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Responses of Fermentation Characteristics and Microbial Communities to Vitamin B_{12} Supplementation in In Vitro Ruminal Cultures

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Abstract: Vitamin B_{12}, an important cofactor involved in propionate formation, is synthesized exclusively by bacteria and archaebacteria. Humans need to intake vitamin B_{12} through food, and dairy products are generally the best source of vitamin B_{12}. In the present study, the effects of vitamin B_{12} supplementation in diets on in vitro ruminal fermentation characteristics and microbial communities were investigated to provide a reference for increasing the vitamin B_{12} content in milk by dietary supplementation. A completely randomized design was carried out using the in vitro rumen culture technique, and 5 vitamin B_{12} dose levels (0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter) were used. The results showed that vitamin B_{12} supplementation in diets decreased acetate: propionate ratio and butyrate concentration. The change in the acetate: propionate ratio can be attributed to the increased relative abundances of the Proteobacteria phylum and the Negativicutes class, both of which are involved in propionate metabolism. The decrease in butyrate concentration can likely be attributed to a reduction in relative abundance of species belonging to the Clostridia class, which are known as the predominant butyrate producers in the mammalian intestine. In addition, vitamin B_{12} supplementation in diets reduced the CH_4 production by altering the species composition of the archaeal community. In conclusion, dietary supplementation of vitamin B_{12} resulted in rumen perturbation. In vivo studies should be conducted cautiously when evaluating the effects of vitamin B_{12} supplementation on the synthesis and absorption of it, as well as its content in milk.

Keywords: vitamin B_{12}; fermentation characteristics; microbial communities; in vitro ruminal cultures

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1. Introduction

Vitamin B_{12}, also known as cobalamin, is a corrinoid compound synthesized exclusively by bacteria and archaebacteria [1]. It acts as a cofactor toward the action of methionine synthase and methylmalonyl CoA mutase, which are involved in two essential metabolic pathways. Methionine synthase catalyzes the transfer of methyl groups during methionine metabolism. Methylmalonyl CoA mutase is a corrinoid-dependent enzyme that catalyzes the interconversion of succinyl-CoA and *R*-methylmalonyl-CoA, a step involved in the Krebs cycle and gluconeogenesis [2]. There are two primary symptoms of vitamin B_{12} deficiency: megaloblastic anemia and neuropathy. A sore tongue enlarged red blood cells, low white blood cell count, low platelet count, hyper-segmented neutrophils leucocytes, and infertility are all common symptoms of megaloblastic anemia [3]. As a result of neuropathy, the lower limbs become weak, spastic, or suffer from depression, and symptoms of degeneration of the spinal cord may occur [3]. Humans are capable of synthesizing vitamin B_{12} through the intestinal microbes; however, it cannot be absorbed since it is synthesized in the colon and absorbed in the small intestine. Therefore, humans need to consume vitamin B_{12} through food to maintain normal metabolic function. Several plant foods, including some edible algae and fermented soybeans, can provide some vitamin B_{12}, but dairy products are

generally the best source of vitamin B_{12} due to the ruminal microbes that synthesize it [4]. One study showed that dairy products have a higher bioavailability of vitamin B_{12} than other sources [5]. The majority of supplements contain cyanocobalamin, a synthetic form of vitamin B_{12} stabilized by cyanide groups. Cyanocobalamin is not biologically active until the cyanide group is removed by enzymes [6]. There are three forms of vitamin B_{12} present in milk: adenosylcobalamin, methylcobalamin, and hydroxocobalamin. Adenosylcobalamin and methylcobalamin are bioactive and have coenzymatic activity in mammal cells, while hydroxocobalamin is the product of photolysis of light-sensitive cobalamins [7]. In addition, the vitamin B_{12} in dairy products is very stable, can withstand pasteurization, and is not destroyed by daylight or prolonged exposure to refrigerator temperatures [8].

It is important to note that the concentration of vitamin B_{12} in milk varies considerably between cows and herds. The concentration of vitamin B_{12} in bovine milk is 0.08–0.49 μg/100 g, while the concentration in goat milk is 0.07–0.10 μg/100 g [3]. The recommended dietary allowance of vitamin B_{12} required for adults and children above 13 years old is 2.4 μg. One glass of bovine milk (250 mL) will provide 0.20–1.22 μg vitamin B_{12}, accounting for 8.3–50.8% of the recommended dietary allowance. As the best source of vitamin B_{12} for humans, milk is expected to contain the higher content of vitamin B_{12} to meet the requirements of humans. Several studies of functional milk, such as Se-enriched milk [9] and CLA-enriched milk [10] have been conducted. Other studies have focused on the effect of vitamin B_{12} on metabolic function of transition dairy cows [11,12], but there have been few studies about vitamin B_{12}-enriched functional products. It has been reported that intramuscular injections of vitamin B_{12} increased its concentration in milk by 50% [13]. Although some vitamin B_{12} added in diets may be degraded in the rumen, dietary supplementation of vitamin B_{12} is a more feasible strategy to increase its concentration in milk than intramuscular injections. Vitamin B_{12} acts as a cofactor for corrinoid-dependent enzymes for microbes [2]. The competition for vitamin B_{12} or corrinoid analogs among microbes may affect the microbial community structure and the fermentation characteristics of the rumen. It has been reported that dietary supplement of vitamin B_8 affects rumen function, possibly through an effect on the microbial community structure of the rumen [11]. To our knowledge, no studies have evaluated the responses of ruminal fermentation characteristics and microbial communities to dietary supplement of vitamin B_{12}. If dietary supplementation of vitamin B_{12} results in rumen perturbation, the synthesis and absorption of vitamin B_{12}, as well as its content in milk, may be affected. Moreover, the performance and health of dairy cows may be adversely affected. Therefore, the objective of the study was to explore the effects of vitamin B_{12} supplementation in diets, using the in vitro rumen culture technique, on ruminal fermentation characteristics and microbial communities, and to provide a reference for increasing the vitamin B_{12} content in milk by dietary supplementation.

2. Material and Methods

2.1. In Vitro Incubation

Three ruminal cannulated lactating Holstein cows were chosen to collect ruminal fluids. The collection procedure was approved by the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China). The donor cows were fed a total mixed ration (TMR) ad libitum, which contained 25.65% corn silage, 18.59% alfalfa hay, 26.02% steam-flaked corn, 7.43% soybean meal, 7.43% cottonseed meal, 5.58% beet pulp, 7.43% distillers dried grains with soluble, and 1.86% minerals and vitamins. The ruminal fluids were obtained approximately 2 h after morning feeding from three donor cows, equal volume pooled in a prewarmed container, and transported to the laboratory in 30 min. The ruminal fluids were filtered through two layers of cheesecloth and then mixed with buffer solution (1:2 *v/v*) to prepare inoculum. The buffer solution was prepared anaerobically as described by Wan, et al. [14]. The inoculum was continuously flushed with CO_2 and constantly stirred at 39 °C in a water bath until in vitro incubation. The TMR fed to donor cows was dried at 55 °C for 48 h and ground to pass a 1 mm

screen as the fermentation substrate. Then, 0.5 g of the substrate was accurately weighed into each 120 mL serum bottle, and 75 mL of inoculum was injected under continuous flushing with CO_2. A completely randomized design was carried out, and 5 vitamin B_{12} dose levels (0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter) were used in the experiment. Rather than adding vitamin B_{12} powder directly to serum bottles, the solution of vitamin B_{12} (2 mg/mL water) was prepared. Distilled water was used to balance the liquid volume of all serum bottles. The serum bottles were closed with butyl rubber stoppers and crimped aluminum seals. The airbags were vacuumed and connected with each serum bottle to collect gases during incubation. All serum bottles were then transferred to a fermentation chamber, which was set at 39 °C with horizontal shaking at 60 rpm. After incubation for 24 h, all serum bottles were placed on ice water to stop the fermentation and then collected samples for analysis. The in vitro incubation was repeated on three different days, totaling three experimental runs and four replicates for each treatment in each run. An extra four serum bottles without substrate were dispensed 75 mL inoculum and used as blank controls to correct the analytes in each run. The experiment of in vitro evaluation for vitamin B_{12} consisted of triplicate runs of 24 h in vitro incubation. Each run included 24 samples: 5 treatments × 4 replicates and 4 blank samples. The vitamin B_{12} power (≥98%) was purchased from Solarbio LIFE SCIENCES (Beijing, China).

2.2. Sample Collection and Analysis

The gas production of each airbag was measured at 24 h of incubation by a calibrated glass syringe (100 mL, Häberle Labortechnik, Lonsee-Ettlenschieß, Germany). To determine CH_4, 0.5 mL of gas was accurately injected into an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal conductivity detector and a packed column (1 m × 2 mm × 3.175 mm; Porapak Q, Agilent Technologies, Santa Clara, CA, USA). The detailed method of determining CH_4 was conducted as described by Wang et al. [15]. The calibration gas was a mixture of CO_2 (900 mmol/mol) and CH_4 (100 mmol/mol). The pH of each serum bottle was measured by a portable pH meter (Seven Go, Mettler Toledo, Greifensee, Switzerland) after the incubation was terminated. Then, 1.5 mL of fermentation material (solid and liquid) from each serum bottle was accurately collected and then frozen in liquid nitrogen for microbial DNA extraction and 16S rRNA genes sequencing and analysis. The whole solid fermentation material of each bottle was filtrated by a nylon bag (8 × 12 cm, 42 μm) and then dried at 55 °C for 48 h to measure the apparent disappearance of dry matter. The filtrate sample of each serum bottle was collected and stored at −20 °C until the analysis of volatile fatty acids (VFA), lactate, oxaloacetate, fumarate, and succinate. The detailed method of determining VFA was conducted as described by Wang et al. [15]. The filtrate sample of each serum bottle (100 μL) was deproteinized in triplicate by adding 400 μL of acetonitrile, centrifuged at 20,000× *g* for 15 min. The supernatant was taken for lactate, oxaloacetate, fumarate, and succinate analysis. The UHPLC-MS/MS analysis was conducted using the SCIEX 6500+ triple quadrupole mass spectrometer system (AB Sciex, Framingham, MA, USA). The Merck ZIC-HILIC column (2.1 × 100 mm, 3.5 μm, H&E Co., Ltd., Framingham, MA, USA) was used. The UHPLC-MS/MS system was performed using 20 mM ammonium formate in water as mobile phase A and pure optima LC/MS-grade acetonitrile as mobile phase B. The column temperature was set at 40 °C, and the injection volume was 5 μL. The total rum time was 20 min. The gradient began at 1:9 of A:B with a flow of 0.25 mL/min and held for 1 min. The gradient was changed to 5:5 of A:B over the course of 13 min and held for 3 min. The gradient was changed to 1:9 of A:B over the course of 0.1 min and held for 3 min to complete the gradient. For mass spectrometer conditions, global parameters were optimized across all analytes: curtain gas pressure, 35 psi; ion spray voltage, 3500 V; source temperature, 550 °C; Gas1 pressure, 55 psi; and Gas2 pressure, 55 psi. The standard curves were established using the standards of lactate, oxaloacetate, fumarate, and succinate with the designated concentrations, which were purchased from Sigma-Aldrich, St. Louis, MO,

USA. Data acquisition and evaluation were performed with the AB Sciex MultiQuant™ (AB Sciex LLC, Framingham, MA, USA) software.

2.3. Microbial DNA Extraction and 16S rRNA Genes Sequencing

Total microbial DNA was extracted using the cetyltrimethylammonium bromide (CTAB) and bead beating method as described by Jin et al. [16]. The purity and concentration of extracted DNA were assessed using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of extracted DNA was detected by agarose gel (1%) electrophoresis. The V3-V4 region of the bacterial 16S rRNA genes was amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The V4–V5 region of the archaeal 16S rRNA genes was amplified using primers 524F10extF (5′-TGYCAGCCGCCGCGGTAA-3′) and Arch958RmodR (5′-YCCGGCGTTGAVTCCAATT-3′). A 20 μL PCR reaction mixture contained 0.4 μL of FastPfu Polymerase, 0.2 μL of BSA, and 10 ng of genomic DNA template, 0.8 μL of 5 μM Primer R, 0.8 μL of 5 μM Primer F, 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, and then adding ddH_2O to 20μL. PCR was performed using an ABI GeneAmp® 9700 (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: 95 °C for 3 min; followed by 27 cycles (bacteria) or 33 cycles (archaea) of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and finished with a final extension at 72 °C for 10 min. Amplicons were further purified with a DNA purification kit (Axygen Biosciences, Glendale, AZ, USA), and the concentrations were measured using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The purified 16S rDNA amplicons were pooled in equimolar ratios and subjected to pair-end sequencing using the Illumina MiSeq platform (2 × 300 bp) at Shanghai Major bio PharmTechnology Biotech Co., Ltd. (Shanghai, China). Raw sequences were deposited into the National Center for Biotechnology Information Sequence Read Archive database under accession numbers SRP327708. The data were analyzed using the online platform program of Majorbio Cloud Platform (www.majorbio.com, accessed on: 25 July 2022). Briefly, the pair-end reads were merged into a sequence according to the overlapping relationship using FLASH (version 1.2.11, Johns Hopkins University, Baltimore, MD, USA). At the same time, FASTP (version 0.19.6, HaploX Biotechnology, Shenzhen, China) was used to control the quality of pair-end reads and merged sequences. Different samples were identified based on the unique barcodes. Then, the primers and barcodes were removed, and chimeras were filtered to obtain clean reads. The clean reads were clustered into operational taxonomic units (OTU) at 97% similar level using UPARSE (version 7.0.1090). The RDP classifier (version 2.11) was used to perform taxonomic analysis with confidence threshold 0.8 and SILVA database (version 138).

2.4. Statistical Analysis

A normal distribution was checked for all data, and some data were transformed into log10(n + 1) if necessary. The fermentation characteristics were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). The data were analyzed according to the following model: $Y_i = \mu + V_i + e_i$, where Y_i was the observation, μ was the overall mean, V_i was the fixed effect of vitamin B_{12} treatment ($I = 5$), and e_{ijk} was the random error term. The linear and quadratic effects of vitamin B_{12} treatment were tested using polynomial contrasts. The effects of run and interaction between run and treatment were not significant. Therefore, they were not included in the model. Alpha diversity indices, such as Simpson, Shannon, Coverage, and ACE, were calculated by Mothur (version v.1.30.2, https://mothur.org/wiki/calculators/, accessed on: 25 July 2022). The bioinformatics software STAMP was used to visualize the relative abundance difference of microbes [17]. Differences were considered statistically significant when $p < 0.05$, and differences were considered a tendency when $0.05 \leq p < 0.10$.

3. Results and Discussion

3.1. Effects of Vitamin B_{12} on Fermentation Characteristics

The effects of vitamin B_{12} supplementation in diets on fermentation characteristics are shown in Table 1. As shown in Table 1, the CH_4 production, total VFA concentrations, and acetate: propionate ratio showed a linear decrease (p = 0.008, 0.017 and <0.001, respectively) in a dose-dependent manner, with a significant decrease observed at 1mg/g and above. The pH of fermentation liquids increased linearly (p = 0.008) in a dose-dependent manner, with a significant increase observed at 1mg/g and above. As the concentrations of vitamin B_{12} increased, the concentration of acetate decreased linearly (p = 0.023) while the concentration of propionate tended to increase linearly (p = 0.084). The butyrate concentration decreased linearly (p < 0.001) in a dose-dependent manner, and a significant decrease was observed at 1mg/g and above. The gas production decreased linearly (p = 0.041) as the concentrations of vitamin B_{12} increased, while the apparent disappearance of dry matter was not significantly affected by vitamin B_{12}.The concentration of lactate decreased linearly (p = 0.006) in a dose-dependent manner, and a significant decrease was observed at 0.5 mg/g and above. The concentration of succinate decreased linearly (p = 0.011) in a dose-dependent manner, with a significant decrease observed at 4 mg/g.

Table 1. Effects of vitamin B_{12} supplementation on fermentation characteristics.

Items	VB_{12} (mg/g DM) [1]					SEM [4]	*p* Value [2]		
	0	0.5	1	2	4		Trt	L	Q
pH	6.57 [b]	6.58 [ab]	6.59 [a]	6.60 [a]	6.60 [a]	0.004	0.048	0.008	0.336
DMD [3]	0.726	0.712	0.711	0.714	0.713	0.003	0.441	0.233	0.224
Gas production (mL)	147.1	144.8	142.0	141.7	141.3	0.998	0.292	0.041	0.447
CH_4 (mL)	9.08 [a]	8.77 [ab]	8.20 [b]	8.22 [b]	8.13 [b]	0.131	0.045	0.008	0.329
Lactate, μg/mL	2.38 [a]	1.55 [b]	1.52 [b]	1.52 [b]	1.51 [b]	0.096	0.009	0.006	0.268
Oxaloacetate, μg/mL	139.9	136.23	136.32	134.74	133.97	1.479	0.761	0.216	0.736
Fumarate, μg/mL	0.03	0.02	0.03	0.02	0.03	0.002	0.607	0.457	0.467
Succinate, μg/mL	0.16 [a]	0.12 [ab]	0.12 [ab]	0.11 [ab]	0.10 [b]	0.007	0.046	0.011	0.422
Concentrations, mM									
Total volatile fatty acids	106.48 [a]	104.62 [ab]	104.22 [b]	103.55 [b]	103.45 [b]	0.421	0.049	0.017	0.348
Acetate	66.58	65.54	65.32	64.89	64.88	0.250	0.184	0.023	0.373
Propionate	21.54	21.56	21.73	21.72	21.73	0.043	0.407	0.084	0.564
Isobutyrate	1.06	1.03	1.03	1.03	1.03	0.006	0.598	0.177	0.420
Butyrate	13.45 [a]	12.72 [ab]	12.41 [b]	12.21 [b]	12.12 [b]	0.135	0.009	<0.001	0.188
Isovalerate	1.94	1.88	1.86	1.85	1.85	0.017	0.394	0.074	0.386
Valerate	1.92	1.88	1.87	1.85	1.85	0.011	0.220	0.027	0.430
Acetate/Propionate	3.09 [a]	3.04 [ab]	3.01 [b]	2.99 [b]	2.99 [b]	0.009	0.008	<0.001	0.194

[a,b] Means within a row with different superscripts differ (p < 0.05). [1] Data were analyzed using vitamin B_{12} dose levels of 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter. [2] Trt = treatment; L = linear; Q = quadratic. [3] DMD = apparent disappearance of dry matter. [4] SEM= standard error of the mean.

Based on the present results, vitamin B_{12} supplementation in diets inhibited rumen fermentation. The gas production and the apparent disappearance of dry matter numerically decreased when vitamin B_{12} was supplemented in diets. The total VFA concentrations was significantly decreased in response to the fermentation inhibition caused by vitamin B_{12} supplementation. The linear increased pH was related to the change of the total VFA concentration. No research has been conducted to investigate the relationship between changes in rumen fermentation characteristics and vitamin B_{12} supplementation in diets. The effects of vitamin B_{12} on ruminal fermentation characteristics could be due to the changes of ruminal microbes. It has been reported that the "cross-feeding" of B vitamins exists in ruminal microbes [18]. A microbial species may require B vitamins produced by another microbial species. Shelton et al. [19] predicted that less than 25% of human gut microbes can synthesize vitamin B_{12} or corrinoid analogs, while more than 86% of microbes rely on corrinoids as cofactors to ensure the viability and stability of the microbial ecology.

Vitamin B_{12} supplementation in diets may disturb the balance of microbial vitamin B_{12} synthesis and consumption in the rumen. Unfortunately, the current experimental design did not allow further evaluation of the mechanism by which vitamin B_{12} supplementation in diets affected the microbial vitamin B_{12} metabolism. We failed to determine vitamin B_{12} in the filtrate of the control group, which may be attributed to the reason why B vitamins primarily exist within bacteria. A study conducted by Santschi et al. [20] found that bacteria contained 10–5000-fold higher concentrations of B vitamins than particle-free supernatants. The amount of vitamin B_{12} in the rumen can be determined by method described by Franco-Lopez et al. [21].

According to the present results, vitamin B_{12} supplementation changed the fermentation pattern, resulting in a decreased acetate: propionate ratio. The changes in CH_4 production may have been related to the changed fermentation pattern. Vitamin B_{12} acts as a cofactor toward the action of methylmalonyl CoA mutase, which is involved in the interconversion of succinyl-CoA and R-methylmalonyl-CoA, a step involved in the propionate metabolism [2]. Vitamin B_{12} supplementation in diets may enhance the activity of microbes related to the production of propionate. In the present study, the linearly decreased concentration of succinate indicated that vitamin B_{12} supplementation promoted propionate production. Compared to acetate, the pathway of propionate formation provided an alternative sink to use metabolic hydrogen. Moreover, a negative correlation between methanogenesis and propionate formation as previously reported [22]. Based on a meta-analysis of batch and continuous culture experiments, Ungerfeld [23] found that the inhibition of methanogenesis redirected metabolic hydrogen from methane toward propionate and H_2. However, a considerable proportion of the reduced equivalents was still missing. In the present study, however, the extent of the increase in propionate production was not stoichiometrically matched to the reduction in CH_4 production. Therefore, the reduction of CH_4 production resulting from vitamin B_{12} was not all attributed to the competition for metabolic hydrogen between methanogenesis and propionate formation. Belanche et al. [24] reported that increased propionate formation resulted from supplementing chitosan explained about one-third of the decrease in CH_4 production. In addition to the competition, propionate was considered to show toxicity to methanogenic archaea [25]. Wang et al. [26] found that propionate showed significant inhibition of the activity of methanogenic archaea, while acetate and butyrate had no negative effects on methanogenesis. In conclusion, vitamin B_{12} supplementation in diets changed ruminal fermentation characteristics, which could be related to changes in ruminal microbial communities.

3.2. Microbial Community

3.2.1. Bacterial Community

The bacterial community compositions of the present study are shown in Figure S1 (https://doi.org/10.6084/m9.figshare.20338692.v2, accessed on: 25 July 2022). The dominant bacteria at the phylum level were Bacteroidetes (50.82%), Firmicutes (43.45%), Actinobacteriota (1.32%), Spirochaetota (0.99%), Proteobacteria (0.92%), and Patescibacteria (0.74%). The Proteobacteria, Firmicutes, and Bacteroidetes were the three main bacterial phyla that changed significantly in response to vitamin B_{12} supplementation in the present study.

Alpha diversity indices of the bacterial community in the present study are shown in Figure 1. There were no significant differences in Coverage, Ace, Shannon, or Simpson, indicating that the sequencing depth was desirable for the analysis in the present and that vitamin B_{12} supplementation did not affect the richness and diversity of the bacterial community.

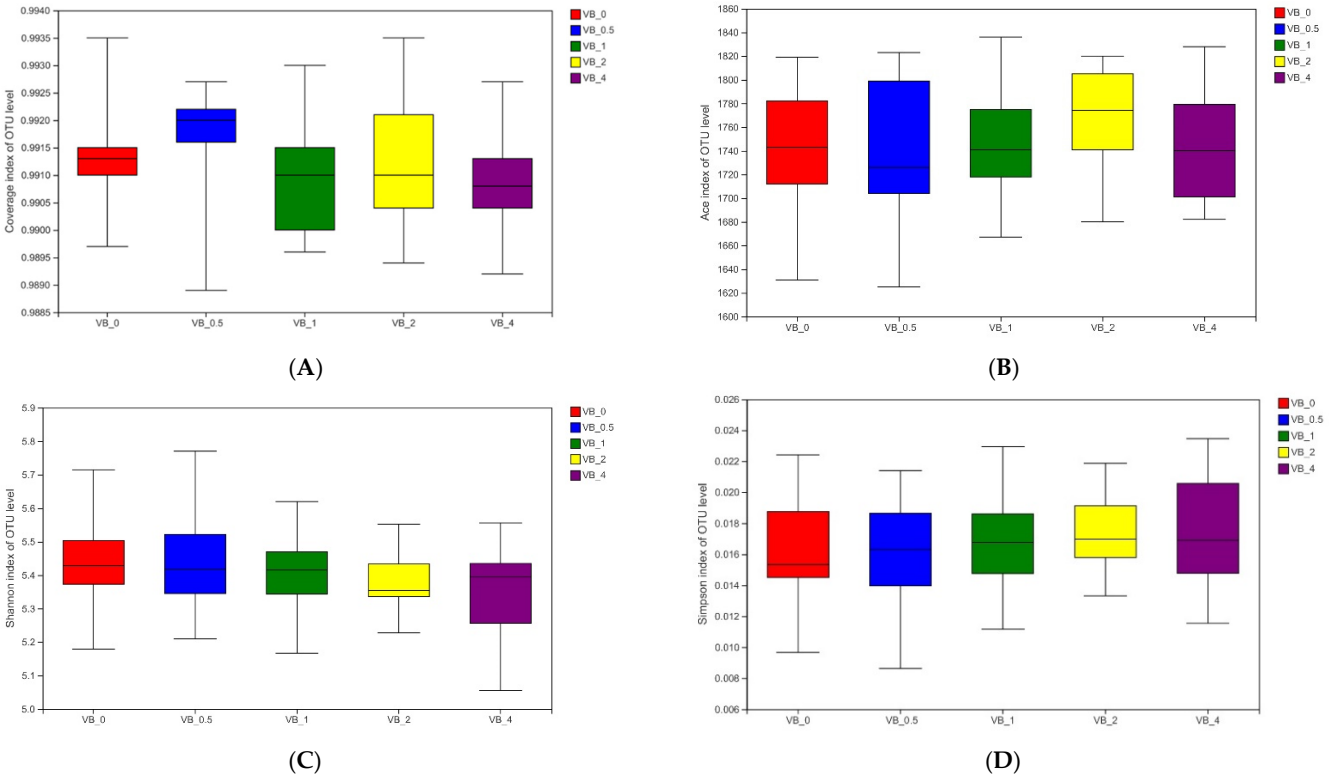
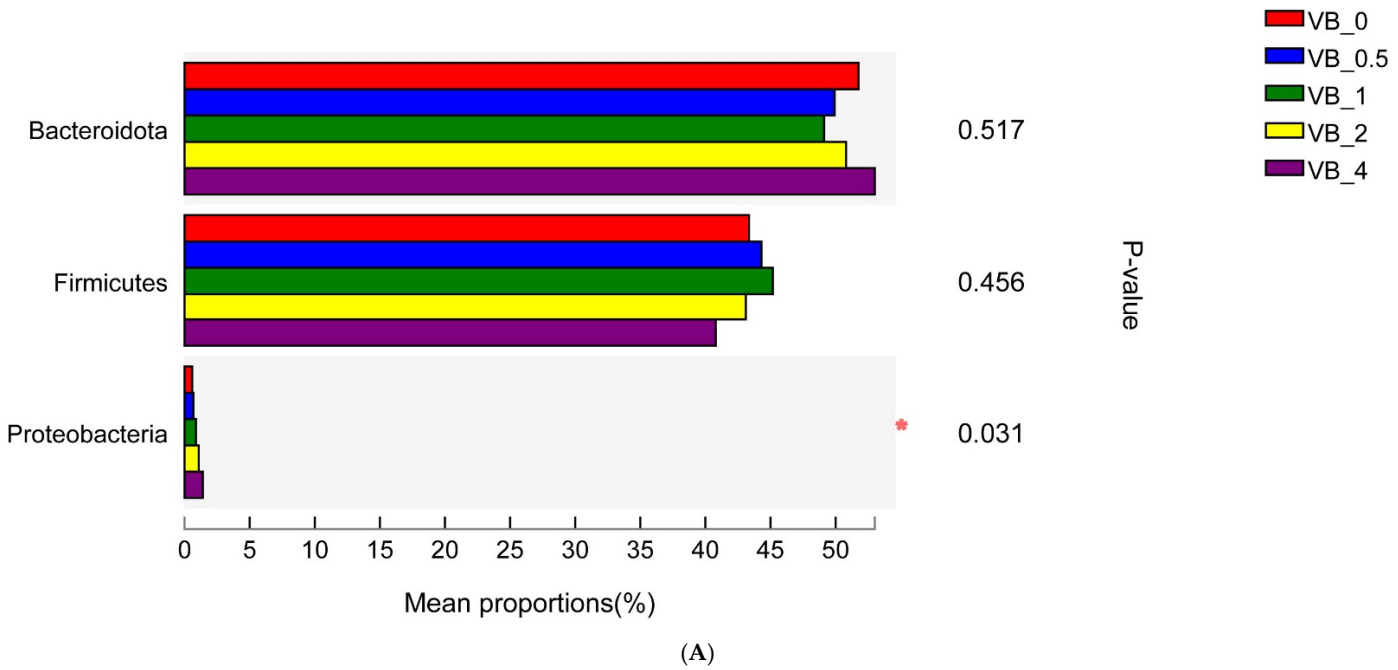


Figure 1. Changes of alpha diversity indices in the bacterial community in the rumen fermentation system with increasing vitamin B_{12} supplementation. (**A**) Coverage index; (**B**) Ace index; (**C**) Shannon index; (**D**) Simpson index.

Proteobacteria

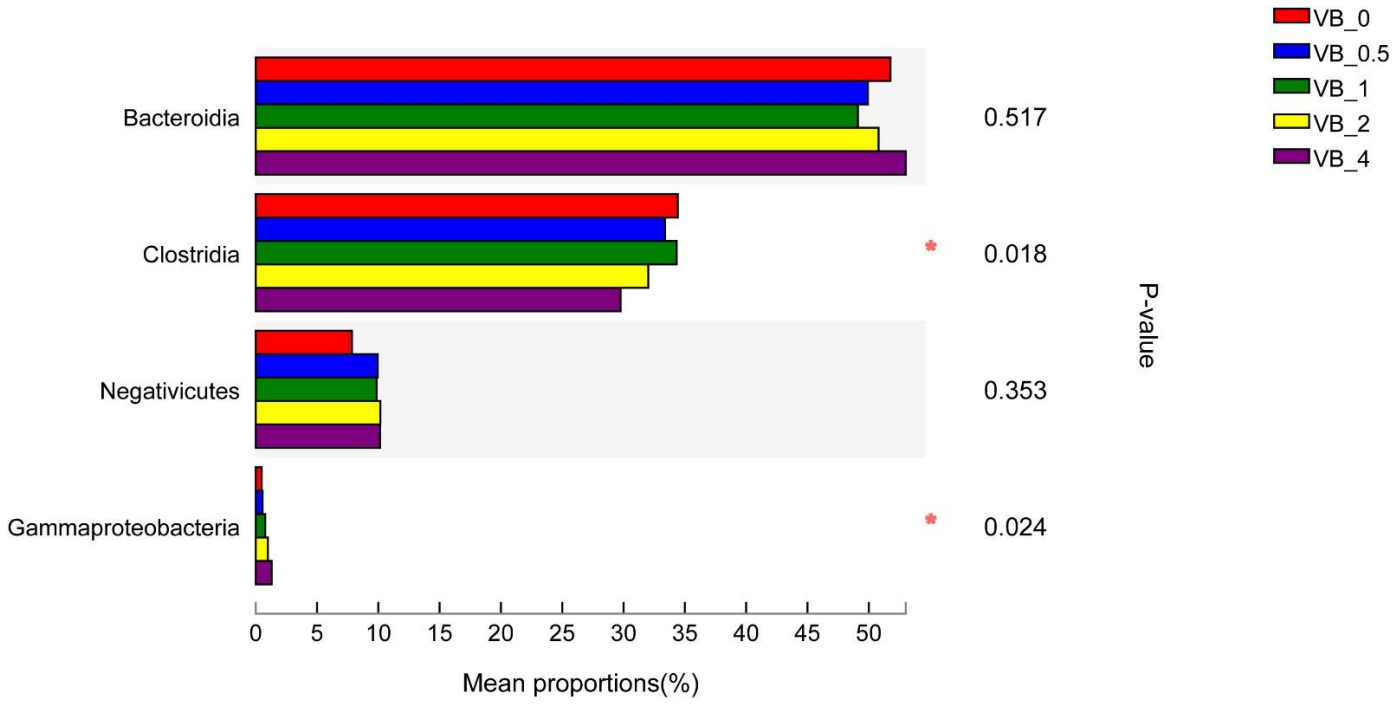
The relative abundance of Proteobacteria phylum was 0.58%, 0.67%, 0.88%, 1.08%, and 1.39% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing an increase in a dose-dependent manner ($p = 0.031$, Figure 2A). Proteobacteria were a major phylum of gram-negative bacteria, which were able to degrade lignocellulosic material in organic wastes [27]. They were also reported to degrade glucose and protein in manure [28] and were associated with the increased rumen succinate production of cows fed with the corn silage-based diet [29]. The relative abundance of the Gammaproteobacteria class was 0.48%, 0.56%, 0.78%, 0.99%, and 1.30% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing an increase in a dose-dependent manner ($p = 0.024$, Figure 2B). Gammaproteobacteria is the most dominant class belonging to the Proteobacteria phylum, and several medically, ecologically, and scientifically important groups of bacteria belong to this class. In the rumen, the increased relative abundance of unclassified or uncultured members of the Gammaproteobacteria class was significantly associated with high concentrate basal diets and linked to the decreased relative abundance of the Methanobacteriaceae family [30]. The relative abundance of Aeromonadales order was 0.44%, 0.53%, 0.75%, 0.94%, and 1.26% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing an increase in a dose-dependent manner ($p = 0.017$, Figure 2C). The Aeromonadales order is an order of the Proteobacteria phylum. It was reported that the relative abundance of Aeromonadales is higher during early lactation than that during the dry period of dairy cows [31]. The early lactation period of dairy cows normally requires more nutrients and energy to meet the high energy demands of milk production. As the main energy precursors for ruminants, propionate is converted to glucose through the gluconeogenesis pathway to provide energy for ruminants. This may be a reason why the relative abundance of the Aeromonadales order is higher during the early lactation. Species belonging to the Aeromonadales order produce succinate, which is then converted

to propionate by other bacteria [29]. The relative abundance of the Succinivibrionaceae family was 0.44%, 0.53%, 0.75%, 0.94%, and 1.26% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing an increase in a dose-dependent manner (p = 0.017, Figure 2D). The Succinivibrionaceae family degraded starch to produce succinate and were found to be abundant throughout the rumen fluid samples of cows fed with the corn silage-based diet, which contained a higher amount of non-fiber carbohydrates, such as starch and sugars [29]. A metagenomics study found that the increased relative abundance of the Succinivibrionaceae family was linked to the decrease of CH_4 production and relative abundance of methanogenic archaea [32] since their members dispose of metabolic hydrogen and produce succinate. The succinate is then converted to propionate, which competes with hydrogenotrophic methanogenesis [33]. At the genus level, the relative abundance of *Succinimonas* was 0.037%, 0.047%, 0.052%, 0.063%, and 0.082% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, and tended to increase in a dose-dependent manner in response to vitamin B_{12} (p = 0.073, Figure 3). The relative abundance of *Succinivibrio* was 0.017%, 0.028%, 0.036%, 0.038%, and 0.045% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing an increase in a dose-dependent manner in response to vitamin B_{12} (p = 0.006, Figure 3). The genera *Succinimonas* and *Succinivibrio* are the typical members of Succinivibrionaceae family and are well known for the degradation of starch to produce succinate, which is an intermediate during the propionate formation and can be decarboxylated by other species to produce propionate [34]. In the present study, vitamin B_{12} enhanced the conversion of succinyl-CoA and R-methylmalonyl-CoA and promoted the utility of succinate, thereby leading to an increase in members in the Proteobacteria phylum producing succinate.

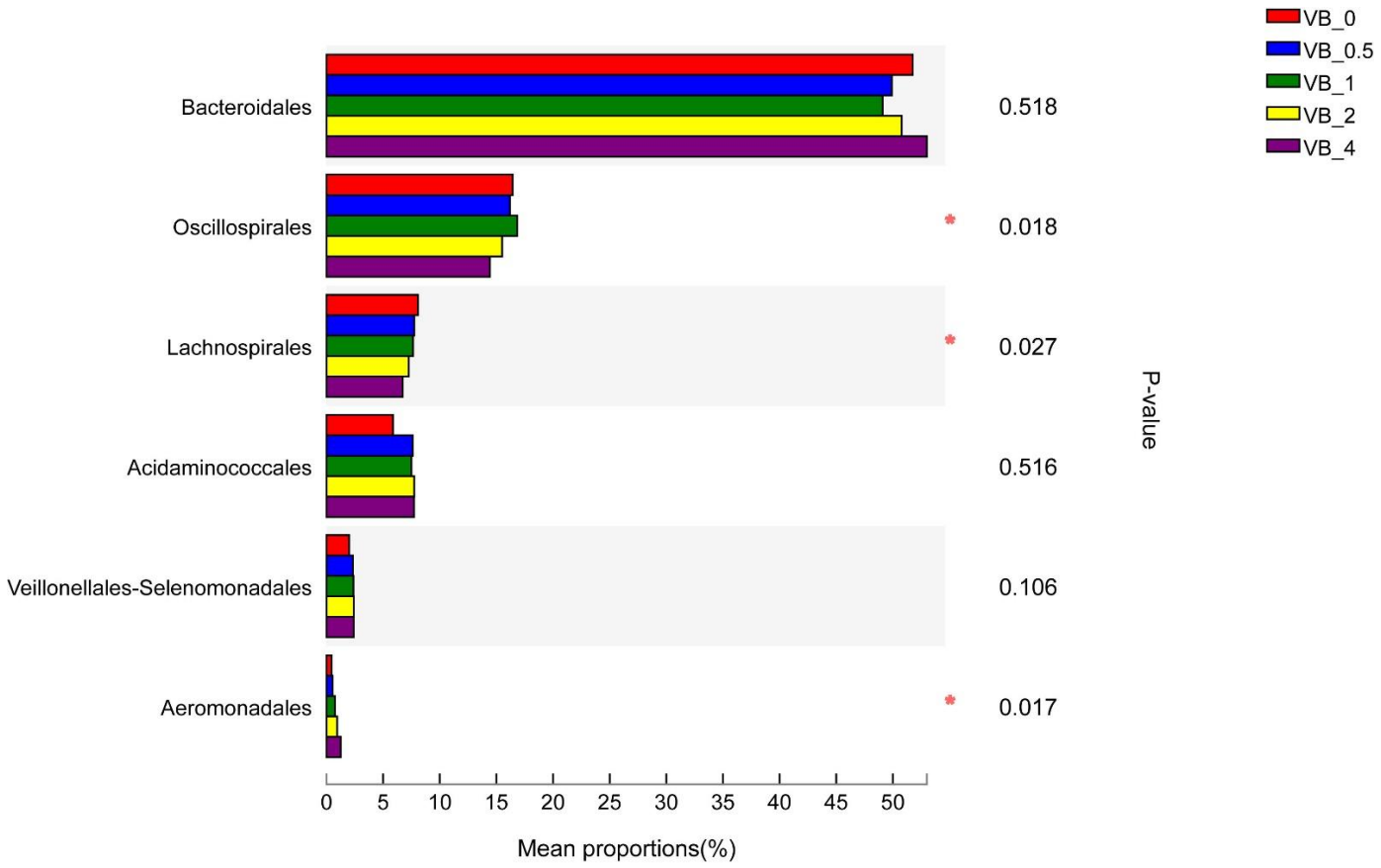


(**A**)

Figure 2. *Cont.*



(B)



(C)

Figure 2. *Cont.*

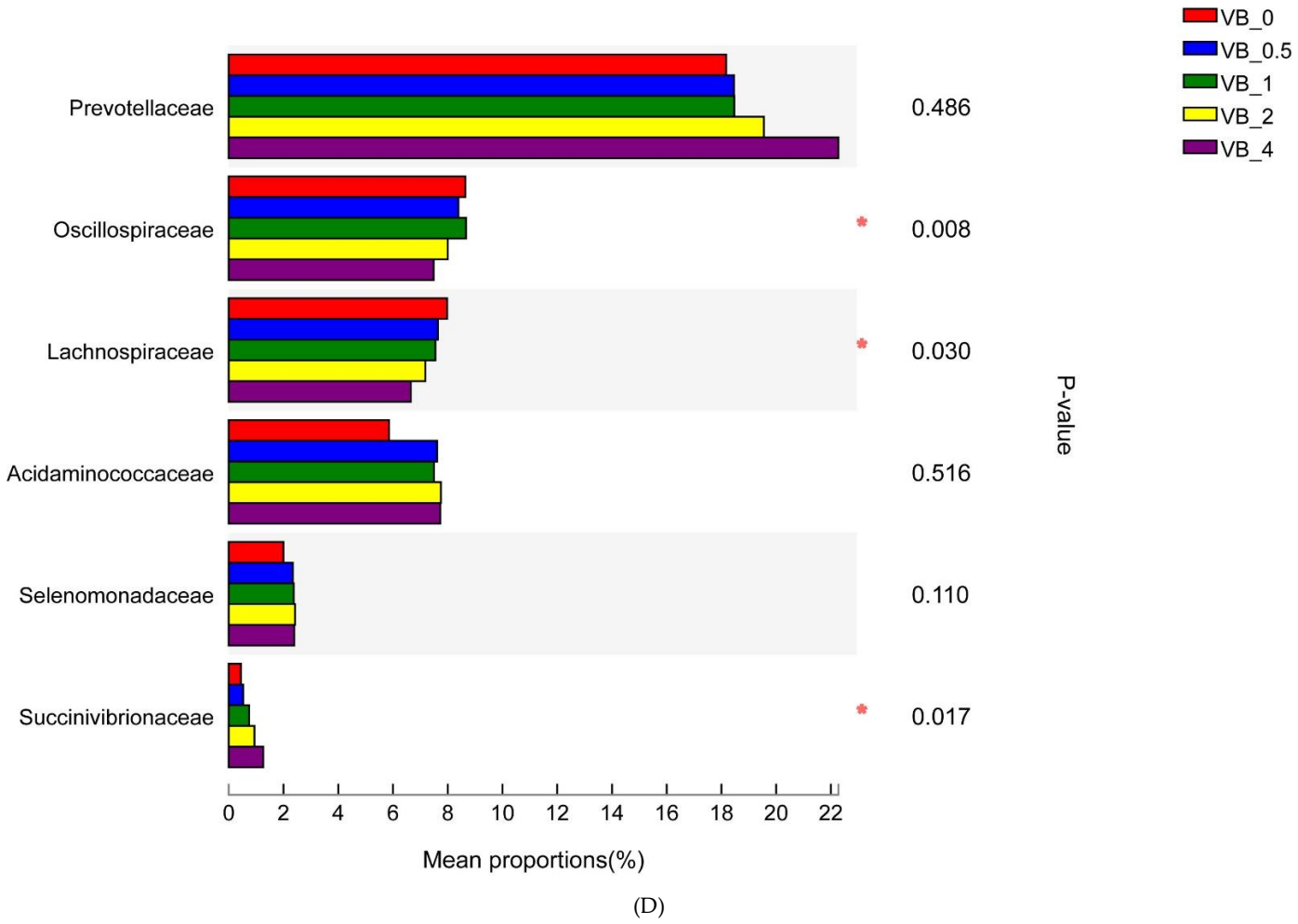


Figure 2. Changes in the bacterial community structure in the rumen fermentation system with increasing vitamin B_{12} supplementation: at the phylum level (**A**), at the class level (**B**), at the order level (**C**), at the family level (**D**). (* $p < 0.05$).

Firmicutes and Bacteroidetes

Firmicutes and Bacteroidetes are the two dominant phyla in the rumen system, and more than 80% of bacteria belong to them. In the present study, the relative abundance of Firmicutes phylum was 43.35%, 44.31%, 45.17%, 43.10%, and 40.80% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively (Figure 2A). The relative abundance of Bacteroidetes phylum was 51.76%, 49.92%, 49.11%, 50.80%, and 53.01% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively (Figure 2A). The phyla of Firmicutes and Bacteroidetes did not show significant changes in response to vitamin B_{12}. The succinate pathway was the major route for propionate formation from carbohydrates, and the Bacteroidetes phylum and the Negativicutes class of the Firmicutes phylum were the main bacteria involved [35]. The Prevotellaceae family and the *Prevotella* genus were the dominant family and genus in the Bacteroidetes. The relative abundance of the Prevotellaceae family was 18.17%, 18.46%, 18.47%, 19.55%, and 22.27% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing numerical increases in response to vitamin B_{12} (p = 0.486, Figure 2D). The relative abundance of the *Prevotella* genus was 13.81%, 13.62%, 13.62%, 14.33%, and 16.51% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, showing numerical increases in response to vitamin B_{12} (p = 0.589, Figure 3). The *Prevotella* species degraded protein, starch, and hemicellulose and produced different products, including acetate, propionate, and succinate [36,37]. Acetate and propionate were both the products of the *Prevotella* species. Therefore, different *Prevotella* species played

significantly different roles in the rumen. Kittelmann et al. [38] found that the *Prevotella* species were linked to a high CH_4 production phenotype, while Danielsson et al. [39] reported that a low CH_4 production was related to the *Prevotella* species. The relative abundance of the Negativicutes class (Figure 2B), Acidaminococcales order (Figure 2C), Veillonellales-Selenomonadales order (Figure 2C), Acidaminococcaceae family (Figure 2D), Selenomonadales family (Figure 2D), *Succiniclasticum* genus (Figure 3), and *Selenomonas* genus (Figure 3) numerically increased in response to vitamin B_{12}. Bacteria belonging to the Negativicutes class were mainly involved in the pathway that converted succinate to propionate. The *Phascolarctobacterium* and *Succiniclasticum* genus, belonging to the Acidaminococcaceae family, have been reported to convert succinate to propionate [35,40]. The *Selenomonas* genus of the Selenomonadales family converted succinate to propionate via the succinate pathway [41]. Taken together, species of the Proteobacteria phylum contributed to succinate production, and then species of the Negativicutes class converted succinate to propionate via the succinate pathway. In conclusion, vitamin B_{12} supplementation in diets changed the fermentation pattern due to the increased relative abundances of microbes related to propionate metabolism. In other words, the supplementation of vitamin B_{12} in diets promoted the proliferation of microbes associated with vitamin B_{12} consumption.

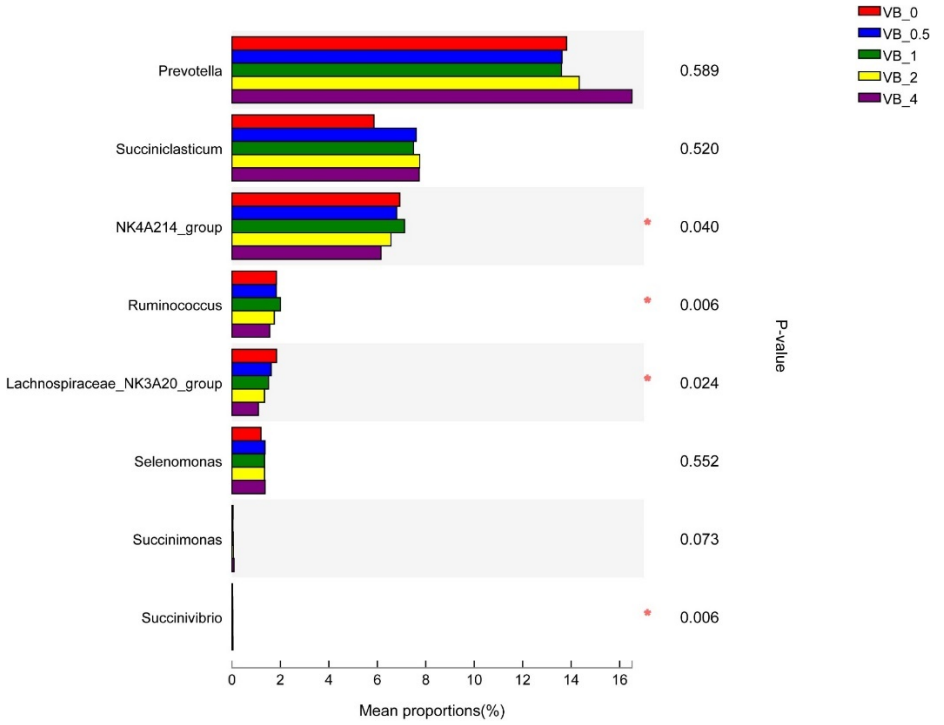


Figure 3. Changes in the bacterial genus level in the rumen fermentation system with increasing vitamin B_{12} supplementation. (* $p < 0.05$).

In the present study, the relative of Clostridia class (Figure 2B), Oscillospirales order (Figure 2C), Lachnospirales order (Figure 2C), Oscillospiraceae family (Figure 2D), Lachnospiraceae family (Figure 2D), NK4A214_group genus (Figure 3), and Lachnospiraceae _NK3A20_group genus (Figure 3) significantly decreased in response to vitamin B_{12}. The Clostridia class is identified as the predominant butyrate producer in the mammalian intestine [42]. The Oscillospiraceae and Lachnospiraceae families are both known butyrate producers [43,44]. It is therefore possible that the decreased concentration of butyrate could be attributed to the reduction in the relative abundance of species belonging to the Clostridia class.

3.2.2. Archaeal Community

Alpha diversity indices of the archaeal community in the present study are shown in Figure 4. There were no significant differences in Coverage and Ace, indicating that the sequencing depth was desirable for the analysis and that vitamin B_{12} supplementation did not affect the richness of the archaeal community in the present study. The Shannon index and Simpson index showed a dose-dependent decrease and increase, respectively, with a significant change observed at 4 mg/g dry matter. The effects on the Shannon index and Simpson index indicate that vitamin B_{12} supplementation decreased the diversity of the archaeal community.

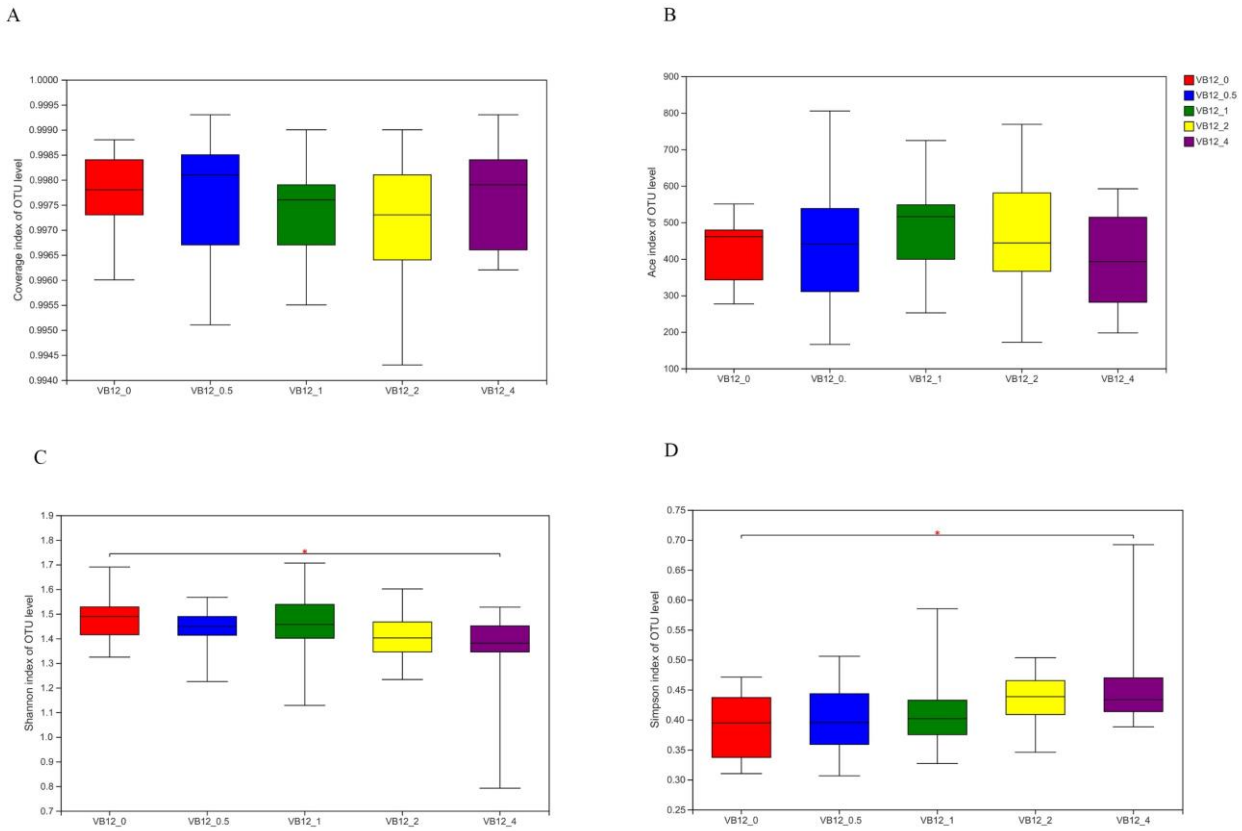


Figure 4. Changes of alpha diversity indices in the archaeal community in the rumen fermentation system with increasing vitamin B_{12} supplementation. (**A**) Coverage index; (**B**) Ace index; (**C**) Shannon index; (**D**) Simpson index.

The archaeal community compositions of the present study are shown in Figure S2 (https://doi.org/10.6084/m9.figshare.20338692.v2, accessed on: 25 July 2022). The Euryarchaeota phylum (98.27%) was the dominant methanogenic archaea in the present study. A minor proportion of the Thermoplasmatota phylum (0.11%) was also detected. At the genus level, only *Methanobrevibacter* (94.24%), *Methanosphaera* (3.5%), and *unclassified_f_Methanobacteriaceae* (0.53%) were detected. Their relative abundances were not significantly affected by vitamin B_{12} (Figure 5A). At the species level, the relative abundance of *uncultured_archaeon_g_Methanobrevibacter* was 59.68%, 61.49%, 63.89%, 65.84%, and 67.73% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, and tended to increase in a dose-dependent manner response to vitamin B_{12} (p = 0.098, Figure 5B). The relative abundance of *unclassified_g_Methanobrevibacter* was 19.90%, 18.96%, 17.07%, 15.32%, and 14.04% at vitamin B_{12} 0, 0.5, 1.0, 2.0, and 4 mg/g of dry matter, respectively, and tended to decrease in a dose-dependent manner response to vitamin B_{12} (p = 0.094, Figure 5B). Methanogenic archaea were the sole producers of CH_4 in the rumen, and their amounts were the main factor that affected CH_4 production [45,46]. However, given the methanogenic efficiency of different methanogens, the composition of archaeal communities

was also an important factor that affected CH_4 production [47]. It has been proposed that methanogens with high mcrA gene expression contribute more to methane production [48]. Wang et al. [49] reported that the change in the composition of the archaeal community was related to the reduction of CH_4 production. In the present study, the change in the composition of the archaeal community at the species level may have been responsible for the reduction of CH_4 production resulting from vitamin B_{12} supplementation.

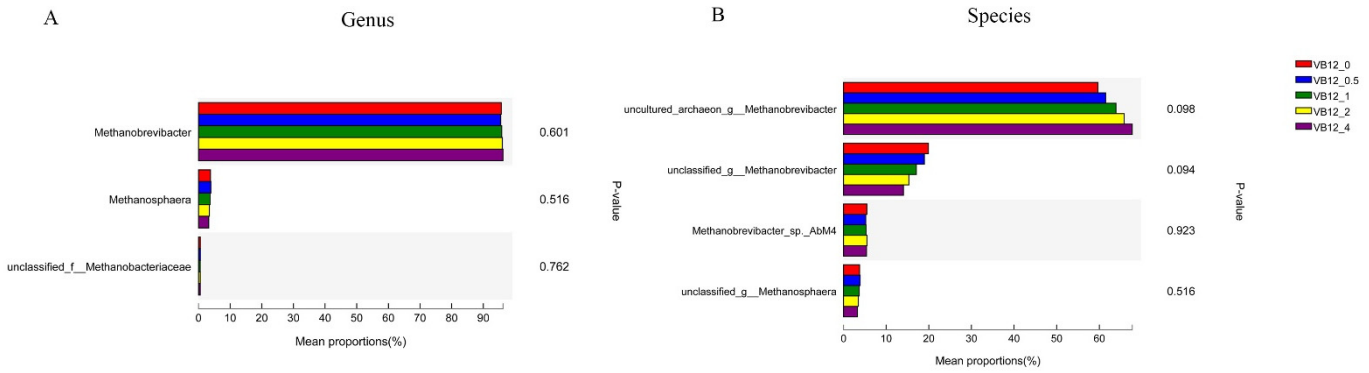


Figure 5. Changes in the archaeal genus (**A**) and species (**B**) level in the rumen fermentation system with increasing vitamin B_{12} supplementation.

4. Conclusions

Vitamin B_{12} supplementation in diets changed the ruminal fermentation pattern, resulting in a decreased acetate: propionate ratio and butyrate concentration. The increased relative abundances of the Proteobacteria phylum and the Negativicutes class and a reduction in relative abundance of species belonging to the Clostridia class were responsible for changes in the production of volatile fatty acids. In addition, vitamin B_{12} supplementation in diets reduced the CH_4 production by altering the species composition of the archaeal community. According to the present results, the dietary supplementation of vitamin B_{12} caused rumen perturbation. There is a need for further research to determine whether the perturbation of the rumen will affect the synthesis and absorption of vitamin B_{12}, as well as its content in milk. It is important to be cautious, as dietary supplementation with vitamin B_{12} may compromise the performance and health of dairy cows since it affects the production of VFA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080406/s1, Figure S1: The composition of the bacterial community in the rumen fermentation system: at the phylum level (A); at the class level (B); at the order level (C); at the family level (D); at the genus level (E).; Figure S2: The composition of the archaeal community in the rumen fermentation system: at the phylum level (A); at the class level (B); at the order level (C); at the family level (D); at the genus level (E); at species level (F).

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Institutional Review Board Statement: All animal care and experiments strictly followed the guidelines for Care and Use of Laboratory Animals of the Chinese Academy of Agricultural Sciences and was approved by the Animal Ethics Committee of Chinese Academy of Agricultural Sciences (Beijing, China; approval number: IAS-2021-14).

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw sequences were deposited into the National Center for Biotechnology Information Sequence Read Archive database under accession numbers SRP327708.

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

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