

# JAST (Journal of Animal Science and Technology) TITLE PAGE

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ARTICLE INFORMATION	Fill in information in each box below
<b>Article Type</b>	Research article
<b>Article Title (within 20 words without abbreviations)</b>	Effects of Italian ryegrass silage-based TMR on rumen fermentation, growth performance, blood metabolites, and bacterial communities of growing Hanwoo heifers
<b>Running Title (within 10 words)</b>	Italian ryegrass silage-based TMR for growing Hanwoo heifers
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<b>Competing interests</b>	No potential conflict of interest relevant to this article was reported.
<b>Funding sources</b> State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This study was supported by Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ013609), Rural Development Administration, Republic of Korea.
<b>Acknowledgements</b>	Not applicable.
<b>Availability of data and material</b>	Upon reasonable request, the datasets of this study can be available from the corresponding author.
<b>Authors' contributions</b> Please specify the authors' role using this form.	Conceptualization: Ku MJ, Jeong CD, Lee SS. Data curation: Ku MJ, Miguel MA, Kim SH, Ramos SC. Formal analysis: Ku MJ, Miguel MA, Kim SH, Jeong CD, Ramos SC. Methodology: Ku MJ, Miguel MA, Kim SH, Jeong CD, Ramos SC, Son AR, Cho YI, Lee SS. Software: Ku MJ, Miguel MA. Validation: Lee SS. Investigation: Ku MJ, Miguel MA, Kim SH, Lee SS. Writing - original draft: Ku MJ, Miguel MA. Writing - review & editing: Ku MJ, Miguel MA, Kim SH, Jeong CD, Ramos SC, Son AR, Cho YI, Lee SS, Lee SS.
<b>Ethics approval and consent to participate</b>	The experimental protocol was approved by the Institutional Animal Care and Use Committee of Suncheon National University (approval number: SCNU-IACUC 2018-01).

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## 1 **Abstract**

2 This study utilized Italian ryegrass silage (IRGS) - based TMR as feedstuff and evaluated its  
3 effects on rumen fermentation, growth performance, blood parameters, and bacterial community in  
4 growing Hanwoo heifers. Twenty-seven Hanwoo heifers (body weight,  $225.11 \pm 10.57$  kg) were  
5 randomly allocated to three experimental diets. Heifers were fed 1 of 3 treatments as follows: TMR  
6 with oat, timothy, and alfalfa hay (CON), TMR with 19% of IRGS (L-IRGS), and TMR with 36% of  
7 IRGS (H-IRGS). Feeding high levels of IRGS (H-IRGS) and CON TMR to heifers resulted in a  
8 greater molar proportion of propionate in the rumen. The impact of different TMR diets on the BW,  
9 ADG, DMI, and FCR of Hanwoo heifers during the growing period did not differ ( $p > 0.05$ ).  
10 Furthermore, the blood metabolites, total protein, albumin, AST, glucose, and total cholesterol of the  
11 heifers were not affected by the different TMR diets ( $p > 0.05$ ). In terms of rumen bacterial  
12 community composition, 264 operational taxonomic units (OTUs) were observed across the three  
13 TMR diets with 240, 239, and 220 OTUs in CON, L-IRGS, and H-IRGS, respectively. IRGS-based  
14 diets increased the relative abundances of genera belonging to phylum *Bacteroidetes* but decreased  
15 the abundances of genus belonging to phylum *Firmicutes* compared with the control. Data showed  
16 that *Bacteroidetes* was the most dominant phylum, while *Prevotella ruminicola* was the dominant  
17 species across the three TMR groups. The relative abundance of *Ruminococcus bromii* in the rumen  
18 increased in heifers fed with high inclusion of IRGS in the TMR (H-IRGS TMR). The relative  
19 abundance of *R. bromii* in the rumen significantly increased when heifers were fed H-IRGS TMR  
20 while *P. ruminicola* increased in both L-IRGS and H-IRGS TMR groups. Results from the current  
21 study demonstrate that the inclusion of Italian ryegrass silage in the TMR is comparable with the  
22 TMR containing high-quality forage (CON). Thus, a high level of IRGS can be used as a replacement  
23 forage ingredient in TMR feeding and had a beneficial effect of possibly modulating the rumen  
24 bacterial community toward mainly propionate-producing microorganisms.

25

26 **Keywords:** bacterial community; growth performance; Hanwoo; Italian ryegrass silage; rumen

## Introduction

Forage is the most economical type of feed utilized for beef and milk production. Feed costs during beef production could be reduced by efficient production and utilization of forage with high yields and high nutritional value [1]. Moreover, forage-based production systems are low-input approaches that are well suited to satisfy the demands of meat retailers and consumers for the production of beef that is ecological and animal-friendly. Hanwoo (*Bos taurus coreanae*), a major beef cattle raised for highly marbled beef in Korea, are reared using a combination of roughage and commercially available feeds [1,2]. The consumption of livestock products in Korea has increased, thus, the demand for roughage also increased. The local farmers opt to utilize domestically grown crops or crop silages in place of or in addition to imported forages in TMR production [2–4]. Hence, in recent years, domestic forage has been utilized to reduce the necessity for imported forage

TMR is generally prepared by mixing concentrates and traditional roughages such as silage, forage, and hay; however, due to pasture shortages, many countries mostly rely on imported feed resources [5]. In 2020, Japan, China, United Arab Emirates, South Korea, and Saudi Arabia were the major importers of high-quality forage crops primarily from the United States, Australia, Canada, Spain, and Italy [6]. Imported forage is more costly than locally grown forage due to higher transportation costs, which account for the majority of production costs [3,5,7]. As of 2020, the overall forage consumption in Korea is 4.82 million tons, wherein the domestic production is 3.92 million tons. Despite the fact that the cultivated area of forage in Korea has expanded greatly compared to the past, the domestic forage self-sufficiency rate has increased to 81.4%, 26.1% of which is accounted for high-quality forage [8]. The livestock sector's reliance on high-quality forage drives the need for imported forages in Korea [9]. In addition, farmers tend to believe that domestically produced forages are of low quality compared to imported forages, which is not always the case [10–12]. Among imported high-quality forages, alfalfa (*Medicago sativa*), timothy (*Phleum pratense*), and oat (*Avena sativa*) hays are commonly used forages in Korea [10,11,13]. Imports of hay from the USA account for more than 80% of overall hay imports [13]. Alfalfa is a very important forage for livestock feeding due to its high crude protein content; however, alfalfa is rarely cultivated in Korea and the main source is imported hay. About 200,000 tons of alfalfa hay per year was imported, which accounts for 20% of the total imported hay in Korea [14,15]. However, there has been great interest in using good quality domestic forages such as Italian ryegrass, whole-crop barley, and whole-crop rice as a replacement for imported forages to reduce the production cost. Italian ryegrass (*Lolium multiflorum* Lam., var. *italicum*, IRG) is a highly valuable crop grown domestically as a winter forage crop due to its feed value, good nutritive quality, high yield, and palatability [2,16–18] and has the potential to reduce the necessity for imported forages in TMR production. In Korea, IRG accounts for 97% of the total cultivated area of winter forage crops and 59.9 % of the total forage production in 2017. In the southern area of Korea, particularly in Jeonnam province, 62% of the production area was used for the cultivation of Italian ryegrass [7,19,20]. Due to its high protein content and energy efficiency, IRG has become increasingly popular among beef producers as a

65 roughage source. It can be provided alone (hay or silage) or as a component of TMR [2,5].  
66 Domestically grown IRG had reduced pasture cost by up to 30 – 50% compared to imported forages  
67 [19]. The replacement of imported forage with domestically grown high-quality roughage or crop  
68 silages has environmental and economic advantages, and it may reduce the feeding costs and the need  
69 for imported feed ingredients. This study was conducted to replace imported forages such as alfalfa  
70 and timothy hays by utilizing domestic IRGS in TMR production. Thus, the study evaluated the  
71 Italian ryegrass silage (IRGS) as a replacement ingredient for imported forages in TMR production  
72 and its effect on rumen fermentation, growth performance, blood metabolites, and rumen microbial  
73 communities of Hanwoo heifers during the growing period.

74

75

## Materials and Methods

76

### 77 **Animals, diets, and experimental design**

78 The experimental protocol was approved by the Institutional Animal Care and Use  
79 Committee (IACUC) of Sunchon National University (approval number: SCNU-IACUC 2018-01).  
80 The study was carried out at the experimental farm of Sunchon National University and in the  
81 Ruminant Nutrition and Anaerobe Laboratory, Department of Animal Science and Technology,  
82 SCNU, Jeonnam, South Korea. The TMR diets were formulated to replace or mix the forages (oat hay,  
83 timothy, and alfalfa hay) with Italian ryegrass and corn silages. Three TMR diets were used: i) TMR  
84 containing oat hay, timothy, and alfalfa hay (CON), ii) TMR containing 19% of IRGS (dry matter  
85 (DM) basis) (L-IRGS), and iii) TMR containing 36% of IRGS (DM basis) (H-IRGS). The  
86 composition of the TMRs is shown in Table 1.

87

### 88 **Growth performance and blood profiles of growing Hanwoo heifers**

89 The feeding trial was conducted for 140 days. Twenty-seven Hanwoo heifers ( $225.11 \pm 10.57$   
90 kg, 8 months old) were used in the study. The heifers were randomly distributed into three groups of  
91 nine heifers each to evaluate three TMR diets (CON, L-IRGS, and H-IRGS), wherein 3 animals were  
92 allotted in each pen. TMR diets were prepared weekly, animals in each pen were fed once daily  
93 (08:00) for a total period of 140 days and had free access to mineral block and water. Individual pens  
94 were equipped with an electronic feed bunk monitoring system that enables monitoring of the feed  
95 intake of the animal.

96 The initial and final body weight (BW) of individual heifers was recorded before feeding.  
97 Average daily gain (ADG) was calculated based on the difference in BW measured in different  
98 periods divided by the number of days of the experimental period, while the feed conversion ratio  
99 (FCR) was expressed as average DMI per ADG. At the end of the experimental period, ruminal fluid  
100 and blood were obtained from each heifer. Ruminal fluid was collected using a stomach tube and the  
101 rumen sample was placed in a sterile 50 ml conical tube. Aliquot samples (1 ml) were prepared in 1.5  
102 ml microcentrifuge tubes and stored at  $-80\text{ }^{\circ}\text{C}$  until analysis for rumen fermentation characteristics

103 and metagenomics sequencing. Meanwhile, approximately 5 mL of blood was taken from the jugular  
104 vein of each heifer and transferred into a vacutainer tube (SSTTM II Advance, BD vacutainer®). The  
105 serum was separated by centrifugation for 5 mins at 4000 rpm using a refrigerated centrifuge.  
106 Following centrifugation, serum was carefully removed using a pipette, transferred to a 1.5 ml  
107 microcentrifuge tube, and stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. Concentrations of aspartate  
108 aminotransferase (AST), blood urea nitrogen (BUN), albumin (ALB), total protein (TP), total  
109 cholesterol (TC), and glucose (GLU) in the serum were analyzed by IDEXX Catalyst One Chemistry  
110 Analyzer (IDEXX Laboratories Inc., USA).

111

### 112 **Analysis of ruminal fermentation parameters**

113 Rumen fluid was analyzed for its fermentation parameters, including pH, ammonia-nitrogen  
114 ( $\text{NH}_3\text{-N}$ ), and volatile fatty acid (VFA) concentrations. The pH of the ruminal fluid was measured  
115 using a pH meter (Mettler Toledo, Greifensee, Switzerland). Aliquot rumen fluid samples were  
116 transferred in 1.5 ml microcentrifuge tubes and stored at  $-80\text{ }^{\circ}\text{C}$  until  $\text{NH}_3\text{-N}$  and VFA analyses. The  
117 frozen rumen samples were thawed at room temperature and centrifuged for 10 min at 14,000 rpm and  
118  $4\text{ }^{\circ}\text{C}$ . Then, the resulting supernatant was used for the  $\text{NH}_3\text{-N}$  and VFA concentration analyses.  
119 Ruminal  $\text{NH}_3\text{-N}$  concentration was determined by a colorimetric method as described by Chaney and  
120 Marbach [21]. The concentration of VFA in the ruminal fluid was analyzed by high-performance  
121 liquid chromatography (HPLC) on an Agilent 1200 Series HPLC System (Agilent Technologies,  
122 USA) equipped with a MetaCarb 87H column ( $300\text{ mm} \times 7.8\text{ mm}$ ) under isocratic conditions (flow  
123 rate =  $0.6\text{ ml/min}$  of  $0.0085\text{ N}$  sulfuric acid ( $\text{H}_2\text{SO}_4$ ); column temperature =  $35\text{ }^{\circ}\text{C}$  [22,23].

124

### 125 **DNA extraction, PCR amplification, and 16S rRNA gene sequencing**

126 Rumen samples were thawed at room temperature and centrifuged at 14,000 rpm for 10 min  
127 at  $4\text{ }^{\circ}\text{C}$ . The supernatant was discarded, and the resulting pellet was used to extract genomic DNA  
128 using a FastDNA SPIN Kit (MP Biomedicals, USA), following the manufacturer's protocol. The  
129 extracted DNA was sent to Macrogen, Korea, for 16S ribosomal RNA (rRNA) gene amplification and  
130 metagenomics sequencing.

131 The amplicon sequencing approach was performed according to the Illumina 16S  
132 Metagenomic Sequencing Library protocols [7]. Amplicon libraries targeting the V3–V4  
133 hypervariable region of the 16S rRNA gene were generated by PCR amplification which consists of  
134 two PCR steps. The genomic DNA was amplified by using a universal primer pair with Illumina  
135 adapter overhang sequences, 16S\_341F ( $5'\text{-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA}$   
136  $\text{CAG CCT ACG GGN GGC WGC AG-3'}$ ) and 16S\_805R ( $5'\text{-GTC TCG TGG GCT CGG AGA TGT}$   
137  $\text{GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3'}$ ). The PCR mixture was  
138 conducted in a final volume of  $25\text{ }\mu\text{L}$ , consisting of  $2.5\text{ }\mu\text{L}$  of DNA sample ( $\sim 5\text{ ng}/\mu\text{L}$ ),  $5\text{ }\mu\text{L}$  of each  
139 forward and reverse primer, and  $12.5\text{ }\mu\text{L}$  of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems,  
140 Wilmington, MA, USA). The cycling condition for the first PCR was performed as follows: initial

141 denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at  
142 55 °C for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The  
143 amplicons were purified using AMPure XP beads (Agencourt Bioscience, Beverly, MA). After  
144 purification, 2 µL of the first PCR product was used as a template for the second PCR using a Nextera  
145 XT Index primer pair (Illumina<sup>®</sup>, USA). The PCR consisted of 5 µL of sample DNA, 5 µL each of  
146 Nextera XT Index primers 1 and 2, 25 µL of 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems,  
147 Wilmington, MA, USA), and 10 µL of PCR Grade Water. The cycle condition was: initial  
148 denaturation at 95 °C for 3 min, followed by 8 cycles of denaturation at 95 °C for 30 s, annealing at  
149 55 °C for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The final  
150 PCR amplicon was cleaned-up using the AMPure XP beads (Agencourt Bioscience, Beverly, MA).  
151 The quantity of the final PCR amplicon was evaluated according to the qPCR Quantification Protocol  
152 Guide (KAPA Library Quantification Kit for Illumina Sequencing platforms), and library quality was  
153 assessed using TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). Finally,  
154 the paired-end sequencing was performed on an Illumina MiSeq platform (San Diego, CA, USA)  
155 using v3 reagents to generate 300 bp paired-end reads.

156

### 157 **Sequencing analyses**

158 The paired-end sequence reads (Fastq files) were obtained using the bcl2fastq package  
159 (Illumina Inc., San Diego, CA, USA). Trimmomatic v0.38 was used to filter the adaptor sequences  
160 and remove low-quality sequences from the raw sequences [24] and assembled using Fast Length  
161 Adjustment of Short Reads (FLASH 1.2.11) [25]. Low-quality sequences, ambiguous sequences, and  
162 chimera sequences from the obtained sequences were removed using CD-HIT-OTU [26]. The filtered  
163 reads were then clustered and identified as OTU at 97% sequence similarity using CD-HIT-OTU [26],  
164 and chimeric sequences were identified and removed using rDnaTools  
165 (<https://github.com/PacificBiosciences/rDnaTool>). The taxonomy of the representative sequences  
166 from the clustered OTU was assigned using Quantitative Insights Into Microbial Ecology (QIIME  
167 Version 1) [27] from the NCBI 16S rRNA database, and the taxonomy composition from phylum to  
168 species level was generated using QIIME-UCLUST [28].

169 Further analysis and data visualization was performed in the MicrobiomeAnalyst tool  
170 (available at: <http://www.microbiomeanalyst.ca>) [29] using a BIOM formatted OTU table [12]  
171 generated in Mothur [30]. Alpha diversity of each sample was assessed using the observed OTUs,  
172 Chao1 estimator, and Shannon index. Venn diagram was generated using jvenn  
173 (<http://jvenn.toulouse.inra.fr/app/index.html>) to illustrate the shared and unique OTUs of rumen  
174 bacterial community in Hanwoo heifers fed different TMR [31]. The hierarchical clustering heat map  
175 was visualized using the MicrobiomeAnalyst tool using the Bray-Curtis dissimilarity test and Ward  
176 clustering algorithm [29]. Linear discriminant analysis (LDA) effect size (LefSe), which uses a non-  
177 parametric Kruskal-Wallis rank sum test and performs a linear discriminant analysis (LDA) to  
178 evaluate the effect size of each taxon, was performed in MicrobiomeAnalyst online tool.

179

## 180 **Statistical analysis**

181 Data were analyzed using the general linear model (GLM) procedure in Statistical Analysis  
182 Systems (SAS) version 9.4 (SAS Institute, Inc. Cary, NC, USA). Statistical comparison of data was  
183 performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test  
184 (DMRT). A  $p$ -value of less than 0.05 indicates a statistically significant result.

185

186

## **Results**

### 187 **Rumen fermentation parameters**

188 The results for rumen fermentation parameters are shown in Table 2. The total VFA,  
189 propionate, butyrate, and A/P ratio showed significant differences between the three TMR diets ( $p <$   
190 0.05). Meanwhile, no significant differences in the rumen pH,  $\text{NH}_3\text{-N}$ , and acetate concentration  
191 between the three TMR diets were observed. However,  $\text{NH}_3\text{-N}$  concentration tended to be lower in  
192 TMR diets containing IRGS (L-IRGS and H-IRGS) than in CON TMR. The total VFA concentration  
193 was significantly higher in CON TMR diet ( $p < 0.05$ ) than in other TMR diets. Meanwhile, heifers fed  
194 CON and H-IRGS TMR diets have a higher molar proportion ( $p < 0.05$ ) of propionate in comparison  
195 to L-IRGS TMR diet. The molar proportion of butyrate in the rumen was greater ( $p < 0.05$ ) when  
196 heifers were fed L-IRGS TMR diet in comparison to other TMR diets. Lower acetate-to-propionate  
197 (A/P) ratio was observed in heifers fed CON and H-IRGS TMR diets compared with L-IRGS ( $p <$   
198 0.05).

199

### 200 **Growth performance and blood profile of Hanwoo heifers**

201 The growth performance and blood metabolites of Hanwoo heifers fed different TMRs are  
202 presented in Tables 3 and 4. The Hanwoo heifers were fed different TMR for 140 days (May to  
203 December 2017). As shown in Table 3, the body weight, ADG, DMI, and FCR of Hanwoo heifers fed  
204 with IRGS-based TMR did not differ ( $p > 0.05$ ) from those heifers fed with CON TMR. These results  
205 suggest that the different TMR diets did not change the overall growth performance of the heifers.  
206 Meanwhile, the total protein, albumin, AST, glucose, and total cholesterol were not affected by the  
207 TMRs diets ( $p > 0.05$ ) (Table 4). The Hanwoo heifers fed with CON TMR had the highest BUN  
208 concentration (14.50 mg/dL), while H-IRGS had the lowest BUN concentration of 12.44 mg/dL ( $p <$   
209 0.05).

210

### 211 **Bacterial diversity and composition**

212 In total, 264 OTUs in the ruminal fluid were identified at 97% similarity. Observed species,  
213 Chao1, and Shannon index did not significantly vary among the rumen samples of the three Hanwoo  
214 heifer groups ( $p > 0.05$ ) (Figure 1). The number of observed OTUs and Chao1, although not  
215 significant, was higher in CON and L-IRGS than in H-IRGS (Figures 1a and 1b). Meanwhile, the  
216 Shannon diversity index showed the most diverse species in CON compared to the L-IRGS and H-

217 IRGS (Figure 1c). As an indicator of microbial diversity, the Good's coverage of all samples was  
218 more than 98% (data not shown), indicating that the obtained sequences could reflect most of the  
219 bacterial community in this study.

220 Venn diagram showed that 194 OTUs were shared by all groups (Figure 2). For OTUs shared  
221 by two groups, CON TMR shared 27 and 13 OTUs with L-IRGS and H-IRGS, respectively, while  
222 only 7 OTUs were shared by L-IRGS and H-IRGS. Meanwhile, 6, 11, and 6 unique OTUs were  
223 observed in the CON, L-IRGS, and H-IRGS groups, respectively. The unique bacterial species found  
224 in the rumen of Hanwoo heifers fed with CON TMR were *Desulfotobacterium dehalogenans*,  
225 *Desulfovibrio simplex*, *Erysipelothrix rhusiopathiae*, *Flavimarina pacifica*, *Oribacterium parvum*, and  
226 *Pelobium manganitolerans*. On the other hand, *Eubacterium hallii*, *Acholeplasma brassicae*,  
227 *Anaerosporebacter mobilis*, *Desulfovibrio longreachensis*, *Fournierella massiliensis*, *Fusibacter*  
228 *paucivorans*, *Lachnobacterium bovis*, *Merdimonas faecis*, *Neglecta timonensis*, *Prevotella enoeca*,  
229 and *Xanthomonas maliensis* were observed only in the rumen of Hanwoo heifers fed with L-IRGS  
230 TMR. Meanwhile, *Clostridium lavalense*, *Desulfovibrio intestinalis*, *Fucophilus fucoidanolyticus*,  
231 *Lactobacillus rogosae*, *Mariniradius saccharolyticus*, and *Robinsoniella peoriensis* were observed  
232 only in the rumen of Hanwoo heifers fed with H-IRGS TMR.

233 The normalized data presented in Figure 3 shows the clustering based on the similarity of  
234 relative abundance between representative genera (row) and different TMR diets (column). The  
235 clustering in the column indicates that the bacterial community composition of the CON is different  
236 compared to the treated group (L-IRGS and H-IRGS). Meanwhile, the bacterial composition between  
237 L-IRGS and H-IRGS is relatively comparable. The genera with high and low abundance are indicated  
238 by the red and blue colors, respectively.

239 The effect of different TMR diets on the bacterial community composition is shown in Figure  
240 4. Illumina analysis of the bacterial community in rumen samples of the three groups of Fifteen  
241 bacterial phyla was classified in the rumen of Hanwoo heifers fed different TMR diets (Figure 3a).  
242 Taxonomic classification showed that *Bacteroidetes* and *Firmicutes* were the predominant phyla,  
243 which accounted for 64.47% and 33.05% of the total sequences, respectively. *Bacteroidetes* was  
244 observed to be higher in L-IRGS-fed Hanwoo heifers accounting for 72.53% of the bacterial  
245 population. Meanwhile, *Firmicutes* was lower in Hanwoo heifers fed L-IRGS TMR accounting for  
246 25.86%. At the genus level, the abundance of *Prevotella* increased in the rumen when Hanwoo heifers  
247 were fed with L-IRGS TMR (Figure 5). In addition, the abundance of *Ruminococcus* increased in the  
248 rumen when Hanwoo heifers were fed with H-IRGS. In contrast, Unclassified *Clostridiaceae* and  
249 Unclassified *Rikenellaceae* decreased in abundance when Hanwoo heifers were fed with TMR  
250 containing IRGS. *Prevotella ruminicola* dominated all treatments at the species level, with relative  
251 abundances of 26.66%, 43.36%, and 40.74%, for CON TMR, L-IRGS, and H-IRGS TMR,  
252 respectively (Figure 6). Meanwhile, the relative abundance of *Ruminococcus bromii* increased when  
253 the H-IRGS TMR was fed to the Hanwoo heifers.

254 To determine the microorganisms that most likely explained significant differences among  
255 samples from the three groups (CON, L-IRGS, H-IRGS), we performed a linear discriminant analysis  
256 of the effect size (LEfSe) (Figure 7). The LEfSe analysis revealed the top 15 significant  
257 microorganisms in the three groups, including five taxa in CON group, four taxa in H-IRGS, and six  
258 taxa in L-IRGS group. The CON group increased the abundance of *Clostridium cellulolyticum*,  
259 *Olivibacter sitiensis*, *Intestinimonas butyriciproducens*, *Bacteroides faecichinchillae*, and  
260 *Christensenella timonensis*. Meanwhile, bacterial species such as *Ruminococcus bromii*, *Blautia*  
261 *caecimuris*, *Mycoplasma muris*, and *Galbibacter mesophilus* was enriched in H-IRGS. In contrast,  
262 *Prevotella ruminicola*, *Paludibacter propionicigenes*, *Prevotella oralis*, *Flintibacter butyricus*,  
263 *Acidaminococcus intestini*, and *Prevotella micans* was increased in L-IRGS fed heifers.  
264

## 265 Discussion

266 This study evaluated the IRGS-based TMR as feedstuff and its effect on rumen fermentation,  
267 growth performance, and bacterial community composition in growing Hanwoo heifers. Three TMR  
268 diets (CON, L-IRGS, and H-IRGS TMR) were used in the study. In the present study, it was observed  
269 that TMR containing oat, alfalfa, and timothy hays as forages resulted in higher total concentration of  
270 VFA in the rumen than other treatments. Several studies reported that feeding TMR with the inclusion  
271 of forages such as oat, timothy, and alfalfa hays resulted in high concentration of total VFA [32–34].  
272 In addition, the molar proportion of propionate in the rumen was significantly higher in the CON and  
273 H-IRGS TMR. Propionate is the most essential VFA precursor of glucose synthesis, and hence has a  
274 significant impact on hormonal release and tissue distribution of nutrients [35,36]. Consequently, as a  
275 result of H-IRGS TMR feeding, the propionate proportion could contribute to the improvement of  
276 nutrient supply and utilization. Moreover, ruminal propionate concentration was reported to be higher  
277 in silage feedings than in hay feedings [37]. Silages contained high value of lactic acid; hence, as  
278 lactic acid is one of the precursors of propionic acid in the rumen, the concentration of propionate is  
279 expected to increase [37,38]. Thus, the increased molar proportion of propionate in this study might  
280 be attributed to the high lactic acid concentration contained in the H-IRGS TMR. Moreover, our  
281 previous study [3] showed that lactate production was higher in TMR containing IRGS than in hay-  
282 based TMR. In addition, the increase in the molar proportion of propionate might be attributed to the  
283 rumen microbes involved in the production of propionic acid, specifically, the *Ruminococcus bromii*  
284 [39]. The proportion of butyrate significantly increased in the L-IRGS TMR diet, apparently due to  
285 the changes in propionate concentration in TMR diets, whereas the acetate proportion remained  
286 constant throughout the study. CON- and H-IRGS-fed heifers had higher propionate and lower acetate  
287 concentrations; thus, a lower acetate-to-propionate ratio was expected [40,41]. Acetate-to-propionate  
288 ratio across experimental diets ranges from 2.84 – 3.06, which is higher than the threshold of 2.78,  
289 this reflects normal ruminal fermentation [42]. Meanwhile, CON had the lowest A/P ratios compared  
290 to the other TMR diets. Russel [43] reported that ruminal fermentation end products are dependent on  
291 diet, and the A/P ratio is generally lower for cereal grains than for forages. He added that the

292 association between the acetate-to-propionate ratio and diet has been explained by the metabolic  
293 characteristics of fiber-digesting and starch-digesting bacteria, this explanation, however, is not  
294 entirely conclusive. Some starch-digesting ruminal bacteria make large amounts of propionate, but  
295 many fiber-digesting bacteria produce large amounts of succinate, which is subsequently converted to  
296 propionate. In the present study, rumen ammonia-N concentrations did not differ among heifers fed  
297 different TMR diets; however, ammonia-N tended to be lower in TMR diets containing IRGS than in  
298 CON. The decrease in ammonia concentrations could be attributed to the increased consumption of  
299 ammonia generated by microbes with access to a readily accessible energy source, which increases  
300 microbial protein synthesis [41]. According to Satter and Slyter [44], a minimum ammonia-N  
301 concentration of 20-50 mg/L was required to sustain efficient microbial production in the rumen. In  
302 this study, ammonia-N concentration was lower than the optimal range. However, they also added that  
303 minimum levels of ammonia-N in ruminants fed fresh forage vary.

304 Different dietary treatments did not change the body weight, ADG, DMI, and FCR of  
305 Hanwoo heifers as there was no difference between CON TMR and IRGS-based TMR diets. These  
306 results imply that the TMR diets did not adversely affect the overall growth performance of the  
307 heifers. Conversely, as the body weight, ADG, DMI, and FCR were not affected, this could suggest  
308 that IRGS can be used as an alternative to high-quality imported forages in TMR production. IRGS  
309 has been utilized as feed due to its good nutritive value, high yield, good palatability, and is cheaper  
310 compared to imported high-quality forages such as alfalfa and timothy. Baldinger et al. [45] reported  
311 that the combination of Italian ryegrass silage and corn silage, which are both energy-rich forage,  
312 improved the DM intake. In this study, the inclusion of IRGS and corn silage in the TMR diet did not  
313 adversely change the rumen fermentation and growth performance of the heifers, implying that it is  
314 comparable to the control diet containing the imported forages. Thus, IRGS with corn silage can  
315 replace the imported forages in the TMR diet without compromising the rumen fermentation  
316 parameters and growth performance of the heifers. Furthermore, the TMR diets in this study have  
317 similar NDF values. Several studies reported that when the NDF content of a diet was the same, the  
318 forage source did not influence the ADG and growth efficiency in cattle [46–48]. Similarly, the NDF  
319 content of the TMR diets in our study was comparable, which might explain why there was no  
320 difference in the ADG and growth performance among the heifers. The blood urea nitrogen (BUN)  
321 was highest in the CON group and lowest in the H-IRGS group. Dietary N-to-energy ratio, forage  
322 intake level, protein degradability in the rumen, as well as dietary carbohydrate amount, and liver and  
323 kidney function in ruminants can directly influence the BUN [49]. The BUN is a good indicator of  
324 rumen ammonia concentrations, closely related to the solubility of nitrogenous compounds fed in the  
325 animal [50]. In addition, it serves as an indicator of microbial protein balance and dietary protein  
326 efficiency [49,51]. Our findings showed that BUN was significantly higher in the heifers fed CON  
327 TMR. Higher concentration of BUN in heifers fed CON TMR were consistent with higher ruminal  
328 NH<sub>3</sub>-N contents, indicating lower dietary nitrogen (N) efficiency. Meanwhile, a lower concentration  
329 of BUN was observed in heifers fed H-IRGS TMR, which indicates high nitrogen metabolism

330 capacity [52]. However, the BUN might be affected by similar starch and fermentable energy content  
331 of diets. According to DePeters and Ferguson [53], the ruminal NH<sub>3</sub>-N and BUN concentrations are  
332 highly correlated. In this study, the ruminal NH<sub>3</sub>-N was not affected by the TMR diets; however,  
333 NH<sub>3</sub>-N tended to be lower in TMR diets containing IRGS than in CON. improve N use efficiency  
334 through lower rumen NH<sub>3</sub>-N However, numerically higher rumen ammonia was observed in heifers  
335 fed TMR containing alfalfa hay. Heifers fed with IRGS-based TMR had higher DMI than heifers  
336 receiving the CON TMR diet. Moreover, the FCR of heifers receiving IRGS-based TMR was higher  
337 than those receiving CON TMR. Consequently, the growth of heifers fed IRGS-based TMR was  
338 comparable with CON TMR.

339 Our study explored the rumen bacterial diversity of Hanwoo heifers fed with different TMR  
340 diets. Alpha diversity indices had no significant differences between the TMR diets; however, species  
341 richness and evenness in the rumen bacterial community were higher in heifers fed with CON, which  
342 suggests that the rumen bacterial diversity of heifers fed TMR containing oat, timothy, and alfalfa  
343 hays were higher than the diversity in heifers fed TMR containing IRGS. In addition, this study  
344 observed significant shifts in the rumen bacterial populations in response to different TMR diets.  
345 *Bacteroidetes* and *Firmicutes* were found to be the most dominant phyla in the rumen of Hanwoo  
346 heifers. These findings are consistent with previous studies that reported *Bacteroidetes* and *Firmicutes*  
347 to be the most prevalent rumen phyla in ruminants [54,55]. Interestingly, the heifers fed a TMR diet  
348 containing IRGS had the highest abundance of *Bacteroidetes* and the lowest abundance of *Firmicutes*.  
349 In contrast, the CON TMR diet had the lowest abundance of *Bacteroidetes* (57.64 %) and the highest  
350 abundance of *Firmicutes*. According to Hu et al. [56], high dietary energy increased the ratio of  
351 *Firmicutes* to *Bacteroidetes* and mainly increased ruminal amylolytic and propionate-producing  
352 bacteria populations. At the genus level, *Prevotella* is the most dominant genus found in the rumen of  
353 heifers. The most significant genera of ruminal bacteria are *Prevotella*, *Ruminococcus*, Unclassified  
354 *Clostridiales*, and *Paludibacter* were among the common genera found in the rumen of Hanwoo  
355 heifers [57]. Meanwhile, fiber-degrading bacteria such as *Fibrobacter succinogenes*, *R. albus*, and *R.*  
356 *flavefaciens* have significantly low abundance in all groups. In this study, *Prevotella*, which belongs  
357 to *Bacteroidetes*, was the dominant bacterial genus in the rumen of Hanwoo heifers. Several studies  
358 have proven that *Prevotella* was the most abundant in the rumen of dairy cows and beef steers and  
359 calves [58–62]. The highest *Prevotella* abundance was in the L-IRGS group, and the lowest  
360 abundance was in the CON TMR. *Prevotella ruminicola* was dominant in all TMR diet groups at the  
361 species level. In addition, the abundance of *Ruminococcus* was increased in the rumen when Hanwoo  
362 heifers were fed with the H-IRGS TMR diet. *Ruminococcus* are highly dominant in the large bowel,  
363 caecum, or rumen of many animals and humans and are one of the primary degraders of plant fiber in  
364 the rumen [63–66]. The relative abundance of *Ruminococcus bromii* was high in Hanwoo heifers fed  
365 with H-IRGS TMR than in other TMR diets. *Prevotella* spp. are amylolytic bacteria that can degrade  
366 starch, xylan, and pectin, while *Ruminococcus* is known as cellulose degrader in the rumen [56,67–  
367 70]. *R. bromii* is a specialized amylolytic bacterium that plays a significant role in utilizing and

368 degrading resistant starch in the rumen and is associated with propionate production [56,67]. In  
369 addition, *Prevotella* spp. contains a membrane-bound electron transfer complex, that facilitates the  
370 reduction of fumarate to succinate, which is the substrate for the synthesis of propionate in the rumen  
371 [71]. TMR diet containing IRGS increased the relative abundance of these bacteria and may have  
372 contributed to the increased propionate production in the rumen. The inclusion of IRGS in the TMR  
373 diet increased the abundance of *Bacteroidetes* and mainly increased propionate-producing bacteria  
374 populations. The present study provided information on the effects of Italian ryegrass silage as an  
375 alternative forage source in TMR production and feedstuff for Hanwoo heifers during the growth  
376 period. Hanwoo heifers fed a TMR diet containing IRGS at 36% DM increased the molar proportion  
377 of propionate and increased the abundance of *R. bromii* in the rumen. This could suggest that the  
378 inclusion of a high amount of IRGS in TMR increased the abundance of propionate-producing  
379 bacteria, as well as the propionate production to enhance the energy harvest in the rumen. In addition,  
380 the effect of IRGS-based TMR on the growth performance of heifers is similar to those heifers fed  
381 CON TMR diet. Therefore, IRGS may be used as an alternative ingredient for imported forages in  
382 TMR production. The H-IRGS TMR decreased the total VFA production, reduced the acetate-to-  
383 propionate ratio, and improve N use efficiency through lower rumen NH<sub>3</sub>-N.

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## Acknowledgments

387 This study was supported by the Cooperative Research Program for Agriculture Science and  
388 Technology Development (Project No. PJ013609), Rural Development Administration, Republic of  
389 Korea.

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## Tables and Figures

615 **Table 1.** Ingredient and chemical composition of different total mixed rations fed on Hanwoo heifer  
616 during the growing period

Items	TMR diets <sup>1</sup>		
	CON	L-IRGS	H-IRGS
<b>Ingredients (% DM)</b>			
Oat hay	21.09	6.91	–
Timothy hay	9.26	7.28	–
Alfalfa hay	13.95	7.31	–
Corn silage	–	4.77	9.11
Italian ryegrass silage	–	19.07	36.43
Corn gluten feed	12.83	12.57	12.54
Lupin seed	10.94	10.74	10.70
Wheat bran	12.45	12.22	12.17
Corn	17.33	17.01	16.95
Vitamin-mineral supplement <sup>2</sup>	0.68	0.67	0.67
Limestone	1.23	1.21	1.20
Salt	0.24	0.24	0.23
<b>Chemical composition (%)</b>			
Crude protein (CP)	16.23	16.36	16.01
Ether extract (EE)	3.74	3.61	3.39
Ash	4.83	5.55	5.11
Neutral detergent fiber (NDF)	41.82	42.27	42.33
Acid detergent fiber (ADF)	23.21	23.05	23.31
Calcium	0.78	1.02	1.25
Phosphorus	0.53	0.67	0.79
Nonstructural carbohydrate (NSC) <sup>3</sup>	35.22	31.26	31.67
Total Digestible Nutrients (TDN)	72.24	72.84	72.74

617 <sup>1</sup> TMR diet: CON, total mixed ration containing oat hay, timothy, and alfalfa hay; L-IRGS, total  
618 mixed ration containing 19% of Italian ryegrass silage; H-IRGS, total mixed ration containing 36% of  
619 Italian ryegrass silage

620 <sup>2</sup> Vitamin-mineral supplement contained vit. A 2,650,000 IU, vit. D3 530,000 IU, vit. E 1,050 IU,  
621 niacin 10,000 mg, Mn 4,400 mg, Zn 4,400 mg, Fe 13,200 mg, Cu 2,200 mg, iodine 440 mg, and Co,  
622 440 mg/kg of Grobic-DC provided from Bayer Health Care (Leverkusen, Germany)

<sup>3</sup> NSC was calculated according to the formula: NSC = 100 – (NDF+CP+EE+Ash) [72]

623 **Table 2.** Effects of different TMR diets on ruminal fermentation parameters

Items	Treatments <sup>1</sup>			<i>p</i> -value
	CON	L-IRGS	H-IRGS	
pH	6.30 ± 0.01	6.35 ± 0.00	6.31 ± 0.00	0.082
NH <sub>3</sub> -N (mg/dL)	14.35 ± 0.37	13.30 ± 0.07	13.90 ± 0.09	0.094
TVFA (mmol/L)	91.88 ± 0.29 <sup>a</sup>	88.30 ± 0.46 <sup>b</sup>	83.90 ± 0.32 <sup>c</sup>	0.001
Individual VFA (mol/100mol)				
Acetate	63.48 ± 0.09	63.09 ± 0.33	63.55 ± 0.05	0.349
Propionate	22.36 ± 0.05 <sup>a</sup>	20.68 ± 0.08 <sup>b</sup>	21.84 ± 0.01 <sup>a</sup>	0.001
Butyrate	14.16 ± 0.05 <sup>b</sup>	16.23 ± 0.41 <sup>a</sup>	14.61 ± 0.03 <sup>b</sup>	0.017
A/P ratio	2.84 ± 0.01 <sup>c</sup>	3.06 ± 0.01 <sup>a</sup>	2.91 ± 0.00 <sup>b</sup>	0.001

624 <sup>1</sup> Treatments: CON, total mixed ration containing oat hay, timothy, and alfalfa hay; L-IRGS, total  
 625 mixed ration containing 19% of Italian ryegrass silage; H-IRGS, total mixed ration containing 36% of  
 626 Italian ryegrass silage

627 <sup>a-c</sup> Means with different superscripts in a row differ significantly (*p* < 0.05). Results are presented as  
 628 mean ± SEM.

629 SEM, standard error of the mean; NH<sub>3</sub>-N, ammonia nitrogen; VFA, volatile fatty acid; A/P ratio,  
 630 acetate-to-propionate ratio

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 632  
 633

634 **Table 3.** Effect of different TMR diets on the growth performance of growing Hanwoo heifers

Parameters	Treatments <sup>1</sup>			<i>p</i> -value
	CON	L-IRGS	H-IRGS	
Initial, kg/head	225.67 ± 7.72	225.44±10.66	224.22±13.33	0.9948
Final, kg/head	356.89±10.82	335.33±10.65	346.00±13.05	0.4321
ADG	0.93±0.05	0.78±0.04	0.87±0.07	0.1936
DMI	5.26±0.01	5.33±0.01	5.36±0.01	0.1688
Feed conversion ratio	5.76±0.32	6.93±0.34	6.42±0.42	0.0943

635 <sup>1</sup> Treatments: CON, total mixed ration containing oat hay, timothy, and alfalfa hay; L-IRGS, total  
 636 mixed ration containing 19% of Italian ryegrass silage; H-IRGS, total mixed ration containing 36% of  
 637 Italian ryegrass silage

638 <sup>a-c</sup> Means with different superscripts in a row differ significantly (*p* < 0.05). Results are presented as  
 639 mean ± SEM.

640 ADG, average daily gain; DMI, dry matter intake

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645 **Table 4.** Effect of different TMR diets on the blood parameters of growing Hanwoo heifers

Parameters	Treatments <sup>1</sup>			<i>p</i> -value
	CON	L-IRGS	H-IRGS	
Albumin (g/dL)	3.10±0.21	3.01±0.18	3.09±0.16	0.6539
AST (U/L)	103.20±27.62	92.70±13.90	103.67±17.40	0.4657
Glucose (mg/dL)	92.00±6.91	95.70±6.83	91.44±11.06	0.0558
Total cholesterol (mg/dL)	100.00±18.89	105.20±13.68	112.44±34.98	0.7052
BUN (mg/dL)	14.50±2.01 <sup>a</sup>	13.50±1.27 <sup>ab</sup>	12.44±1.88 <sup>b</sup>	0.0453
Total protein (mg/dL)	7.16±0.39	6.90±0.29	7.09±0.39	0.2901

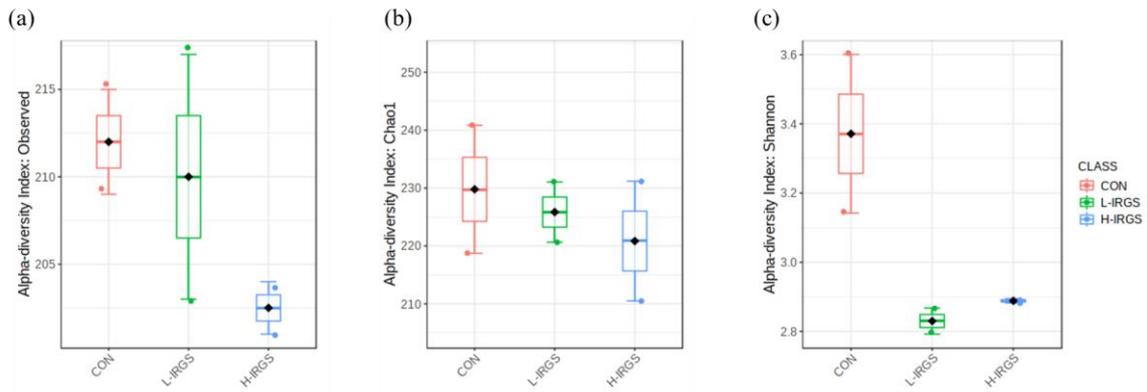
646 <sup>1</sup> Treatments: CON, total mixed ration containing oat hay, timothy, and alfalfa hay; L-IRGS, total  
 647 mixed ration containing 19% of Italian ryegrass silage; H-IRGS, total mixed ration containing 36% of  
 648 Italian ryegrass silage

649 <sup>a-c</sup> Means with different superscripts in a row differ significantly (*p* < 0.05). Results are presented as  
 650 mean ± SEM.

651 AST, aspartate aminotransferase; BUN, blood urea nitrogen

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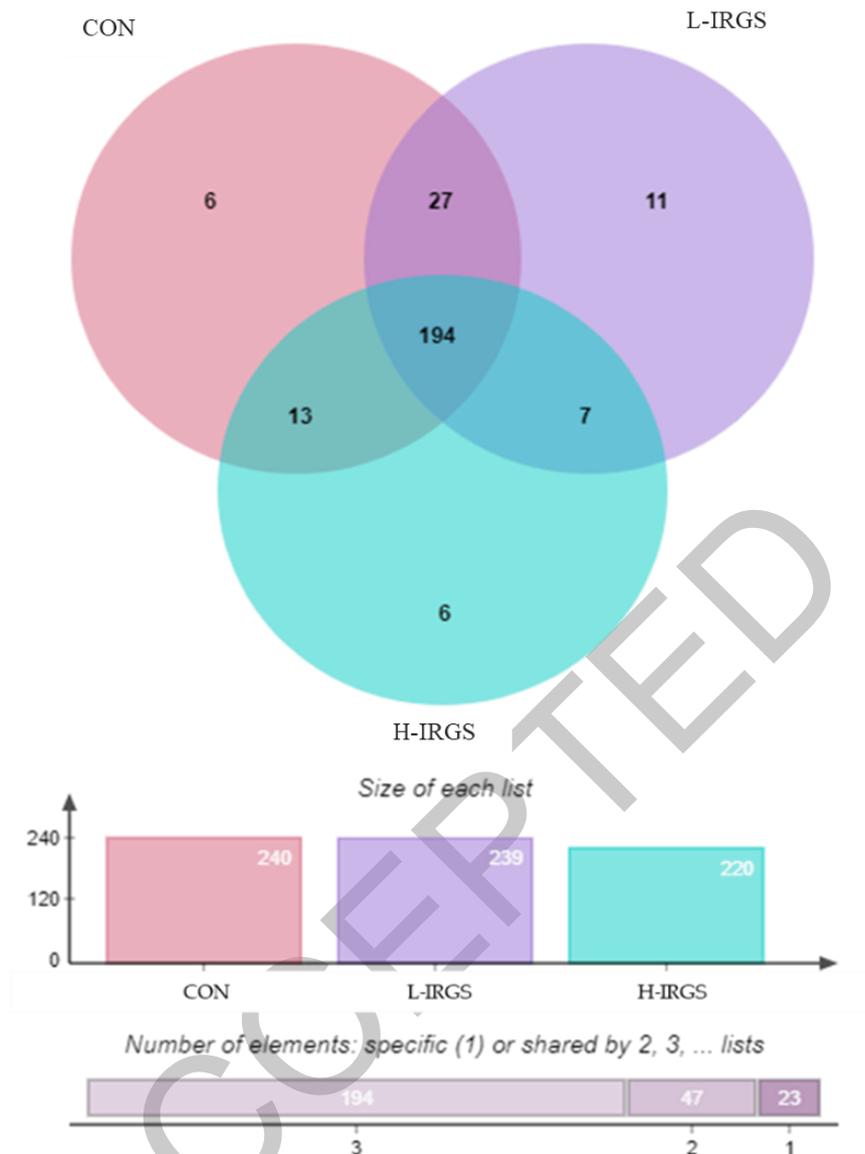


657

658 **Figure 1.** Effects of different TMRs on the rumen bacterial alpha diversity of growing Hanwoo  
659 heifers. (A) observed OTUs, (B) chao1, and (C) Shannon, between treatment groups. (Treatments:  
660 CON, total mixed ration containing oat hay, timothy, and alfalfa hay; L-IRGS, total mixed ration  
661 containing 19% of Italian ryegrass silage; H-IRGS, total mixed ration containing 36% of Italian  
662 ryegrass silage)

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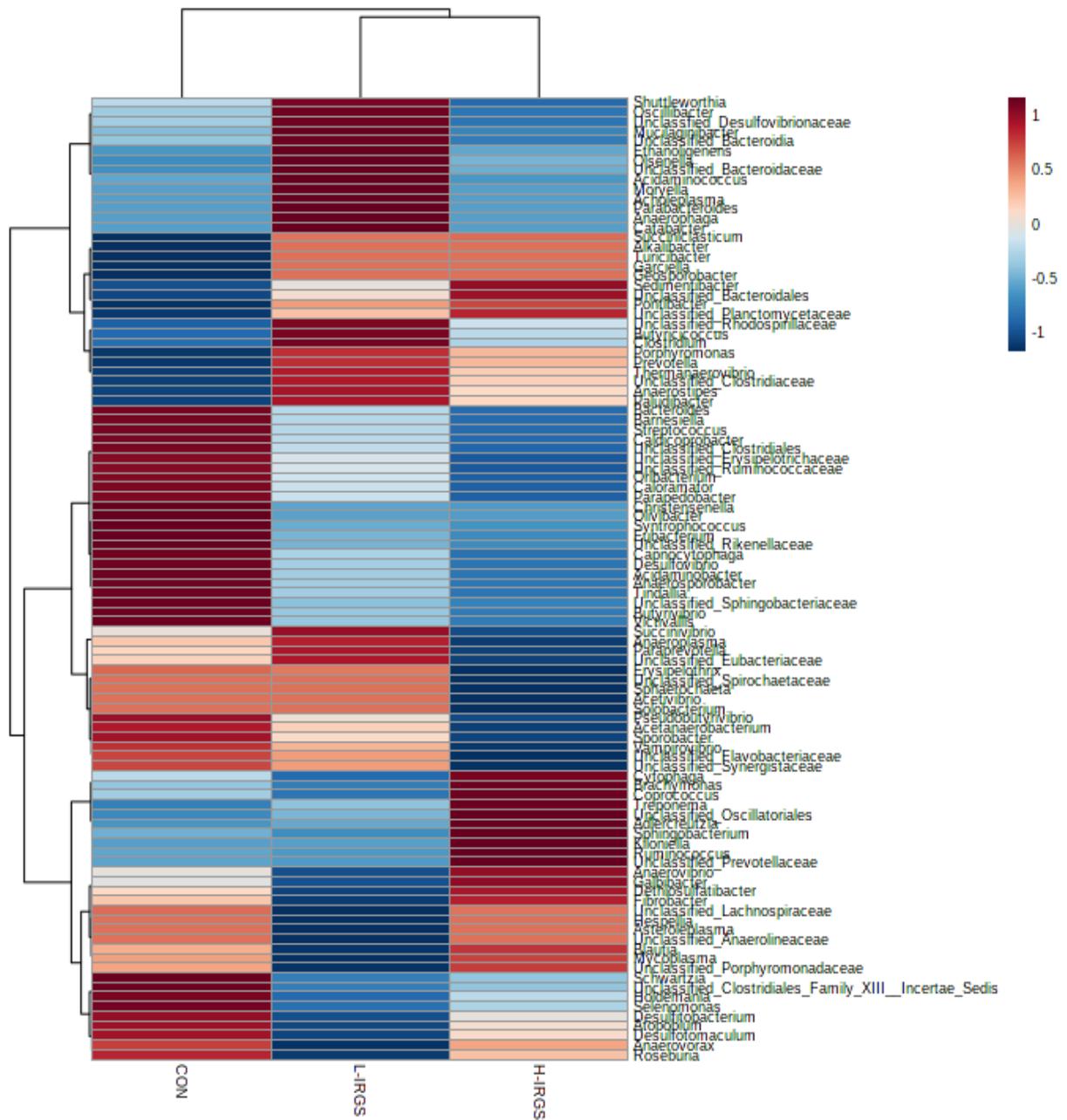
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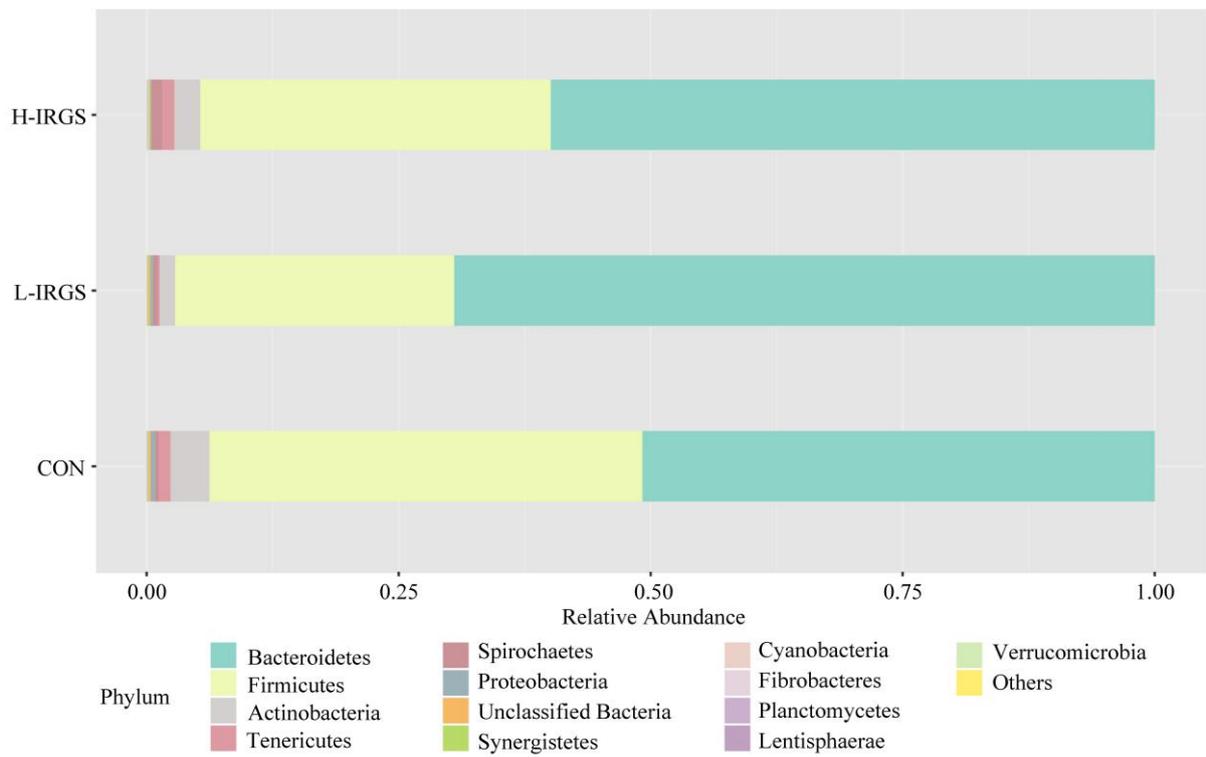
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665 **Figure 2.** Venn diagram showing the unique and common OTUs in the rumen of Hanwoo heifers fed  
 666 with different TMR diets. The three TMR diets used in this study each showed a number of unique  
 667 OTU's. Each circle represents each of the TMR diets (CON, L-IRGS, and H-IRGS) with numbers  
 668 within circles or overlapping areas indicating the number of OTU's in common to the corresponding  
 669 diets.

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**Figure 3.** Hierarchical clustering heatmap of the bacterial genus from the set-up generated in MicrobiomeAnalyst using Bray-Curtis dissimilarity test and Ward clustering algorithm. Normalized relative abundances are plotted from low (blue), mid (peach), and high (red). (CON, total mixed ration containing 21 % of oat hay; L-IRGS, total mixed ration containing 19% of Italian ryegrass silage; H-IRGS, total mixed ration containing 36% of Italian ryegrass silage).

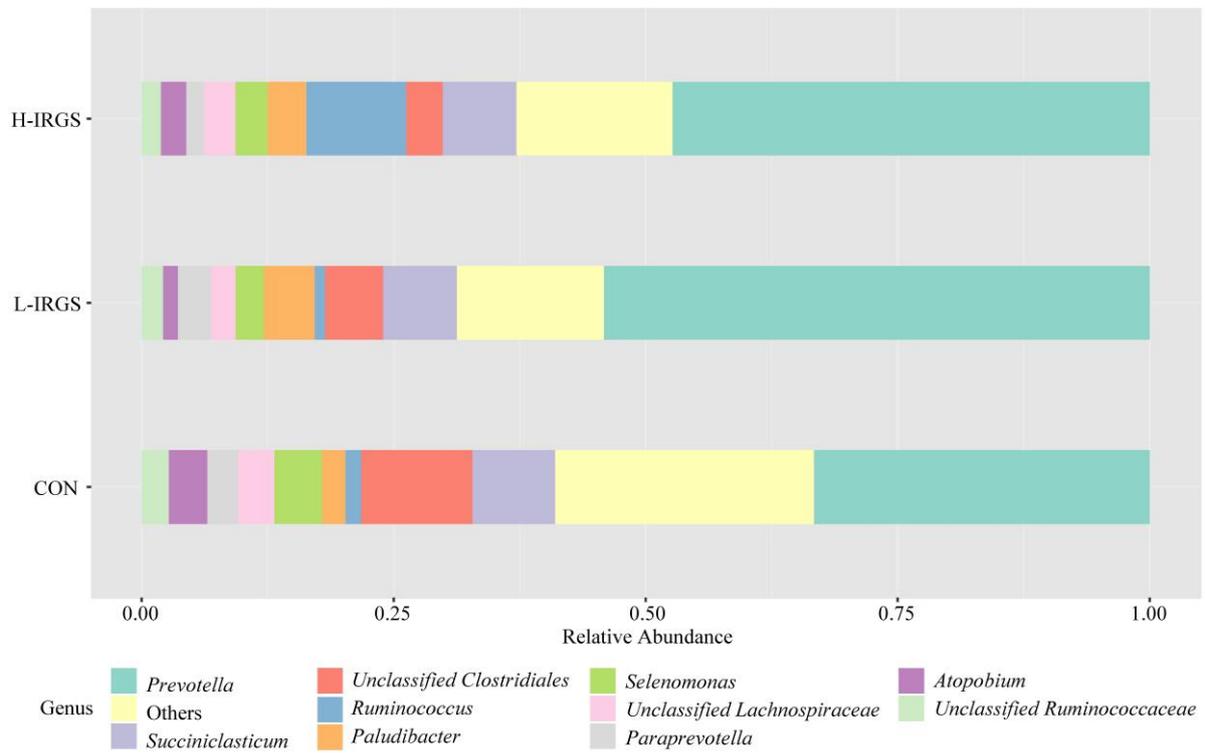


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679 **Figure 4.** Effect of different TMR diets on the rumen bacterial composition of growing Hanwoo

680 heifers at the phylum level.

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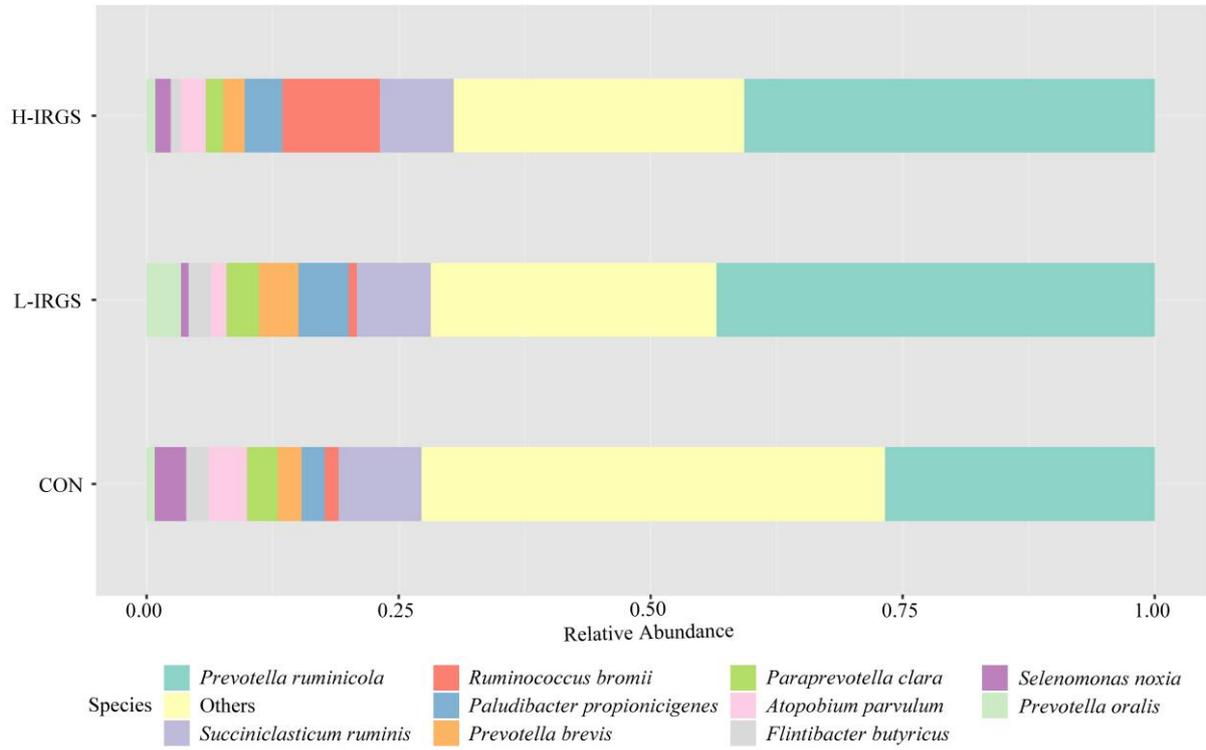


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684 **Figure 5.** Effect of different TMR diets on the rumen bacterial composition of growing Hanwoo

685 heifers at the genus level.

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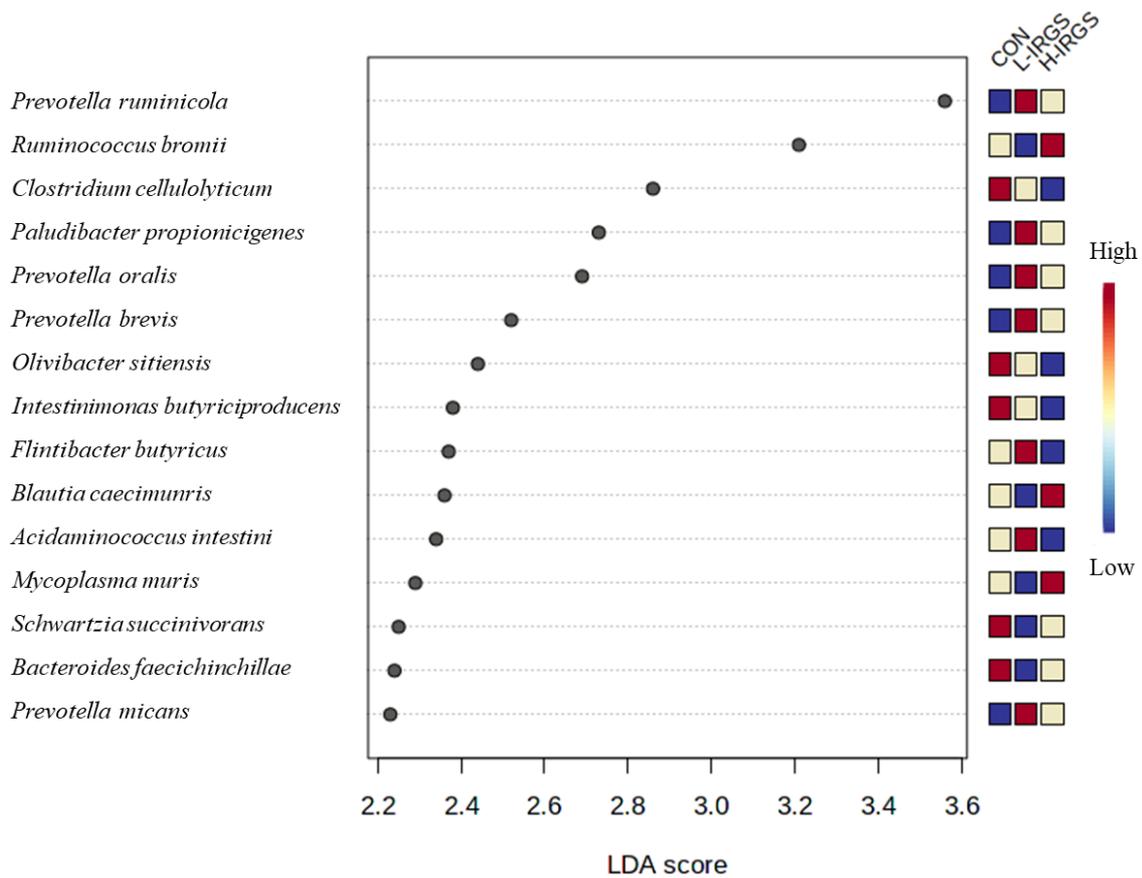


688

689 **Figure 6.** Effect of different TMR diets on the rumen bacterial composition of growing Hanwoo

690 heifers at the species level.

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693 **Figure 7.** Linear discriminant analysis (LDA) effect size (LEfSe) plot comparing the rumen microbial  
 694 communities in growing Hanwoo heifers fed different TMRs. Only the top 15 species with  $p < 0.05$   
 695 and an effect size cut-off of 2.0 are plotted.