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Effects of Italian Ryegrass (IRG) Supplementation on Animal Performance, Gut Microbial Compositions and Odor Emission from Manure in Growing Pigs

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Received: 21 March 2020; Accepted: 30 April 2020; Published: 2 May 2020



Abstract: Fermentable carbohydrate (FC) is a promising material to reduce odor emission from pig manure. This study was conducted to investigate the impact of diets containing Italian ryegrass (IRG), as a FC, on animal performance, odorous chemical and bacterial composition of manure. Pigs were weighed and fed diets containing various levels of IRG powder (0%, 0.5%, 1.0% and 1.5%) for 28 days. At the end of the trial, manure was collected to analyze the chemical composition, odorous compounds and bacterial community structure. As dietary IRG levels increased, concentrations of phenols and indoles were decreased by 12% and 37% compared with control, respectively, without changes in growth performance. IRG treatment increased the relative abundances of genera belong to the family *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, *Peptostreptococcaceae* and *Lactobacillaceae*, in order *Clostridiales* of phylum *Firmicutes*, but decreased the relative abundances of genus *Sphaerochaeta* in phylum *Spirochaetes* and genus *AB243818_g* of family *Porphyromonadaceae* in phylum *Bacteroidetes* when compared with control. Results from the current study demonstrate that IRG supplemented diets had a beneficial effect of reducing the odorous compounds in manure, possibly by altering the bacterial community structure towards predominantly carbohydrate utilizing microorganisms in the large intestine.

Keywords: bacterial compositions; Italian ryegrass; phenols and indoles; pig manure

1. Introduction

Swine manure accounts for the highest ratio (40.6%) of total manure discharging from animal facility [1]. Odor emitting from swine manure accounts for 34.6% from that of total livestock facilities and contributed to civil complaints, affecting environment, animal health and production [2]. The Korea's national regulation of malodor emission from livestock facilities was enacted in 2005 [3]. Odor mitigation management systems have been produced in the context of bio-chemical agents and mechanical technologies [4]. Equipment systems such as ventilation, biofilters and wet scrubbers are difficult to adopt in open-type ventilation systems that account for 54% of total swine farms in Korea [5]. Therefore, modulation of diet formulation is a more desirable strategy for odor mitigation than that of equipment systems [4].

Control of odor generation and attenuation of nutrient excretion depends on the essential formulation and balance of nutrients in the animal diet. De Lange et al. [6] showed that feed ingredients and nutrient factors are closely associated with gut fermentation and odor formation. In particular, fermentable carbohydrate (FC)-containing dietary fiber has been known to relieve constipation, increase

nutrient digestibility and improve gut health, resulting in reducing odor emission via incorporating excessive nitrogen into the microbial biomass nitrogen [7–9]. Italian ryegrass (IRG) can be cultivated abundantly in Korea and used as a feed ingredient which levels of acid detergent fiber, neutral detergent fiber, acid detergent lignin and soluble and insoluble dietary fiber and non-structural carbohydrates are similar to those of sugar beet pulp, thus can provide abundant FC [10]. However, there are limited data on dietary IRG fiber content and the effects of supplementation on odor generation and gut microbial compositions in pigs. We hypothesized that IRG—rich in dietary fiber and FC content—would effectively decrease odor emission by improving gut fermentation. Therefore, this study was conducted to evaluate the effects of IRG powder on growth performance, manure chemical materials, odorous composition and bacterial community in growing-finishing pigs.

2. Materials and Methods

2.1. Animal and Manure Management

All procedures were conducted in accordance with guidelines No. 16 set by the National Institute of Animal Science Animal Care and Use Committee in 2008. Twenty cross-bred growing-finishing barrows [(Landrace × Yorkshire) × Duroc] with an average initial body weight (BW) of 38.70 kg, 5 for 4 treatments (IRG 0%, 0.5%, 1.0% and 1.5%), were randomly allotted to each pen equipped with a stainless steel feeder, a nipple waterer and slatted plastic floor. Ambient temperature in the facility was automatically maintained at approximately 25 °C by a ventilation control system. Pigs were fed a diet typical for this BW, which was formulated to be adequate in all nutrients (Table 1) [11].

Table 1. Composition of experimental diets, as-fed basis.

Items	IRG ¹				Raw ⁶
	0%	0.5%	1.0%	1.5%	
Ingredients (%; as fed basis)					
Corn, yellow	72.34	69.74	66.91	64.26	-
IRG ¹ , powder	0.00	0.50	1.00	1.50	-
Soybean oil	0.67	1.30	1.97	2.62	-
Molasses	0.07	1.29	2.69	4.00	-
Soy meal (CP ¹ 44%)	24.07	24.34	24.64	24.85	-
Limestone	0.53	0.48	0.42	0.37	-
DCP ¹	1.52	1.55	1.57	1.60	-
Mineral mixture ²	0.20	0.20	0.20	0.20	-
Vitamin mixture ³	0.20	0.20	0.20	0.20	-
Salt	0.30	0.30	0.30	0.30	-
Endopower ⁴	0.05	0.05	0.05	0.05	-
BioPlus 2B ⁵	0.05	0.05	0.05	0.05	-
Total	100	100	100	100	-
Analyzed chemical compositions					
Moisture (%)	12.77	12.87	13.10	13.69	10.78
Crude protein (%)	15.44	16.23	14.98	15.52	13.48
Crude fiber (%)	1.99	1.75	2.08	2.19	23.62
Crude ash (%)	4.21	4.15	3.88	3.82	11.50
DE ¹ (kcal/kg)	4438	4127	4110	4050	4103
Ca (%)	1.01	0.91	0.92	1.06	0.56
P (%)	0.66	0.64	0.63	0.71	0.32
Lysine (%)	0.70	0.75	0.78	0.71	0.61
Methionine (%)	0.22	0.22	0.24	0.21	0.17
Threonine (%)	0.69	0.58	0.58	0.56	0.63
Tyrosine (%)	0.54	0.61	0.57	0.57	0.46

¹ Abbreviations: IRG—Italian ryegrass; CP—crude protein; DCP—dicalcium phosphate; DE—digestible energy.

² Provided the following amounts per kilogram of diet: selenium 0.15 mg; manganese 0.03 g; zinc 0.1 g; iron 0.1 g; iodine 0.5 mg; magnesium 0.1 g. ³ Provided the following amounts per kilogram of diet: vitamin A 5500 IU; vitamin D₃ 550 IU; vitamin E 27 IU; menadione sodium bisulfate 2.5 mg; pantothenic acid 27 mg; niacin 33 mg; riboflavin 5.5 mg; vitamin B₁₂ 0.04 mg; thiamin 5 mg; pyridoxine 3 mg; biotin 0.24 mg; folic acid 1.5 mg; choline chloride 700 mg. ⁴ Mixture including α-galactosidase, galactomannanase, xylanase, β-glucanase, α-amylase, etc. ⁵ Mixture including bacillus subtilis and bacillus licheniformis. ⁶ RAW: only IRG not included with anything.

IRG was seeded in October and harvested at the heading stage in April of the next year. After naturally dry under the sun, IRG hay was ground into particles with less than 2 mm size. Dietary adaptation period was given to pigs for a 25 day in pen. Thereafter, they were transferred to individual metabolism crates ($1.6 \times 0.8 \text{ m}^2$) that allowed for collection of manure with another 3-day adaptation period. Pigs were weighed at the beginning and end of the experimental period to assess BW change and to ensure the gain weight. During the trial, pigs were fed twice daily (09:00 and 16:00) with feed being mixed with water at a 1:2.5 ratio based on BW. Feed intake was recorded daily for calculating feed efficiency. From each metabolism crates manure was collected at twice weekly, added in enclosed manure storage container and stored in the freezer ($-20 \text{ }^\circ\text{C}$) until analysis. A detailed scheme of the feeding trial is presented in Figure 1.

2.2. Manure Analysis

Manure pH was measured using a pH meter (Model 850C, Schott, Mainz, Germany). Biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS) and ammonium nitrogen ($\text{NH}_4\text{-N}$) of manure were tested according to Standards Methods ES 04305, ES 04315, ES 04303 and ES 04908 respectively [12]. BOD concentration was measured using a dissolved oxygen meter (Orion Star A, electrode #086030MD, Thermo Fisher Scientific, MA, USA). The samples for COD measurement were prepared with 0.005 M potassium permanganate solution (Crown Analytical Reagent, Tokyo, Japan) in flask and boiled at $97 \text{ }^\circ\text{C}$ for 30 min in water bath. Concentrations were calculated by amount of titrated with 0.005 M potassium permanganate solution. SS was analyzed that 20 mL of manure was poured into a pre-weighed crucible, dried at $105 \text{ }^\circ\text{C}$ for 2 h and cooled in a desiccator. Total nitrogen (TN) was analyzed using an UV-Visible spectrophotometer (Model Cary 300, Agilent, CA, USA). Manure 50 mL was added in beaker with 10 mL of sodium hydroxide–potassium persulfate solution (Sigma-Aldrich, MO, USA). After boiling for 30 min at $120 \text{ }^\circ\text{C}$, 25 mL of supernatant was mixed with 5 mL of hydrochloric acid solution (hydrochloric acid (Merck, Darmstadt, Germany):water = 1:16). $\text{NH}_4\text{-N}$ was analyzed by Kjeldahl method using steam distillation system (Vapodest 50s, Gerhardt, Königswinter, Denmark). The Kjeldahl procedure for total nitrogen determination is widely used for animal feed, fertilizer quality, environmental samples and biologic analysis [13]. Nitrogen was quantified to three steps of digestion, distillation and titration. Organic nitrogen containing samples are digested with sulfuric acid to ammonium sulfate; the ammonium is then liberated by raising the pH and measured by titration [14]. Manure was boiled with the solution which mixed perchloric acid, sulfuric acid and distilled water. Concentrations were calculated using the values of titrated with solutions of 40% sodium hydroxide, 2% boric acid and 0.1 N hydrogen chloride. All chemical materials were used the products from Junsei company (Tokyo, Japan).

Odorous compounds, including volatile fatty acid (VFA; short chain fatty acid (SCFA; acetic acid, propionic acid, butyric acid and valeric acid) and branched-chain fatty acid (BCFA; iso-butyric acid and iso-valeric acid)), phenols (phenol and p-cresol) and indoles (indole and skatole), were measured by gas chromatography (GC; 6890 N, Agilent, CA, USA) equipped with a flame ionization detector. VFA, phenols and indoles were analyzed by the previous methods reported by Hwang et al [15]. Briefly, 5 mL of manure was added to a 15 mL polypropylene centrifuge tube (Nunc, Thermo Fisher Scientific, MA, USA) along with 1 mL of 25% meta-phosphoric acid solution (Sigma-Aldrich, MO, USA) and 0.05 mL of saturated mercury (II) chloride solution (Sigma-Aldrich, MO, USA). Tubes were vortexed and centrifuged at $13,800 \times g$ for 10 min after at $3134 \times g$ for 20 min at $20 \text{ }^\circ\text{C}$. The supernatant was filtered through a $0.2 \text{ }\mu\text{m}$ syringe filter (Merck, Darmstadt, Germany) and analyzed to VFA. Concentrations of phenols and indoles were analyzed that supernatant (4 mL) of centrifuged manure at $3134 \times g$ for 20 min at $20 \text{ }^\circ\text{C}$ was mixed with 4 mL of chloroform (Merck, Darmstadt, Germany) and 60 μL of 4 M sodium hydroxide solution (Sigma-Aldrich, MO, USA). The supernatant was removed 2.0 mL GC vials (Agilent, CA, USA) after it was centrifuged at $3134 \times g$ for 20 min at $20 \text{ }^\circ\text{C}$. Concentrations of VFA, phenols and indoles were determined by the external standard quantitation method with standard reagents of each compounds from Sigma-Aldrich (MO, USA).

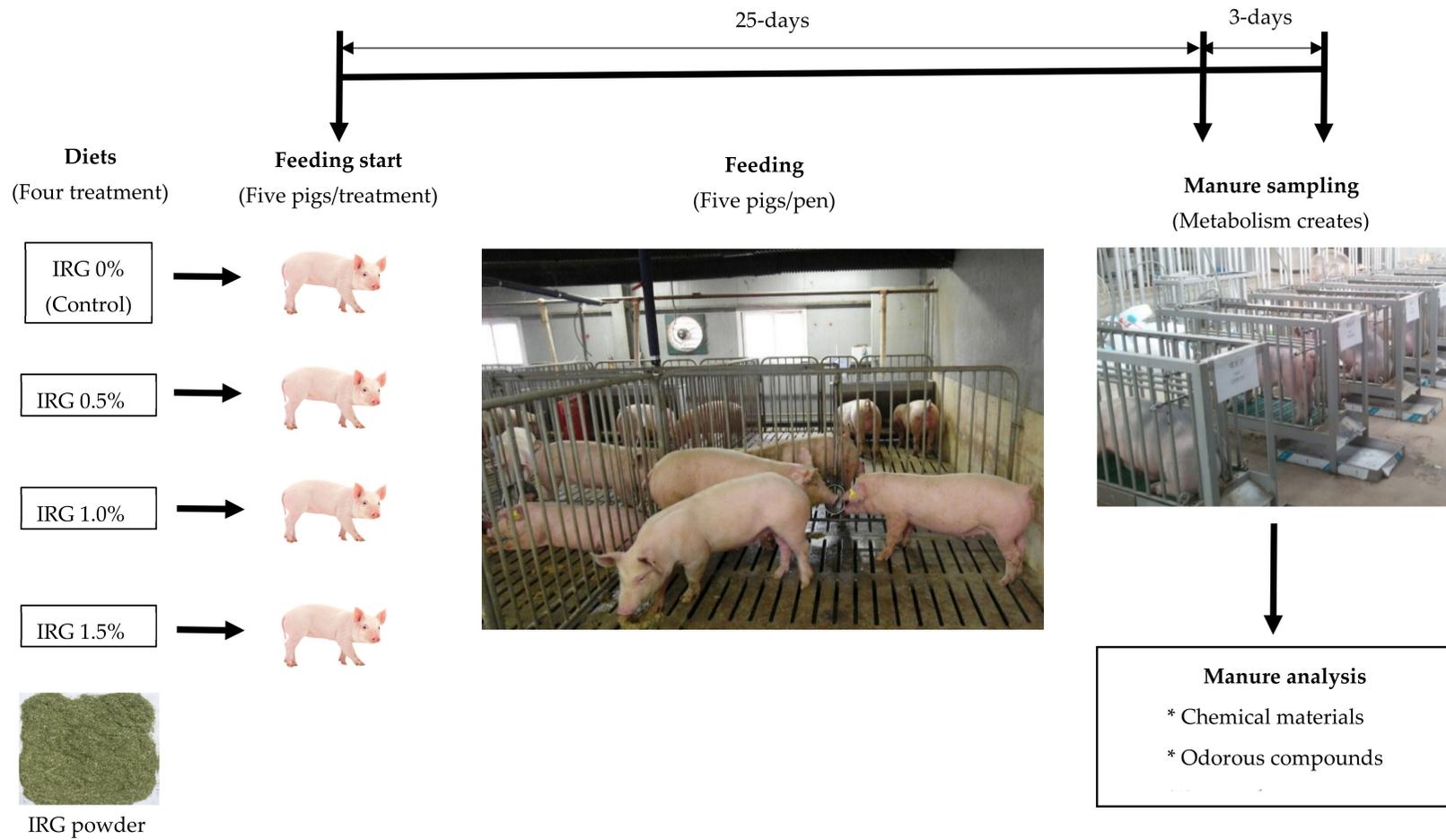


Figure 1. Schematic view of the experimental feeding trial.

2.3. Bacterial Community Analysis

Total genomic DNA from manure was extracted using a Fast-DNA Spin Kit (MP Biomedicals, CA, USA) according to the manufacturer's instructions with modification [16]. Humic acid was removed using a Power-Clean DNA Clean-Up Kit (MP Biomedicals, CA, USA). Bacterial 16S ribosomal RNA genes (about 500–700 bp containing variable regions V1 to V3) were amplified using a bar-coded primer set [17]. Pyrosequencing was done by ChunLab (Seoul, Korea) using a 454 GS FLX Titanium Sequencing System (Roche, Basel, Switzerland). Taxonomic assignment of the bacterial high-quality reads was performed using the EzTaxon-e DB [18]. Sequences that cannot be matched to the EzTaxon-e DB at the species level (>97%) are subjected to a secondary process checking for chimeric sequences using the UCHIME program [19]. Operational taxonomic units (OTUs) were generated by CD-HIT program at a 97% similarity level, the Shannon–Weaver diversity index, Chao1 richness index and Goods library coverage were calculated by the Mothur package [20]. Bacterial taxonomic compositions concerning levels of phylum and genus were classified at a cut off level of > 0.05% relative abundance.

2.4. Statistical Analysis

Statistical analyses on animal performance, chemical materials and odor compounds were performed with SAS Enterprise Guide 7.13 HF4 (SAS Institute, Inc., NC, USA) [21]. Data were analyzed as a completely randomized design with each treatment containing experimental units with individual pigs. The general linear model procedure and Duncan's multiple range test were used to test for significant differences at $p < 0.05$. Treatment trends were discussed when $0.05 > p > 0.10$.

3. Results & Discussion

3.1. Animal Performance

The effect of dietary IRG powder on animal performance is shown in Table 2. Dietary inclusion of IRG resulted in no significant differences ($p > 0.05$). The amount and the physicochemical composition of fiber source in the diet influence the energy, nutrient digestion and growth of animal. Although IRG diet contains about 8% less digestible energy (DE) than control diet (0% IRG diet), pigs in IRG group showed similar levels of average daily gain (ADG) and gain:feed (G:F) to those in control. In study of Gutierrez et al. [22], diet with corn bran including low DE did not affect the average daily feed intake (ADFI), ADG and G:F. The levels of dietary fiber and crude protein in corn bran are similar to those of IRG. Pig can tolerate quite a wide range of dietary fiber; if the dietary energy density is adequately provided, animals could maintain a growth performance [23]. Concomitant to these data, pigs fed IRG diet which contains 0.1%–0.2% more crude fiber than control diet did not show detrimental effect on their growth performance.

Table 2. Animal performance of pigs fed increasing levels of IRG.

Items	IRG				SEM	Linear	Quadratic
	0%	0.5%	1.0%	1.5%			
Initial body weight (kg)	37.40	38.96	37.60	40.85	1.18	0.46	0.74
Final body weight (kg)	57.50	61.20	59.20	62.13	1.71	0.50	0.92
Average daily gain (kg day ⁻¹)	0.33	0.36	0.35	0.33	0.01	0.83	0.48
Gain/feed	0.19	0.20	0.19	0.18	0.01	0.69	0.48

Abbreviations: IRG—Italian ryegrass; SEM—standard error of the mean.

3.2. Chemical Materials and Odor Compounds of Manure

Manure characteristics including chemical materials and odor compounds are depicted in Tables 3 and 4, respectively. Compare to control group, both 1.0% and 1.5% IRG treatments increased significantly ($p < 0.05$) COD (17% and 28%, respectively) and SS (38% and 50%, respectively). COD and SS are the parameters for determining the amount of typical organic substance and level of odor release from

manure [24]. Pigs fed the diets with fiber had higher levels of organic indicator including solids, carbon and nitrogen in the manure [25]. Degradation of greater molecular weight components like fibers leads to an increase in the production of biodegradable soluble intermediates that can be metabolized by microbes [26].

Table 3. Chemical characterization of manure from pigs fed diets increasing levels of IRG.

Items (mg L ⁻¹)	IRG				SEM	Linear	Quadratic
	0%	0.5%	1.0%	1.5%			
pH	9.07	9.16	9.13	8.84	0.06	0.16	0.13
BOD	30,184	29,580	32,681	36,774	1318	0.05	0.39
COD	13,845	14,607	16,717	19,099	642	<0.01	0.45
SS	23,333	29,600	37,500	46,400	2881	<0.01	0.79
TN	7908	8533	8457	8974	276	0.22	0.93
NH ₄ -N	2174	2882	2937	2552	98	0.08	<0.01

Abbreviations: IRG—Italian ryegrass; BOD—biologic oxygen demand; COD—chemical oxygen demand; SS—suspended solids; TN—total nitrogen; NH₄-N—ammonium nitrogen; SEM—standard error of mean.

Table 4. Concentrations of volatile fatty acids and odorous compounds in manure from pigs fed diets with increasing levels of IRG.

Items (mg L ⁻¹)	IRG				SEM	Linear	Quadratic
	0%	0.5%	1.0%	1.5%			
AA	3604	2618	2745	3842	150.30	0.49	<0.01
PA	488	415	487	673	25.86	<0.01	0.01
BA	341	287	347	507	24.11	0.01	0.03
VA	86	75	83	138	7.85	0.01	0.04
i-BA	71	59	64	88	3.02	0.02	<0.01
i-VA	189	143	154	207	7.95	0.33	<0.01
SCFA	4520	3395	3662	5160	199.83	0.18	<0.01
BCFA	260	203	219	295	10.77	0.18	<0.01
Phenol	10.21	6.24	6.42	8.54	0.46	0.18	<0.01
<i>p</i> -Cresol	143.32	156.00	132.32	95.43	4.56	<0.01	<0.01
Indole	6.83	4.29	3.27	4.45	0.44	0.02	0.03
Skatole	2.18	1.98	1.60	1.33	0.09	<0.01	0.81
Phenols	153.53	162.24	138.74	103.97	4.48	<0.01	0.01
Indoles	9.01	6.28	4.87	5.78	0.47	<0.01	0.05

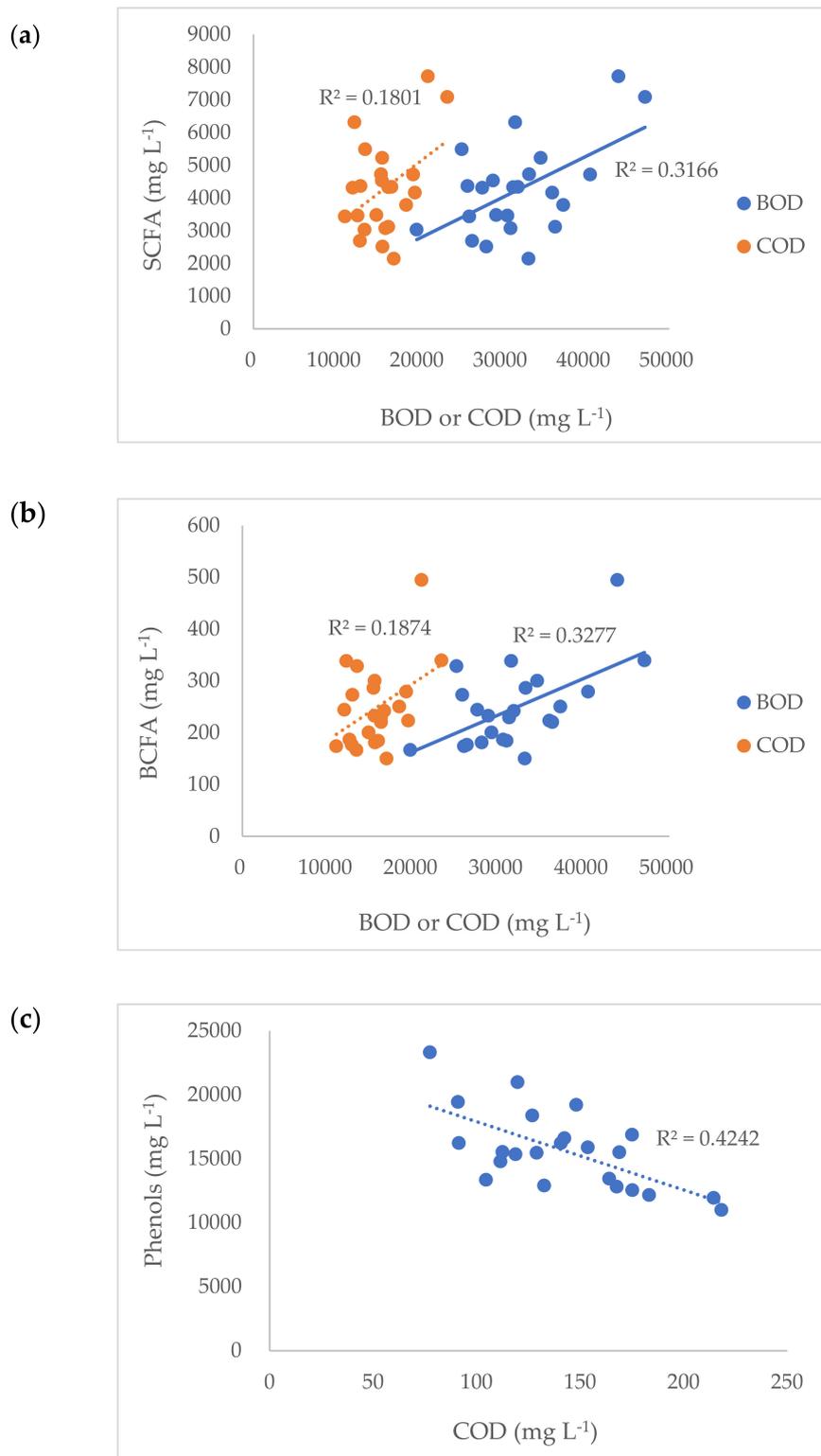
Abbreviations: IRG—Italian ryegrass; AA—acetic acid; PA—propionic acid; BA—butyric acid; VA—valeric acid; i-BA—iso-butyric acid; i-VA—iso-valeric acid; SCFA—straight chain fatty acid (sum of acetic acid, propionic acid, butyric acid and valeric acid); BCFA—branched chain fatty acid (sum of iso-butyric acid and iso-valeric acid); Phenols—sum of phenol and *p*-cresol; Indoles—sum of indole and skatole; SEM—standard error of mean.

Concentrations of SCFA and BCFA from manure were quadratically treatment effects ($p < 0.05$) upon dietary IRG (Table 4). Administration of 1.5% IRG increased the levels of both SCFA and BCFA by 12% relative to control, respectively. However, decreasing effect in VFA was found in 0.5% or 1.0% IRG group. Dietary fiber contains considerable amount of FC. Between 10 and 60 g day⁻¹ of dietary carbohydrate reaches in the large intestine [27]. The principal sources are resistant starch (RS; 8–40 g day⁻¹), non-starch polysaccharides (NSP; 8–18 g day⁻¹), unabsorbed sugars (2–10 g day⁻¹) and oligosaccharides (2–8 g day⁻¹) [28]. Carbohydrates are metabolized into SCFA (40%), lactate (15%), CO₂ (5%) and bacterial biomass (40%) [29]. SCFA level was remarkably increased by supplementation of inulin, a rich FC source, in pigs [30,31]. When pigs fed diets supplemented with 10% soybean hull, SCFA increased by 32%, but other odorants decreased by 10%–20% [32]. VFA concentrations depend on the type and level of FC included in diets [33]. SCFA can provide about 24%–30% of total energy needed for growing pigs [34]. The amount of carbohydrate consumed by animals has a profound influence on the rate of SCFA absorption by regulating the fermentation process in the large

intestine [35–37]. Excessive consumption of FC, however, results in excretion of larger amount of SCFA that has not been converted to metabolic energy.

Dietary IRG supplementation effected the phenols and indoles levels in manure showing both linear ($p < 0.05$) and quadratic ($p < 0.05$) effects (Table 4). Manure from 1.0% and 1.5% IRG groups had lower 10% and 32% phenols than those in control. Level of *p*-cresol, which is the main compound of phenols, was significantly lower in manure of finishing pigs fed diets with RS than in control [38]. Indoles were decreased by 46% and 36% in 1.0% and 1.5% IRG treatment, respectively. In particular, skatole concentration was linearly decreased (9%–39%) by increasing IRG levels (0.5%–1.5%). Dietary fiber content is closely associated with protein excretion levels [39]. The lower degradability of fiber is in the hindgut, the greater excretion of amino acids is [22]. However, dietary FC decreased the excretion of indoles in pig manure on the low digestion rate of tryptophan in the large intestine [40–42]. Five percent chicory inulin supplementation decreased the skatole level in manure of growing pig [30]. The inclusion of FC supports the growth of beneficial bacteria in that highly activated bacterial population accelerates protein synthesis thus decreases proteolytic fermentation [43]. Consequently, odorous chemical production which is originated from protein excretion is reduced in manure. In addition, butyrate, which is known to decrease mucosal apoptosis, limits the availability of endogenous tryptophan [44,45]. We found significant decrease in skatole level with increased SCFA by 1.5% IRG treatment. The antimicrobial effect of acidic conditions by VFA suppresses the rate of skatole synthesis by inhibiting the growth of skatole-producing bacteria [42,46,47].

Biochemical and physical factor of manure presented the linear correlation coefficients with odor production [48,49]. In this study, BOD and COD showed significantly ($p < 0.05$) positive related to SCFA (Figure 2a) and BCFA (Figure 2b), but COD had a negative correlation to phenols (Figure 2c). The chemical parameters of manure are used to detect the organic compounds in wastewater. BOD is an indicator of dissolved oxygen content that is consumed by bacteria [50]. The decreasing BOD levels have shown to inhibit the production of odorous compounds, resulting from decrease in the degradation rate of organic matter by bacteria [51]. Highly significant correlation coefficients were found in linear relationship between VFA and BOD produced during manure storage [52,53]. BOD exists a definite correlation with COD under wastewater treatment systems. In anaerobic treatment, COD is a factor to estimate the removal efficiencies of phenol compounds from wastewater [54,55]. Phenolic compounds could release from the degradation of lignocellulose that contains up to 40%–50% in total solids of pig manure. However, substantial amount of lignocellulose is difficult to biodegrade by bacteria [56]. COD is measured by chemical digestion of organic matter including lignocellulosic substances (cellulose, hemicellulose and lignin) with acidic reagents [50]. Therefore, phenols have the positively relation with COD, but in this study showed opposite pattern. In the study of Birkett et al. [57], total excretion of phenols decreased significantly from the high RS diets, whereas nitrogen output increased. Because bacteria require the nitrogen sources to growth and also acts as nitrogen sinks in manure, increased bacterial growth by FC is higher manure excretion of nitrogen as organic matter. In this study, concentration of TN increased by adding IRG, whereas phenols decreased (Table 3). Consequently, negatively correlation would be found between COD and phenols.



Abbreviations: SCFA—short chain fatty acid (sum of acetic acid, propionic acid, butyric acid and valeric acid); BCFA—branched chain fatty acid (sum of iso-butyric acid and iso-valeric acid); Phenols—sum of phenol and *p*-cresol; BOD—biochemical oxygen demand; COD—chemical oxygen demand; $p < 0.05$.

Figure 2. Relationship between chemical materials and odorous compounds in pigs' manure. (a) BOD or COD with SCFA; (b) BOD or COD with BCFA; (c) COD with phenols.

3.3. Bacterial Community Composition

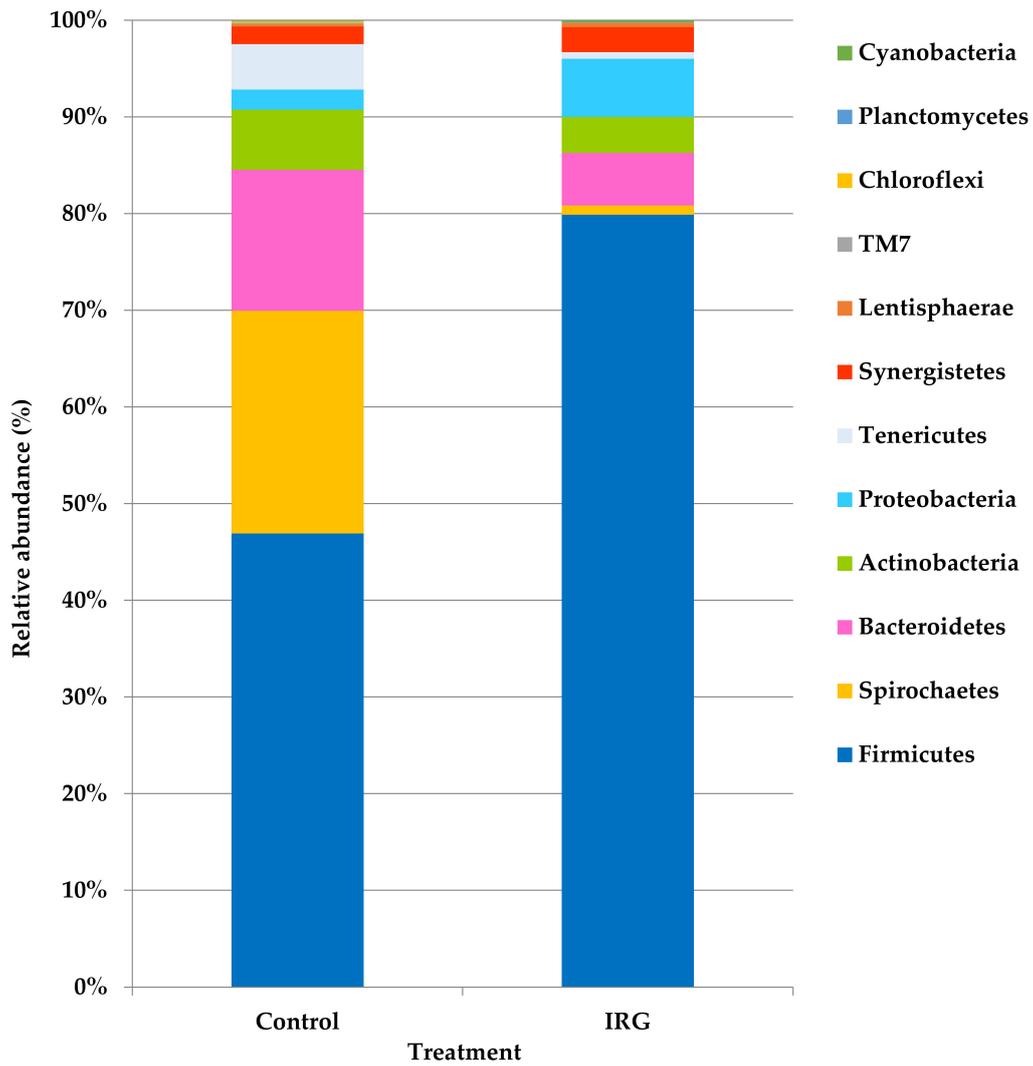
Formation of odor compounds are influenced by changes in bacterial diversity and composition from diet formulation [58]. The IRG 1.0% group was chosen for analysis of the changes in bacterial community based on overall odor reducing efficiency of manure in the current study. Concomitant to previous result, control (IRG 0%) and IRG 1.0% treatment group showed different correlation in the level of odorous compounds with the structure of bacterial community. Total reads of pyrosequencing were 9344 and 6552 in the control and IRG group, respectively (Table 5). After the removal of low-quality reads, 6589 (control) and 4041 (IRG) sequences were used for further analysis. Although the IRG group had a lower number of bacterial reads than control, the diversity indices as OTUs, Shannon–Weaver and Chao1 were not different between control and IRG group.

Table 5. Bacterial enumeration and diversity indices for manure of pigs fed a diet with control (0% IRG) or IRG (1.0%).

Items	Treatment	
	Control	IRG
No. of total reads	9344	6552
No. of high-quality reads	6589	4041
Read length (bp)	504	504
OTUs	1336	1100
Shannon–Weaver index	5.06	5.31
Chao1 index	2780	2230
Goods library coverage	0.92	0.90

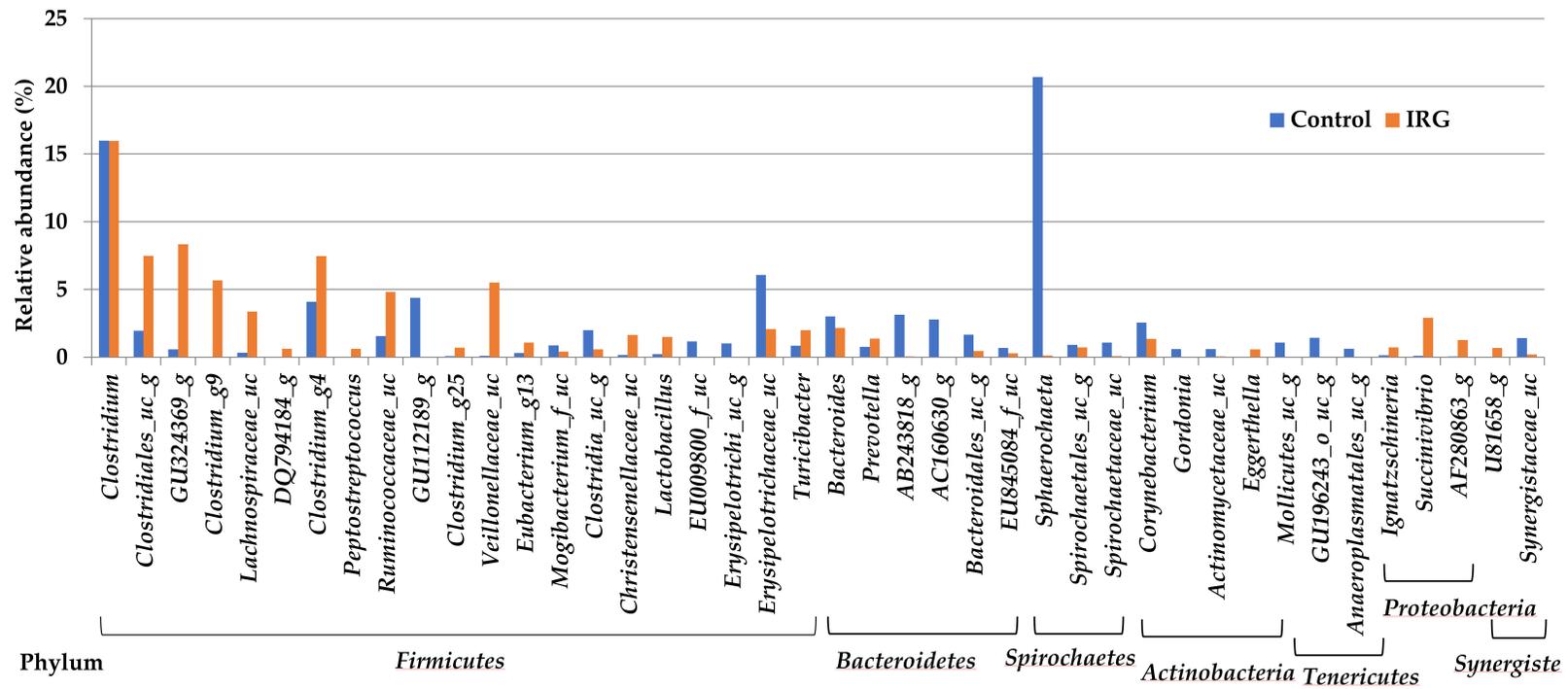
Abbreviations: IRG—Italian ryegrass; OTUs—operational taxonomic units.

Taxonomic profiles for bacterial communities from treatment groups are shown in Figures 3 and 4. Twenty-one phyla were represented among the OTUs with the vast majority classified to 8 major phyla: *Firmicutes*, *Spirochaetes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Tenericutes*, *Synergistetes* and *Lentisphaerae* (Figure 3). The relative abundances of *Firmicutes* and *Proteobacteria* were higher by 33% and 4%, respectively, in IRG group than control. *Spirochaetes*, *Bacteroidetes*, *Actinobacteria* and *Tenericutes* were lower by 22%, 9%, 2% and 4%, respectively, in IRG group than control. Differences between treatment groups were identified on the genus level (Figure 4). Six hundred and three bacterial genera were represented and distributed to the following phyla: *Firmicutes* (43%), *Proteobacteria* (20%), *Bacteroidetes* (11%), *Actinobacteria* (10%), *Tenericutes* (3%), *Lentisphaerae* (3%), *Synergistetes* (2%) and *Spirochaetes* (1%). Bacteria of Gram-positive by 60% and Gram-negative by 40% were classified in these genera. Among the dominant genera, the relative abundances of genera belong to the families *Lachnospiraceae* (*GU324369_g*, *Clostridium_g9*, *Lachnospiraceae_uc* and *DQ794184_g*), *Ruminococcaceae* (*Ruminococcaceae_uc*, *GU112189_g* and *Clostridium_g25*), *Veillonellaceae* (*Veillonellaceae_uc*), *Peptostreptococcaceae* (*Clostridium_g4* and *Peptostreptococcus*) and *Lactobacillaceae* (*Lactobacillus*) of phylum *Firmicutes* were higher in IRG group than control.



Abbreviations: IRG—Italian ryegrass; Bacterial phyla were classified at a cut off level of >0.05% relative abundance.

Figure 3. Bacterial taxonomic compositions at phylum level for manure of pigs fed a diet with control (0% IRG) or IRG (1.0%).



Abbreviations: IRG—Italian ryegrass; Bacterial genera were classified at a cut off level of >0.05% relative abundance.

Figure 4. Bacterial taxonomic compositions at genus level for manure of pigs fed a diet with control (0% IRG) or IRG (1.0%).

Firmicutes account for 50%–80% in the community of core intestinal bacteria. *Lachnospiraceae* and *Ruminococcaceae* are the most abundant families in order *Clostridiales* of phylum *Firmicutes*, accounting for roughly 50% and 30% of *Firmicutes*, respectively [59,60]. These families obligated fibrolytic and saccharolytic microbes produce SCFA [61,62], in particular butyric acid [63], by the fermentation of dietary fibers. *Veillonellaceae* produces acetate and propionate from NSP supplemented diets [64,65] and has a positive correlation with isobutyrate and isovalerate production in deer [66]. *Peptostreptococcaceae* and *Lactobacillales* were also associated with the increased VFA concentrations by FC supplementation. The abundance of *Peptostreptococcus spp.* was increased in feces of pigs fed a diet including 8.7% RS [67]. It has been shown that *Lactobacillales* has grown up a large amount in the colon of humans (Oligosaccharides) [68], rats (Sorghum bran) [69] and dogs (Mixture of fiber and resistant starch) [70] fed carbohydrate supplemented diets. In addition, the increase of *Lactobacillus* supports the observation of increased VFA levels in the colon and feces of humans (Apple) [71], pigs and rats (RS and arabinoxylan) [72] fed FC diets. However, our results suggest that an increase in relative abundances of the genera belonging to five families are not associated with the changes of VFA levels (Table 4 and Figure 4) implicating that other bacterial groups may be involved with the changes of VFA levels.

Genus *Sphaerochaeta* in phylum *Spirochaetes* was decreased in IRG (1%) group compared with control (IRG 0%). *Spirochaetes* are frequently detected in the wastewater of livestock production, industry and anaerobic digestion systems [73]. SCFA is mainly originated from carbohydrate fermentation [74] and also used by *Sphaerochaeta* under anaerobic conditions [75,76]. More than 10% of the core genes and presumably more than 50% of the auxiliary and secondary metabolism genes of *Sphaerochaeta* are acquired from members of *Firmicutes*, especially order *Clostridiales*. Several genes related to carbohydrate metabolism of *Spirochetes spp.* have been acquired from co-occurring members of *Clostridiales* in the large intestine of pig [77]. In addition, two bacterial members have a common ecological niche [10]. Biomass yields of cellulose degradation by *Clostridiales* increase by co-culture with *Spirochaeta* [78]. In our current study, an increase in the proportion of order *Clostridiales* (from 30% to 62%) belonging to representative families of *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae* and *Peptostreptococcaceae* under the IRG supplemented condition may be associated with a decrease of *Spirochaetes* (from 22% to 1%).

In addition, order *Clostridiales* were also identified in many studies as aromatic hydrocarbon degraders [79,80]. Families belonging to this order were dominated in the waste with an anaerobic treatment and subsequent lower level of phenol [81]. Decrease in phenols in IRG treatment (IRG 1%) may be associated with enrichment of these families. By contrast, genus *AB243818_g* of family *Porphyromonadaceae* decreased in IRG group compared with control. This bacterial strain, which is dominant family of the phylum *Bacteroidetes*, produces skatole during the process of decarboxylation and deamination of tryptophan [82]. The increased relative abundance of order *Clostridiales* as acid producing bacteria inhibits the growth of skatole producing bacteria [42,46,47]. These results suggest that mechanism by which IRG treatment decreases the skatole level possibly via modulating the bacterial proportion in the current study.

4. Conclusions

Our results demonstrate that IRG supplementation in pig diets significantly affected the composition of odorous compounds, as well as the bacterial community in manure. Levels of odorous compounds—including phenols and indoles—were lower in manure of pigs fed supplementary IRG diets. In particular, skatole concentration dramatically decreased with increasing levels of dietary IRG. This is associated with changes in composition of bacterial community by IRG supplementation (IRG 1%) in that decrease the concentration of odorous compounds. IRG supplementation (IRG 1%) in pig diets modulate relative abundances of the genera belong to the family *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, *Peptostreptococcaceae* and *Lactobacillaceae* in order *Clostridiales* of phylum *Firmicutes*, which predominantly utilize carbohydrate and produce acidic compounds as byproducts of fermentation. The high levels of this bacterial strains led to decrease of skatole levels by inhibit the population

of skatole producing bacteria. Consequently, IRG supplementation (IRG 1%) has a beneficial role for controlling the odorous compound formations via improving gut fermentation in pigs. Further investigation on the land-applied manure is needed in order to comprehensively assess the impact of FC on manure odor reduction.

Author Contributions: Conceptualization, O.H. and S.C.; methodology, O.H. and S.C.; validation, O.H., S.C. and S.P.; formal analysis, O.H. and S.C.; investigation, O.H. and S.C.; data curation, O.H. and S.P.; writing—original draft preparation, O.H. and S.P.; writing—review and editing, O.H. and S.P.; visualization, O.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Institute of Animal Science (NIAS)—Rural Development Administration (RDA) Grant Number PJ90711003.

Acknowledgments: The authors would like to acknowledge the technical support provided by NIAS-RDA. This research was carried out with the support of “Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ90711003)” NIAS-RDA, Republic of Korea.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ADFI—average daily feed intake; ADG—average daily gain; BCFA—branched chain fatty acid; BOD—biochemical oxygen demand; BW—body weight; COD—chemical oxygen demand; DE—digestible energy; FC—fermentable carbohydrate; GC—gas chromatography; G:F—gain:feed; IRG—Italian ryegrass; NH₄-N—ammonium nitrogen; NSP—non-starch polysaccharides; OTUs—operational taxonomic units; RS—resistant starch; SCFA—short chain fatty acid; SS—suspended solids; TN—total nitrogen; VFA—volatile fatty acid.

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