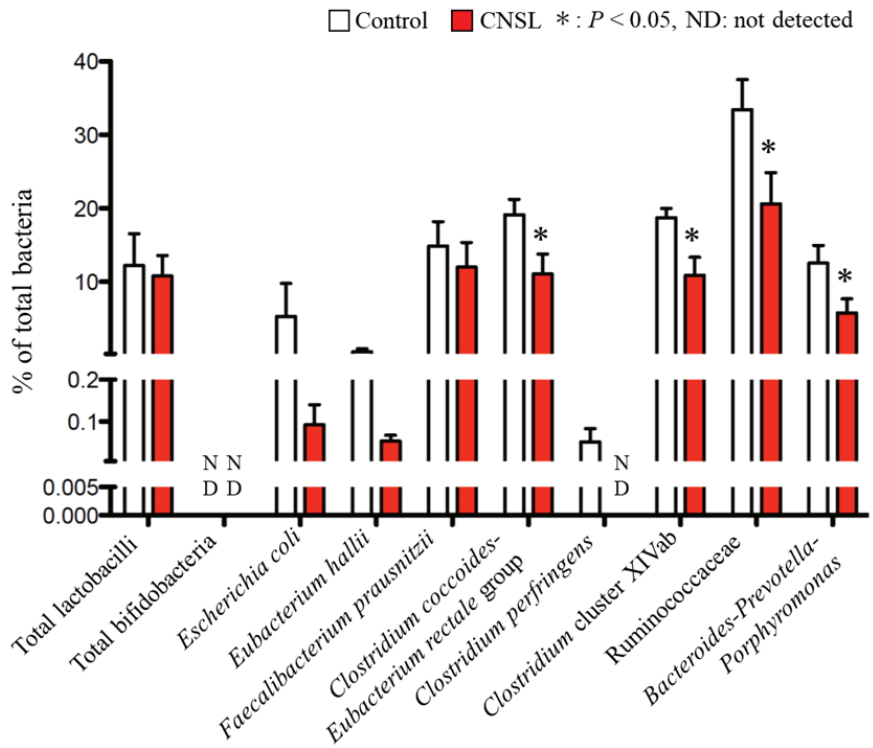


Figure 1: Effect of cashew nut shell liquid (CNSL) feeding on relative abundance of cecal bacteria in broiler chickens slaughtered on day 42.
ND, not detected (lower than quantifiable level in the assay used).
The target group (total lactobacilli) includes the genera of *Lactobacillus, Lacticaseibacillus, Latilactobacillus, Ligilactobacillus, Lactiplantibacillus, Limosilactobacillus, Levilactobacillus, Fructilactbacillus,* and *Apilactobacillus* that have been recently reclassified.

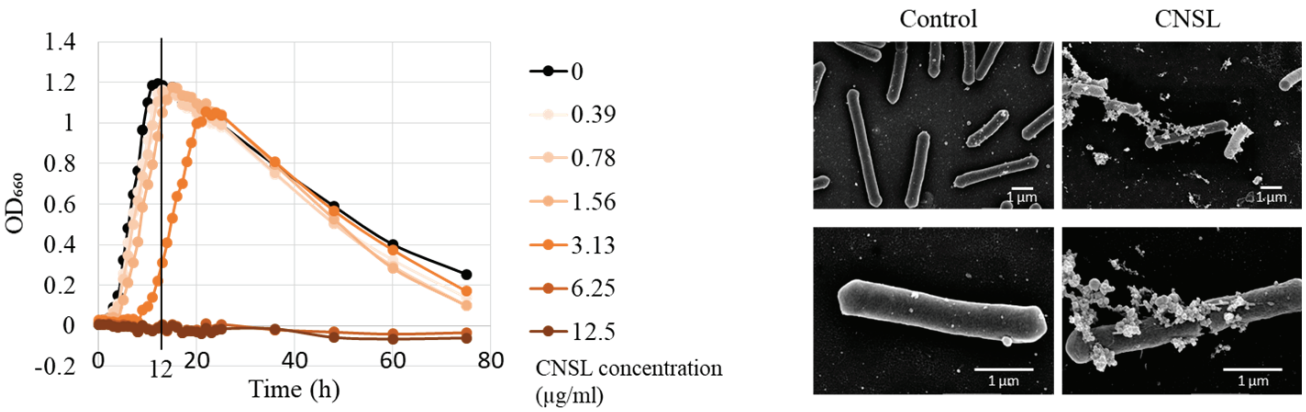


Figure 2: Effect of cashew nut shell liquid (CNSL) supplementation on growth (left) and morphology (right) of *Clostridium perfringens* in pure culture.
Observation by scanning electronic microscopy was performed at ×10,000 (upper) and ×30,000 (lower) magnifications.

The above discussion assumes that the amount of CNSL reaching the hindgut is sufficient to act as an anti-*C. perfringens* surfactant. Assuming that the CNSL fed to broiler chickens (experiment 1) was not degraded or absorbed throughout the GI tract, the CNSL concentration in feces could be around 99.2 μg/g; this value was calculated from levels of dietary CNSL (100 μg/g) and feed intake (129 g/d) in experiment 1, and the reported excreta level (130 g/day) [46]. Thus, CNSL is thought to reach the hindgut at much higher concentrations than the MIC for *C. perfringens* (6.25 μg/ml) defined in experiment 2, supporting the suppressive effect of CNSL on *C. Perfringens* in the birds in experiment 1. Meanwhile, the detected level of cecal *C. perfringens* in the present study was quite low, i.e., at such a level to ensure the health of the birds (10^2~10^6 copies/g) [47]. Therefore, CNSL function needs to be assessed in birds harbouring *C. perfringens* at a higher level, i.e., a level that has negative health impacts.

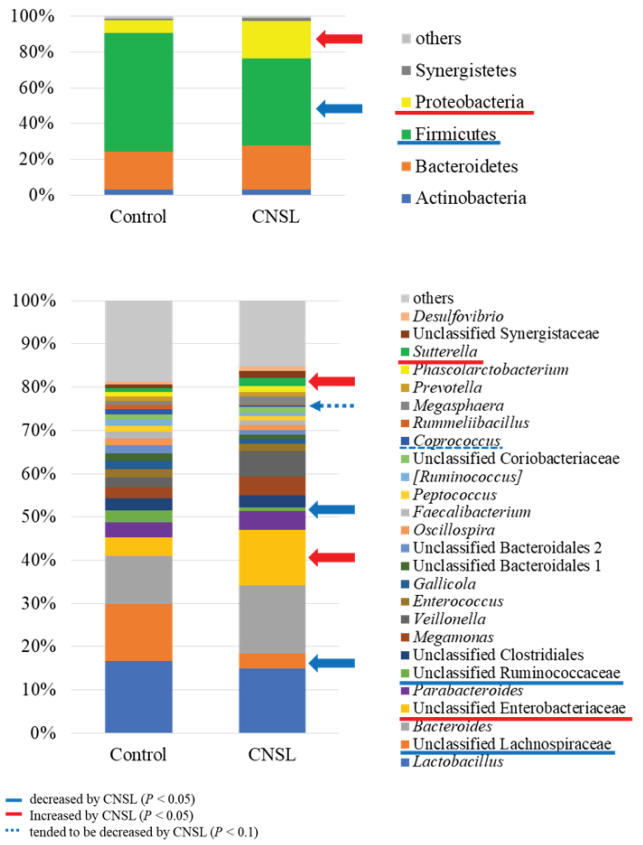


Figure 3: Effect of cashew nut shell liquid (CNSL) supplementation on bacterial community in fecal culture of laying hens determined at phylum (upper) or genus (lower) level by MiSeq analysis.

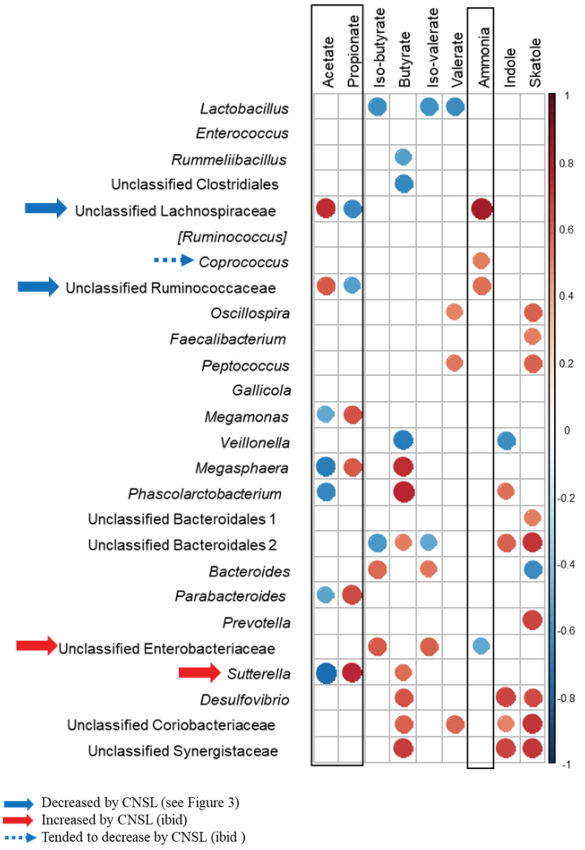
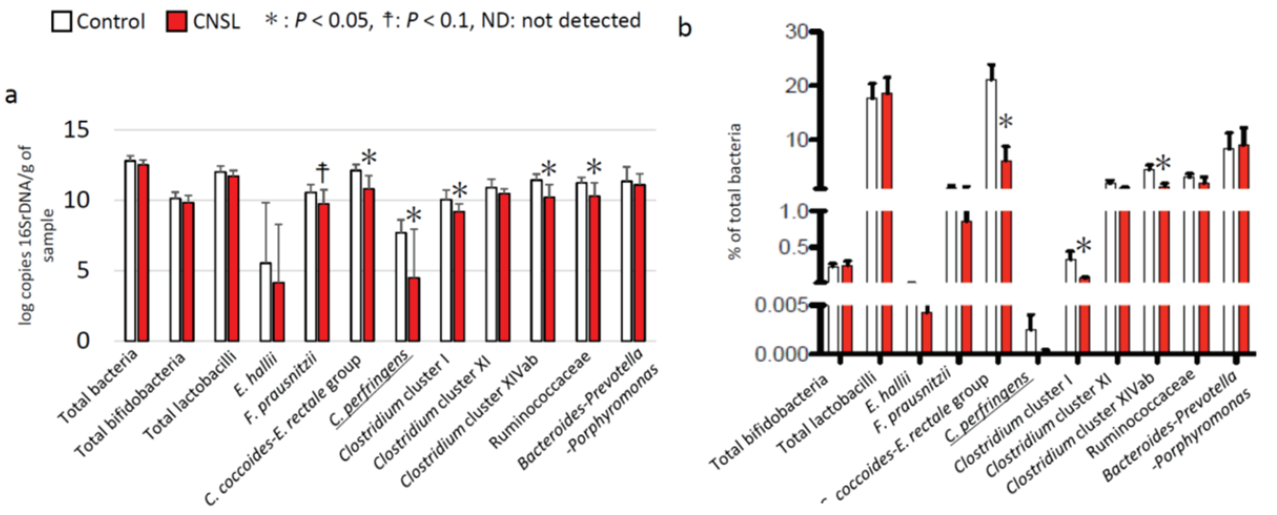


Figure 4: Correlations between relative abundance of bacteria and fermentation parameter in fecal culture of laying hens.
Circles indicate correlation coefficients; red for positive while blue for negative. Darker colors denote higher correlations.



Supplemetary figure: Effect of cashew nut shell liquid (CNSL) supplementation on bacteria in fecal cultures
a. expressed in log copy number/g culture
b. expressed in % in total bacteria

In conclusion, CNSL could alter the hindgut microbiota of chickens to favorably modify fermentation profiles in relation to SCFAs and ammonia production. These modifications might improve the hindgut environment, leading to enhanced animal health and productivity. CNSL was clearly indicated to possess inhibitory action against *C. perfringens*; however, the preventive effect of CNSL on GI inflammation due to this pathogen should be verified using birds with *C. perfringens* infection at levels sufficient to induce inflammation.

Acknowledgments

The authors gratefully acknowledge the staff of Field Science Center, Hokkaido University for the collection of fecal samples from laying hens.

Conflict of Interest

A part of the funding for the present study was provided by Idemitsu Co. Ltd. The funding source had no role in the design, practice or analysis of this study.

References

1. Yeoman CJ, Chia N, Jeraldo P, Sipos M, Goldenfeld ND, et al. (2012) The microbiome of the chicken gastrointestinal tract. Anim Health Res Rev 13: 89-99.
2. Rinttilä T, Apajalahti J (2013) Intestinal microbiota and metabolites-Implications for broiler chicken health and performance. J Appl Poultry Res 22: 647-658.
3. Apajalahti J, Vienola K (2016) Interaction between chicken intestinal microbiota and protein digestion. Anim Feed Sci Technol 221: 323-330.
4. Clavijo V, Flórez MJV (2018) The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. Poultry Sci 97: 1006-1021.
5. Gou ZY, Li L, Fan QL, Lin XJ, Jiang ZY, et al. (2018) Effects of oxidative stress induced by high dosage of dietary iron ingested on intestinal damage and caecal microbiota in Chinese Yellow broilers. J Anim Physiol Anim Nutr 102: 924-932.
6. Oh MH, Paek SH, Shin GW, Kim HYE, Jung GY, et al. (2009) Simultaneous identification of seven foodborne pathogens and *Escherichia coli* (pathogenic and nonpathogenic) using capillary electrophoresis–based single-strand conformation polymorphism coupled with multiplex PCR. J Food Protec 72: 1262-1266.
7. Mehdi Y, Montminy MPL, Gaucher ML, Chorfi Y, Suresh G, et al. (2018) Use of antibiotics in broiler production: Global impacts and alternatives. Anim Nutr 4: 170-178.
8. Food and Agriculture Organization of the United Nations (FAO) (2006). Livestock's Long Shadow. Part 3. Livestock's role in climate change and air pollution, 80-99.
9. Nava GM, Attene-Ramos MS, Gaskins HR, Richards JD (2009) Molecular analysis of microbial community structure in the chicken ileum following organic acid supplementation. Vet Microbiol 137: 345-353.
10. Peng QY, Li JD, Li Z, Duan ZY, Wu YP (2016) Effects of dietary supplementation with oregano essential oil on growth performance, carcass traits and jejunal morphology in broiler chickens. Anim Feed Sci Technol 214: 148-153.
11. Wu Y, Shao Y, Song B, Zhen W, Wang Z, et al. (2018) Effects of *Bacillus coagulans* supplementation on the growth performance and gut health of broiler chickens with *Clostridium perfringens*-induced necrotic enteritis. J Anim Sci Biotechnol 9: 9.
12. Tavaniello S, Maiorano G, Stadnicka K, Mucci R, Bogucka J, et al. (2018) Prebiotics offered to broiler chicken exert positive effect on meat quality traits irrespective of delivery route. Poultry Sci 97: 2979-2987.
13. Moraes PO, Cardinal KM, Gouvêa FL, Schroeder B, Ceron MS, et al. (2019) Comparison between a commercial blend of functional oils and monensin on the performance and microbiota of coccidiosis-challenged broilers. Poultry Sci 98: 5456-5464.
14. Watanabe Y, Suzuki R, Koike S, Nagashima K, Mochizuki M, et al. (2010) *In vitro* evaluation of cashew nut shell liquid as a methane-inhibiting and propionate-enhancing agent for ruminants. J Dairy Sci 93: 5258-5267.
15. Paramashivappa R, Kumar PP, Vithayathil PJ, Rao AS (2001) Novel method for isolation of major phenolic constituents from cashew (*Anacardium occidentale* L.) nut shell liquid. J Agric Food Chem 49: 2548-2551.
16. Kubo I, Ochi M, Vieira PC, Komatsu S (1993) Antitumor agents from the cashew (*Anacardium occidentale)* apple juice. J Agric Food Chem 41: 1012-1015.
17. Kubo I, Muroi H, Himejima M, Yamagiwa Y, Mera H, et al. (1993) Structure-antibacterial activity relationships of anacardic acids. J Agric Food Chem 41: 1016-1019.
18. Shinkai T, Enishi O, Mitsumori M, Higuchi K, Kobayashi Y, et al. (2012) Mitigation of methane production from cattle by feeding cashew nut shell liquid. J Dairy Sci 95: 5308-5316.
19. Kang S, Suzuki R, Suzuki Y, Koike S, Nagashima K, et al. (2018) Rumen responses to dietary supplementation with cashew nutshell liquid and its cessation in sheep. Anim Sci J 89: 1549-1555.
20. Konda S, Onodera R, Kanchanasatit E, Boonsean P, Sawanon S, et al. (2019) Effect of cashew nut shell liquid feeding on fermentation and microbiota in the rumen of Thai native cattle and swamp buffaloes. Livestock Sci 226: 99-106.
21. Maeda K, Nguyen VT, Suzuki T, Yamada K, Kudo K, et al. (2021) Network analysis and functional estimation of the microbiome reveal the effects of cashew nut shell liquid feeding on methanogen behaviour in the rumen. Microbial Biotechnol 14: 277-290.
22. Bjerrum L, Engberg RM, Leser TD, Jensen BB, Finster K, et al. (2006) Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poultry Sci 85: 1151-1164.
23. Zhu XY, Zhong T, Pandya Y, Joerger RD (2002) 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl Environ Microbiol 68: 124-137.
24. Stanley D, Hughes RJ, Moore RJ (2014) Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Appl Microbiol Biotechnol 98: 4301-4310.
25. McDougall EI (1948) Studies on ruminant saliva. Biochem J 43: 1780-1783.
26. Oh S, Shintani R, Koike S, Kobayashi Y (2017) Ginkgo fruit extract as an additive candidate to modify rumen microbiota and fermentation for mitigating methane production. J Dairy Sci 100: 1923-1934.
27. Weatherburn MW (1967) Phenol-hypochlorite reaction for determination of ammonia. Anal Chem 39: 971-974.
28. Walstra P, Claudi-Magnussen C, Chevillon P, von Seth G, Diestre A, et al. (1999) An international study on the importance of androstenone and skatole for boar taint: levels of androstenone and skatole by country and season. Livestock Prod Sci 62: 15-28.

29. Yu Z, Morrison M (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36: 808-812.

30. Koike S, Yabuki H, Kobayashi Y (2007) Validation and application of real-time polymerase chain reaction assays for representative rumen bacteria. Anim Sci J 78: 135-141.

31. Myint H, Iwahashi Y, Koike S, Kobayashi Y (2017) Effect of soybean husk supplementation on the fecal fermentation metabolites and microbiota of dogs. Anim Sci J 88: 1730-1736.

32. Yamada H, Watabe Y, Suzuki Y, Koike S, Shimamoto S, et al. (2021) Chemical and microbial characterization for fermentation of water-soluble cellulose acetate in human stool cultures. J Sci Food Agric 101: 2950-2960.

33. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6: 1621-1624.

34. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460-2461.

35. van der Wielen PW, Biesterveld S, Notermans S, Hofstra H, Urlings BA, et al. (2000). Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl Environ Microbiol 66: 2536-2540.

36. Lee IK, Gu MJ, Ko KH, Bae S, Kim G, et al. (2018) Regulation of $CD4^+$ $CD8^-$ $CD25^+$ and $CD4^+$ $CD8^+$ $CD25^+$ T cells by gut microbiota in chicken. Sci Rep 8: 8627.

37. Byrne C, Chambers ES, Morrison DJ, Frost G (2015) The role of short chain fatty acids in appetite regulation and energy homeostasis. Int J Obes 39: 1331-1338.

38. Gilbert MS, Ijssennagger N, Kies AK, van Mil SWC (2018) Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs, and poultry. Amer J Physiol Gastrointes Liver Physiol 315: G159-G170.

39. Zhou Y, Liu QX, Li XM, Ma DD, Xing S, et al. (2020) Effects of ammonia exposure on growth performance and cytokines in the serum, trachea, and ileum of broilers. Poultry Sci 99: 2485-2493.

40. Vince AJ, Burridge SM (1980) Ammonia production by intestinal bacteria: the effects of lactose, lactulose and glucose. J Medical Microbiol 13: 177-191.

41. Goldin BR (1986) *In situ* bacterial metabolism and colon mutagens. Annual Rev Microbiol 40: 367-393.

42. Skinner JT, Bauer S, Young V, Pauling G, Wilson J (2010) An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis 54: 1237-1240.

43. Shojadoost B, Vince AR, Prescott JF (2012) The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet Res 43: 74.

44. Whelan RA, Doranalli K, Rinttilä T, Vienola K, Jurgens G, et al. (2019) The impact of Bacillus subtilis DSM 32315 on the pathology, performance, and intestinal microbiome of broiler chickens in a necrotic enteritis challenge. Poult Sci 98: 3450-3463.

45. Himejiama M, Kubo I (1991). Antibacterial agents from the cashew anacardium occidentale (anacardiaceae) nut shell oil. J Agric Food Chem 39: 418-421.

46. Tsuiki M, Harada Y (1997) A computer program for estimating the amount of live stock wastes. J Jap Agr Sys Soc 13: 17-23.

47. Wei S, Gutek A, Lilburn M, Yu Z (2013) Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. Vet Microbiol 166: 595-601.