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Utilization of Sake lees as Broiler Feedstuff and its Effects on Growth Performance and Intestinal Immunity

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Increasing food loss and waste (FLW) is a global problem, and efforts are being made to use waste food as potential livestock feed material. The amount of self-supplied feed is lower in Japan than in other countries, and the government recommends FLW use for animal feed. Sake (Japanese rice wine) is a traditional alcoholic beverage. During the sake manufacturing process, large amounts of squeezed solids or "lees" (sake lees) are generated. Sake lees are nutritious and functional, but are prone to spoilage. In this study, we investigated whether sake lees should be mixed with animal feed immediately or after drying. To assess the usefulness of sake lees as a poultry feed ingredient and determine the effect of sake lees on intestinal immunity, we performed a feeding trial with three treatments: a raw sake lees (RSL) diet, dried sake lees (DSL) diet, and control diet. Three-week-old broilers were fed these diets (n=8 per group) for two weeks. We then calculated feed efficiency and performed RT-qPCR to assess the effects of diet on intestinal immunity. The growth performance in the RSL diet group was equivalent to that in the control diet group. The DSL diet became difficult for broilers to eat, resulting in decreased growth performance. In the ileum of RSL-diet broilers, the mRNA expression levels of TGF-β1 and avian β-defensin (AvBD)12 were significantly increased compared to those of control diet broilers ($p<0.05$), and a significant correlation was observed between the two genes ($p<0.05$). Our results indicated that sake lees should not be dried and should be mixed immediately with feed, and this sake lees when fed to chicken activates the intestinal immunity. However, sake lees have a lower fat content than corn, and it is thus important to combine sake lees with high-energy feed.

Key words: broiler, food loss and waste, intestinal immunity, sake lees

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Introduction

Food loss and waste (FLW) arises during food production, handling and storage, processing, distribution, marketing, and consumption. The increasing levels of FLW are a global problem. The annual quantity of FLW is estimated at 1.6 billion tons globally, and the estimated annual economic cost is USD $750 billion (FAO, 2013; Ishangulyyev *et al*., 2019). If the trend of increasing FLW continues, its adverse effects on the natural environment (e.g., greenhouse gas emissions, wastewater, and land) will become untenable (Lipinski *et al*., 2013). However, the food demand of the world's population also increases annually, and it is estimated that the demand could reach approximately 150−170% of the current demand by 2050 (Ishangulyyev *et al*., 2019). One approach to decrease global FLW and reduce the environmental load and economic cost of FLW is to reuse the components of FLW that can be consumed by humans and use what is not edible as animal feed (Garcia *et al*., 2017).

Japan imports most of the corn and soybeans used in animal feed; the imports of corn and soybeans are approximately 15 million metric tons and 3 million tons, respectively (Nakai *et al*., 2015). Japan's domestic production of corn is nil, and all its domestic consumption needs depend on overseas imports (15.65 million tons) (MAFF, 2020a). Domestic animal feed ingredients that do not depend on overseas imports are desirable.

The Government of Japan is promoting the use of FLW for animal feed as a product referred to as "eco-feed." Most of the existing eco-feed in Japan is used for pigs and cattle; however, eco-feed for chickens has not been well investigated (MAFF,

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2020b). In their study on overseas poultry, Cho *et al*. (2004) used dried leftover food (DLF) in feed for broilers, and they observed that feed comprising 10% DLF provided the same growth performance level as the control diet. The addition of DLF also lowered the cost of the feed compared to that of the control diet. In another study, single food-waste products were added to broiler feed as alternatives to the addition of pooled FLW, including bakery waste, dried tomato pomace, dried carrot and carrot pulp, cornflakes waste, oyster mushroom waste, and meat meal (Truong *et al*., 2019). The results revealed that in most cases, it was possible to add the products to provide approximately 3–30% of the feed, however up to 60% cornflake waste could be used.

Although these products provide high nutritional value, they also have high moisture content, which makes them easily perishable (Sugiura *et al*., 2009). Most FLW is dried before use in poultry feed, but the drying process is costly. In one report, to obtain 1 ton of dehydrated product, 250−300 L of fuel and 200 kWh of electricity were required for drying (Truong *et al*., 2019).

Sake, also known as Japanese rice wine, is specific to Japan and is produced by fermenting a specific variety of rice with brewer's microbe (*Aspergillus oryzae* and *Saccharomyces cerevisiae*) and followed by straining. Sake rice solids, or sake lees, are generated in this straining process. Although good quality sake lees products are marketed as foods, sake lees that cannot be used as food are discarded as industrial waste. The costs associated with this disposal are large, as is the environmental impact.

Sake lees are derived from rice and contain large amounts of proteins and carbohydrates, with their amino acid composition closely resembling that of rice. When feeding tests were conducted with rice replacing the corn feed, the growth performance of chicken was the same as that obtained with the corn-containing feed (Honda *et al*., 2011; Nanto *et al*., 2012). Sake lees are also rich in indigestible ingredients, such as resistant proteins, resistant starch, and β-glucan-derived yeast. These indigestible ingredients, called luminacoids, have the same physiological effects as dietary fiber (Kiriyama *et al*., 2006). β-Glucan and resistant starch are known to promote immune activation and improve tight junction proteins in the intestines of mono-gastric animals (Adebowale *et al*., 2019).

Considering the above findings, we speculated that sake lees could replace corn in poultry feed and enhance immune activation and tight junction proteins in the chicken intestine. In the present study, we investigated the effects of undried and dried sake lees on chicken growth and intestinal immunity.

Materials and Methods

Obtaining and Drying the Sake Lees

Sake lees were obtained from a local sake brewery (Hiraizumi Honpo Co., Akita, Japan). The grade of the sake lees was *Honjozo-shu* (sokujo starter culture), and the ratio of sake lees was 23%. In this state they are designated as raw sake lees (RSL). The sake lees obtained were frozen and stored at −30℃ until use. To prepare dried sake lees (DSL), raw sake lees were heated at 60℃ in a forced-air dryer for 24 h. After drying, the DSL was pulverized using a liquidizer and passed through a 3-mm sieve. In contrast, the RSL was passed through a 3-mm sieve after being mixed with other feedstuffs because of its high viscosity and moisture.

General Component Analysis and Amino Acid Composition of the Feed

The crude protein (CP), ethanol extract (EE), and crude ash (CA) of the feed material used in this study were determined using the Kjeldahl method, Soxhlet extraction, and dry ashing method, respectively. Crude fiber (CF) content was determined under the following conditions: samples were boiled with 1.25% H_2SO_4 and 1.25% NaOH before being washed with ethanol and diethyl ether. The dried samples were ashed for 2 h at 600℃, and the CF content was calculated according to the following equation:

$$\text{CF (\%)} = \frac{\textit{acidic-alkaline and organic solvent residues- ashed sample}}{\textit{weighted sample}} \times 100.$$

Moisture content was determined using a heat drying type moisture meter (MX-50, A&D, Tokyo, Japan). The nitrogen-free extract (NFE) was calculated as follows:

NFE (%)=100−(CP+EE+CF+CA).

Triplicate measurements were made and the means calculated.

To determine the amino acid content, the samples were hydrolyzed with 6 N HCl for 24 h at 110℃. The samples were then evaporated and re-dissolved in 0.02 N HCl before being analyzed with an auto amino acid analyzer (JLC-500/V, JEOL, Tokyo).

Animal Care and Feeding Experiment

Day-old Chunkey broiler chicks (without chicken sexing) were obtained from Prifoods Co. (Aomori, Japan). All chicks were raised in a brooder maintained at 32℃ and lowered by 1℃ every 2 days. Commercial feed (CP 24%, ME 3.0 Mcal/kg, Chubushiryo Co., Aichi, Japan) and tap water were freely available. When the chicks reached the age of 3 weeks, they were transferred to metabolism cages (80 cm×35 cm×35 cm per bird) for 2 days to adapt to the experimental environment. We selected 24 chickens with body weight of 1.1 kg±0.01 SEM and assigned eight to each of the three treatment groups: (1) the Control diet group, (2) the raw sake lees (RSL) diet group, and (3) the dried sake lees (DSL) diet group. At the time of dissection, the sex of the chickens in all groups was determined.

The experimental diets were designed to meet the Japanese feeding standards for poultry (NARO, 2011) (Table 1). The experimental feed and tap water were provided *ad libitum* for 2 weeks under a 16 h light and 8 h dark schedule. At the end of the 2-week experimental period, the chickens were euthanized using isoflurane hyperanesthesia, after which each chicken was decapitated, and a blood sample collected in a VENOJECT®II vacuum blood collecting tube (Terumo Corp., Tokyo, Japan) containing blood coagulant. The collected blood samples were immediately placed on ice and incubated for 1 h. To obtain serum, blood samples were centrifuged (3,000×*g*, 4℃, 30 min), and the supernatant was stored at

Table 1. **Composition of the Experimental Diets**

g/kg	Control	RSL	DSL
Corn	420	—	—
Soybean meal	375	375	375
RSL	—	240	—
DSL	—	—	127
Mineral mixture[a]	60	60	60
Vitamin mixture[b]	2	2	2
Cellulose[c]	15	72	193
Rapeseed oil	128	246	243
CP, %	19.5	19.5	19.5
ME, Mcal/kg	3.2	3.2	3.2
Moisture, %	11.4	19.5	8.0

[a] Mineral mixture (/kg of diet): $CaHPO_4 \cdot 2H_2O$ 20.7 g, $CaCO_3$ 14.8, KH_2PO_4 10.0 g, KCl 3.0 g, NaCl 6.0 g, $MgSO_4$ 3.0 g, $FeSO_4 \cdot 7H_2O$ 500 mg, $MnSO_4 \cdot 5H_2O$ 350 mg, KI 2.6 mg, $CuSO_4 \cdot 5H_2O$ 40 mg, ZnO 62 mg, $CoCl_2 \cdot 6H_2O$ 1.7 mg, $NaMoO_4 \cdot 2H_2O$ 8.3 mg, Na_2SeO_3 400 µg.

[b] Vitamin mixture (/kg of diet): thiamin hydrochloride 3 mg, riboflavin 6 mg, pyridoxine hydrochloride 4 mg, nicotinic acid 40 mg, calcium pantothenate 15 mg, folic acid 1.5 mg, biotin 200 µg, cyanocobalamine 20 µg, cholecalciferol 5 µg, menadione 500 µg, D-glucose 1.9 g, retinol acetate 1 mg, D,L-a-tocopherol acetate 10 mg.

[c] KC Flock W-100G (Nippon Paper Industries Co., Tokyo).

RSL: raw sake lees. DSL: dried sake lees.

−50℃. The breast muscle, tender thigh, heart, liver, and gizzard were weighed. The jejunum and ileum were washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS) and immersed in RNAlater™ overnight at 4℃. The intestinal samples were stored at −50℃ until total RNA was extracted. The protocols for animal experimentation were approved by the Animal Care and Use Committee of Akita Prefectural University (approval no. 18-16).

Serum Amino Acid Analysis

Each sample was mixed with an equal volume of 3% sulfosalicylic acid and stored at 4℃ for 1 h for deproteinization. The sample was then centrifuged (10,000×*g*, 10 min, 4℃), and the supernatants were passed through a 0.45-µm membrane filter. The amino acid concentration of the filtered sample was determined using a JLC-500/V auto amino acid analyzer.

RT-qPCR for the Assessment of Intestinal Immunity and Tight-junction Protein

Total RNA was isolated from the intestinal samples using a QuickGene RNA Tissue Kit S II (Kurabo Industries, Osaka, Japan) and treated with DNase I (Nippon Gene, Tokyo, Japan). The extracted total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the A260/280 and A260/A230 ratios were confirmed to be ≥2.0.

cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix (Toyobo, Tokyo) under the following cycling conditions: 37℃ for 30 min, 50℃ for 5 min, 98℃ for 5 min, and an infinite hold at 12℃. A real-time quantitative polymerase chain reaction (RT-qPCR) was performed as follows: denaturing at 95℃ for 1 min; 40 cycles at 95℃ for 15 s, 62℃ for 30 s, and 72℃ for 30 s. The reaction volume was 20 µL, containing THUNDERBIRD® SYBR® qPCR Mix (Toyobo), 25 ng cDNA, 0.2 pM left primer, and 0.2 pM right primer. The reactions were performed on a LightCycler® 96 System (Hoffmann-La Roche, Basel, Switzerland) using the intercalation method. Since the gene expression levels of interleukin (IL)-14 and IL-17A were low, the amount of cDNA was set to 100 ng for analysis.

After calculating the cycle threshold (Ct) value, amplification was determined using a relative quantification model (Pfaffl, 2001). Primer specificity was confirmed using 3% agarose electrophoresis and melting curve analysis using RT-qPCR. The determination coefficients (R^2) of the calibration curves for all primers were ≥ 0.98, and the amplification efficiency was 100−110%. The housekeeping genes used were beta-2-microglobulin (B2M) and hypoxanthine phophoribosyltransferase 1 (HPRT1). We selected HPRT1, with the most stable expression, for relative quantification in this study.

When the primers were designed, the target gene information was obtained from the GenBank database (https://www.ncbi.nlm.nih.gov). The primers were designed to contain an exon–exon junction using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), and at the same time their specificity was confirmed using the *Gallus gallus* (taxid: 9031) database.

To assess intestinal immunity, we targeted genes associated with Th1, Th2, Th17, inflammatory, anti-inflammatory, intestinal antibacterial, tight-junction, and lipopolysaccharide (LPS) signaling pathways. Among the AvBD1–14 genes of the avian β-defensin (AvBD) family, we targeted AvBD10/12, which is highly expressed in the chicken gastrointestinal tract (Nii *et al*., 2020; Mohammed *et al*., 2015). The primer sequences used are listed in Table 2.

Statistical Analysis

The results had their mean±SEM calculated. We used the statistical analysis software R 4.0.2 (R Core Team, 2020) to conduct a generalized linear model (GLM) analysis, create a heatmap, and test for associations. When GLM analysis was performed, dietary treatment was used as the explanatory value, and the concentration of amino acids, growth performance, and gene expression were defined as the response variables. "Gaussian" was selected as the distribution, and "Identity" was selected as the link function. The fitted model was evaluated using Akaike's information criterion (AIC). Statistical significance was determined using Tukey's honest significant difference (HSD) test for multiple comparisons ($p<0.05$).

For the identification of the genes that are most affected by sake lees, a heatmap was created by using the 'Complex Heatmap' package (Gu *et al*., 2016). The relationship between the genes most affected by sake lees and other genes was determined by a network analysis using the 'qgraph' package (Epskamp *et al*., 2012). We also created scatter plots and regression lines to clarify the strength of the connections between the pairs of genes. The correlations were obtained as

Table 2. **Primer sequences for RT-qPCR**

Target gene	NCBI RefSeq ID	Primer sequence	Product Size, bp
Th1 cytokines			
IFN-γ	NM_205149.1	F: 5′-AAGTCAAAGCCGCACATCAAACA-3′ R: 5′-GGATTCTCAAGTCGTTCATCGGG-3′	130
IL-2	NM_204153.1	F: 5′-TCTGCAGTGTTACCTGGGAGAAG-3′ R: 5′-TCCGGTGTGATTTAGACCCGTAA-3′	139
Th2 cytokines			
IL-4	NM_001007079.1	F: 5′-GCTCTTATGCAAAGCCTCCACAAT-3′ R: 5′-CGTGGGACATGGTGCCTTGAG-3′	130
IL-13	NM_001195791.1	F: 5′-CCTGCACGGCATGACGAACT-3′ R: 5′-GTACAGCGCCTGGGTGTAGT-3′	97
Th17 cytokines			
IL-17A	NM_204460.1	F: 5′-CCGATCCCTTATTCTCCTCTGTTCA-3′ R: 5′-CTTCCCATGTGCAGAAATGCTGG-3′	84
IL-22	NM_001199614.1	F: 5′-CTAGAATCACAGCAAAGCGCTG-3′ R: 5′-AGCAACAACAGCAGAAGACAACC-3′	125
Inflammatory cytokines			
IL-1β	NM_204524.1	F: 5′-CCTCTGCCTGCAGAAGAAGCC-3′ R: 5′-CTCCGCAGCAGTTTGGTCATGG-3′	141
IL-6	NM_204628.1	F: 5′-CAGGACGAGATGTGCAAGAAGTT-3′ R: 5′-GTCAGGCATTTCTCCTCGTCGAA-3′	137
TNF-α	MF801626.1	F: 5′-GAAGGAACAAATTGGTGGTCCCC-3′ R: 5′-GGACGTCTTTGGGGTACTCCTC-3′	141
Anti-inflammatory cytokines			
IL-10	NM_001004414.2	F: 5′-CGTTCGAGAAGATGGATGAGAACG-3′ R: 5′-CTCCTCCTCATCAGCAGGTACTC-3′	97
TGF-β1	NM_001318456.1	F: 5′-GATGGACCCGATGAGTATTGGGC-3′ R: 5′-GGGACACGTTGAACACGAAGAAG-3′	124
Intestinal antibacterial proteins			
AvBD10	NM_001001609.2	F: 5′-AAACTGCTGTGCCAAGATTCCG-3′ R: 5′-CTCAAGGCAGTGGAAATGTTGCT-3′	88
AvBD12	NM_001001607.2	F: 5′-TCCCTGCTCGCTCACGGAAG-3′ R: 5′-CAGCAGAGAATGACGGGTTCAAA-3′	131
DEFB4A	NM_204992.2	F: 5′-TCATCTAATATCCGCAGCTCAGCA-3′ R: 5′-GGCGAAGACAACCCTGGAGAA-3′	106
Lyz	NM_205281.1	F: 5′-TGGGGAAAGTCTTTGGACGATGT-3′ R: 5′-TTTGCAACACACACCCAGTTTCC-3′	103
Muc2	JX284122.1	F: 5′-TGCTCACACTTGGAAGTCAGCAGCC-3′ R: 5′-TCCATGGAGTCTGCAGGAGCACTGG-3′	138
Tight-junction proteins			
Claudin1	NM_001013611.2	F: 5′-ATGAAGTGCATGGAGGATGACCA-3′ R: 5′-GTGCTGACAGACCTGCAATGATG-3′	88
Claudin5	NM_204201.1	F: 5′-GATCTTTGTGCCCTGGCTCCAGCAC-3′ R: 5′-TGCTCAGCAAGAAGGCCACGAAGC-3′	132
E-cad	NM_001039258.2	F: 5′-TGAATAGGCAGCCCTCGTCCCCTTG-3′ R: 5′-GGAGGGATGCGAGTGGTGGATCCAA-3′	130
Occludin	NM_205128.1	F: 5′-TGTGCTGAGATGGACAGCATCAA-3′ R: 5′-TCCTCTGCCACATCCTGGTATTG-3′	101
ZO-1	XM_015278975.2	F: 5′-TACCTGACTGTCTTGCAGATGGC-3′ R: 5′-ATGGAGTTACCCACAGCTTCCTC-3′	91
LPS signaling pathway			
LITAF	NM_204267.1	F: 5′-ACCCGTAGTGCTGTTCTATGACC-3′ R: 5′-CTATGCACCCCAGCAGGAAGAG-3′	140
PTP4A3	XM_004940067.3	F: 5′-CATCATCCTTCTCTTGTCCCACC-3′ R: 5′-GCACCATACTTCTTCAGATCCTCC-3′	148
TLR2A	NM_204278.1	F: 5′-CAACGGTCATCTCAGCTACACCA-3′ R: 5′-CCTGTCTCAGGGCTTGTTCTTCA-3′	134
TLR4	NM_001030693.1	F: 5′-CCTGCTGAAATCCCAAACACCAC-3′ R: 5′-TGTATGGATGTGGCACCTTGAAA-3′	132
House-keeping genes			
B2M	NM_001001750.3	F: 5′-GCAGTACTCCGACATGTCCTTCA-3′ R: 5′-AACTCGGGATCCCACTTGTAGAC-3′	150
HPRT1	NM_204848.1	F: 5′-TGGGATATCGGCCAGACTTTGTT-3′ R: 5′-TTTGTACTTCTGCTTCCCCGTCT-3′	137

Primers were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast). All primers were confirmed to be specific by agarose gel electrophoresis and a melting curve analysis. AvBD: avian β-defensin, B2M: β-2-microglobulin, DEFB4A: defensin β 4A, HPRT1: hypoxanthine phosphoribosyltransferase 1, LITAF: liposaccharide-induced TNF factor, LPS: lipopolysaccharide, Lyz: lysozyme, Muc2: mucin 2, PTP4A3: protein tyrosine phosphatase 4A3, TGF-β1: transforming growth factor-beta1, TLR: Toll-like receptor, TNF-α: tumor necrosis factor-alpha, ZO-1: zonula occludens protein 1.

Spearman's rank-order correlation coefficients, which were calculated using the 'ggpubr' package (Kassambara, 2020). The Smirnov–Grubbs test for outliers was conducted with the 'outliers' package in R (Komsta, 2011).

Results

Nutritional Analysis of the RSL and DSL

Table 3 summarizes the results of the nutritional analysis of the RSL and DSL feedstuffs. The RSL had a high water content (58.8%), but the water content of the DSL decreased to 23.4% after drying. Each nutritional component was concentrated approximately two-fold after drying. However, compared to corn, the EE values of both RSL and DSL were low, and the GE values were accordingly low. Heat treatment of sake lees increased the content of many amino acids but decreased that of Lys.

Effects of the Experimental Diets on Growth Performance and Serum Amino Acid Concentrations

During the 2-week feeding period, body weight gain, feed intake, feed efficiency, and final body weight of the RSL diet group were equivalent to those of the control diet group (Table 4). In contrast, the growth performance of the DSL diet group was significantly lower than that of the control group. The RSL and control diet groups showed comparable growth performance; however, the gizzard tissue weight of the RSL diet group was significantly lower than that of the control diet group. Sex determination revealed the male-to-female ratio in all the groups to be 1:1. In addition, there was no statistically significant difference in the growth performance between male and female chickens.

The effects of the experimental diets on the serum amino acids of broiler chickens are shown in Table 5. The serum concentrations of most of the amino acids in the RSL diet group were almost the same as those of the control diet group, with the exceptions of Phe and Val; compared to the control group, the serum Phe in the RSL group was significantly lower and the serum Val was significantly higher. The serum concentrations of Ala, Arg, Cys, Gln, Glu, Gly, His, Leu, Phe, and Tyr were significantly lower in the DSL group than in the control diet group.

Expression of Immune-related and Tight Junction Protein Genes in the Intestinal Tract

The results of RT-qPCR analysis are provided in Table 6. The gene expression levels of TGF-β1 in the jejunum and ileum of the RSL diet group were significantly higher than those of the control diet group. In addition, the gene expression level of AvBD12 in the ileum of the RSL group was significantly higher than that of the control diet group (Table 6). The expression level of IL-10 in the ileum of the RSL diet group tended to be higher than that of the control diet group (p=0.0595). In contrast, the gene expression levels of Muc2, E-cad, and occludin in the jejunum and ileum of the RSL diet

Table 3. **Nutrient and Amino Acid Composition of the Feed Ingredients**

	RSL	DSL	Corn	Soybean meal
General component, %:				
Moisture	58.8	23.4	14.5	13.6
CP	12.9	25.0	7.6	43.6
EE	0.5	1.0	3.8	0.9
CF	2.6	4.7	1.7	13.5
CA	0.4	0.9	1.2	6.2
NFE	24.8	45.1	71.7	22.2
GE (Mcal/kg)	1.96	3.65	3.92	4.16
Amino acid content, mg/100 g:				
Ala	983	1540	410	1800
Arg	1019	1363	218	2831
Asp	1376	2183	375	4795
Glu	2173	3530	1012	7568
Gly	708	1126	205	1738
His	351	395	158	1035
Ile	584	921	183	1699
Leu	1214	1941	672	3172
Lys	738	580	160	2560
Phe	716	1161	264	2074
Pro	714	991	495	2070
Ser	758	1074	188	2050
Thr	652	864	236	1780
Tyr	763	1241	96	1120
Val	828	1324	260	1799

The data represent the means of triplicate measurements. Bold amino acids are essential in broiler chickens. CA, crude ash; CF, crude fiber; CP, crude protein; DSL, dried sake lees; EE, ethanol extract; GE, gross energy; NFE (nitrogen-free extract), 100−(CP+EE+CF+CA), RSL: raw sake lees.

Table 4. **Effects of RSL and DSL Diets on the Growth Performance of Broiler Chickens**

	Control diet	RSL diet	DSL diet
At 1 week			
Body weight gain, g	510.5±34.6[a]	535.2±22.0[a]	273.6±23.3[b]
Feed intake, g	787.7±24.5[a]	802.2±27.5[a]	590.5±10.7[b]
Feed efficiency, %	64.3±2.9[a]	66.9±2.6[a]	46.0±3.4[b]
Body weight, g	1561.8±43.9[a]	1587.3±29.9[a]	1327.5±15.1[b]
At 2 weeks			
Body weight gain, g	1101.5±59.6[a]	1140.9±71.9[a]	690.8±50.8[b]
Feed intake, g	1836.6±47.0[a]	1840.2±83.7[a]	1448.8±42.9[b]
Feed efficiency, %	59.8±2.2[a]	61.6±1.8[a]	47.3±2.4[b]
Final body weight, g	2152.8±64.3[a]	2192.9±76.7[a]	1744.7±41.8[b]
Tissue weight, g			
Breast	183.1±8.5[a]	182.0±9.4[a]	154.5±3.7[b]
Tender	42.7±1.3[a]	44.5±1.5[a]	35.7±0.6[b]
Thigh	180.3±8.9[a]	173.9±9.3[a]	136.7±4.8[b]
Heart	12.6±0.7[a]	13.0±0.5[a]	9.7±0.4[b]
Liver	47.0±2.0[a]	44.2±5.9[ab]	34.2±1.2[b]
Gizzard	20.1±1.0[a]	16.7±1.1[b]	16.0±0.6[b]

[a,b] Means with different superscript letters in the same row are significantly different ($P<0.05$).
Values are mean±SEM. $n=8$ per diet group.

Table 5. **Influence of Experiment Diet on the Serum Concentrations (mM) of Amino Acids**

	Control diet group	RSL diet group	DSL diet group	Pooled SE
Ala	959.0[a]	953.31[a]	680.62[b]	300.4
Arg	471.7[a]	460.65[a]	343.55[b]	95.9
Asn	167.5	139.7	121.1	144.2
Asp	145.5[ab]	158.6[a]	95.5[b]	78.5
Cys	49.2[a]	48.9[a]	39.9[b]	9.8
Gln	934.4[a]	953.4[a]	656.6[b]	234.9
Glu	190.9[a]	189.4[ab]	155.3[b]	45.7
Gly	889.6[a]	780.7[ab]	704.9[b]	177.4
His	123.9[a]	106.8[a]	74.5[b]	35.3
Ile	129.7	153.7	127.7	37.1
Leu	235.5[a]	231.3[a]	195.6[b]	47.8
Lys	510.2	543.8	418.6	222.0
Met	32.1	37.7	33.4	11.8
Phe	139.6[a]	120.0[b]	101.0[c]	23.8
Pro	312.8	347.4	291.2	177.4
Ser	964.5	938.1	920.0	206.2
Thr	756.4	788.0	752.3	283.0
Tyr	241.4[a]	197.4[ab]	163.2[b]	88.3
Trp	53.8	56.8	49.2	13.2
Val	249.1[b]	306.6[a]	262.9[ab]	71.3

[a–c] Means with different superscript letters in the same row are significantly different ($P<0.05$). $n=8$ per diet group. Bold letters indicate essential amino acids in broiler chickens. RSL: raw sake lees. DSL: dried sake lees.

group were significantly lower, as were the ZO-1 and LITAF levels in the jejunum.

In the DSL diet group, the expression levels of E-cad, occludin, Muc2, and LITAF in the jejunum and ileum were significantly lower than those in the control diet group, while the expression level of AvBD12 in the ileum was significantly higher than that in the RSL diet group. However, the expression levels of TNF-α, IL-1β, IL-6, TGF-β1, TLR2A, and TLR4 in the jejunum of the DSL diet group were significantly lower, as were the expression levels of claudin 1/5 and AvBD10 in the ileum. The heat map results are illustrated in Fig. 1. Of all genes, AvBD12 showed the highest value in the RSL and DSL diet groups (Fig. 1).

Network analysis and scatter plot revealed a close relationship between ileal AvBD12 and TGF-β1 in the RSL diet group ($p<0.05$) (Figs. 2, 3). In the DSL diet group, no direct relationship between ileal AvBD12 and TGF-β1 was found (Fig. 2). As shown in Figure 4, AvBD12 in the ileum of the DSL diet group was significantly correlated with PTP4A3, TRL2A, and LITAF ($p<0.05$), and TGF-β1 was significantly correlated with PTP4A3 and TLR2A ($p<0.05$). These data suggest that ileal AvBD12 and TGF-β1 in the DSL diet group were indirectly related to PTPT4A3 and TLR2A.

Discussion

Improving the Preservability of Sake Lees and its Effects on the Growth Performance of Broilers

Although FLW is generally highly nutritious and has high water content, it has a poor shelf life. Thus, two methods have been applied to use FLW for livestock. One method was to immediately mix FLW with other feed ingredients (e.g., total mixed ration [TMR]) or liquid feed without drying. Although this method does not entail high drying costs, it requires immediate use to avoid spoilage.

Another method is to heat-dry FLW and then use it in the

Table 6. **Influence of Sake-Lees on Intestinal Inflammatory- and Barrier-related Genes**

	Jejunum			Ileum		
	Control diet group	RSL diet group	DSL diet group	Control diet group	RSL diet group	DSL diet group
Th1 cytokines (proinflammatory cytokine)						
IFN-γ	1.76±0.48	1.20±0.39	1.01±0.18	0.63±0.07	0.79±0.17	0.46±0.05
IL-2	1.16±0.10	1.24±0.30	1.50±0.26	1.01±0.14	0.88±0.11	0.75±0.04
Th2 cytokines						
IL-4	0.84±0.09	0.64±0.09	0.95±0.13	1.38±0.26	2.09±0.58	1.64±0.27
IL-13	1.15±0.26	0.95±0.34	0.86±0.27	0.92±0.24	0.81±0.26	0.53±0.15
Th17 cytokines (proinflammatory cytokine)						
IL-17A	0.85±0.22*	0.53±0.18	0.28±0.11*	0.78±0.31	0.80±0.62	0.10±0.04
IL-22	2.08±0.88	0.77±0.19	1.51±0.81*	1.26±0.51	0.60±0.10*	0.47±0.11
Inflammatory cytokines						
TNF-α	1.34±0.22^{a}	1.57±0.31^{a}	0.57±0.06^{b}	0.71±0.17	0.76±0.13	0.57±0.08
IL-1β	1.13±0.25^{a}	1.13±0.15^{a}	0.47±0.05^{b}	0.66±0.16	0.87±0.13	0.59±0.06
IL-6	1.89±0.35^{a}	1.53±0.32^{ab}	0.62±0.11^{b}	0.79±0.09	0.79±0.24	0.44±0.09
Treg (anti-inflammatory cytokines)						
IL-10	0.85±0.10^{ab}	1.30±0.23^{a}	0.49±0.11^{b}	0.65±0.07	0.89±0.25	0.54±0.09
TGF-β1	1.56±0.12^{b}	2.18±0.22^{a}	0.71±0.05^{c}	0.97±0.12^{b}	1.62±0.22^{a}	1.16±0.08^{ab}
Intestinal antibacterial proteins						
Lyz	1.11±0.17^{ab}	1.87±0.37^{a}	0.44±0.14^{b}	0.89±0.22	1.39±0.34	0.54±0.18
Muc2	1.15±0.16^{a}	0.27±0.07^{b}	0.40±0.13^{b}	1.17±0.13^{a}	0.39±0.06^{b}	0.25±0.08^{b}
DEFB4A	2.06±0.75	1.22±0.24	2.02±0.49	0.60±0.16	0.43±0.18	1.78±0.68
AvBD10	1.22±0.19	0.77±0.23	0.73±0.11	1.37±0.23^{a}	0.85±0.12^{ab}	0.66±0.10^{b}
AvBD12	1.78±0.45	2.82±0.95	1.96±0.42	1.39±0.15^{b}	3.72±0.44^{a}	4.99±1.07^{a}
Tight-junction proteins						
E-cad	1.25±0.21^{a}	0.58±0.06^{b}	0.63±0.12^{b}	1.20±0.06^{a}	0.55±0.07^{b}	0.53±0.06^{b}
Claudin1	1.67±0.18	1.58±0.17	1.31±0.17	0.89±0.08^{b}	0.88±0.08^{b}	1.33±0.16^{a}
Claudin5	1.45±0.10	1.47±0.11	1.26±0.08	0.93±0.11^{b}	1.14±0.14^{ab}	1.41±0.15^{a}
Occludin	1.15±0.18^{a}	0.61±0.09^{b}	0.63±0.11^{b}	1.19±0.07^{a}	0.61±0.08^{b}	0.49±0.07^{b}
ZO-1	1.42±0.06^{a}	1.07±0.07^{b}	1.17±0.05^{b}	1.07±0.05	0.95±0.03	0.96±0.05
LPS signaling pathway						
LITAF	1.64±0.20^{a}	1.05±0.11^{b}	0.72±0.09^{b}	0.94±0.12^{a}	0.72±0.09^{ab}	0.56±0.08^{b}
TLR2A	1.63±0.19^{a}	1.42±0.09^{ab}	1.02±0.08^{b}	0.77±0.05	0.91±0.21	0.90±0.09
TLR4	1.68±0.20^{a}	1.36±0.08^{ab}	1.11±0.06^{b}	0.91±0.09	0.96±0.18	0.90±0.04
PTP4A3	1.35±0.07^{ab}	1.59±0.16^{a}	1.09±0.03^{b}	0.85±0.07	0.99±0.12	1.06±0.07

$^{a, b}$ Means with different superscript letters in the same row are significantly different ($P<0.05$). $n=8$ per diet group. AvBD, avian β-defensin; B2M, β-2-microglobulin; DEFB4A, defensin β 4A; HPRT1, hypoxanthine phosphoribosyltransferase 1; LITAF, liposaccharide-induced TNF factor; LPS, lipopolysaccharide; Lyz, lysozyme; Muc2, mucin 2; PTP4A3, protein tyrosine phosphatase 4A3; TGF-β1, transforming growth factor-beta1; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; ZO-1, zonula occludens protein 1.

same way as the raw material for the concentrate feed. Although this method has high drying costs, it is easy to perform and does not pose the risk of spoilage. In addition, the weight reduction of the FLW reduces the transportation costs.

For the present investigation, both the aforementioned methods were used, we designated an RSL diet group in which sake lees were immediately added to chicken feed and a DSL diet group in which sake lees subjected to drying treatment were added. The sake lees had a 58% water content (Table 3), but by mixing with other feed ingredients, the water content of the RSL diet was reduced to 19.5% (Table 1). Despite this reduction, the water content of the feed material remained high. However, no spoilage or mold generation was observed for more than 2 weeks during the feeding period, and the feed could be used as poultry feed without any complications.

Bacon *et al*. (1973) reported that mold activity in poultry feed increases with increasing moisture content; specifically, when the moisture content in feed reaches 18%, the amount of ochratoxin A increases, and when it reaches 32%, the amount of penicillic acid increases. The fact that the experimental feed in our present study did not spoil despite the high water content could be attributed to the presence of 8% alcohol in the sake lees (MEXT, 2015), and the yeasts that grew during fermentation were still alive.

Rice, the raw material of sake lees, is a gramineous grain that is similar to corn; thus, Lys is the limiting amino acid. However, the Lys content in sake lees was higher than that of the other amino acids (Table 3). Sake is produced by fer-

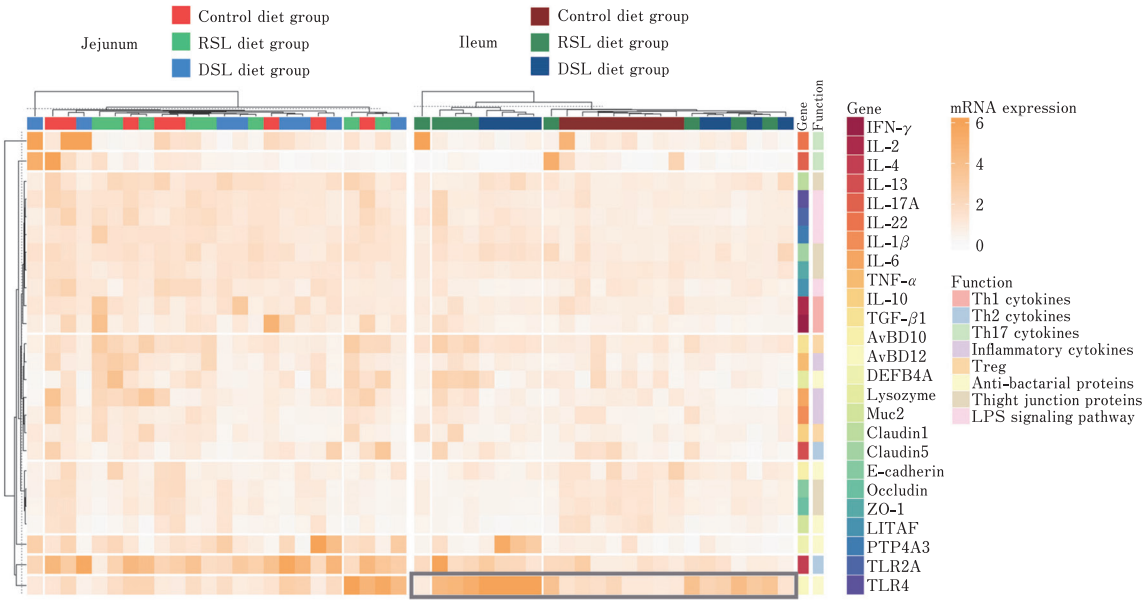

Fig. 1. **Heatmap of intestinal immunity and tight junction protein-related genes in the jejunum and ileum.** The *orange color density* indicates the levels of fold change. The *gray box* at the lower right indicates the gene (AvBD12) with the highest gene expression in the sake lees feeding groups.

menting rice, and Lys contained in the bacterial cell protein increases with the growth of *A. oryzae* during the fermentation stage (Tsutui *et al*., 1998). We suspect that the sake lees used in the present study also had a higher Lys content than corn because of the Lys derived from the bacterial cell protein. Sake lees also have a high Thr content, which is a limiting amino acid for chickens (Kubo and Sugahara, 1992). Thus, Sake lees are superior to corn in terms of amino acid content.

The DSL drying process concentrated many amino acids by approximately 1.6-fold compared to the RSL. However, Lys, His, and Arg showed low enrichment rates of 0.8-, 1.1-, and 1.3-fold, respectively (Table 3). Lys, Arg, and His are called "basic" amino acids and serve as substrates for the Maillard reaction that bind to sugars. Ashoor and Zent (1984) reported that when 21 types of amino acids and monosaccharides (D-glucose, D-fructose, D-ribose, and α-lactose) were reacted at 121℃ for 10 min, Lys showed the highest reaction. Sun *et al*. (2021) reported that His and Lys are more reactive with monosaccharides (dihydroxyacetone) than Arg is. Lys has also been shown to be a substrate closely involved in the Maillard reaction of carbohydrates in foods (Lund and Ray, 2017). A comparison of these reports with the present results confirms that the order of the loss rates of basic amino acids, which are likely to be substrates for the Maillard reaction, are in agreement. Therefore, it is highly possible that the amino acids in the DSL were lost via the Maillard reaction, in which the amino acids in the sake lees would react with carbohydrates during heat treatment.

The growth performance of broilers fed the DSL diet declined (Table 4). We also observed decreased serum amino acid levels and feed intake in the DSL diet group (Tables 4 and 5). These results suggest that the reduction in protein intake, rather than the deficiency of certain amino acids due to the Maillard reaction, reduces broiler growth performance. The reason for the decrease in feed intake was that the amount of cellulose added to the DSL diet increased, and the palatability of the diet decreased. Because the water content of the DSL was reduced by the drying process, it was necessary to substitute this amount with cellulose. As a result, the proportion of cellulose in the total feed was high, and the resulting mealy feed had a reduced palatability.

In contrast, the growth performance and most of the tissue weight in the RSL diet group were equivalent to those in the control diet group, and only the gizzard weight was significantly lower in the RSL diet group. The size of the chicken gizzard changes depending on the physical stimulation by the shape of the feed (Svihus, 2011). We speculate that the gizzard was lighter because of the softer sake lees compared to corn and the lower physical irritation of the feed containing sake lees.

In a study similar to our present experiment, Mahfudz *et al*. (1996) revealed that the addition of lees derived from shochu (another distilled alcohol beverage produced in Japan) to broiler feed improved growth efficiency and it was noted that the factor involved in this improvement was BBA, that is, 23-O-(1,4'-bipiperidine-1-carbonyl)betulinic acid. It was also observed that by adding 0.05% of *Aspergillus luchuensis* cells that are used in the production of shochu to the feed, the gene expression levels of ubiquitin, proteasome, and calpain, which are proteolysis-related genes in skeletal muscle, were suppressed, and chicken growth efficiency was improved (Kamizono *et al*., 2010; Saleh *et al*., 2012). In addition, Saleh

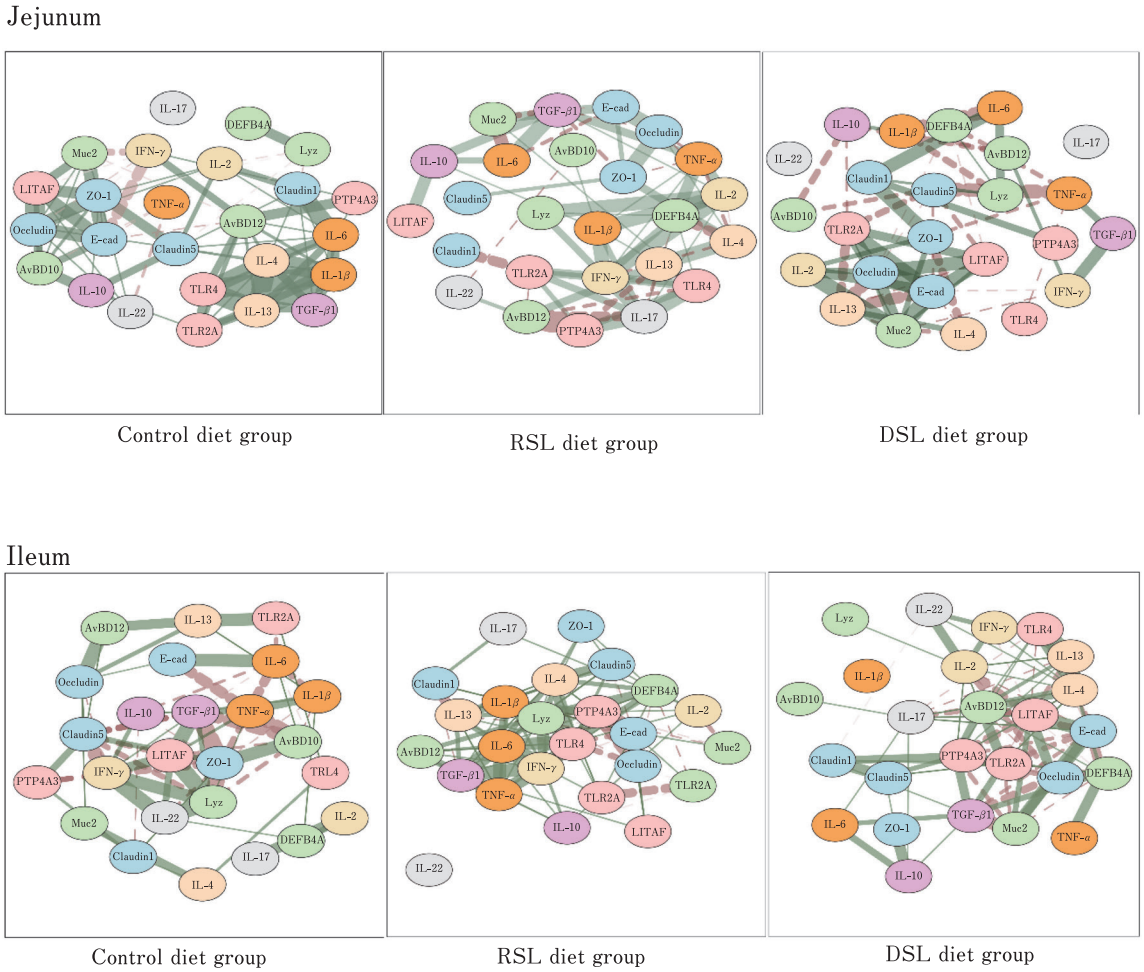

Fig. 2. **Network analysis of intestinal immunity and tight junction protein-related genes in the jejunum, and ileum.** Genes with a Spearman's rank correlation coefficient ≥0.5 and showing a positive correlation are connected using *green lines*. Genes showing a negative correlation are connected using *red dashed lines*. The thickness of the line represents the height of the correlation coefficient.

et al. (2013) demonstrated that *A. luchuensis* and *S. cerevisiae* had a synergistic effect on the growth performance of broilers and that *A. luchuensis* significantly increased the weight of breast muscle. Sake and shochu use the same yeast, *S. cerevisiae*, but the types of yeasts and ingredients are different. *Aspergillus oryzae* is used to brew sake as malted rice (rice-*koji*), but in shochu, *A. luchuensis* is used with rice or wheat *koji*. Moreover, sake production uses only rice as the raw material, whereas shochu production uses rice, wheat, and sweet potatoes as raw materials. The same phenomenon was not observed in the sake lees in the present study, but we speculate that this was due to the difference in the raw materials and bacterial species used in the sake lees and shochu-lees.

Increased Intestinal TGF-β1 Expression Level Due to the Intake of Sake Lees

As shown in Table 6, gene expression levels of TGF-β1 (which is also known as TGF-β4; Halper *et al*., 2004) in the jejunum and ileum were significantly higher in the RSL diet group. The gut barrier is reinforced by TGF-β1 by driving lymphocyte development and/or function (Bauché and Marie, 2017). Subsequent reports suggested that the increase in TGF-β1 was because of the components of sake lees on *Lactobacillus* spp. in the broiler intestine. Kawakami *et al*. (2020) revealed that feeding sake cake (which is the same as sake lees) to mice significantly increased *Lactobacillus* in the ileum; however, the components responsible for this are unknown. Slawinska *et al*. (2019) reported that feeding chickens galacto-oligosaccharides (GOS) increased the amount of *Lactobacillus* spp. in the ileum. Torii *et al*. (2007) reported that oral administration of *Lactobacillus acidophilus* to mice significantly increased the expression level of TGF-β in Peyer's patches and increased total IgA production. The above findings and the inclusion of GOS in sake lees

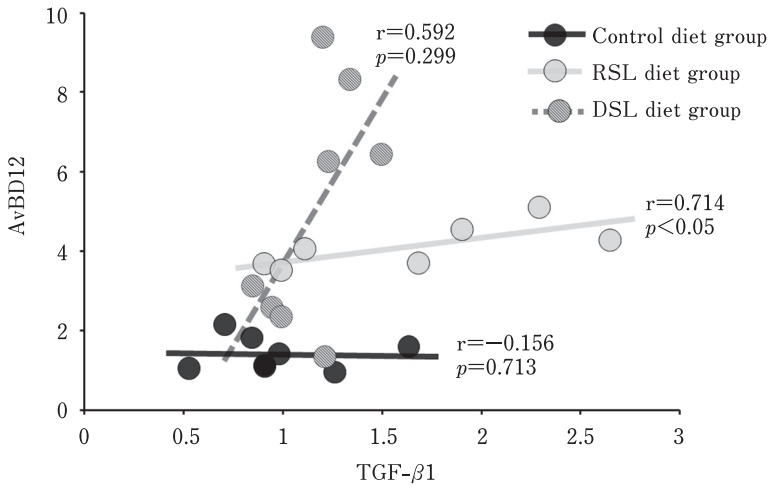

Fig. 3. **Scatterplot and correlation of the gene expression levels of TGF-β1 and AvBD12 in the ileum.** Control diet group: $n=8$, RSL diet group: $n=7$, DSL diet group: $n=8$.

(Kurahashi, 2021) suggest that GOS derived from sake lees increased *Lactobacillus* spp. in the intestine and, as a result, increased the expression level of TGF-β1. Investigations of the relationship between GOS in sake lees and intestinal bacteria are limited.

Expression Regulation Mechanism of AvBD12 in the RSL and DSL Diet Groups

The gene expression level of AvBD12 was significantly higher in the ileum of the RSL diet group than in the ileum of the control group. In addition, the heatmap results demonstrated that AvBD12 in the ileum was the gene that was most influenced by sake lees (Fig. 1). β-defensin is an antibacterial protein; AvBDs, which are β-defensins in chickens, have a broad antibacterial spectrum and microbicidal and microbiostatic activities against bacteria such as gram-negative bacteria, gram-positive bacteria, mycoplasma, and Candida (van Dijk *et al*., 2008). This is due to detergent-like action that disrupts the cell membrane and also acts on DNA and RNA to disrupt protein synthesis and function.

The network analysis that we conducted to search for the causes of the increased AvBD12 expression in the RSL diet group was affected by TGF-β1, and a significant correlation was observed in the ileum (Figs. 2, 3). This result suggests that the RSL diet increased the expression of AvBD12 via TGF-β1. Although AvBD12 expression increased in the DSL diet group, no increase in TGF-β1 was observed, and no relationship was confirmed. As shown in Figure 2, AvBD12 in the ileum was related to LPS signaling pathway genes such as LITAF, suggesting that the effect of increasing TGF-β1 was different between the RSL and DSL diet groups. A large amount of cellulose was added to the DSL diet to compensate for the decrease in water content caused by the drying process (Table 1). It is highly possible that the DSL diet group experienced a greater effect from cellulose than sake lees because the intake of sake lees decreased due to the increase in the proportion of cellulose and subsequent decrease in palatability.

One of the effects of cellulose is the increase in *Enterobacteriaceae* family members owing to cellulose feeding (Berer *et al*., 2018). Biofilm components (curil fibrils and bacterial cellulose) derived from *Enterobacteriaceae* have been shown to regulate intestinal mucosal immunity via the LPS signaling pathway gene TLR2 (Ellermann and Sartor, 2018). Among the biofilm components, peptidoglycan regulates the secretion of TGF-β via TLR2 in dendritic cells and increases the secretion of β-defensin via TLR2 (Kumar *et al*., 2006; Kashiwagi *et al*., 2015). In the present study, TGF-β1 and chicken defensin AvBD12 were associated with the LPS signaling pathway genes (Fig. 4). We concluded that changes in *Enterobacteriaceae* in the intestine due to cellulose in the DSL diet group led to a change in the expression level of AvBD12. From these findings, it is highly possible that the difference in the regulation of AvBD12 expression between the RSL and DSL is due to the difference in their effect on the intestinal biota. Further research focusing on changes in intestinal bacterial biota is required.

We observed that the expression level of TGF-β1 in the jejunum was increased in the RSL diet group. Network analysis of the jejunum in the RSL diet group revealed that TGF-β1 was related to AvBD10 and Muc2 (Fig. 2). However, no significant effect was observed for intestinal antibacterial proteins (Table 6). The expression level of AvBDs varies depending on the site in the intestinal tract (Lyu *et al*., 2020). Thus, AvBDs corresponding to jejunal TGF-β1 are likely to be other than AvBD10 and AvBD12. It is necessary to identify the localization of TGF-β1-expressing cells, their interaction with intestinal antibacterial proteins (especially AvBDs), and the corresponding antibiotic type for each site in the intestinal tract in future studies.

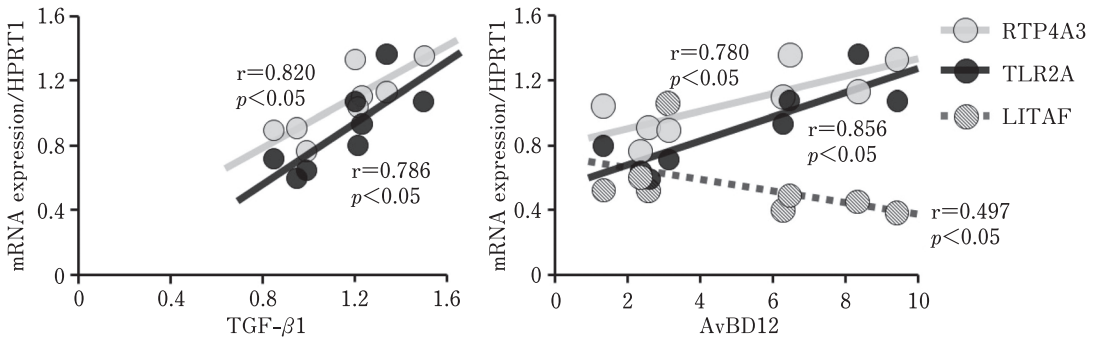

Fig. 4. **Correlation between TGF-β1 or AvBD12 and LPS signaling pathway genes in the ileum of the DSL diet group:** $n=8$.

Reduction of Tight Junction Protein-related Genes Due to Sake Lees in the Diet

We hypothesized that the cause of the decrease in tight junction proteins in the intestine (Table 6) was due to inflammatory effects, but we observed that the expression levels of TNF-α, which is involved in tight junctions during inflammation (Ma *et al*., 2003; Al-Sadi *et al*., 2013, 2016) in the sake lees groups were not significantly different from those in the control diet group. Similar to the gizzard, the small intestine is known to change in weight and structure depending on the properties of the feed. Zaefarian *et al*. (2016) tested mashed and pelleted diets (which are softer than mashed diets), and observed that the gizzard and intestinal weights of broilers fed pelleted feed were reduced. It has also been reported that mashed feed reduces duodenal villus height and jejunum crypt depth compared with pelleted feed (Mohammadi Ghasem Abadi *et al*., 2019). Although sake lees are solids, they become liquid due to the addition of water, and it can thus be expected that after they are eaten, they liquify in the digestive tract, and the need for physical stimulation is reduced. Considering the findings of the above-cited studies, the decrease in physical stimulation by the addition of sake lees affected villus formation and decreased the expression level of tight junction protein-related genes. Since there are no reports that mention a direct relationship between physical stimulation and the expression level of tight junction proteins, we cannot reach any conclusion regarding the effect of sake lees on tight junction proteins. However, considering the aforementioned findings, we infer that the decrease in physical stimulation by the addition of sake lees affected intestinal morphology, resulting in a decrease in the expression level of tight junction proteins.

In conclusion, dried sake lees are more storable, but their growth performance may be inadequate if they are not combined with other feed ingredients. If raw sake lees are immediately mixed into feed, they will have good storage stability and provide the same growth performance as corn. Since the deficiency of physiologically active substances is suppressed, sake lees can be used as a feed with functionality, and an intestinal immunostimulatory effect can be observed. However, low fat content of sake lees provides a low amount of energy; therefore, it is necessary to combine sake lees with a feed material that has a high fat content.

Future studies should focus on morphological evaluation of the chicken intestinal tract and the relationship between intestinal microbiota and intestinal immunity.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

KRI, TS, HG, KS, JW, and MY were involved in study design and data interpretation. KRI, TS, and HG were involved in the data analysis. All authors revised the manuscript, approved the manuscript to be published, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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