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Effect of Isopropyl Ester of Hydroxy Analogue of Methionine on Rumen Microbiome, Active Enzymes, and Protein Metabolism Pathways of Yak

Xirui Zhang [1], Yao Liu [1], Zizhen Zuo [2], Chenxi Wang [1], Zhongli Peng [1], Jincheng Zhong [2] and Haibo Wang [2,*]

[1] College of Animal and Veterinary Sciences, Southwest Minzu University, Chengdu 610041, China; 13548197974@163.com (X.Z.); liuyao100141931@gmail.com (Y.L.); wcx199787@163.com (C.W.); leo3131@163.com (Z.P.)

[2] Key Laboratory of Qinghai-Tibetan Plateau Animal Genetic Resource Reservation and Utilization, Sichuan Province and Ministry of Education, Southwest Minzu University, Chengdu 610041, China; cairangji120@163.com (Z.Z.); zhongjincheng518@126.com (J.Z.)

* Correspondence: wanghaibo@swun.edu.cn; Tel.: +86-028-8916-5877

Abstract: This study aims to investigate the effect of methionine analogue 2-hydroxy-4-methylthiobutanoic acid isopropyl ester (HMBi) on the rumen microbial community, microbial carbohydrate-active enzymes (CAZy), and protein metabolism pathways in yak. Twenty-four male Maiwa yaks (252.79 ± 15.95 kg) were selected and randomly divided into groups that received the basal diet alone, or a diet supplemented with different amounts of HMBi (5 g, 10 g or 15 g). At the phylum level, the group receiving 5 g of HMBi showed a considerably higher relative abundance of *Lentisphaerae* than the other treatment groups ($p < 0.05$). The relative abundance of *Actinobacteria* decreased linearly with the increase in HMBi supplemental levels ($p < 0.05$). The relative abundance of *Prevotella* increased linearly with the increasing level of HMBi supplementation ($p < 0.05$). The relative abundance of *Butyrivibrio* linearly decreased ($p < 0.05$), and the relative abundance of *Alistipes* tended to linearly decrease ($p = 0.084$). The addition of HMBi had linear or quadratic effects on the relative abundance of CAZy enzymes and functional proteins in the rumen of yak ($p < 0.05$). Conclusively, these results indicated that feeding yaks a diet supplemented with HMBi is an excellent strategy to enhance carbohydrate breakdown, and improve rumen microbial structure and function.

Keywords: yak; methionine analogues; rumen microbial metagenome; carbohydrate-active enzymes; protein metabolism pathways

Citation: Zhang, X.; Liu, Y.; Zuo, Z.; Wang, C.; Peng, Z.; Zhong, J.; Wang, H. Effect of Isopropyl Ester of Hydroxy Analogue of Methionine on Rumen Microbiome, Active Enzymes, and Protein Metabolism Pathways of Yak. *Fermentation* **2024**, *10*, 94. https://doi.org/10.3390/fermentation10020094

Academic Editor: Tao Shao

Received: 22 December 2023
Revised: 1 February 2024
Accepted: 2 February 2024
Published: 4 February 2024

1. Introduction

The yak (*Bos grunniens*) is internationally acknowledged as an interesting species of domestic animal with unique physiological characteristics [1]. They live in a harsh environment with low temperatures, high altitude (Qinghai-Tibetan Plateau), and low food availability [2]. Yaks often spend two-thirds of the year without adequate nutrition [3]. As a cultural symbol of the Tibetan people, the yak plays a vital role in the alpine ecosystem [4]. Although there is significant interest in yak breeding, development of this industry is significantly limited by the feed cost. The high cost of feed for yaks is due to the fact that protein, which is an important nutrient component that makes up around 40% of the total cost, accounts for 12–17% of feed dry matter [5].

The rumen contains a variety of microflora. Ruminal microorganisms can degrade plant cell walls and fibrous materials, and convert them into nutrients for host uptake, including microbial crude protein (MCP) and volatile fatty acids (VFA). Methionine (Met), which serves as one of the first two restricted amino acids for ruminants, is a crucial sulfur-containing amino acid in the animal body that is essential for enhancing animal production performance and feed protein utilization. Beta-hydroxy-beta-methylbutyrate

(HMB) is a commonly used addition to supplement Met in swine and poultry [6]. It is a hydroxyl group that replaces the amino group in methionine. A material known as isopropyl 2-hydroxy-4-methylthiobutyrate (HMBi) is created when isopropyl alcohol and HMB are esterified. HMBi, which has more branched chains of alcohols than HMB from the standpoint of chemical structure, is less polar overall and can more easily penetrate biofilms. HMBi is hence more biologically potent [7]. Therefore, HMBi has been studied for improving milk production in ruminants [8].

To create effective feeding and management plans for yaks, it is crucial to thoroughly comprehend their rumen microbial environment. Although some recent studies have evaluated the ruminal microbial community of yaks [9,10], there has been little work to leverage metagenomic approaches for this purpose. Metagenomic sequencing, as opposed to 16S rRNA sequence analysis, focuses on the species composition in environmental materials, identifies microorganisms down to the level of species or even strains, and reflects the functional composition, metabolic pathways, and other data of the microorganisms [11]. By better understanding the functions of the rumen microbes, it may be possible to optimize rumen fermentation and improve the efficiency of nutrient utilization in ruminant animals. Thus, the purpose of this study was to investigate the effects of supplemental HMBi on yak rumen microbial flora, carbohydrate-active enzymes, and protein metabolism pathways. We hypothesized that HMBi would optimize the rumen microbial community structure for healthy and efficient yak breeding.

2. Materials and Methods

2.1. Animals, Diets, and Experimental Design

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol number: 2023MDLS010) of Southwest Minzu University (Chengdu, China). The study was performed at Aba Prefecture, Sichuan Province (2500 m above sea level 7). The temperature in the barn was between 5–12 °C during the experiment. Diets were supplemented with the commercial product MetaSmart (MS; Adisseo Inc., Antony, France), containing HMBi as the main ingredient, with an effective content of 57% and a rumen protection efficiency of approximately 50% [12,13]. A completely randomized design was used in this study. We randomly selected 24 male yaks (weight: (252.79 ± 15.95 kg) and gave them four different dietary treatments, 6 yaks per group: a basal diet (CON); an increase of 5 g MS supplementation over the CON (MS1); an increase of 10 g MS supplementation over the CON (MS2); and an increase of 15 g MS supplementation over the CON (MS3). Each treatment was added to a basal diet consisting of 55% maize stover silage and 45% concentrate, on a dry matter (DM) intake basis. The ingredients and nutrient composition of the basal diet are listed in Table 1. Following 10 days of adaption, the 70-day experimental diet was given. Prior to the trial, all animals were vaccinated against parasites and common infectious diseases.

2.2. Sample Collection

We inserted the rumen fluid extractor into each yak's stomach to obtain the sample of rumen fluid at 3 h after feeding. According to Wang's procedures, this time period is an efficient stage for microbial feed digestion [14]. Between sample collections, the tubes were thoroughly washed with fresh warm water. To minimize the possibility of contamination of the sample with saliva, around 50 mL of the rumen fluid sample was thrown away before the collection procedure. The samples were then quickly and carefully stored in liquid nitrogen to maintain their biological activity for further evaluation.

2.3. DNA Extraction, Library Construction, and Metagenomic Sequencing

Total genomic DNA was extracted from every rumen liquid sample using the FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The concentration and purity of extracted DNA were evaluated using TBS-380

and NanoDrop2000. The concentration of DNA was 50 ng/μL, and the ratio of 260 to 280 nm were between 1.8 and 2.0. DNA extract quality was checked on 1% agarose gel.

The DNA extracts were fragmented to an average size of about 400 bp using a Covaris M220 (Gene Company Limited, Shanghai, China). Paired-end libraries were constructed using NEXTFLEX® Rapid DNA-Seq (Bio Scientific, Austin, TX, USA). Paired-end sequencing was performed on Illumina Novaseq (Illumina Inc., San Diego, CA, USA). The raw reads were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession Number: PRJNA953596, http://www.ncbi.nlm.nih.gov/sra, accessed on 5 April 2023).

Table 1. Experimental diet ingredients and nutrition composition (DM Basis).

Items	Content
Ingredients	[g kg^{-1}]
Corn straw silage	550
Corn meal	157.5
Soybean meal	76.5
Sprayed corn bran	45
Soybean	45
Corn germ meal	36
Soybean hull	31.5
Rapeseed meal	22.5
Molasses	22.5
Premix [1]	13.5
Nutrition composition [2]	[g kg^{-1}]
OM	907.5
CP	131.3
NDF	439.2
ADF	156.3
NE_m (MJ/kg)	3.52
NE_g (MJ/kg)	5.58

Note: [1] Premix contained (per kg): vitamin A, 2500 IU; vitamin D, 550 IU; vitamin E, 10 IU; Fe, 40 mg; Zn, 40 mg; Cu, 10 mg; Mn, 40 mg; I, 0.5 mg; Co, 0.2 mg; and Se, 0.2 mg. [2] OM: organic matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; NEm: net energy for maintenance; NEg: net energy for gain.

2.4. Sequence Quality Control and Genome Assembly

Additional data are accessible at Majorbio Cloud Portal (www.majorbio.com, accessed on 28 February 2022), and may be evaluated in real time on the web platform. Fastp [15] was used to remove low-quality hits from paired Illumina reads by removing adapters (https://github.com/OpenGene/fastp, accessed on 28 February 2022, version 0.20.0).

Metagenomics data sets were collected and presented in a de Bruijn plot using MEGAHIT [16]. Alleles less than 300 base pairs (bp) are considered fully assembled. Assignment of alleles allowed genetic predictions and commentary (https://github.com/voutcn/megahit, accessed on 28 February 2022, version 1.1.2).

2.5. Gene Prediction, Taxonomy, and Functional Annotation

MetaGene [17] (http://metagene.cb.k.u-tokyo.ac.jp/, accessed on 28 February 2022) was used to examine the assembly's open reading frames (ORFs) with projected contigs. The predicted ORFs (length of at least 100 bp) were obtained using the NCBI translation table. The numerical values were used to infer the amino acid sequences of the translated proteins.

CD-HIT [18] (http://www.bioinformatics.org/cd-hit/, accessed on 28 February 2022, version 4.6.1) was used to effectively generate a non-redundant gene catalog. SOAPaligner was used to compare high-quality reads to a database of genes without duplications [19] (http://soap.genomics.org.cn/, accessed on 28 February 2022, version 2.21). This allowed us to calculate gene abundance at 95% identity.

Diamond [20] (http://www.diamondsearch.org/index.php, accessed on 28 February 2022, version 0.8.35) was used to analyze and compare extracts from the whole gene

database and the NR library with an e-value threshold of 1×10^{-5} for taxonomy classification and to annotate example sequences with orthologous groups (COGs).

2.6. Statistical Analysis

SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used for one-way ANOVA analysis. If the main impact was significant at the 0.05 level, then a TukeyeKramer test for multiple comparisons was performed. We also used the orthogonal polynomial comparison method to determine if there were linear and quadratic effects on the responses to the different treatments. To confirm potentially significant differences, we used $p < 0.05$, and for reporting trends, we used $0.05 \leq p < 0.10$. The Spearman rank correlation in SAS was also used. The previous correlation levels were used to test the association of the proportion of rumen microorganisms. Phyla and genera present at less than 1% of all data were removed from further analysis. The results were expressed by means and pooled SEM.

3. Results

3.1. Microbial Metagenomic Sequence Data

A total of 685,214,172 unique reads were obtained by sequencing all the rumen samples from 24 yaks. There was an average of 28,550,591 reads per sample. First, MEGAHIT v1.1.2 was used to assemble the sequences (the shortest allele length $\geq$ 300). After assembly was completed, sequences with the best allele effect were selected and the ORF prediction results of the hybrid assembly were obtained. The catalog of non-redundant genes was obtained by analysis. A total of 5,217,957 non-redundant genes were predicted, with a total of 6.89 base pairs and a ratio of 40.14%.

3.2. Analysis of Rumen Microbial Community Composition

NR species annotation detected 235 phyla, 459 classes, 966 orders, 1941 families, 5442 genus, and 28,466 species. At phylum level, the rank order of abundance was *Bacteroidetes* (41.99%) > *Firmicutes* (34.70%) > *Euryarchaeota* (2.34%) > *Cillophora* (1.85%) > *Lentisphaerae* (1.65%) > *Proteobacteria* (1.35%) in the CON group (Figure 1a). In the MS1 group, the rank order of abundance was *Bacteroidetes* (40.57%) > *Firmicutes* (32.58%) > *Lentisphaerae* (2.85%) > *Cillophora* (2.73%) > *Euryarchaeota* (1.99%) > *Proteobacteria* (1.62%). In the MS2 group, the rank order of abundance was *Bacteroidetes* (47.25%) > *Firmicutes* (27.46%) > *Cillophora* (3.23%) > *Euryarchaeota* (1.95%) > *Lentisphaerae* (1.73%) > *Proteobacteria* (1.48%). In the MS3 group, the rank order of abundance was *Bacteroidetes* (41.15%) > *Firmicutes* (31.90%) > *Cillophora* (3.08%) > *Euryarchaeota* (2.28%) > *Proteobacteria* (1.54%) > *Lentisphaerae* (1.47%). *Prevotella* was the most common genus across all categories: (CON: 16.00%, MS1: 14.52%, MS2: 23.26%, MS3: 21.22%), followed by *Bacteroides*, *Methanobrevibacter*, *Ruminococcus*, *Clostridium*, *Butyrivibrio*, *Alistipes* and *Fibrobacter* (Figure 1b).

At the phylum level, it can be considered that *Bacteroidetes* was the most represented taxon in each group (Table 2). The abundance of other phyla also varied considerably across the four categories. The relative abundance of *Lentisphaerae* in MS1 was significantly higher than those in other groups ($p < 0.05$). The data indicated a quadratic trend from high to low abundance ($p < 0.05$). Similarly, the relative abundance of *Actinobacteria* was associated with a linear reduction in increasing concentrations of MS supplementation ($p < 0.05$).

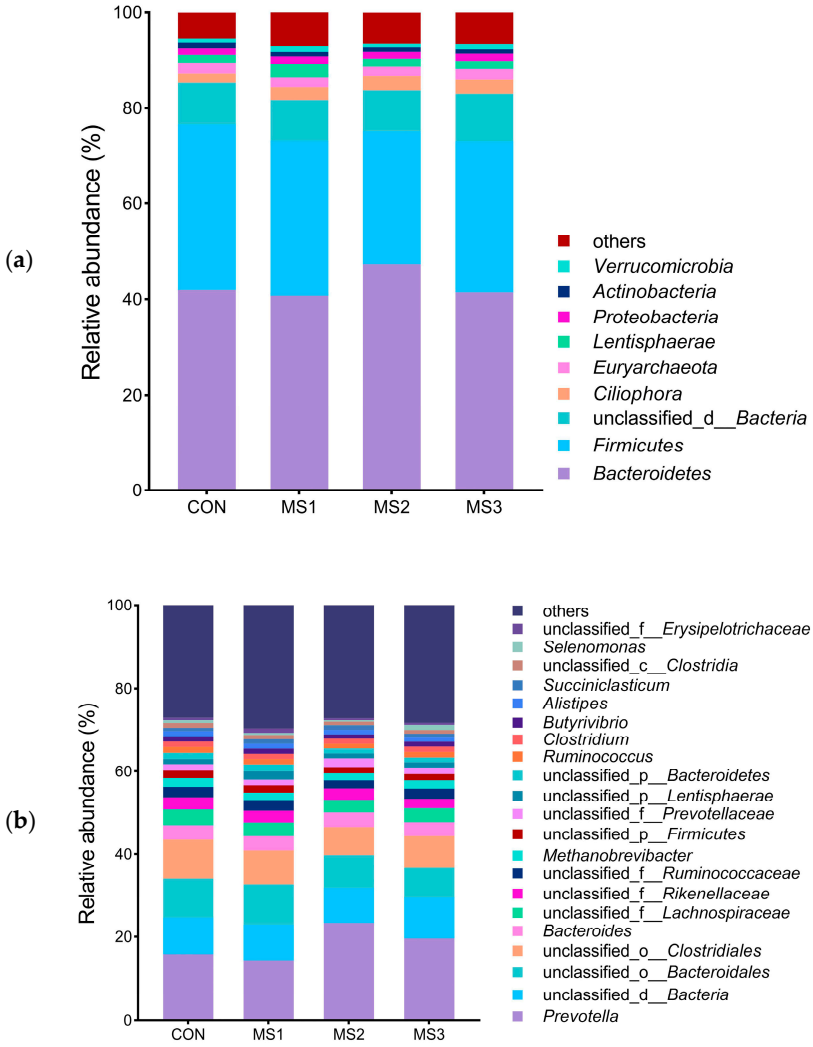

Figure 1. Components of the rumen bacteria in different 2-hydroxy-4-methylthiobutanoic acid isopropyl ester supplementation levels. ((**a**) phylum level, (**b**) genus level).

Table 2. Effect of 2-hydroxy-4-methylthiobutanoic acid isopropyl ester supplementation in diets on rumen bacterial composition of yak (Phylum level, %).

Items	Treatments [1]				SEM	*p*-Value [2]		
	CON	MS1	MS2	MS3		Treat	Linear	Quadratic
Bacteroidetes	41.99	40.59	47.25	41.15	3.018	0.372	0.103	0.247
Firmicutes	34.70	32.58	27.46	31.90	2.505	0.251	0.197	0.799
Cillophora	1.85	2.73	3.23	3.08	1.231	0.859	0.939	0.278
Euryarchaeota	2.34	1.99	1.95	2.28	0.493	0.918	0.909	0.212
Lentisphaerae	1.65 ^{a}	2.85 ^{b}	1.73 ^{a}	1.47 ^{a}	0.258	0.005	0.467	0.022
Proteobacteria	1.35	1.62	1.48	1.54	0.107	0.350	0.393	0.206
Actinobacteria	0.98	0.94	0.82	0.79	0.076	0.251	0.029	0.308
verrucomicrobia	0.84	1.14	0.68	0.87	0.161	0.244	0.484	0.578
Spirochaetes	0.71	0.79	0.57	0.49	0.119	0.331	0.138	0.893
planctomycetes	0.54	0.53	0.43	0.56	0.095	0.759	0.937	0.527
Fibrobacteres	0.36	0.45	0.56	0.43	0.066	0.158	0.216	0.212
Chytridiomycota	0.27	0.38	0.47	0.45	0.174	0.845	0.747	0.277

[1] CON: yaks receiving a basic diet; MS1: yaks receiving a basic diet supplementation with 5 g MetaSmart (MS); MS2: yaks receiving a basic diet supplementation with 10 g MS; MS3: yaks receiving a basic diet supplementation with 15 g MS; SEM: standard error of mean. [2] Means with different superscript letters in the same row are significantly different ($p < 0.05$).

At the genus level, the most highly represented taxon in each group was *Prevotella* (Table 3). Other genus' abundances also varied considerably across the four categories. There was a linear rise in the relative abundance of *Prevotella* across all MS supplementation doses ($p < 0.05$). Both MS2 and MS3 groups showed higher abundance of *Prevotella* compared to other groups. The relative abundance of *Butyrivibrio* decreased in a linear decrease ($p < 0.05$) with the supplementation of MS, and a similar trend was seen for those of *Alistipes* ($p = 0.084$).

Table 3. Effect of 2-hydroxy-4-methylthiobutanoic acid isopropyl ester supplementation in diets on rumen bacterial composition of yak (genus level, %).

Items	Treatments [1]				SEM	*p*-Value		
	CON	MS1	MS2	MS3		Treat	Linear	Quadratic
Prevotella	16.00	14.52	23.26	21.22	2.518	0.068	0.013	0.770
Clostridium	1.29	1.38	1.27	1.29	0.125	0.919	0.818	0.800
Bacteroides	3.52	3.41	3.52	3.33	0.243	0.937	0.434	0.972
Butyrivibrio	1.30	1.15	0.99	1.19	0.140	0.501	0.014	0.450
Fibrobacter	0.35	0.45	0.57	0.42	0.060	0.114	0.227	0.213
Alistipes	1.27	1.15	1.12	0.97	0.093	0.187	0.084	0.999
Succiniclasticum	0.77	1.11	1.08	0.98	0.220	0.709	0.127	0.118
Ruminococcus	1.43	1.30	1.12	1.32	0.085	0.104	0.555	0.944
Methanobrevibacter	1.84	1.80	1.76	1.42	0.320	0.790	0.436	0.786

[1] CON: yaks receiving a basic diet; MS1: yaks receiving a basic diet supplementation of 5 g MetaSmart (MS); MS2: yaks receiving a basic diet supplementation of 10 g MS; MS3: yaks receiving a basic diet supplementation of 15 g MS; SEM: standard error of mean.

3.3. Relationship between Rumen Bacteria and Fermentation Parameters

The findings of a scientific investigation into the relationship between the number of the first 30 species found in the yak rumen and digestive parameters are shown in Figure 2 and Table S1. These results show a favorable correlation between *Succiniclasticum* relative abundance and NH_3-N concentration ($r = 0.618$, $p = 0.0013$). *Selenomonas* abundance was favorably linked with MCP concentration ($r = 0.546$, $p = 0.0006$). *Parabacteroides* ($r = 0.685$, $p = 0.0002$); *Bacteroides* ($r = 0.654$, $p = 0.0005$); *Fibrobacter* ($r = 0.552$, $p = 0.0052$); unclassified_*p__Bacteroidetes* ($r = 0.523$, $p = 0.0088$); and unclassified_*f__Prevotellaceae* ($r = 0.493$, $p = 0.0144$) were positively correlated with acetic acid concentration, while *Stylonychia* ($r = -0.522$, $p = 0.0089$) and *Stentor* ($r = -0.514$, $p = 0.0103$) were negatively correlated with acetic acid concentration. Propionic acid concentration was negatively correlated with the abundance of *Prevotella* ($r = 0.433$, $p = 0.0345$); *Bacteroides* ($r = 0.641$, $p = 0.0008$); unclassified *_f__ Prevotellaceae* ($r = 0.504$, $p = 0.0121$); unclassified_*p__Bacteroidetes* ($r = 0.568$, $p = 0.0038$); *Parabacteroides* ($r = 0.701$, $p = 0.0001$); and *Fibrobacter* ($r = 0.740$, $p = 0.00004$), and had a significant negative correlation with the relative abundance of *Butyrivibrio* ($r = -0.439$, $p = 0.0321$); *Stylonychia* ($r = -0.482$, $p = 0.0170$; and *Stentor* ($r = -0.473$, $p = 0.0195$). The abundances of *Clostridium* ($r = 0.492$, $p = 0.0147$); *Succiniclasticum* ($r = 0.441$, $p = 0.0311$); and *Selenomonas* ($r = 0.499$, $p = 0.0130$) were all favorably linked with the isobutyric acid concentration. The butyric acid concentration was positively correlated with *Bacteroides* ($r = 0.572$, $p = 0.0035$); unclassified_*f__Prevotellaceae* ($r = 0.431$, $p = 0.0353$); unclassified_*p__Bacteroidetes* ($r = 0.495$, $p = 0.0140$); *Parabacteroides* ($r = 0.614$, $p = 0.0014$); and *Fibrobacter* ($r = 0.672$, $p < 0.01$) abundances, but was negatively correlated with *Butyrivibrio* ($r = -0.536$, $p = 0.0003$) abundances. The abundance of *Succiniclasticum* was favorably linked with the isovaleric acid content ($r = 0.476$, $p = 0.0188$). The valeric acid concentration was positively correlated with *Prevotella* ($r = 0.408$, $p = 0.0481$); *Bacteroides* ($r = 0.540$, $p = 0.0065$); unclassified_*f__Prevotellaceae* ($r = 0.417$, $p = 0.0427$); unclassified_*p__Bacteroidetes* ($r = 0.511$, $p = 0.0108$); *Parabacteroides* ($r = 0.613$, $p = 0.0015$); and *Fibrobacter* ($r = 0.783$, $p = 0.00001$) abundances, and negatively correlated with *Butyrivibrio* ($r = -0.477$, $p = 0.0183$) abundance. The total volatile fatty acid concentration was positively correlated with *Bacteroides* ($r = 0.653$, $p = 0.0006$); un-

classified_*f*__*Prevotellaceae* ($r = 0.492$, $p = 0.0146$); unclassified_*p*__*Bacteroidetes* ($r = 0.539$, $p = 0.0066$); *Parabacteroides* ($r = 0.693$, $p = 0.0002$); and *Fibrobacter* ($r = 0.623$, $p = 0.0011$) abundances, and was negatively correlated with *Butyrivibrio* ($r = -0.434$, $p = 0.0339$); *Stylonychia* ($r = -0.503$, $p = 0.0123$); and *Stentor* ($r = -0.494$, $p = 0.0142$) abundances.

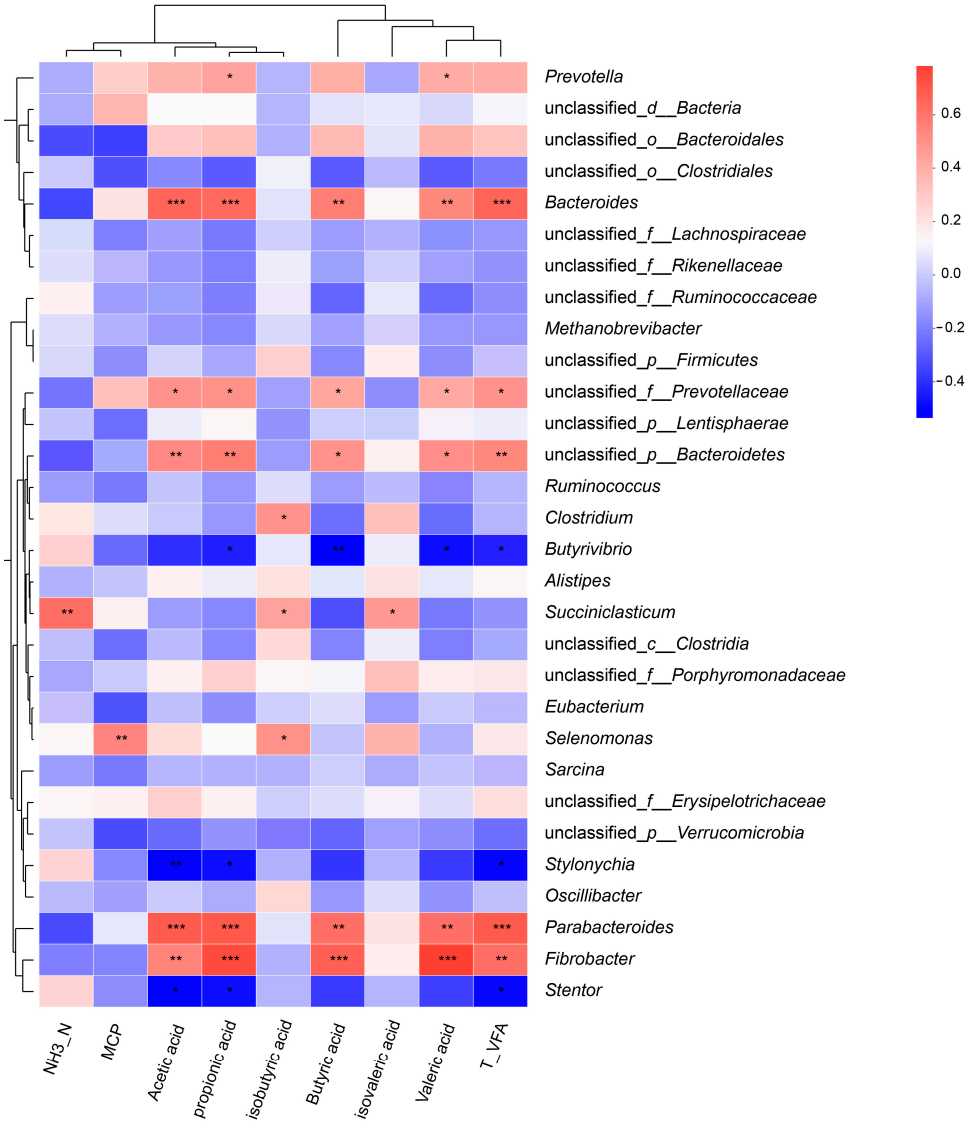

Figure 2. Correlation between relative abundances of bacteria and rumen fermentation parameters. The color and the color intensity of the squares correspond to the direction and strength of the correlation based on the scale to the right. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. CAZy Functional Annotation

A totally of 13,421,742 genes were compared with the CAZy database. The results showed the glycoside hydrolase (GH) gene had the highest representation (7,055,684); followed by glycosyltransferase (GT; 3,387,410); carbohydrate esterase (CE; 1,931,618); and carbohydrate-binding module (CBM; 469,702) (Figure 3). Polysaccharide lyase (PL) and auxin activity (AA) were discovered to be synonymous with a smaller number of genes (282,176 and 295,152, respectively). In terms of the GH, GT, CBM, or AA genes, there was no noticeable variation amongst each of the groups (Table 4). There was a linear decline in the number of CE genes ($p < 0.05$) as the MS replenishment levels increased. There was a correlation between rising levels of MS replenishment and a quadratic rise in PL gene expression ($p = 0.076$).

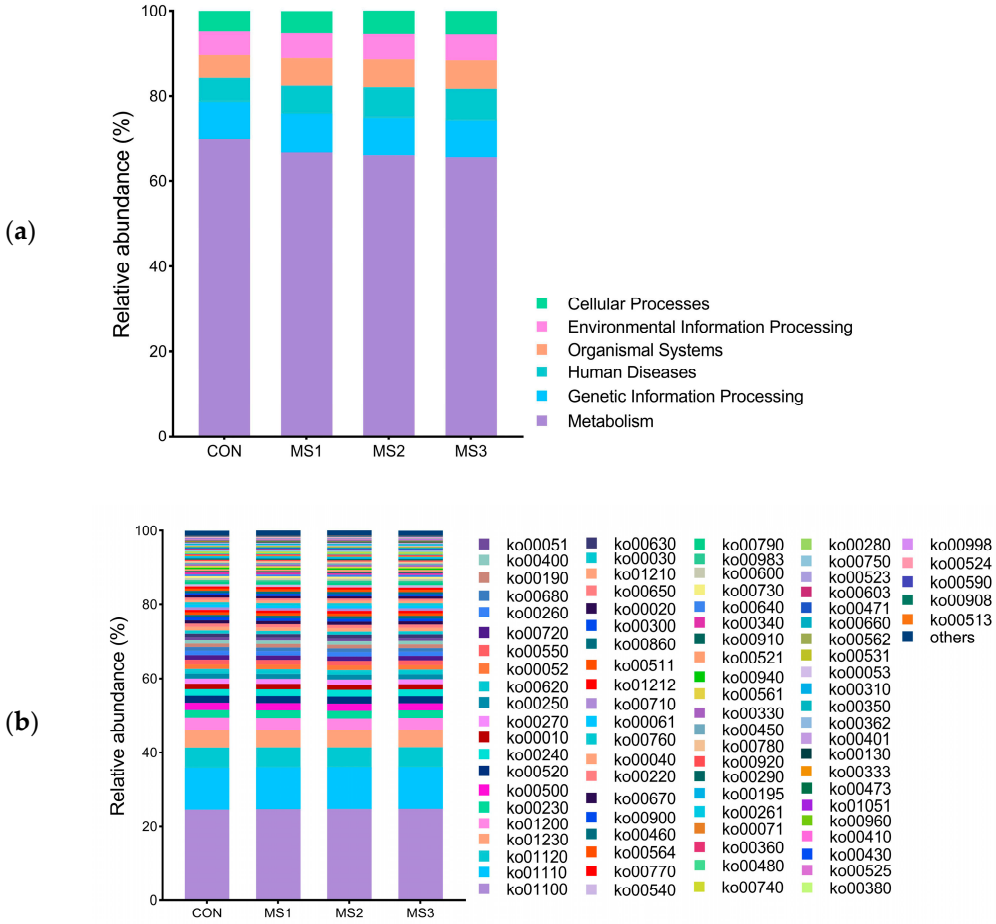

Figure 3. KEGG pathway classification of the rumen microorganisms in different rumen-protected methionine supplementation levels of yak. Pathway ID indicates the number of pathways corresponding to KO. That is, ko01100: metabolism pathways; ko01110: secondary metabolites biosynthesis; ko01120: microbial metabolism in different environments; ko01230: biosynthesis of amino acids; ko01200: carbon metabolism; ko00230: purine metabolism; ko00500: starch and sucrose metabolism; ko00520: amino sugar and nucleotide sugar metabolism; ko00240: pyrimidine metabolism; ko00010: glycolysis/gluconeogenesis. ((**a**) level 1, (**b**) level 2).

Table 4. Effect of 2-hydroxy-4-methylthiobutanoic acid isopropyl ester supplementation in diets on relative abundance of CAZy enzymes in rumen microbes of yak (%).

Items	Treatments [1]				SEM	*p*-Value		
	CON	MS1	MS2	MS3		Treat	Linear	Quadratic
Glycoside Hydrolases (GH)	49.56	49.61	49.54	48.29	0.828	0.623	0.304	0.507
Glycosyl Transferases (GT)	25.06	25.18	26.14	27.00	1.033	0.519	0.144	0.698
Carbohydrate Esterases (CE)	15.21	14.58	14.23	14.51	0.331	0.235	0.040	0.911
Carbohydrate-Binding Modules (CBM)	5.07	5.52	5.06	5.04	0.209	0.312	0.473	0.944
Auxiliary Activities (AA)	2.76	2.59	2.52	2.64	0.081	0.249	0.822	0.653
Polysaccharide Lyases (PL)	2.36	2.38	2.51	2.52	0.190	0.890	0.434	0.076

[1] CON: yaks receiving a basic diet; MS1: yaks receiving a basic diet supplementation of 5 g MetaSmart (MS); MS2: yaks receiving a basic diet supplementation of 10 g MS; MS3: yaks receiving a basic diet supplementation of 15 g MS; SEM: standard error of mean.

3.5. The eggNOG Functional Annotation

The corresponding NOG annotations have been summarized by comparison with the eggNOG database. We found the most enriched genes in cellular processes, and signalling

was enriched in the functions of replication, recombination, and repair. Similarly, information storage and processing also exhibits similar properties. The next most abundant genes found in the yak rumen were grouped into the carbohydrate transport and metabolism categories of the eggNOG database (Table 5). The proportion of genes classified in the categories of amino acid transport and metabolism (E), and energy production and conversion (C), linearly decreased with the MS supplemental level ($p < 0.05$). The proportion of genes classified under carbohydrate transport and metabolism (G), tended to decrease linearly with increasing MS supplemental level ($p = 0.080$).

Table 5. Effect of 2-hydroxy-4-methylthiobutanoic acid isopropyl ester supplementation in diets on relative abundance of eggNOG functional protein in rumen microbes of yak (%).

Items	Treatments [1]				SEM	*p*-Value		
	CON	MS1	MS2	MS3		Treat	Linear	Quadratic
L	7.33	7.39	7.05	7.47	0.242	0.643	0.429	0.673
G	7.10	6.84	6.87	6.60	0.171	0.258	0.080	0.232
M	6.67	6.48	6.84	6.64	0.286	0.952	0.521	0.356
E	6.15	5.95	5.73	5.74	0.173	0.300	0.006	0.194
C	4.53	4.36	4.27	4.27	0.115	0.347	0.017	0.501
T	3.82	4.08	4.37	4.34	0.413	0.762	0.956	0.244
O	3.72	3.88	3.98	3.96	0.205	0.801	0.810	0.191
K	3.92	3.86	3.71	3.82	0.085	0.354	0.835	0.955

[1] CON: yaks receiving a basic diet; MS1: yaks receiving a basic diet supplementation of 5 g MetaSmart (MS); MS2: yaks receiving a basic diet supplementation of 10 g MS; MS3: yaks receiving a basic diet supplementation of 15 g MS; SEM: standard error of mean; L: replication, recombination, and repair; G: carbohydrate transport and metabolism; M: cell wall/membrane/envelope biogenesis; E: amino acid transport and metabolism; J: translation, ribosomal structure, and biogenesis; C: energy production and conversion; T: signal transduction mechanisms; O: posttranslational modification, protein turnover, and chaperones; K: transcription.

3.6. KEGG Functional Annotation

For further functional annotation, the KEGG database was used to sort a total of 539,706 genes into 157 KEGG pathways. Comparing the four different treatment groups, there were no obvious changes in any of the pathways of different systems. The top 10 enriched KEGG pathways were related to molecular metabolism (Figure 3), including ko01110: secondary metabolite production; ko01230: biosynthesis of amino acids; ko01200: carbon synthesis; and ko00240: pyrimidine metabolism.

4. Discussion

Rumen microbes include bacteria, protozoa, fungi, and other microorganisms, and are crucial for the digestion and utilization of food nutrients in ruminants. Numerous studies have demonstrated that variations in rumen microbiota may affect energy efficiency [21]. Rumen microorganisms degrade amino acids in large quantities through their deaminating activity [22]. Because some amino acids are utilized for microbial metabolism and MCP synthesis in the rumen, lower amounts of amino acids reach the small intestine. Thus, supplements such as essential amino acids may be broken down by the rumen microbes, resulting in low availability to the animal and little improvement to ruminant health. Previous work found that Met improves bacterial nitrogen incorporation and the protein synthesis efficiency of rumen bacteria in vitro [22,23]. In this study, a 50% rumen-passing efficiency of dietary MS was assumed based on previous work [13], with 50% of the substance being released in the rumen and utilized by rumen microorganisms, which has potential effects on the composition and metabolism of the rumen microbial community.

The results of this research came to the conclusion that *Bacteroidetes* and *Firmicutes* were the dominant phyla, which is in line with the report on yaks [24]. All rumen bacteria contribute to the overall metabolic processes of the host animal. For example, *Firmicutes* participate in energy absorption, whereas *Bacteroidetes* primarily act in the metabolic reaction of carbohydrates [25]. Studies on cattle [26], dairy cows [27,28], and growing yaks [29]

identified *Bacteroidetes* and *Firmicutes* as making up the majority of bacteria in rumen. *Bacteroidetes* members produce short-chain fatty acids (SCFAs) by fermenting indigestible polysaccharides, thus converting this material to energy for the animal [30]. In this study, the relative abundance of *Bacteroidetes* among the treatments were similar.

Microbiomes, including *Cillophora*, *Euryarchaeota* and *Lentisphaerae*, can serve as functional extensions of the host genome and help regulate the physiology and metabolism of the host [31]. *Lentisphaerae* have a strong ability to metabolize polysaccharides, and starch can promote the growth of *Lentisphaerae*. When starch was added to the culture medium, the expression levels of genes encoding a large number of GH and GT were significantly upregulated [32]. The relative abundance of *Lentisphaerae* in the MS1 group was significantly higher than that of the control group. The explanation for this was supplementation with MS can improve the utilization of carbohydrates in the feed and optimize rumen fermentation. *Actinobacteria* can grow at pH = 5–9, and grow faster under alkaline conditions (pH = 7–9) with higher ATP concentration [33]. Carbohydrate in the diet was fermented and decomposed into organic acids in the rumen after yak intake. Thus, the pH decreased after the organic acids increased, caused the relative abundance of *Actinobacteria* to decrease linearly with the increase in MS supplement. Instead, adding MS can promote the degradation of nutrients in the feed in rumen. This study also found higher relative abundance of *Fibrobacteres* in the MS2 group than in the other groups. This finding was consistent with the early experimental results of this experiment, which showed higher digestibility of NDF and ADF of yaks in the MS2 group than in other groups, because the cellulase in the periplasm of *Fibrobacteres* can decompose cellulose and improve the utilization efficiency of cellulose in ruminants.

At the genus level, the most common species of bacteria in each group was *Prevotella*. *Prevotella ruminicola* can degrade protein, starch, oligosaccharides, and hemicellulose, a key component of plant cell walls [34]. In a factorial experimental design, a high concentrate diet was able to increase the *Prevotella* abundance in buffalo [35]. In feedlot lambs, increased abundance of *Prevotella* was associated with augmented concentrations of vitamin B12, and was found inversely correlated with methane emissions [36]. These results show that a high abundance of *Prevotella* in ruminants is associated with a healthy microbiome, in which carbohydrates and free amino acids are limiting factors. In this work, the abundance of the *Prevotella* genus increased linearly with the addition of MS, with the MS2 group showing higher relative abundance of *Prevotella* compared to the other groups, showing that addition of 10 g/d MS to the diet supplies sufficient free amino acids for yaks. The relative abundance of *Butyrivibrio* decreased linearly with the increase in MS supplemental level. *Butyrivibrio* has been linked to hemicellulose degradation, and its abundance decreases as the rumen's degradable carbohydrate content increases [37]. We hypothesized that the lipid-containing MS coating material, and an increase in degradable carbohydrates in the rumen, reduces its relative abundance. The relative abundance of *Alistipes* tended to decrease linearly with the increase in MS supplemental level. *Alistipes* deficiency can lead to a lack of SCFA, and is related to progress to a state of decompensated cirrhosis [38]. The results suggest that lower abundance of *Alistipes* associated with MS supplementation may cause liver burden. In other words, excessive addition of MS may cause liver burden, but this experimental treatment (5 g, 10 g, 15 g) was safe when provided to yaks.

Relative abundance of rumen bacteria genera and their relationship with the rumen fermentation parameters has been widely studied [39]. Positive correlations were found between rumen fermentation parameters as total VFA concentration and pH, and the relative abundance of *Prevotella* [40]. Data analysis in the current investigation revealed a favorable correlation between acetic acid and valeric acid concentrations, and the relative abundance of *Prevotella*. Acetic acid, lactic acid, butyric acid, and valeric acid were positively correlated with the relative abundance of the unclassifiable *_f__ Prevotellaceae*. It was shown that all *Prevotella* species are connected to one another. Chiquette et al. also observed that feeding cows with members of the *Prevotella* species as direct-fed microbes dramatically raised rumen concentrations of acetic and butyric acids; therefore, our results were consistent with

their findings [41]. This may be because *Prevotella* can efficiently degrade hemicellulose and other complex carbohydrates, resulting in the production of VFAs that are important sources of energy for the host animal. The higher butyrate concentration indicates an increased rate of fiber fermentation. In a study of Bangladeshi children, *Bacteroides* promoted fermentation of glycans and generated SCFAs [42]. *Parabacteroides* is considered an important bacterial genus for VFA generation [43]. A study in cows indicated the positive relationship of *Fibrobacter*, a primary cellulolytic bacteria, to key enzymes in propionate formation via the succinic pathway [44]. In this work, isobutyric concentration was positively correlated with the *Clostridium*, consistent with the finding of Miguel et al. that *Clostridium* can improve ruminal fermentation by increasing butyrate concentrations [45]. *Butyrivibrio* is known as a fibrolytic bacterium, but it can also utilize starch and produce butyrate [46,47]. Previous studies found that a high-concentrate diet decreased the population of *Butyrivibrio* in cattle [48]. The drop in the *Butyrivibrio* population may be due to an increased number of fermentable substrates in the rumen. The relative abundances of *Stylonychia* and *Stentor* genera were negatively correlated with concentrations of acetic acid and total VFA. There are numerous CAZymes and peptidases in *Stylonychia* [49], and the ability to depolymerize starch, hemicellulose, and pectin may reduce the concentration of total VFA. The *Alveolata* clade, the most abundant among the eukaryotes, is largely associated with methane emissions [50]. Little is known about the metabolism of *Stylonychia* in the *Alveolata* clade. *Succiniclasticum* generates propionate via succinate decarboxylation [51]. The relative abundance of *Succiniclasticum* was positively correlated with the concentrations of NH_3-N, isobutyric acid and isovaleric acid. Isovalerate can be used by fiber-degrading ruminal bacteria, and may be related to the elevation of ammonia levels [52]. We concluded that *Succiniclasticum* may be involved in protein degradation. The observed positive correlation between *Selenomonas* abundance, and the concentrations of MCP and isovaleric acid suggests that this genus highlights its potential as a target for optimizing rumen fermentation in ruminant animals.

The CAZymes, produced by the rumen microbiota breakdown of rumen cellulose, participate in many biological processes in the body, including carbohydrate metabolism, protein glycosylation, and degradation processes [53]. Gong et al. reported that the fecal microbiota of yaks varies depending on the kind of food that they ingest [54]. Additionally, the abundances of CAZymes in the rumen microbiota of yaks can show seasonal variations due to seasonal variation in their feed composition [55]. CE catalyze the acylation of substituted sugars. *Prevotella* ruminant 23, grown on an ester-rich substrate, had 16 CE genes upregulated relative to the non-esterified portion of corn oligosaccharides [56]. In this experiment, the relative abundance values of CE decreased linearly with the increase in MS supplemental level, perhaps due to the MS supplementation inhibiting the ester hydrolysis of plant polysaccharides. PL cleaves the polysaccharide-containing uronic acid to produce unsaturated hexalonic acid and a new reducing end [57]. There were 15 CAZymes belonging to PL, which correspond to *Prevotella* genomes in the CAZy database [36]. *Prevotella,* which contain a group of coregulatory genes, can encode enzymes that specifically degrade noncellulosic plant fibers [58]. The relative abundances of PL in the MS2 and MS3 groups were higher than those in other groups, showing a trend of secondary increase. This was in line with the results of the abundance of the *Prevotella* genus increasing linearly with the addition of MS.

NOG annotations were obtained from analysis with the eggNOG database and revealed enrichment in the categories of metabolism, cellular processes, and information storage and processing. Next, the annotations of several proteins with relatively high abundances were statistically analyzed with the NOG database, and the results showed that genes related to L were the most abundant, followed by genes related to G. This could be due to the yak's adaption to a high-altitude environment, as well as its coarse feed diet. There were also relatively large enrichments in genes related to M, E, J, and T, which is consistent with previous studies on Indian water buffalo [59]. We found decreased genes involved in E and C, and decreased genes involved in G, with increased MS supplemen-

tation levels. A study on yak found higher numbers of genes involved in E and C in older yaks [60]. MS, as a supplement of concentrate feed, may reduce the stress of yaks in their harsh habitat. The underlying mechanisms connecting Met supplementation and carbohydrate, energy, and amino acid transport and metabolism in ruminants, deserves further research.

KEGG analysis is frequently utilized to investigate specific molecular operations, cellular constituents, and biological and signaling activities of genes [61]. Enrichment of KEGG pathways can suggest possible important metabolic pathways and enzymes required for various reactions [62–64]. The KEGG analysis revealed the highest abundances of genes associated with metabolism in all groups of yaks in this study. These genes were followed by those associated with genetic information processing, disease, organismal systems, environmental information processing, and other cellular processes. Most of the identified pathways are related to the metabolism of nutrients and small molecules, a finding consistent with the results of a cattle–yak study [60].

5. Conclusions

MS supplementation was able to affect yak rumen microbiota and CAZy enzyme classes, promote the degradation of carbohydrates, and optimize rumen fermentation. Hence, MS supplementation is an effective method to optimize rumen microbial community structure and improve feed nutrient utilization of yaks. Further research should explore the detailed mechanism through which Met supplementation alters gene functional changes in ruminants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10020094/s1, Table S1: The relationship involving the number of the first 30 bacteria species found in the yak rumen and rumen fermentation parameters.

Author Contributions: H.W., Z.P. and J.Z. conceived the study; Y.L. and X.Z. performed the bioinformatics and statistical analysis; Z.Z. and C.W. conducted the investigation; H.W., Z.P. and J.Z. oversaw the project's management; H.W., Y.L. and X.Z. wrote the first manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the National Natural Science Foundation of China (32102577); the China Agriculture Research System (CARS-37); the Natural Science Foundation of Sichuan Province (2022NSFSC1623); the Fundamental Research Funds for the Central Universities; the Southwest Minzu University (ZYN2023004); the National Modern Agricultural Industrial Technology System Sichuan Beef Cattle Innovation Team Building Project (sccxtd-2023-13); the research on Key Technology and Integrated Application of efficient yak breeding (20230044); the Qinghai Science and Technology Program (2022-NK-110); the Innovation Team Development Funds for Sichuan Beef (cxtd2019-13); and the National Key R&D Program of China (2022YFD1601601).

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee (protocol number: 2023MDLS010) of Southwest Minzu University (Chengdu, Sichuan, China).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw sequencing files and associated metadata have been deposited at NCBI's Sequence Read Archive (accession PRJNA953596), http://www.ncbi.nlm.nih.gov/sra (accessed on 5 April 2023).

Acknowledgments: We are grateful to Shengyuan Yak Husbandry Co., Ltd., and Adisseo Inc.

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

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