fermentation

MDPI

Article

Corn Straw Total Mix Dietary Supplementation of Bacillus Subtilis-Enhanced Growth Performance of Lambs by Favorably Modulating Rumen Bacterial Microbiome

Yuan Gao [1], Wurilege Wei [2], Feng Tian [2], Jiuyue Li [2], Yufei Wang [2], Jingwei Qi [1,*] and Shuyuan Xue [2,*]

[1] College of Animal Science, Inner Mongolia Agricultural University, Hohhot 010018, China
[2] Institute of Animal Husbandry, Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, Hohhot 010031, China
* Correspondence: qijingwei_66@126.com (J.Q.); shuyuanxue@163.com (S.X.)

Abstract: In this experiment, *B. subtilis* was added to pelletized straw-based total mixed ration, and the rumen microbial diversity of house-fed Duhan hybrid sheep was compared. Ten 3-month-old weaned Duhan hybrid lambs were separated into two groups and fattened for 80 days using a single-factor trial design. During the fattening period, the control and the experiment groups were fed with the same ration, except that the experiment group was supplemented with *B. subtilis*. The results showed that the addition of *B. subtilis* could significantly increase the daily weight gain, total weight gain, rumen microbial abundance, and rumen microbial diversity of the Duhan lamb. Among them, the proportion of microbial flora such as *Bacteroidetes* was significantly increased, producing more acetate, iso-butyrate, and butyrate, obtaining higher energy efficiency.

Keywords: *Bacillus subtilis*; rumen microbes; Duhan lamb

Citation: Gao, Y.; Wei, W.; Tian, F.; Li, J.; Wang, Y.; Qi, J.; Xue, S. Corn Straw Total Mix Dietary Supplementation of Bacillus Subtilis-Enhanced Growth Performance of Lambs by Favorably Modulating Rumen Bacterial Microbiome. *Fermentation* **2023**, *9*, 32. https://doi.org/10.3390/fermentation9010032

Academic Editor: Qing Zhang

Received: 13 November 2022
Revised: 23 December 2022
Accepted: 26 December 2022
Published: 30 December 2022

1. Introduction

The use of natural alternatives in the livestock industry is rapidly increasing due to the global ban of the use of in-feed antibiotics as growth promoting agents, which include probiotics, prebiotics, enzymes, and plant nutraceuticals [1–3]. Direct-fed microbials (DFM) are products that contain live (viable) probiotic microorganisms (bacteria and fungi) with the primary goal of enhancing animal health and improving production in ruminants, and therefore has led to increasing interest in the animal industry. However, the efficacy of DFM in promoting animal health and production performance varies greatly owning to the different microbial species compositions of the DFM products, animal species and diets, and feeding conditions [4–6].

Bacterial DFM for ruminants mostly consist of propionate-forming bacteria and lactic acid-producing bacteria from a range of genera. Recently, interest in the use of *Bacillus* species as DFM in ruminants has been increasing owing to their specific properties, e.g., heat stability and ability to survive the low pH of the digestive tract. *Bacillus subtilis* (BS) is one of the species from genus *Bacillus* with strong environmental adaptability [7] that produces polymyxin, nystatin, gramicidin, and other active substances such as enzymes (alpha-amylase, protease, lipase, cellulase, etc.) and vitamins during growth. These active substances possess strong inhibitory effects on pathogens and the subsequent infection as well as favorably modulating nutrient metabolism and immune function [8,9]. *B. subtilis* rapidly consumes free oxygen in the intestine, resulting in hypoxia, which promotes the growth of beneficial anaerobic bacteria while preventing the growth of harmful bacteria. Therefore, BS could be a promising DFM for ruminants. Several strains of BS (e.g., BS natto, BS PB6, BS 10071, and BS C-3102) have been assessed in cattle and in vitro for their effects on rumen fermentation and productive performance [10–15]. However, there is scarce information about the effects of BS on the rumen microbiome that is crucial for

elucidating the mechanism by which BS improves rumen metabolism, animal health, and productive performance.

The objectives of this study were to evaluate the effects of the supplementation of BS on the rumen metabolism and growth performance of lambs and to determine its effects on the rumen bacterial microbiome.

2. Materials and Methods

2.1. Experimental Design, Animals, and Diet Preparation

Ten (five male and five female) lambs (3 months old) with an initial body weight (BW) of 24 ± 1.01 kg were randomly divided into two groups stratified by gender, and were randomly allocated to two dietary treatments. The treatments were basal diet only (Control; C) and basal diet supplemented with *B. subtilis* C-3102, *B. subtilis* at the concentration of 300 g/ton; 3×10^8 CFU/kg (experimental group; BS) [16]. The basal diet was a corn, corn stalk, and DDG-based total mixed ration (TMR) that was formulated to meet the nutrient requirements of growing sheep (NRC; 2012) for two growing stages (Table 1). The ingredients of the TMR were mixed thoroughly, tempered at 90 °C for 45 s, and then pelletized at 60 °C to an average size of 0.5 × 5 cm pellets using a YPM508E Granulator (Jiangsu Yongli Machinery Co., Ltd., Liyang City, Jiangsu Province, China). The *B. subtilis* product was obtained from Calpis Trading Co., Ltd. of Japan Asahi Group (Shanghai, China) that contained minimum 1×10^9 CFU/g of viable cells and was mixed with other dietary ingredients prior to pelleting. All diets were made in one batch at the beginning of the experiment and stored in covered containers for the entire experiment period.

Table 1. Ingredients and nutrient composition of the diet fed to the lambs.

Raw Material	Control (C) Group		*Bacillus subtilis* (BS) Group	
	First Stages	Later Stages	First Stages	Later Stages
Corn (%)	45.00	53.08	45.00	53.08
DDGS (%)	23.00	19.54	23.00	19.54
Soybean meal (%)	4.28	1.67	4.28	1.67
1% additive (%)	0.76	0.76	0.76	0.76
Stone powder (%)	1.00	1.00	1.00	1.00
NH_4Cl (%)	0.42	0.42	0.42	0.42
NaCl (%)	0.50	0.50	0.50	0.50
$NaHCO_3$ (%)	0.42	0.42	0.42	0.42
Bacteria preparation (%)	0	0	0.03	0.03
Soybean hulls (%)	6.93	2.79	6.90	2.76
Corn stalks (%)	17.69	19.82	17.69	19.82
Total (%)	100	100	100	100
Nutrient content				
Digestive energy (MJ/kg)	89.13	88.27	88.63	87.98
Ash (%)	6.15	6.11	6.24	6.10
CP (%)	14.91	13.86	14.67	13.80
EE (%)	5.09	3.63	5.02	3.83
NDF (%)	29.89	25.72	28.17	25.40
DNF (%)	15.24	12.14	15.19	12.03
ADF (%)	3.51	2.95	3.68	2.88
Ca (%)	0.51	0.42	0.60	0.48
P (%)	0.40	0.35	0.45	0.35

2.2. Experimental Procedure and Sampling

A total of 87 d feeding experiment was conducted with a 7 d adaptation followed by an 80 d data collection period. The lambs were individually fed twice daily for ad libitum intake throughout the adaptation and experimental periods. The lambs were fed the Stage 1 diet for the first 35 d followed by the Stage 2 diet for the remaining experimental period. The orts were weighed weekly for measuring dry matter intake (DMI). The lambs were

weighed twice after overnight fast at the beginning and at the end of the experiment, and weekly between. The animals had free access to water during the entire experimental period. The animal care protocol was approved by the Animal Care Committee, Inner Mongolia Autonomous Region Academy of Agriculture and Animal Husbandry, Hohhot, Inner Mongolia, China.

At the end of the experiment (d 87), all lambs were slaughtered after being fasted for 18 h using the procedure described by Geng et al. [12]. The carcass characteristics including carcass weight, carcass ratio, and weights of the heart, liver, lung, spleen, kidney, rumen, reticulum, ovum, true stomach, and intestines were determined using the procedures described by Geng et al. [17].

The rumen was opened immediately upon removal and the rumen content was strained through four layers of cheesecloth to obtain the rumen fluid. The rumen fluid from each lamb was divided into two portions, one being stored at −20 °C in 50 mL centrifuge tubes for the determination of the total volatile fatty acids (VFA), microbial protein, and ammonia nitrogen and the other being stored at −80 °C in 5 mL cryopreservation tubes for DNA extraction and high-throughput sequencing analysis.

2.3. Laboratory Analyses

2.3.1. Determinations of Rumen Ammonia Nitrogen (NH_3-N), VFA, and Microbial Protein

The rumen fluid samples were processed and analyzed for NH_3-N as described by Feng et al. [18], microbial protein using procedures described by Bradford [19], and for VFA using gas chromatography [20].

2.3.2. Determination of Microbial Compositions by Sequencing

Genomic DNA was extracted from the rumen fluid samples using the CTAB method described by Rogers et al. [21]. The purity and concentration of the extracted DNA were determined by agarose gel electrophoresis, as described by Lee et al. [22]. The extracted rumen microbial DNA was diluted to 1 ng/μL and the genome sequencing was conducted by Nuohe Zhiyuan Technology Co., Ltd. (Beijing, China). Then, 16S rRNA genes of the distinct regions V3-V4 were amplified using a specific primer (341F 5′-CCTAYGGGRBGCASCAG-3′; 806R5′-GGACTACNNGGGTATCTAAT–3′) with the barcode. All PCR reactions were carried out in a 30 μL reaction system with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μmol of forward and reverse primers, and 10 ng of DNA. The thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s.

The PCR product obtained from the above procedure was then mixed with the same amount of buffer containing SYB green and was electrophoresed on 2% agarose gel and purified with a Gene JETTM Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA).

Sequencing libraries were generated using an Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, Waltham, MA, USA) following the manufacturer's recommendations. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific). The library was sequenced on an Ion S5 TM XL platform and 400 bp/600 bp single-end reads were generated.

2.4. Calculation and Statistical Analysis

The operational taxonomic unit (OTU) database single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on the raw reads was performed under specific filtering conditions to obtain the high-quality clean reads according to the Cutadapt quality control process [23]. The reads were compared with the reference database using the UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html accessed on 20 December 2022) [24] to identify chimera sequences. The clean reads were obtained after the removal of the chimera sequences [25]. Sequence analysis was

performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/ accessed on 20 December 2022) [26]. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequences from each OTU were screened by Chao1, observed species, and Shannon's index to obtain α diversity. QIIME was employed to calculate both weighted and unweighted UniFrac to obtain beta diversity. Unweighted UniFrac was used for principal coordinate analysis (PCoA).

The data were analyzed by analysis of variance using the PROC MIXED procedure of SAS with the individual animal as a statistical unit. Differences were determined using the PDIFF option in SAS 9.4. Significance was declared at $p < 0.05$ and the tendency of significant differences was described at p values between 0.05 and 0.1.

3. Results

3.1. Growth Performance and Carcass Characteristics

Lambs had similar BW (kg) at the beginning of the experiment (25.3 ± 2.07 kg vs. 25.9 ± 2.11 kg; $p = 0.140$). Dietary supplementation of *B. subtilis* had no effects on DMI (1.52 ± 0.15 kg vs. 1.53 ± 0.20 kg; $p = 0.830$), but increased average daily gain (255.8 ± 56.22 g vs. 284.3 ± 58.78 g; $p < 0.02$) of the lambs, resulting in significantly heavier (48.7 ± 5.82 vs. 45.7 ± 5.53 kg; $p < 0.05$) lambs in the BS than in the C group at the end of the experiment.

All lambs had similar carcass weight, carcass ratio, and weights of heart, liver, lung, spleen, kidney, rumen, reticulum, ovum, true stomach, and small intestine ($p > 0.05$). However, the large intestine was significantly heavier ($p < 0.05$) for the BS (265.1 ± 11.12 g)-supplemented group than for the C group (224.9 ± 32.61 g) lambs. The total weight of the digestive tract of the lambs in the BS group (2.4 ± 0.14 kg) was also significantly higher ($p < 0.05$) than that in the C group (2.2 ± 0.16 kg).

3.2. Rumen Metabolites

The dietary supplementation of *B. subtilis* did not affect ($p > 0.05$) ruminal pH or concentrations of NH_3-N and total VFA, but increased ($p \leq 0.05$) the concentration of microbial protein (Tables 1 and 2). The concentrations of acetate, iso-butyrate, and butyrate were higher ($p < 0.01$), but that of propionate was lower ($p < 0.01$) for the BS group than for the C group, resulting in a higher ratio of acetate to propionate ($p < 0.01$) for BS than for C lambs.

Table 2. pH and concentrations (mean ± SE; $n = 5$) of ammonia-N (NH_3-N), volatile fatty acids (VFA), and microbial protein in the rumen fluid of lambs fed basal diet only (Control; C) or basal diet supplemented with *Bacillus subtilis* at the concentration of 3×10^8 CFU/kg DM (BS).

Index	C	BS	*p*-Value
pH	6.6 ± 0.31	6.7 ± 0.32	0.164
NH_3-N; mg/100 mL	13.2 ± 0.98	11.6 ± 0.81	0.133
Microbial protein; mg/L	3.9 ± 0.64	5.8 ± 0.36	0.050
Total VFA; mmol/L	27.1 ± 7.48	23.9 ± 3.85	0.450
Acetate; %	70.9 ± 2.77	74.2 ± 1.87	<0.001
Propionate; %	21.4 ± 2.96	14.7 ± 4.97	<0.001
Iso-butyrate; %	2.5 ± 0.90	4.8 ± 2.31	0.011
Butyrate; %	5.1 ± 1.15	6.4 ± 1.20	0.010
Acetate/propionate	3.4 ± 0.51	5.6 ± 1.89	<0.001

3.3. Bacterial Microbiome of the Rumen Fluid

Effects of *B. Subtilis* on Rumen Microbiota

An average of 56,934 clean read sequences with an average length of 417 bp were obtained from all 10 rumen fluid samples, which sufficiently covered the bacterial communities in these samples (Figure 1). There were 17 phyla and 27 genera (eight unidentified families) among these samples. Overall, *Firmicutes* was the most dominant phylum for the

rumen fluid from both the BS group (49.9%) and the C group (57.7%), and at the genus level, *Succiniclasticum* was relatively the most abundant genus, although on average, 86.1% of sequences could not be classified to a particular genus.

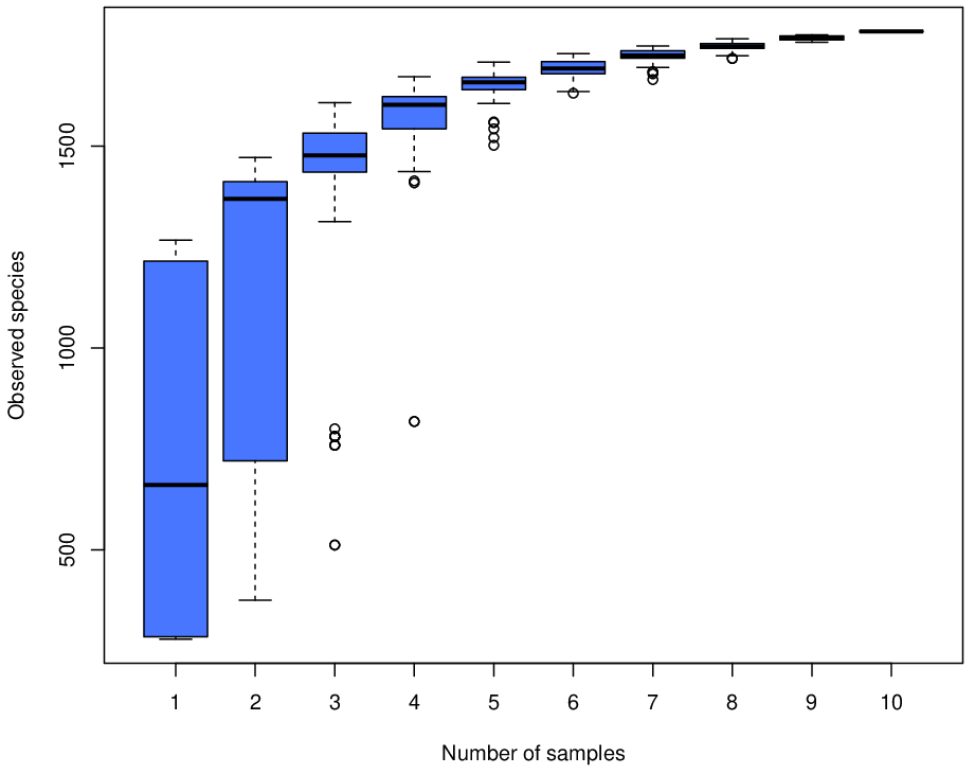

Figure 1. Horizontal axis is the sample size; vertical axis is the number of OTUs after sampling.

The alpha diversity analysis showed that the OTUs, Chao1, ACE, PD whole tree, and Shannon indices of BS group were significantly higher ($p < 0.01$) than that of the C group (Tables 1 and 3).

Table 3. Alpha indices (mean ± SE; $n = 5$) of observed species, Shannon, Chao1, ACE, and PD whole tree in the rumen fluid of lambs fed basal diet only (Control; C) or basal diet supplemented with *Bacillus subtilis* at the concentration of 3×10^8 CFU/kg DM (BS).

Indices	C	BS	*p*-Value
Observed species	397.6 ± 155.89	1215.6 ± 67.60	<0.001
Shannon	5.0 ± 0.56	8.0 ± 0.54	<0.001
Chao1	484.6 ± 36.15	1279.4 ± 82.33	<0.001
ACE	498.1 ± 41.92	1282.4 ± 79.94	<0.001
PD whole tree	39.7 ± 10.30	88.6 ± 3.39	<0.001

The Venn diagram (Figure 2) revealed significant differences in the OTUs between the C group and the BS group. The two groups shared 660 OTUs, but the C group had 184, whilst the BS group had 862 unique OTUs, respectively.

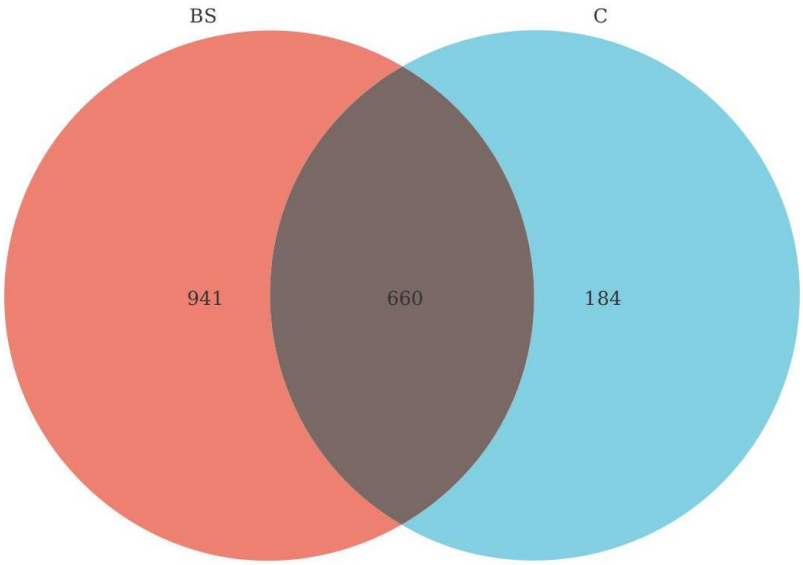

Figure 2. Venn diagram of OTU distribution in GIT. Each petal in the diagram represents a sample, and different colors represent different samples. The core number in the middle represents the total number of mutual OTUs in all samples, and the number on the Venn represents the number of unique OTUs in the sample.

At the phylum level, the abundances of *Firmicutes*, *Actinobacteria*, *Oxyphotobacteria*, and *Proteobacteria* in the BS group were significantly lower than the C group ($p < 0.05$), whereas the abundances of *Bacteroidetes* and *Tenericutes* were higher ($p < 0.05$) for the BS group than for the C group of sheep. Other taxonomies less than 1% and those not annotated only accounted for 1.6% and 2.2% for the C and BS groups, respectively (Tables 1 and 4). The heat map of the phylum-level classification also showed the same trend (Figure 3). *Firmicutes* and *Bacteroidetes* were the most abundant phylum in both group of sheep, although the abundance of microorganisms in each phylum varied between the two groups (Figure 4). The total abundances of *Firmicutes* and *Bacteroidetes* were 94.9% in the BS group and 84.6% in the C group. Interestingly, the *Firmicutes/Bacteroidetes* (F/B) ratios were 1.11 and 2.14 for the BS and C groups, respectively.

Table 4. Abundance (mean ± SE; $n = 5$; >1%) at phylum level of bacteria in the rumen fluid of lambs fed basal diet only (Control; C) or basal diet supplemented with Bacillus subtilis at the concentration of 3×10^8 CFU/kg DM (BS).

Taxonomy	C	BS	*p*-Value
Firmicutes	57.7 ± 14.20	49.9 ± 8.76	0.322
Bacteroidetes	26.9 ± 10.10	45.0 ± 8.67	0.016
Proteobacteria	10.8 ± 9.45	1.3 ± 0.37	0.052
Actinobacteria	2.8 ± 2.61	0.0 ± 0.01	0.071
Tenericutes	0.1 ± 0.035	1.1 ± 0.13	<0.001
Others	1.6 ± 1.34	2.2 ± 0.13	0.241

At the genus level, the abundances of unidentified Lachnospiraceae, Rikenellaceae and Bacteroidales, and Saccharofermentans were higher ($p < 0.05$), but the abundances of Succiniclasticum, Succinivibrio, Dialister, Syntrophococcus, and Shuttleworthia were lower ($p < 0.05$) in the rumen fluid of the BS lambs than that of the C group lambs. The abundances of Ruminococcaceae, Pseudoscardovia, Roseburia, Selenomonas, and unidentified Prevotellaceae and Clostridiales were similar between the two groups (Tables 1 and 5). The taxonomy accounting for less than 1% of the abundance with no annotation was higher ($p < 0.05$) for the BS than for the C group of lambs. The heat map of the genus-level classification also showed the same trend (Figure 5).

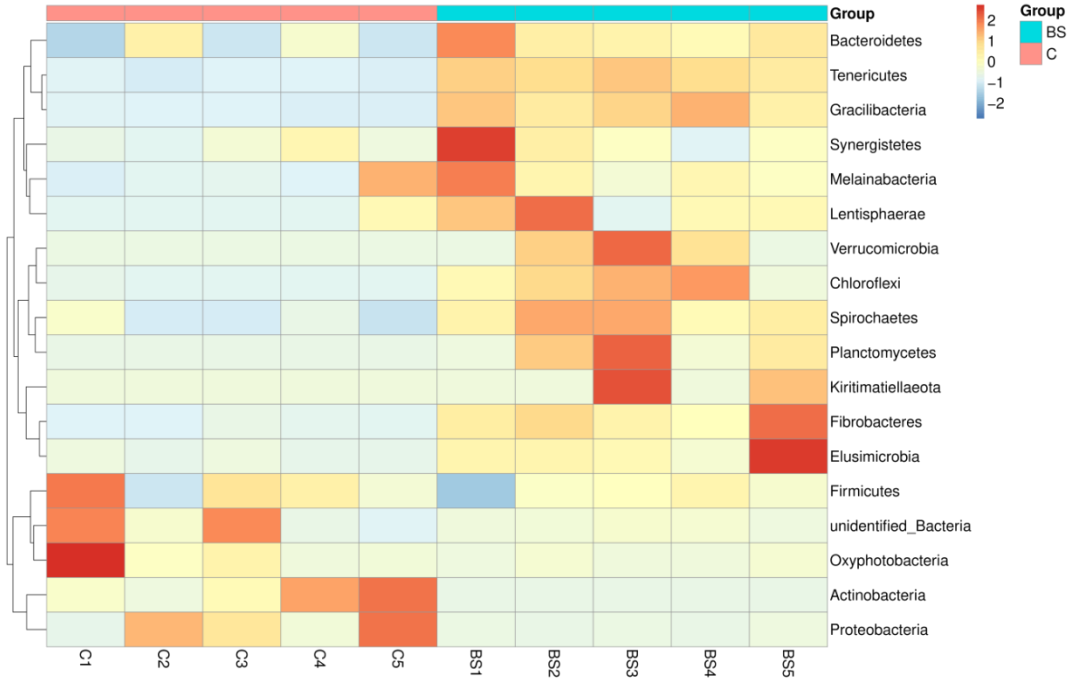

Figure 3. Analysis of rumen bacterial populations at the phylum classification level.

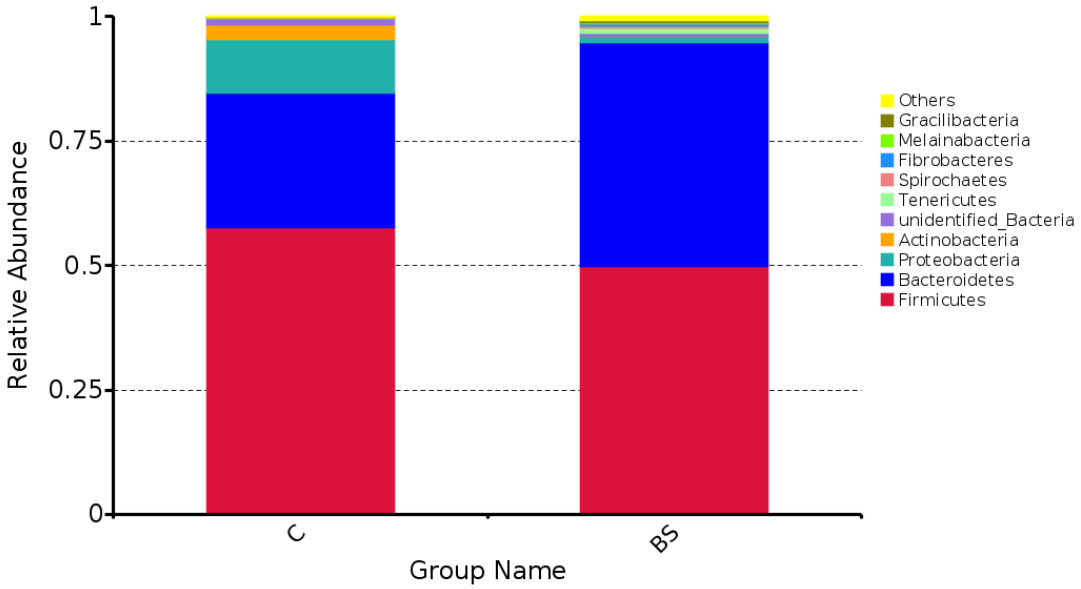

Figure 4. Species relative abundance column chart at the phylum level.

Table 5. Abundance (mean ± SE; n = 5; >1%) at genus level of bacteria in the rumen fluid of lambs fed basal diet only (Control; C) or basal diet supplemented with Bacillus subtilis at the concentration of 3×10^8 CFU/kg DM (BS).

Index	C	BS	*p*-Value
unidentified_*Ruminococcaceae*	6.5 ± 3.02	9.9 ± 2.63	0.092
unidentified_*Lachnospiraceae*	2.2 ± 1.72	4.8 ± 1.46	0.036
unidentified_*Rikenellaceae*	0.1 ± 0.13	2.7 ± 0.75	<0.001

Table 5. *Cont.*

Index	C	BS	*p*-Value
unidentified_*Prevotellaceae*	6.2 ± 5.60	2.1 ± 0.41	0.138
unidentified_*Bacteroidales*	0.1 ± 0.03	1.6 ± 0.31	<0.001
Saccharofermentans	0.0 ± 0.02	1.50 ± 0.51	0.003
Succiniclasticum	7.7 ± 5.44	1.0 ± 0.33	0.025
Succinivibrio	7.8 ± 6.86	0.0 ± 0.04	0.035
Dialister	6.2 ± 2.97	0.0 ± 0.01	0.004
Pseudoscardovia	2.0 ± 2.45	0.0 ± 0.00	0.23
unidentified_*Clostridiales*	1.9 ± 1.95	0.58 ± 0.16	0.176
Syntrophococcus	1.7 ± 1.42	0.1 ± 0.035	0.036
Roseburia	1.4 ± 1.66	0.0 ± 0.01	0.108
Selenomonas	1.4 ± 0.88	0.0 ± 0.00	0.095
Shuttleworthia	1.3 ± 0.73	0.1 ± 0.03	0.005
Others	53.50 ± 0.64	75.62 ± 5.27	0.002

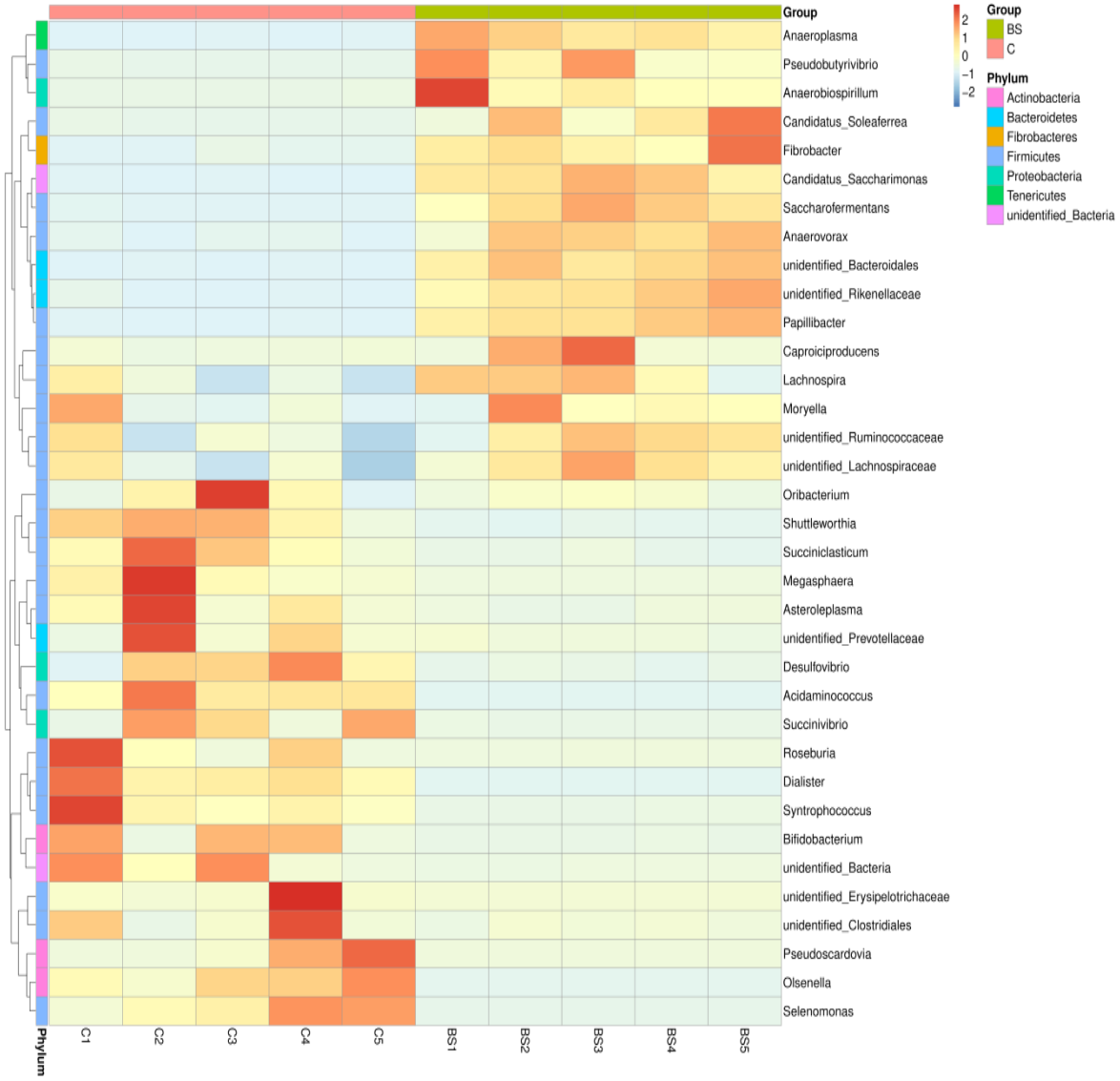

Figure 5. Heat map of genus-level classification.

The evolutionary cladogram revealed that the species playing an important role were *Rikenellaceae*, *Bacteroidales*, *Bacteroidia*, *Bacteroidetes*, *Christensenellaceae*, and *Ruminococcaceae*

in the BS group, whereas these were *Bifidobacteriaceae*, *Bifidobacteriales*, *Actinobacter*, *Acidaminococcaceae*, *Veillonellaceae*, *Selenomonadales*, *Negativicutes*, and *Lachnospiraceae* for the C group (Figure 6). The other taxonomies that accounted for less than 1% in both groups were excluded.

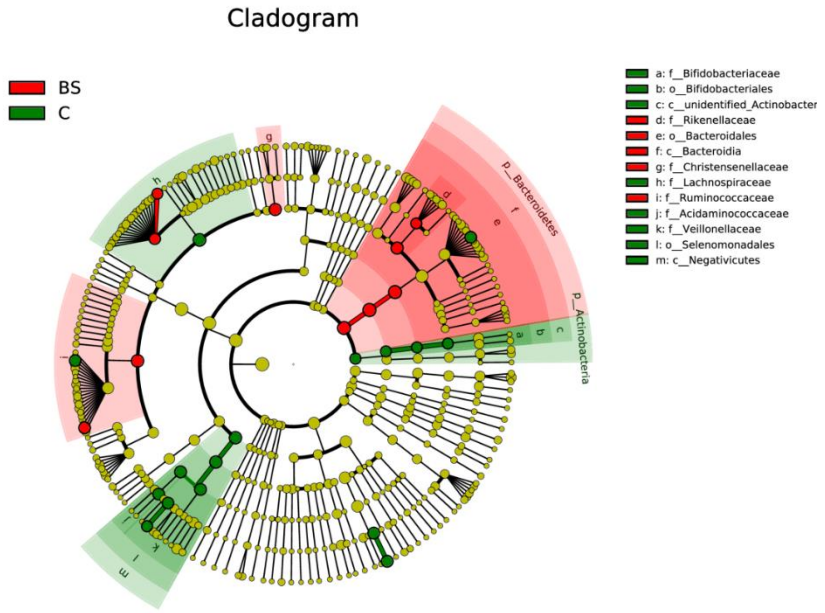

Figure 6. Taxonomic cladogram reporting the different taxon abundances among GIT groups.

4. Discussion

4.1. Effects of BS Supplementation on Ruminal Fermentation

Although feed intake was not affected, the ADG and final body weight were increased by *B. subtilis*, indicating that the supplementation of *B. subtilis* increased the growth rate and the feed efficiency of the lambs in this study. It needs to be pointed out that only five lambs were used in each group because this study focused on the effects of treatment on the rumen microbiome. The animal replicate number was relatively small compared with a typical growth performance study. Therefore, this part of the results was only indicative, and a large-scale feeding experiment needs to be conducted to confirm the findings of this study. Nevertheless, our previous study also showed that supplementation of the same *B. subtilis* product promoted the growth and development and increased the feed intake of Duhan hybrid sheep [27]. The higher growth rate of the lambs supplemented with *B. subtilis* might due to the greater microbial protein synthesis, as indicated by its higher microbial protein concentration in the rumen than that of the C group lambs. This is also consistent with the microbiome analysis results that showed that bacterial populations, diversity, and abundance were all increased by *B. subtilis* supplementation. Although the total VFA concentration was not affected, the lambs supplemented with *B. subtilis* had higher concentrations of acetate and butyrate than those in the C group. This may indicate that *B. subtilis* increased the fiber digestion because it is known that the digestion of fiber is prone to produce acetate and butyrate [28]. It has been shown that short-chain fatty acids are an important source of energy for ruminants, with up to 70% of the energy requirements of adult animals being absorbed as short-chain fatty acids through the stratified squamous epithelium of the rumen [29]. It has been shown that at the same nutritional level, feeding *B. subtilis* could improve energy efficiency and increase the butyric acid content [30], which is consistent with the observations in this study.

4.2. Effects of BS Supplementation on Bacterial Diversity

The bacterial microbiome analysis showed that the OTU number in the BS group was more than two times higher ($p < 0.01$) than that in the C group, demonstrating that more

bacterial species were observed in the BS group lambs than the C group lambs. Higher Shannon index and PD whole tree values were observed for the *B. subtilis*-supplemented lambs (BS group) than for the C group lambs. Shannon and PD whole tree are positively correlated with bacterial diversity, demonstrating that the supplementation of *B. subtilis* under the conditions of this study increased the bacterial phylogenetic diversity. Increased bacterial diversity by BS supplementation was also observed for cattle and broilers [31]. It has been shown that *B. subtilis* could produce a variety of secondary metabolites that inhibit and kill pathogenic bacteria such as *Staphylococcus aureus*, *Candida albicans*, *Listeria monocytogenes*, *Escherichia coli*, and *Enterococcus* when it was supplemented to sheep and lambs [32]. This suggested that the supplementation of *B. subtilis* has the potential to promote intestinal health via the modification of the microbiome in the digestive tract [33]. Research has shown that rumen microbial diversity is positively correlated with the resistance of animals to environmental influences [34]. Therefore, the increased bacterial diversity achieved by BS supplementation may have partially contributed to the enhanced growth performance of the lambs observed in this study. Zhang et al. also reported a similar relationship between rumen bacterial diversity and animal growth performance in cattle [35].

4.3. Effects of BS Supplementation on Bacterial Abundances

Chao1 and ACE are positively correlated with bacterial abundances. The higher values of Chao1 and ACE observed for the *B. subtilis*-supplemented lambs than for the C group lambs indicated that the supplementation of *B. subtilis* increased rumen bacterial abundance under the conditions of this study. This study showed that *Firmicutes* and *Bacteroidetes* were the two most dominant phyla regardless of the treatment, which is consistent with the observations of other studies [35], indicating that these two phyla play a key role in rumen metabolism. It has been suggested that the F/B ratio in the gut impacts intestinal homeostasis and the energy metabolism of the body [36]. This study found that the supplementation of BS decreased the F/B ratio mainly due to its effect on increasing the abundance of Bacteroidetes. A literature review showed that the dietary supplementation of probiotics affected the F/B ratio depending on the specific probiotics [37]. The phylum Bacteroidetes includes approximately 7000 different species of Gram-negative bacteria that are predominantly from the genera Bacteroides, Alistipes, Parabacteroides, and Prevotella. Bacteroidetes express a relatively large number of genes encoding carbohydrate active enzymes, thus promoting the breakdown of rumen structural polysaccharides. They can also ferment amino acids into acetate [35]. These are consistent with the increased acetate proportion by BS supplementation in this study.

Although the decrease of the abundances of the phyla *Proteobacteria* and *Actinobacteria* by BS supplementation did not reach statistical difference, the large scale of the reduction and its potentially biological implications still need to be noted. Further analysis showed that the genera *Succinivibrio* and *Pseudoscardovia* accounted for more than 70% of the genera in the phyla *Proteobacteria* and *Actinobacteria* for the C group of lambs, whereas it decreased to less than 1% for the BS group of lambs, indicating that the negative effect of BS supplementation on the phyla *Proteobacteria* and *Actinobacteria* was mainly due to its effects on decreasing the abundance of the genera *Succinivibrio* and *Pseudoscardovia*, respectively. Both *Succinivibrio* and *Pseudoscardovia* are opportunistic pathogens possessing health implications to animals [35,38]. *Tenericutes* were significantly higher ($p < 0.05$) for the BS group than for the C group. It is one of the inhabitants of the gastrointestinal tract; many studies have focused on pathogenic species, but recent studies found that *Tenericutes* expressed more carbon metabolism genes. Sugars such as xylose, galactose, and fructose might be fermented to lactate, formate, and acetate. These are consistent with the increased acetate proportion by BS supplementation in this study [39].

Within the phylum *Firmicutes*, the abundances of unidentified *Ruminococcaceae*, unidentified *Lachnospiraceae*, and *Succiniclasticum* each accounted for more than 1% of the total bacterial populations. In both groups, the abundances of *Dialister*, unidentified *Clostridiales*,

Syntrophococcus, *Roseburia*, *Selenomonas*, and *Shuttleworthia* each exceeded 1% of the total bacterial populations for the C group of lambs only. On the contrary, the abundance of *Saccharofermentans* was greater than 1% in the BS group only. This study found that the supplementation of BS increased the abundances of unidentified *Lachnospiraceae*, *unidentified Lachnospiraceae*, and *Saccharofermentans,* but decreased the abundances of *Dialister*, unidentified *Clostridiales*, *Syntrophococcus*, *Roseburia*, *Selenomonas*, and *Shuttleworthia.* Unidentified *Ruminococcaceae* and unidentified *Lachnospiraceae* belong to the genera *Ruminococcaceae* and *Lachnospiraceae*, respectively, that have been shown to be positively correlated to gut health [40,41] and *Saccharofermentans* was reported to be positively correlated with feed efficiency [40]. In contrast, *Syntrophococcus*, *Shuttleworthia*, unidentified *Clostridiales*, and *Roseburia* have been suggested to be positively correlated with diseases. Therefore, the increased abundances of the beneficial bacteria that promote animal health and feed efficiency and decrease the abundances of those disease-causing bacteria by BS supplementation would have promoted the overall health status and production efficiency of the lambs, thereby contributing to the enhanced growth performance of the BS-supplemented lambs in this study.

Within the phylum *Bacteroidetes*, BS supplementation increased the abundances of unidentified *Rikenellaceae* and unidentified *Bacteroidales. Unidentified Rikenellaceae* belongs to *Rikenellaceae* that has been shown to possess immune-improving effects [42]. *Unidentified Bacteroidales* belongs to *Bacteroidales* that possess unique and powerful carbohydrate-utilization systems, and *Bacteroidales* species are considered potential probiotics [43].

5. Conclusions

The supplementation of BS at the dietary concentration of 3×10^8 CFU/kg enhanced the lamb growth rate and feed efficiency via increasing ruminal microbial protein synthesis and VFA, and favorably modulating the rumen microbiota under the conditions of this study. Both rumen bacterial diversity and the abundances of microflora that are positively related to rumen metabolism and health condition (probiotic populations) were increased, whereas the abundances of those bacteria that are negatively correlated with health conditions were decreased by the BS supplementation. Overall, this study showed that *B. subtilis* has potential as a direct-fed microbial to enhance the sheep production efficiency and requires further evaluation under the commercial production situation.

Author Contributions: Data curation, F.T., J.L. and Y.W.; Writing—original draft, Y.G. and W.W.; Writing—review & editing, J.Q.; Funding acquisition, S.X. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the China Agriculture Research System of MOF and MARA (CARS-02-55).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

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

References

1. Wang, Y.; Nan, X.; Zhao, Y.; Jiang, L.; Wang, H.; Zhang, F.; Hua, D.; Liu, J.; Yao, J.; Yang, L.; et al. Dietary Supplementation of Inulin Ameliorates Subclinical Mastitis via Regulation of Rumen Microbial Community and Metabolites in Dairy Cows. *Microbiol. Spectr.* **2021**, *9*, e0010521. [CrossRef] [PubMed]
2. Chen, H.; Guo, B.; Yang, M.; Luo, J.; Hu, Y.; Qu, M.; Song, X. Response of Growth Performance, Blood Biochemistry Indices, and Rumen Bacterial Diversity in Lambs to Diets Containing Supplemental Probiotics and Chinese Medicine Polysaccharides. *Front. Vet. Sci.* **2021**, *8*, 681389. [CrossRef] [PubMed]
3. Zeitz, J.O.; Guertler, P.; Pfaffl, M.W.; Eisenreich, R.; Wiedemann, S.; Schwarz, F.J. Effect of non-starch-polysaccharide-degrading enzymes as feed additive on the rumen bacterial population in non-lactating cows quantified by real-time PCR. *J. Anim. Physiol. Anim. Nutr.* **2013**, *97*, 1104–1113. [CrossRef]

4. Maake, T.W.; Aiyegoro, O.A.; Adeleke, M.A. Effects of *Lactobacillus rhamnosus* and *Enterococcus faecalis* Supplementation as Direct-Fed Microbials on Rumen Microbiota of Boer and Speckled Goat Breeds. *Vet. Sci.* **2021**, *8*, 103. [CrossRef] [PubMed]
5. Monteiro, H.F.; Lelis, A.L.J.; Fan, P.; Calvo Agustinho, B.; Lobo, R.R.; Arce-Cordero, J.A.; Dai, X.; Jeong, K.C.; Faciola, A.P. Effects of lactic acid-producing bacteria as direct-fed microbials on the ruminal microbiome. *J. Dairy Sci.* **2022**, *105*, 2242–2255. [CrossRef] [PubMed]
6. Jeyanathan, J.; Martin, C.; Morgavi, D.P. Screening of bacterial direct-fed microbials for their antimethanogenic potential in vitro and assessment of their effect on ruminal fermentation and microbial profiles in sheep. *J. Anim. Sci.* **2016**, *94*, 739–750. [CrossRef]
7. Nie, L.; Zhang, A.; Jiang, N.; Yang, Z.N. The application of probiotics in the production of juvenile ruminants. *Feed Expo.* **2017**, *9*, 11–14.
8. Pan, K.; He, M. Research and application of microbial additives in my country. *Vet. Med. Feed Addit.* **2002**, *4*, 35–37.
9. Chen, G.; Pan, K.; Yuan, C.; Shen, J. Study on the effect of Bacillus PAS38 and β-mannan on 5-HT in rabbit small intestine. *Chin. Anim. Husb. Newsl.* **2008**, *22*, 11–13.
10. Choi, P.; Rhayat, L.; Pinloche, E.; Devillard, E.; De Paepe, E.; Vanhaecke, L.; Haesebrouck, F.; Ducatelle, R.; Van Immerseel, F.; Goossens, E. *Bacillus Subtilis* 29784 as a Feed Additive for Broilers Shifts the Intestinal Microbial Composition and Supports the Production of Hypoxanthine and Nicotinic Acid. *Animals* **2021**, *11*, 1335. [CrossRef]
11. Chang, M.; Ma, F.; Wei, J.; Liu, J.; Nan, X.; Sun, P. Live *Bacillus subtilis* natto promotes rumen fermentation by modulating rumen microbiota in vitro. *Animals* **2021**, *11*, 1519. [CrossRef] [PubMed]
12. Sun, P.; Wang, J.Q.; Deng, L.F. Effects of *Bacillus subtilis* natto on milk production, rumen fermentation and ruminal microbiome of dairy cows. *Animal* **2013**, *7*, 216–222. [CrossRef] [PubMed]
13. Wang, Z.; He, Z.; Beauchemin, K.A.; Tang, S.; Zhou, C.; Han, X.; Wang, M.; Kang, J.; Odongo, N.E.; Tan, Z. Comparison of two live Bacillus species as feed additives for improving in vitro fermentation of cereal straws. *Anim. Sci. J.* **2016**, *87*, 27–36. [CrossRef]
14. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Bampidis, V.; Azimonti, G.; Bastos, M.L.; Christensen, H.; Dusemund, B.; Fašmon Durjava, M.; Kouba, M.; López-Alonso, M.; López Puente, S.; et al. Safety and efficacy of a feed additive consisting of *Bacillus subtilis* strains CNCM I-4606, CNCM I-5043 and CNCM I-4607 and *Lactococcus lactis* CNCM I-4609 for all animal species (Nolivade). *EFSA J.* **2021**, *19*, e06907. [CrossRef]
15. Mingmongkolchai, S.; Panbangred, W. *Bacillus* probiotics: An alternative to antibiotics for livestock production. *J. Appl. Microbiol.* **2018**, *124*, 1334–1346. [CrossRef]
16. Zheng, D.Z. Application of Calsporin in livestock feed. *Anim. Husb. Mark.* **2007**, *10*, 49–51.
17. Geng, C. Comparison of Growth Performance, Carcass Index and Beef Quality of Fattening Cattle Fed with Active Yeast and Yeast Culture. Ph.D. Dissertation, China Agricultural University, Beijing, China, 2015.
18. Feng, Z.; Gao, M. Improvement of the method for determination of ammonia nitrogen content in rumen liquid by colorimetry. *Inn. Mong. Anim. Husb. Sci.* **1993**, *4*, 40–41.
19. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
20. Stewart, C.S.; Duncan, S.H. The effect of avoparcin on cellulolytic bacteria of the ovine rumen. *J. Gen. Microbiol.* **1985**, *131*, 427–435. [CrossRef]
21. Rogers, S.O.; Bendich, A.J. Extraction of DNA from plant tissues. In *Plant Molecular Biology Manual*; Springer: Dordrecht, The Netherlands, 1989; pp. 73–83.
22. Lee, P.Y.; Costumbrado, J.; Hsu, C.Y.; Kim, Y.H. Agarose gel electrophoresis for the separation of DNA fragments. *J. Vis. Exp.* **2012**, *62*, 3923. [CrossRef]
23. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10–12. [CrossRef]
24. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]
25. Haas, B.J.; Gevers, D.; Earl, A.M.; Feldgarden, M.; Ward, D.V.; Giannoukos, G.; Ciulla, D.; Tabbaa, D.; Highlander, S.K.; Sodergren, E.; et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* **2011**, *21*, 494–504. [CrossRef]
26. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef]
27. Wurilege. The Effect of Adding *Bacillus subtilis* to the Diet on the Growth Performance, Blood Indicators and Rumen Microbes of Fattening Sheep. Master's Thesis, Inner Mongolia Agricultural University, Hohhot, China, 2019. [CrossRef]
28. Xiros, C.; Shahab, R.L.; Studer, M.H. A cellulolytic fungal biofilm enhances the consolidated bioconversion of cellulose to short chain fatty acids by the rumen microbiome. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 3355–3365. [CrossRef] [PubMed]
29. Baldwin, R.L., VI; Connor, E.E. Rumen Function and Development. *Vet. Clin. N. Am. Food Anim. Pract.* **2017**, *33*, 427–439. [CrossRef] [PubMed]
30. Kawauchi, D.; Angthong, W.; Keaokliang, O.; Ishida, T.; Takahashi, T.; Kawashima, T. Effect of feeding *Bacillus subtilis* on rumen fermentation, blood metabolites, nutrient digestibility, and energy and nitrogen balances in non-lactating crossbred cows. *Anim. Sci. J.* **2021**, *92*, e13531. [CrossRef] [PubMed]
31. Xu, Y.; Yu, Y.; Shen, Y.; Li, Q.; Lan, J.; Wu, Y.; Zhang, R.; Cao, G.; Yang, C. Effects of *Bacillus subtilis* and *Bacillus licheniformis* on growth performance, immunity, short chain fatty acid production, antioxidant capacity, and cecal microflora in broilers. *Poult. Sci.* **2021**, *100*, 101358. [CrossRef] [PubMed]

32. Caulier, S.; Nannan, C.; Gillis, A.; Licciardi, F.; Bragard, C.; Mahillon, J. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Front. Microbiol.* **2019**, *10*, 302. [CrossRef]
33. Devyatkin, V.; Mishurov, A.; Kolodina, E. Probiotic effect of *Bacillus subtilis* B-2998D, B-3057D, and *Bacillus licheniformis* B-2999D complex on sheep and lambs. *J. Adv. Vet. Anim. Res.* **2021**, *8*, 146–157. [CrossRef]
34. Konopka, A. What is microbial community ecology? *ISME J.* **2009**, *3*, 1223–1230. [CrossRef] [PubMed]
35. Zhang, Y.K.; Zhang, X.X.; Li, F.D.; Li, C.; Li, G.Z.; Zhang, D.Y.; Song, Q.Z.; Li, X.L.; Zhao, Y.; Wang, W.M. Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* **2021**, *15*, 100161. [CrossRef] [PubMed]
36. Okazaki, Y.; Sekita, A.; Chiji, H.; Kato, N. Consumption of lily bulb modulates fecal ratios of firmicutes and bacteroidetes phyla in rats fed a high-fat diet. *Food Sci. Biotechnol.* **2016**, *25* (Suppl. S1), 153–156. [CrossRef] [PubMed]
37. Stojanov, S.; Berlec, A.; Štrukelj, B. The Influence of Probiotics on the Firmicutes/Bacteroidetes Ratio in the Treatment of Obesity and Inflammatory Bowel disease. *Microorganisms* **2020**, *8*, 1715. [CrossRef]
38. Ren, Z.; Fang, H.; Zhang, J.; Wang, R.; Xiao, W.; Zheng, K.; Yu, H.; Zhao, Y. Dietary *Aronia melanocarpa* Pomace Supplementation Enhances the Expression of ZO-1 and Occludin and Promotes Intestinal Development in Pigs. *Front. Vet. Sci.* **2022**, *9*, 904667. [CrossRef]
39. Wang, Y.; Huang, J.M.; Zhou, Y.L.; Almeida, A.; Finn, R.D.; Danchin, A.; He, L.S. Phylogenomics of expanding uncultured environmental Tenericutes provides insights into their pathogenicity and evolutionary relationship with Bacilli. *BMC Genom.* **2020**, *21*, 408. [CrossRef]
40. Santarossa, S.; Sitarik, A.R.; Johnson, C.C.; Li, J.; Lynch, S.V.; Ownby, D.R.; Ramirez, A.; Yong, G.L.M.; Cassidy-Bushrow, A.E. Associations of physical activity with gut microbiota in pre-adolescent children. *Phys. Act. Nutr.* **2021**, *25*, 24–37. [CrossRef]
41. Sun, D.; Bai, R.; Zhou, W.; Yao, Z.; Liu, Y.; Tang, S.; Ge, X.; Luo, L.; Luo, C.; Hu, G.F.; et al. Angiogenin maintains gut microbe homeostasis by balancing α-Proteobacteria and *Lachnospiraceae*. *Gut* **2021**, *70*, 666–676. [CrossRef]
42. Lozupone, C.A.; Li, M.; Campbell, T.B.; Flores, S.C.; Linderman, D.; Gebert, M.J.; Knight, R.; Fontenot, A.P.; Palmer, B.E. Alterations in the gut microbiota associated with HIV-1 infection. *Cell Host Microbe* **2013**, *14*, 329–339. [CrossRef]
43. Xu, Y.; Ai, C.; Jiang, P.; Sun, X.; Liu, Y.; Jiang, G.; Song, S. Oligosaccharides from *Gracilaria lemaneiformis* better attenuated high fat diet-induced metabolic syndrome by promoting the *Bacteroidales* proliferation. *Food Funct.* **2020**, *11*, 1049–1062. [CrossRef]