

Article

Impact of Exposure of Dairy Cow Feed to Polystyrene Microplastics on 24 h In Vitro Rumen Fermentation Responses, Microbiota Biodegradation Potential and Metabolic Pathways

Xitong Guan ^{1,†} , Haokai Ma ^{1,2,†}, Rui Liu ¹, Yiou Xu ¹, Diene Gnagna ¹, Xiujie Yin ¹ , Yonggen Zhang ¹ and Yang Li ^{1,3,*}

¹ College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, China; gxt20010827@163.com (X.G.)

² Laboratory of Gastrointestinal Microbiology, Jiangsu Key Laboratory of Gastrointestinal Nutrition and Animal Health, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

³ State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

* Correspondence: liyang1405053@neau.edu.cn

† These authors contributed equally to this work.

Simple Summary

Microplastic pollution is rising worldwide and may harm both animals and people. Dairy cows rely on a large stomach compartment, called the rumen, to break down feed with the help of many microbes, and this process produces gases and acids that reflect healthy digestion. In this study, we tested whether tiny plastic particles made of polystyrene, in different sizes and amounts, change rumen digestion and whether rumen microbes can break these plastics down. We found that exposure to these particles reduced fermentation activity, shown by lower gas production and lower levels of volatile fatty acids, and the negative effects became stronger as the amount of microplastics increased. At the same time, higher amounts and larger particles increased the activity of some digestive enzymes, suggesting a stress response in the microbial system. Microplastics also lowered the abundance of certain microbes and changed the chemicals produced during digestion. After 24 h, the amount of microplastic particles decreased, indicating that rumen microbes can partially degrade them and generate small chemical products that could further disturb rumen balance. These findings help explain how microplastics may influence ruminant health and suggest a possible biological route to reduce microplastic contamination.

Abstract

Microplastic pollution is increasingly serious worldwide, threatening human and animal health. The cow rumen is a key organ for nutrient digestion and absorption, and its fermentation is closely related to rumen microorganisms. Here, we investigated how polystyrene microplastics (PS-MPs) with varying particle sizes and concentrations affect rumen fermentation and the biodegradability of PS-MPs by rumen fermentation. The results reveal that exposure to PS-MPs lowered gas production and gas concentrations, as well as volatile fatty acid content, and these decreases were positively correlated with PS-MP concentration. However, higher PS-MP concentration and larger particle size increased the activity of carboxymethyl cellulase, β -glucosidase, and xylanase. Furthermore, PS-MP exposure reduced the abundance of certain rumen microorganisms and altered metabolic pathways and metabolites linked to PS-MP biodegradation. It was also found that PS-MP content decreased significantly after 24 h fermentation. Therefore, PS-MPs can inhibit rumen fermentation by affecting the rumen microbiome, and rumen microorganisms and their



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secreted enzymes can biodegrade PS-MPs to produce styrene and derivatives; such small molecules may further disrupt rumen homeostasis, thereby affecting lactation performance. In addition, rumen microbial degradation of PS-MPs provides a new idea to resolve future microplastic contamination challenges.

Keywords: polystyrene microplastics; in vitro rumen fermentation; metabolome; biodegradation of microplastics

1. Introduction

Due to the extensive usage of plastic products in industry and daily life, plastic pollution has become an increasingly serious global problem [1,2]. Large quantities of plastic waste fragment into particles smaller than 5 mm through physicochemical and biological processes, generating microplastics [3]. Microplastics (MPs) have been detected in human stool [4], placenta [5], thrombi [6], blood [7], and breastmilk [8], raising concerns regarding chronic exposure. Owing to their high surface area, MPs can adsorb environmental contaminants and additives, potentially amplifying toxicological risks [9]. Therefore, increasing attention has been directed toward understanding how MPs affect human and animal health.

Experimental evidence in aquatic organisms and rodents suggests that MPs can induce inflammation, immune responses, and oxidative stress [10–12]. In livestock systems, exposure is plausible because feedstuffs can be contaminated during production, processing, packaging, and transportation, and plastic materials used in feed storage and handling may further contribute to MP entry into diets. Recent studies have quantified MPs in common ruminant feeds such as corn silage, hay, and total mixed ration (TMR), supporting feed as a realistic exposure route for ruminants [13,14]. Because dairy cows consume large amounts of feed daily [15], MPs may accumulate in the rumen and adversely affect rumen fermentation and microbiota, thereby compromising productivity.

The rumen is a key organ for nutrient digestion and absorption, and its fermentation depends on a diverse microbial community. Ruminal bacteria, fungi, and protozoa generate volatile fatty acids (VFAs) and ammonia nitrogen, which are major precursors for host energy supply and protein synthesis [16]. Fermentation also produces abundant metabolites and small molecules; accordingly, metabolomics has been used to investigate MP-related toxicity mechanisms in animals [10]. Thus, defining MP-induced changes in rumen microbial composition, metabolic pathways, and metabolites is crucial to understanding their effects on rumen fermentation.

Recent evidence has started to address MPs in ruminants. In vivo, polystyrene exposure has been linked to gastrointestinal injury, inflammation, and reduced growth performance in lambs [17]. In vitro, polyethylene terephthalate (PET), low-density polyethylene (LDPE), and polyamide (PA) microplastics have been reported to inhibit rumen fermentation and compromise feed utilization [18,19]. Moreover, in vitro ruminal incubation has provided evidence that MPs can undergo measurable changes over time, indicating potential microbial transformation or biodegradation in the ruminal environment [20,21]. Despite these advances, the effects of polystyrene microplastics (PS-MPs) on 24 h end-point in vitro rumen fermentation responses, together with associated changes in gas production, fermentation parameters, microbial community structure, and the metabolite landscape, remain insufficiently characterized, particularly across different particle sizes and concentrations. In addition, whether biodegradation-related small molecules such as styrene and derivatives could further perturb rumen homeostasis warrants investigation.

Therefore, we hypothesized that PS-MPs with different particle sizes and concentrations would affect rumen fermentation parameters in a dose- and size-dependent manner and that rumen microorganisms and their secreted enzymes could contribute to PS-MP biodegradation. In this study, 16S amplicon sequencing and untargeted metabolomics were adopted in conjunction with the in vitro gas production technique (IVGPT) to explore the impacts of PS-MPs on gas production, fermentation parameters, microorganism composition, metabolic pathways and differential metabolites and to preliminarily reveal the biodegradation effect of rumen microorganisms on PS-MPs, thereby providing a methodological and mechanistic reference for future studies evaluating microplastic–rumen interactions and mitigation strategies in ruminant production systems.

2. Materials and Methods

2.1. Animals, Diet, and Experimental Design

The experimental procedures involving animals were reviewed and approved by the Animal Care and Use Committee of Northeast Agricultural University (approval No. NEAUEC20220266). Three lactating rumen-fistulated Chinese Holstein cows (120 ± 11 d in milk; milk yield, 28.9 ± 2.3 kg/d; parity = 2) were kept in individual tethered stalls with unrestricted access to water and served as long-term rumen fluid donors for the in vitro rumen fermentation assays. All cows were offered the same total mixed ration (TMR) formulated to meet the nutrient requirements described in [22] and were milked twice per day at 06:30 and 18:30. The ingredients and nutrient composition of the diet are provided in Table 1. For the in vitro rumen fermentation assays, this basal diet without added polystyrene microplastics (PS-MPs) was used as the fermentation substrate.

Table 1. Ingredient and nutrient composition of the substrate (DM basis).

Ingredient	Content, %
Corn silage	15.59
Alfalfa hay	30.29
Steam-flaked corn	19.15
Soybean meal	11.14
Beet pulp pellet	12.03
Dry corn gluten feed	5.35
DDGSs ¹	5.35
Premix ²	1.11
Chemical composition, %	
NEL (Mcal/kg of DM) ³	1.72
CP	16.67
NDF	32.75
ADF	21.11
Ash	7.14
Starch	27.12
EE	4.62
NFC ⁴	38.82

¹ DDGSs, distillers dried grains with solubles. ² The premix contained (on a DM basis): 99.17% ash, 14.25% Ca, 5.40% P, 4.93% Mg, 10.64% Na, 2.95% Cl, 12 mg kg^{−1} Co, 1750 mg kg^{−1} Cu, 125 mg kg^{−1} I, 4000 mg kg^{−1} Mn, 50 mg kg^{−1} Se, 6500 mg kg^{−1} Zn, 1,000,000 IU kg^{−1} vitamin A, 250,000 IU kg^{−1} vitamin D3 and 4000 IU kg^{−1} vitamin E. ³ NEL was calculated using the Cornell–Penn–Miner (CPM) Dairy model (version 3.1.10). ⁴ NFC = OM – (CP + NDF + fat). DM, dry matter; NEL, net energy for lactating cow; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; NFC, non-fibrous carbohydrate; “OM” refers to organic matter (typically calculated as DM – ash).

Anaerobic culture techniques followed Xin et al. [23]. The IVGPT experiment was carried out in three independent experimental runs on separate days using freshly collected rumen inoculum. Rumen fluid was collected via fistula from three cows pre-feeding, purged

with O₂-free CO₂, and filtered through four-layer cheesecloth. Within each run, rumen fluid was pooled from the three donor cows in equal volumes to generate one representative inoculum for that run; therefore, the cow was not considered an experimental unit. It was then mixed with pre-warmed buffer (39 °C) in a 2:1 buffer-to-rumen-fluid ratio under continuous CO₂ flow [24,25]. Subsequently, 150 mL was dispensed into 200 mL glass bottles containing 2 g of TMR substrate. PS-MPs was purchased from Mingshuo Chemical Company, Guangdong, China. PS-MPs with different particle sizes (1 µm, 5 µm, and 10 µm) were added to the rumen fluid to make 0.0, 0.5, 5.0, and 50.0 mg/L final concentrations. The PS-MP concentrations (0.5–50 mg/L) were set as a low-to-high gradient and were informed by plausible inputs from drinking water and feed [26]. Sample bottles were sealed with silicone stoppers and gas-tight collection bags, then incubated at 39 °C in a shaking water bath (40–50 rpm) for 24 h. In each run, fermentation bottles were prepared for a 3 (particle sizes: 1, 5, and 10 µm) × 4 (PS-MP concentrations: 0, 0.5, 5, and 50 mg/L) factorial design, with two parallel bottles per size × concentration combination. The entire experiment was repeated in three independent runs on separate days, yielding six bottles in total for each treatment across runs (n = 6 per size × concentration).

2.2. Determination of Rumen Fermentation Parameters and Total Hydrogen, Carbon Dioxide and Methane Gas Production

After incubation, we transferred gases to air collector bags, measured volumes with syringes, and analyzed 0.5 mL subsamples for H₂, CO₂, and CH₄. Gas chromatography (GC-8A; Shimadzu Co., Ltd., Tokyo, Japan) followed Kim et al. [27]. After gas measurement, we recorded culture fluid pH with a Sartorius Basic meter (Sartorius AG, Göttingen, Germany). Samples were strained through 4-layer cheesecloth, treated with 2 mL of metaphosphoric acid (25%) per 10 mL of rumen fluid, and frozen (−20 °C) for later analysis. Ammonia-N was analyzed by the phenol-hypochlorite assay [28] using a Hitachi U-2900 spectrophotometer (Hitachi High-Tech Corporation, Tokyo, Japan) and VFAs by Shimadzu GC-8A gas chromatography (Shimadzu Corporation, Kyoto, Japan) [29].

Carboxymethyl cellulase (CMCase), xylanase, and β-glucosidase activities were assayed following the procedures described by Wood and Bhat [30] and Jiang et al. [31], with minor formatting adjustments. Briefly, frozen samples were taken out, allowed to thaw at 4 °C, and then centrifuged at 1000× g for 10 min; the supernatant was subsequently removed as reported previously [32]. For substrate preparation, 1.000 ± 0.001 g of sodium carboxymethyl cellulose (Fu Chen Chemical Reagent, Tianjin, China), xylan (Sigma-Aldrich, Shanghai, China), or salicin (Aladdin, Shanghai, China) was weighed separately in 100 mL volumetric flasks. Each flask was brought to volume using 0.2 mol/L sodium phosphate buffer (pH 7.0) to obtain 10 g/L substrate solutions (carboxymethyl cellulose, xylan, and salicin-based substrates), which were pre-incubated in a 40 °C water bath prior to analysis. A volume of 1 mL of rumen fluid in 3 test tubes (1 tube as a blank tube and 2 tubes as sample tubes) was preheated in the same water bath for 5 min. Following that, 1 mL of the substrate solution was added to the sample tubes, while 3 mL of 3,5-dinitro salicylic acid (DNS) solution was added to the blank tube. The tubes were shaken for 3 s before placing them in the water bath at 40 °C for 60 min. Immediately after removing, 3 mL of DNS solution was added to the sample tubes and 1 mL of the substrate solution to the blank tube, and the tubes were shaken for 3 s. The 3 test tubes were then placed in a boiling water bath at the same time; the timer was started at the boiling point of the water, and the reaction was terminated immediately at 7 min. Then, the tubes were removed and quickly cooled to room temperature, after which 10 mL of distilled water was added to the three test tubes, which were shaken for 3 s. According to the dinitro salicylic acid method [33], the enzymatic release of reducing sugars, expressed as the absorbance, was used to quantify glucose equivalents at 540 nm through an ultraviolet–visible spectrophotometer (U-2900;

Hitachi, Ltd., Tokyo, Japan). Hydrolysis of cellulose-like substrates in 1 min yielded an enzyme amount equivalent to 1mol of reducing sugar, which is 1 enzyme active unit and is denoted by U.

2.3. DNA Extraction, Sequencing, and Diversity Analysis

Upon completion of 24 h incubation, culture liquor was collected from the maximum-dose treatment (50 mg/L) for each particle size and the corresponding control (0 mg/L). From each bottle, 20 mL of culture liquor was aliquoted and stored at -80°C for subsequent 16S rRNA amplicon sequencing and untargeted metabolomics. Across the three independent runs, each group comprised six bottles, and five bottles per group were randomly selected for sequencing, yielding a total of 20 sequenced samples. Total genomic DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) [34]. Polymerase chain reaction amplifications targeted distinct microbial groups: bacterial communities employed primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for the V3–V4 region of 16S rRNA; archaeal populations utilized primers F341 (5'-GYGCASCAGKCGMGAAG-3') and R806 (5'-GGAC TACVSGGGTATCTAAT-3') spanning the same region; protozoan diversity was assessed via TAREuk454FWD1 (5'-CCAGCASCYGC GGTAATTCC-3') and TAREukREV3 (5'-ACTTTCCG TTCTTGATYRA-3') amplifying the 18S rRNA V3–V4 region; fungal communities were characterized using ITS-specific primers F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and R (5'-GCTGCGTTCTTCATCGATGC-3'). Sequencing libraries were constructed following manufacturer protocols and paired-end sequenced (2×250 bp) on an Illumina NovaSeq 6000 platform (Biomarker Technologies, Beijing, China). Raw reads underwent merge processing in FLASH v1.2.7 [35], discarding reads with >6 mismatches. Quality control implemented Trimmomatic [36] to eliminate sequences with average Phred scores < 20 over a 50 bp sliding window and reads shorter than 350 bp. Chimeric sequences were removed via USEARCH v10.0, followed by the clustering of denoised sequences into operational taxonomic units (OTUs) at a 97% similarity threshold. Taxonomic classification against SILVA database v128 was performed using QIIME (version 1.9.1). Alpha diversity of the bacterial community was assessed using the Shannon, Chao1, ACE, and Simpson indices based on the OTU table in QIIME. To reduce potential bias from uneven sequencing depth, the feature table was rarefied to an even sampling depth before diversity calculation. Pairwise comparisons between each PS-MP group and the control were performed using the Wilcoxon rank-sum test (two-sided). Beta diversity analysis employed partial least squares discriminant analysis (PLS-DA), while genus-level abundance differences between polystyrene exposure groups and controls were evaluated statistically.

2.4. Non-Targeted Metabolomics Analysis

For quality control, 10 μL aliquots of prepared rumen fluid from each sample were pooled. Untargeted metabolomics employed an LC/MS system (Waters Acquity I-Class PLUS UPLC coupled to a Xevo G2-XS QTOF high-resolution mass spectrometer (Waters Corporation, Milford, MA, USA) with an Acquity UPLC HSS T3 column ($1.8\ \mu\text{m}$, 2.1×100 mm) [23]. Chromatographic separation used mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile), with a 1 μL injection volume in both positive and negative ionization modes.

Mass spectrometry data were acquired in MSe mode under MassLynx V4.2 control (Waters Corporation, Milford, MA, USA). Primary and secondary spectra were collected via dual-channel acquisition: low collision energy (2 V) for molecular ions and high-collision-energy ramping (10–40 V) for fragment ions, with a 0.2 s/scan frequency. ESI parameters

followed [37]: capillary voltage of 2000 V (+)/−1500 V (−), cone voltage of 30 V, source temperature of 150 °C, desolvation gas at 800 L/h at 500 °C, and cone gas at 50 L/h.

Raw data were processed in Progenesis QI (version 3.0) using METLIN database annotation, incorporating peak extraction, alignment, and Biomark's custom library matching. Metabolite identifications required mass error < 100 ppm with theoretical fragment verification. Combined positive/negative mode datasets underwent total peak area normalization prior to multivariate analysis. Model reliability was validated by 200 permutation tests using principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA). Differential metabolites were identified by implementing a tripartite filtering criterion: (1) absolute fold change threshold $|FC| > 2.0$ (equivalent to $|\log_2 FC| > 1.0$), (2) variable importance in projection (VIP) scores derived from OPLS-DA exceeding 1.0, and (3) statistically significant differences (Student's *t*-test $p < 0.05$). Metabolites fulfilling these criteria were subsequently subjected to KEGG pathway enrichment analysis using hypergeometric distribution testing. Biological interpretation was augmented by generating volcano plots via the ggplot2 package (version 3.5.1) in R (version 4.5.2), wherein each metabolite was visualized according to its $\log_2$ (fold change) versus $-\log_{10}(p\text{-value})$ coordinates to highlight compounds of significant interest.

2.5. Scanning Electron Microscopy (SEM)

The surface morphology of PS-MPs of different particle sizes was analyzed by SEM at WEIPU Testing Technology Group Co., Ltd. (Shanghai, China). The SEM investigations were carried out on a scanning electron microscope (ZEISS Sigma 500, ZEISS, Oberkochen, Germany) under high-vacuum and variable-high-tension (3–10 kV) conditions. Sample surfaces were sputter-coated with a 10 nm gold layer to ensure adequate electrical conductivity prior to imaging [38]. Micrographs were acquired in secondary electron (SE) mode using an Everhart–Thornley detector (ZEISS, Oberkochen, Germany). PS-MP diameters were quantified with Nano Measurer 1.2.5 software (Nano measurer 1.2.5, Jie Xu, Fudan University, Shanghai, China), and graphical representations were generated in Origin 2022 (Origin Lab, Northampton, MA, USA).

2.6. Py-GC/MS Analysis

The concentration of microplastics in rumen fluid was determined using Py-GC/MS at WEIPU Testing Technology Group Co., Ltd. (Shanghai, China).

The pyrolysis–GC/MS instrumentation had a Single-shot Pyrolyzer EGA/Py-3030 micro-furnace (Frontier Laboratories Ltd., Fukushima, Japan) fitted to a gas chromatograph (GCMS-QP2020, Shimadzu Co., Ltd., Kyoto, Japan) that was equipped with a chromatographic column (Rtx-5MS, Shimadzu Co., Ltd., Kyoto, Japan) [39]. The rumen fluid was homogenized firstly with the vortex oscillator (Vortex-Genie 2 SI-0246, Scientific Industries, Inc., Bohemia, NY, USA); then a portion of each sample was taken, and an appropriate amount of concentrated nitric acid was added for digestion for 1–2 h. A certain amount of deionized water was added to ensure that the solution was weakly acidic. The solution was concentrated and dropped into the injection crucible of pyrolysis–GC/MS.

After the solvent in the crucible was completely volatilized, the crucible was inserted in the micro-furnace and pyrolyzed for 0.20 min at 550 °C. A single quadrupole mass spectrometer (GCMS-QP2020 NX, Shimadzu Co., Ltd., Kyoto, Japan) operated in electron ionization mode (EI) at 70 eV, scanning in positive mode in the 40–600 m/z range for detection. Both the MS quadrupole and the MS ion source were at 230 °C. The injection port temperature was 320 °C, the injection port had a split ratio of 5:1, and the interface temperature was 320 °C. The pyrolysis products were maintained for 14 min after being flushed in constant flow mode at 1.0 mL/min (carrier gas, He; purity, 99.995%) at a rate of

20 °C/min. The total procedure time was 30 min. The data collected were processed by GCMS Post-run Analysis (Shimadzu Co., Ltd., Kyoto, Japan).

2.7. Statistical Analysis

Data were analyzed using SAS (Version 9.4; SAS Institute Inc., Cary, NC, USA). Gas production and concentration, rumen fermentation parameters and enzyme activity data were assessed for normality and homogeneity of variance using histograms and formal tests with the SAS UNIVARIATE procedure. These data were analyzed using PROC MIXED (SAS 9.4) with microplastic size, concentration, and their interaction as fixed effects; an experimental run was included as a random blocking factor, and the bottle was considered the experimental unit. Least squares means were compared using the PDIFF option. To evaluate the effects of PS-MP size on relative rumen microbiome abundance (%) after 24 h exposure, the non-parametric Kruskal–Wallis Test was applied; results for this analysis are presented as means $\pm$ standard deviations. Residual PS-MP concentration data were analyzed using PROC MIXED in SAS, with PS-MP concentration as the fixed effect and an experimental run included as a random effect. Statistical significance was declared at $p \leq 0.05$, while $0.05 < p \leq 0.10$ indicated a trend.

3. Results

3.1. PS-MP Exposure Affects Ruminal In Vitro Gas Production, Concentration, and Fermentation Parameters

As indicated in Figure 1, in order to comprehensively understand how varying PS-MP sizes and concentrations influence rumen fermentation, an in vitro gas production assay was adopted to investigate the effect of PS-MPs on gas production, gas composition, ruminal pH, enzyme activity, ammonia nitrogen, and VFA content (Tables 2 and 3). Compared with the control group, in vitro gas production and concentration were not affected in the rumen exposed to 1 μ m polystyrene microplastics. However, the production ($p < 0.05$) and concentration ($p < 0.05$) of methane in the 5 μ m and 10 μ m groups were reduced significantly. At the maximum concentration, adding 5 μ m and 10 μ m PS-MPs significantly reduced the hydrogen content ($p < 0.05$). Moreover, adding 10 μ m polystyrene microplastics significantly lowered the total gas production, carbon dioxide production, and hydrogen production at 5 mg/L and 50 mg/L ($p < 0.05$) and significantly decreased the carbon dioxide concentration at 50 mg/L ($p < 0.05$).

In comparison to the control group, rumen fermentation parameters, the supplementation of 1 μ m polystyrene microplastics and the maximum concentration of 5 μ m and 10 μ m polystyrene microplastics significantly decreased the content of total VFAs ($p < 0.05$). When the rumen was exposed to 1 μ m of PS at 5 mg/L and 50 mg/L, pH and the molar proportions of propionate, valerate, and isovalerate increased significantly ($p < 0.05$), while the molar ratio of acetate and the ratio of acetate and propionate decreased significantly ($p < 0.05$). Moreover, carboxymethyl cellulose and xylanase activities decreased when the rumen was exposed to PS-MPs of all particle sizes, and the activity increased with the increase in PS-MP concentration and particle size ($p < 0.05$). The activity of β -glucosidase also increased gradually with the increase in PS-MPs supplemental level and particle size, and it was significant at 5 mg/L and 50 mg/L ($p < 0.05$).

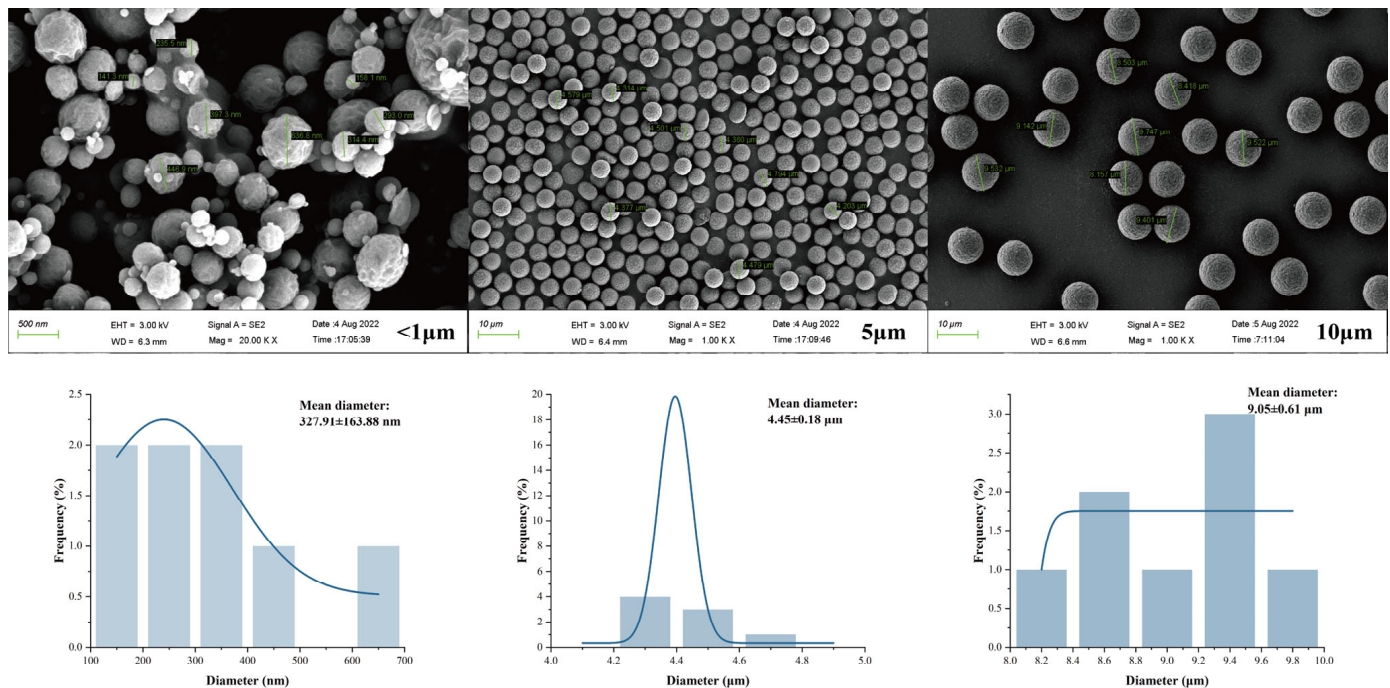


Figure 1. Images of PS-MPs and average diameter of PS-MPs (<1 μm, 5 μm, and 10 μm) used in this study observed by scanning electron microscopy. Abbreviations: EHT, extra-high tension; Mag, magnification; WD, working distance.

3.2. PS-MP Exposure at the Maximum Concentration (50 mg/L) Affects the Structure and Relative Abundance of the Rumen Microbiome

We further assessed rumen microbial alpha diversity using four indices (Figure 2). Compared with the control group, the PSMP5 group showed significantly higher richness and diversity, with increases in Chao1 ($p = 0.016$), Shannon ($p = 0.0079$), ACE ($p = 0.032$), and Simpson ($p = 0.016$). In contrast, PSMP1 did not differ from CON in Chao1 ($p = 0.22$), Shannon ($p = 0.095$), ACE ($p = 0.15$), or Simpson ($p = 0.12$). Likewise, PSMP10 did not differ from CON in Chao1 ($p = 0.056$), Shannon ($p = 0.42$), ACE ($p = 0.095$), or Simpson ($p = 0.17$). To further explore how PS-MPs affect the rumen microbiome at the maximum concentration (50 mg/L), 16S amplicon sequencing was used to measure the relative abundance and β -diversity of the bacterial, fungal, archaeal, and protozoal communities in the rumen. In comparison with the control group, exposing the rumen to PS-MPs significantly reduced the relative abundance of *Succinivibrionaceae* ($p = 0.019$) and *Isotricha* ($p = 0.0084$), and that of *Methanomassiliicoccus* ($p = 0.097$), *Methanoculleus* ($p = 0.097$), *unclassified_Nitrosotaleaceae* ($p = 0.097$) and *Thermoascus* ($p = 0.08$) showed a decreasing trend. However, this study found that PS-MPs significantly elevated the abundance of *unclassified_Colpodida* ($p = 0.048$) while *Butyrivibrio* ($p = 0.07$) had an increasing trend (Table 4). As indicated by the partial least squares discriminant analysis (PLS-DA), there were variations in the microbial community, and the distance between the control group and the 1 μm, 5 μm, and 10 μm groups was large (Figure 3).

Table 2. The effects of PS-MPs with different sizes and different exposure concentrations on in vitro gas production and concentration after exposure for 24 h.

Item	1 μm				5 μm				10 μm				SEM	<i>p</i> -Value		
	0	0.5 mg/L	5 mg/L	50 mg/L	0	0.5 mg/L	5 mg/L	50 mg/L	0	0.5 mg/L	5 mg/L	50 mg/L		Size	Conc.	Size $\times$ Conc.
Gas production (mL)	111.50 ^a	110.83 ^{ab}	112.33 ^a	112.83 ^a	111.50 ^a	107.00 ^{ab}	103.33 ^{ab}	104.17 ^{ab}	111.50 ^a	106.67 ^{ab}	98.00 ^{bc}	87.50 ^c	2.689	<0.0001	0.0002	0.0005
Methane production (mL)	27.45 ^a	26.16 ^{ab}	26.63 ^a	26.40 ^a	27.45 ^a	23.01 ^{bc}	21.06 ^{cd}	19.97 ^{cd}	27.45 ^a	22.17 ^{cd}	19.07 ^d	15.63 ^e	0.695	<0.0001	<0.0001	<0.0001
Carbon dioxide production (mL)	68.86 ^a	68.33 ^a	69.69 ^a	68.19 ^a	68.86 ^a	63.68 ^{ab}	61.51 ^{ab}	61.11 ^{ab}	68.86 ^a	63.80 ^{ab}	57.61 ^{bc}	50.13 ^c	2.034	<0.0001	<0.0001	0.0014
Hydrogen production (mL)	1.10 ^a	1.06 ^a	1.06 ^a	1.03 ^{ab}	1.10 ^a	1.04 ^{ab}	0.97 ^{ab}	0.94 ^{ab}	1.10 ^a	1.00 ^{ab}	0.89 ^{bc}	0.74 ^c	0.033	<0.0001	<0.0001	0.0016
Methane concentration (%)	24.59 ^a	23.59 ^a	23.71 ^a	23.41 ^a	24.59 ^a	21.52 ^b	20.38 ^{bc}	19.16 ^d	24.59 ^a	20.81 ^b	19.45 ^{cd}	17.85 ^e	0.252	<0.0001	<0.0001	<0.0001
Carbon dioxide concentration (%)	61.69 ^a	61.63 ^a	62.06 ^a	60.41 ^{ab}	61.69 ^a	59.50 ^{ab}	59.54 ^{ab}	58.64 ^{ab}	61.69 ^a	59.80 ^{ab}	58.78 ^{ab}	57.37 ^b	0.883	0.0046	0.0024	0.53
Hydrogen concentration (%)	0.99 ^a	0.96 ^{ab}	0.94 ^{ab}	0.92 ^{abc}	0.99 ^a	0.97 ^{ab}	0.93 ^{ab}	0.91 ^{bc}	0.99 ^a	0.94 ^{ab}	0.91 ^{abc}	0.84 ^c	0.017	0.0115	<0.0001	0.41

Means within a row with different superscript letters are significantly different ($p < 0.05$). Means with the same letter are not significantly different. Comparisons were made among all size $\times$ concentration treatment means within each row. PS-MPs, polystyrene microplastics; SEM, standard error of the mean; Conc., concentration.

Table 3. The effects of PS-MPs with different sizes and different exposure concentrations on rumen fermentation parameters in vitro after exposure for 24 h.

Item	1 μm				5 μm				10 μm				SEM	<i>p</i> -Value		
	0	0.5 mg/L	5 mg/L	50 mg/L	0	0.5 mg/L	5 mg/L	50 mg/L	0	0.5 mg/L	5 mg/L	50 mg/L		Size	Conc.	Size $\times$ Conc.
pH	6.49 ^c	6.49 ^c	6.54 ^{ab}	6.54 ^a	6.49 ^c	6.50 ^{bc}	6.51 ^{abc}	6.52 ^{abc}	6.49 ^c	6.52 ^{abc}	6.51 ^{abc}	6.53 ^{abc}	0.008	0.19	<0.0001	0.0210
Carboxymethyl cellulose, U/mL	1.03 ^a	0.36 ^f	0.49 ^{de}	0.56 ^{cd}	1.03 ^a	0.42 ^{ef}	0.61 ^c	0.77 ^b	1.03 ^a	0.58 ^{cd}	0.65 ^c	0.78 ^b	0.0243	<0.0001	<0.0001	<0.0001
Xylanase, U/mL	6.55 ^a	4.75 ^{ef}	5.18 ^{de}	5.25 ^{cde}	6.55 ^a	4.54 ^f	5.85 ^b	5.53 ^{bcd}	6.55 ^a	5.44 ^{bcd}	5.45 ^{bcd}	5.71 ^{bc}	0.103	<0.0001	<0.0001	<0.0001
β -glucosidase, U/mL	0.25 ^e	0.37 ^e	0.61 ^{bcd}	0.66 ^{abc}	0.25 ^e	0.46 ^{cde}	0.73 ^{ab}	0.73 ^{ab}	0.25 ^e	0.41 ^{de}	0.67 ^{abc}	0.84 ^a	0.0454	0.047	<0.0001	0.2819
NH ₃ -N	33.16	33.33	32.66	33.18	33.16	33.41	32.75	34.26	33.16	34.98	34.23	35.18	1.261	0.32	0.68	0.99
Total VFAs (mmol/L)	74.58 ^a	67.51 ^{cd}	66.37 ^{de}	63.16 ^e	74.58 ^a	72.46 ^{ab}	71.96 ^{ab}	68.88 ^{bcd}	74.58 ^a	71.38 ^{abc}	71.24 ^{abc}	70.38 ^{bcd}	0.846	<0.0001	<0.0001	0.0002
VFA profile (mol/100 mol)																
Acetate	56.09 ^a	55.35 ^{abc}	54.13 ^{bc}	53.74 ^c	56.09 ^a	55.50 ^{abc}	55.03 ^{abc}	54.93 ^{abc}	56.09 ^a	55.91 ^a	55.54 ^{ab}	54.92 ^{abc}	0.370	0.0117	<0.0001	0.44
Propionate	25.42 ^c	25.91 ^{bc}	26.33 ^{ab}	26.63 ^a	25.42 ^c	25.71 ^{bc}	25.82 ^{bc}	26.03 ^{abc}	25.42 ^c	25.76 ^{bc}	25.94 ^{bc}	26.20 ^{ab}	0.141	0.0052	<0.0001	0.43
Isobutyrate	1.28 ^b	1.36 ^b	1.40 ^{ab}	1.52 ^a	1.28 ^b	1.32 ^b	1.33 ^b	1.35 ^b	1.28 ^b	1.30 ^b	1.29 ^b	1.33 ^b	0.026	<0.0001	<0.0001	0.014
Butyrate	13.44	13.42	13.97	13.88	13.44	13.57	13.83	13.66	13.44	13.20	13.40	13.57	0.200	0.13	0.08	0.78
Isovalerate	1.97 ^b	2.08 ^{ab}	2.21 ^a	2.24 ^a	1.97 ^b	2.05 ^{ab}	2.10 ^{ab}	2.12 ^{ab}	1.97 ^b	2.00 ^b	2.00 ^b	2.07 ^{ab}	0.040	0.0007	<0.0001	0.22
Valerate	1.80 ^b	1.87 ^{ab}	1.97 ^a	1.99 ^a	1.80 ^b	1.86 ^{ab}	1.89 ^{ab}	1.90 ^{ab}	1.80 ^b	1.82 ^b	1.82 ^b	1.90 ^{ab}	0.028	0.0018	<0.0001	0.21
Acetate/propionate	2.21 ^a	2.14 ^{abc}	2.06 ^{bc}	2.02 ^c	2.21 ^a	2.16 ^{ab}	2.13 ^{abc}	2.11 ^{abc}	2.21 ^a	2.17 ^{ab}	2.14 ^{ab}	2.10 ^{abc}	0.025	0.011	<0.001	0.46

Means within a row with different superscript letters are significantly different ($p < 0.05$). Means with the same letter are not significantly different. Comparisons were made among all size $\times$ concentration treatment means within each row. PS-MPs, polystyrene microplastics; SEM, standard error of mean; Conc., concentration; NH₃-N, ammonia nitrogen; VFAs, volatile fatty acid.

Table 4. The effects of PS-MPs with different sizes on relative abundance (%) of the 15 most predominant genera of rumen microbiome in vitro after exposure for 24 h.

Rumen Microbiome	Control	1 µm	5 µm	10 µm	p-Value
Bacteria					
<i>Succiniclasticum</i>	15.64 ± 0.42 ^a	13.63 ± 0.81 ^b	13.29 ± 1.78 ^b	14.26 ± 1.19 ^b	0.019
<i>Rikenellaceae_RC9_gut_group</i>	8.43 ± 0.56	8.66 ± 0.54	8.82 ± 0.55	8.54 ± 0.70	0.59
<i>NK4A214_group</i>	8.21 ± 0.60	8.13 ± 0.59	8.12 ± 0.71	8.65 ± 0.84	0.79
<i>unclassified_F082</i>	7.03 ± 0.47	7.33 ± 0.51	7.20 ± 0.34	7.25 ± 0.73	0.73
<i>Prevotella</i>	6.68 ± 1.27	7.13 ± 2.18	7.06 ± 3.10	6.48 ± 2.22	0.98
<i>uncultured_rumen_bacterium</i>	6.42 ± 0.67	7.11 ± 0.46	6.68 ± 0.30	6.25 ± 0.79	0.16
<i>Christensenellaceae_R_7_group</i>	4.54 ± 0.51	4.47 ± 0.45	4.50 ± 0.47	4.90 ± 0.64	0.53
<i>Succinivibrio</i>	3.86 ± 0.88	3.29 ± 1.05	3.78 ± 0.79	3.18 ± 1.61	0.77
<i>unclassified_Clostridia</i>	3.29 ± 0.19	3.19 ± 0.34	3.23 ± 0.18	3.33 ± 0.33	0.96
<i>unclassified_Lachnospiraceae</i>	2.08 ± 0.17	2.20 ± 0.14	2.08 ± 0.10	2.15 ± 0.26	0.64
<i>Lachnospiraceae_XPB1014_group</i>	1.81 ± 0.11	1.79 ± 0.07	1.79 ± 0.20	1.68 ± 0.14	0.34
<i>Butyrivibrio</i>	1.36 ± 0.06	1.42 ± 0.16	1.60 ± 0.11	1.54 ± 0.21	0.07
<i>Prevotellaceae_UCG_003</i>	1.45 ± 0.19	1.52 ± 0.17	1.46 ± 0.25	1.42 ± 0.33	0.71
<i>Veillonellaceae_UCG_001</i>	1.44 ± 0.09	1.37 ± 0.09	1.41 ± 0.21	1.48 ± 0.15	0.67
<i>Desulfovibrio</i>	1.18 ± 0.06	1.03 ± 0.16	1.08 ± 0.35	1.23 ± 0.15	0.37
Methanogens					
<i>Methanobrevibacter</i>	94.06 ± 2.25	95.69 ± 0.78	94.55 ± 0.78	94.57 ± 1.24	0.32
<i>unclassified_Methanomethylophilaceae</i>	4.72 ± 1.74	3.45 ± 0.68	4.57 ± 0.62	4.29 ± 0.74	0.20
<i>Methanosphaera</i>	0.98 ± 0.30	0.82 ± 0.12	0.84 ± 0.19	1.11 ± 0.5	0.45
<i>Candidatus_Methanomethylophilus</i>	0.036 ± 0.02	0.015 ± 0.01	0.016 ± 0.01	0.015 ± 0.01	0.28
<i>Methanomassiliicoccus</i>	0.072 ± 0.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.097
<i>Methanomicrobium</i>	0.015 ± 0.017	0.016 ± 0.021	0.020 ± 0.013	0.0075 ± 0.0066	0.26
<i>Methanoculleus</i>	0.049 ± 0.082	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.097
<i>Methanobacterium</i>	0.019 ± 0.028	0.00068 ± 0.0015	0.00 ± 0.00	0.00067 ± 0.0015	0.41
<i>Natronomonas</i>	0.0098 ± 0.013	0.0010 ± 0.0015	0.0021 ± 0.0032	0.0017 ± 0.0024	0.39
<i>Candidatus_Halobonum</i>	0.011 ± 0.024	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.39
<i>Methanimicrococcus</i>	0.0017 ± 0.0039	0.0014 ± 0.0019	0.0028 ± 0.0038	0.0010 ± 0.0015	0.93
<i>Candidatus_Nitrococcus</i>	0.0049 ± 0.011	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.39
<i>unclassified_Archaea</i>	0.0018 ± 0.0024	0.00 ± 0.00	0.00071 ± 0.0016	0.00069 ± 0.0015	0.41
<i>uncultured_archaeon_Arc.201</i>	0.0022 ± 0.0050	0.00 ± 0.00	0.020 ± 0.013	0.00 ± 0.00	0.39
<i>unclassified_Nitrosotaleaceae</i>	0.0022 ± 0.0031	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.097
Protozoa					
<i>unclassified_Litostomatea</i>	41.52 ± 5.22	44.55 ± 1.83	46.10 ± 3.12	44.51 ± 8.65	0.46
<i>Entodinium</i>	24.17 ± 2.24	24.82 ± 3.71	23.85 ± 2.37	25.72 ± 1.77	0.54
<i>Dasytricha</i>	13.52 ± 7.37	8.75 ± 4.30	5.35 ± 1.26	10.00 ± 9.34	0.24
<i>Ophryoscolex</i>	7.85 ± 1.21	8.37 ± 4.30	12.62 ± 3.03	8.15 ± 2.05	0.12
<i>Ostracodinium</i>	5.13 ± 0.87	4.25 ± 1.65	4.88 ± 1.23	6.71 ± 1.54	0.16
<i>Isotricha</i>	5.01 ± 1.65 ^a	2.08 ± 1.05 ^b	2.21 ± 0.26 ^b	1.68 ± 0.33 ^b	0.0084
<i>unclassified_Eukaryota</i>	0.44 ± 0.19	0.77 ± 0.43	0.42 ± 0.29	0.53 ± 0.18	0.34
<i>Entamoeba</i>	0.63 ± 0.31	0.55 ± 0.19	0.36 ± 0.23	0.47 ± 0.41	0.34
<i>unclassified_Entodiniomorphida</i>	0.42 ± 0.13	0.61 ± 0.15	0.39 ± 0.11	0.58 ± 0.39	0.20
<i>unclassified_Gnathostomata</i>	0.099 ± 0.079	0.31 ± 0.55	1.12 ± 2.37	0.028 ± 0.027	0.22
<i>unclassified_Rotifera</i>	0.00030 ± 0.00067	0.065 ± 0.12	0.080 ± 0.14	0.54 ± 1.19	0.32
<i>unclassified_Calanoida</i>	0.066 ± 0.056	0.34 ± 0.58	0.16 ± 0.33	0.0043 ± 0.0096	0.12
<i>unclassified_Colpoda</i>	0.0049 ± 0.011 ^b	0.26 ± 0.42 ^a	0.077 ± 0.055 ^a	0.15 ± 0.20 ^a	0.0477
<i>unclassified_Mediphyceae</i>	0.025 ± 0.057	0.18 ± 0.28	0.23 ± 0.50	0.00 ± 0.00	0.21
<i>unclassified_DinoflagellataDinophyceae</i>	0.050 ± 0.051	0.17 ± 0.24	0.17 ± 0.34	0.00061 ± 0.0014	0.37
Fungi					
<i>Piomyces</i>	30.32 ± 26.79	39.49 ± 16.26	52.87 ± 2.67	33.75 ± 21.69	0.29
<i>unclassified_Fungi</i>	13.32 ± 3.17	12.68 ± 4.87	10.28 ± 3.40	8.66 ± 4.98	0.23
<i>Orpinomyces</i>	1.83 ± 1.86	7.66 ± 9.79	7.05 ± 4.64	3.34 ± 2.74	0.12
<i>unclassified_Basidiomycota</i>	3.11 ± 1.03	3.73 ± 2.80	2.84 ± 0.71	4.33 ± 2.46	0.86
<i>Thermoascus</i>	3.05 ± 1.45	2.52 ± 1.11	2.99 ± 0.72	2.40 ± 2.68	0.39
<i>Neocallimastix</i>	0.78 ± 0.97	1.72 ± 1.41	2.52 ± 0.78	4.63 ± 6.51	0.18
<i>Byssoschlamys</i>	2.54 ± 1.83	2.42 ± 1.70	1.85 ± 0.95	2.53 ± 2.10	0.90
<i>Ascochyta</i>	3.03 ± 2.91	2.23 ± 2.06	2.42 ± 1.25	1.58 ± 1.22	0.71
<i>unclassified_Neocallimastigaceae</i>	1.08 ± 0.95	2.77 ± 1.86	1.22 ± 1.15	3.97 ± 3.88	0.33
<i>Cladosporium</i>	1.50 ± 1.88	2.96 ± 1.94	0.66 ± 0.40	2.77 ± 5.21	0.15
<i>Pecoramyces</i>	1.27 ± 1.22	1.21 ± 0.63	1.54 ± 0.96	1.36 ± 1.44	0.98
<i>Thermomyces</i>	1.54 ± 0.54	0.52 ± 0.54	0.97 ± 0.81	2.06 ± 1.80	0.08
<i>Mortierella</i>	3.71 ± 6.05	0.44 ± 0.53	0.11 ± 0.15	0.36 ± 0.28	0.26
<i>Debaryomyces</i>	0.83 ± 0.85	1.29 ± 1.31	1.34 ± 1.11	0.43 ± 0.51	0.37
<i>unclassified_Ascomycota</i>	1.99 ± 1.65	0.56 ± 0.38	0.52 ± 0.35	0.52 ± 0.71	0.15

Means within a row with different superscript letters are significantly different ($p < 0.05$). Means with the same letter are not significantly different. Comparisons were made among all size × concentration treatment means within each row. PS-MPs, polystyrene microplastics.

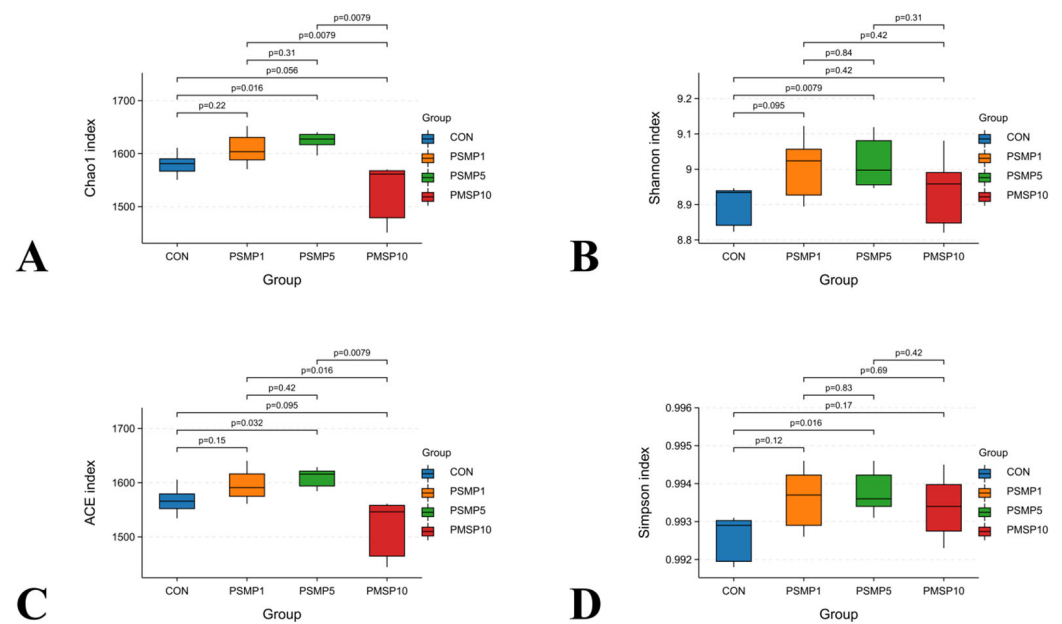


Figure 2. Alpha diversity indices of the rumen microbiome after 24 h exposure to polystyrene microplastics. Treatments were defined as follows: CON, control (no PS-MPs); PSMP1, 1 μm PS-MPs; PSMP5, 5 μm PS-MPs; PSMP10, 10 μm PS-MPs. Panels show (A) Chao1 index, (B) Shannon index, (C) ACE index, and (D) Simpson index. Pairwise comparisons were performed using the Wilcoxon rank-sum test ($p < 0.05$). PS-MPs, polystyrene microplastics; μm , micrometer.

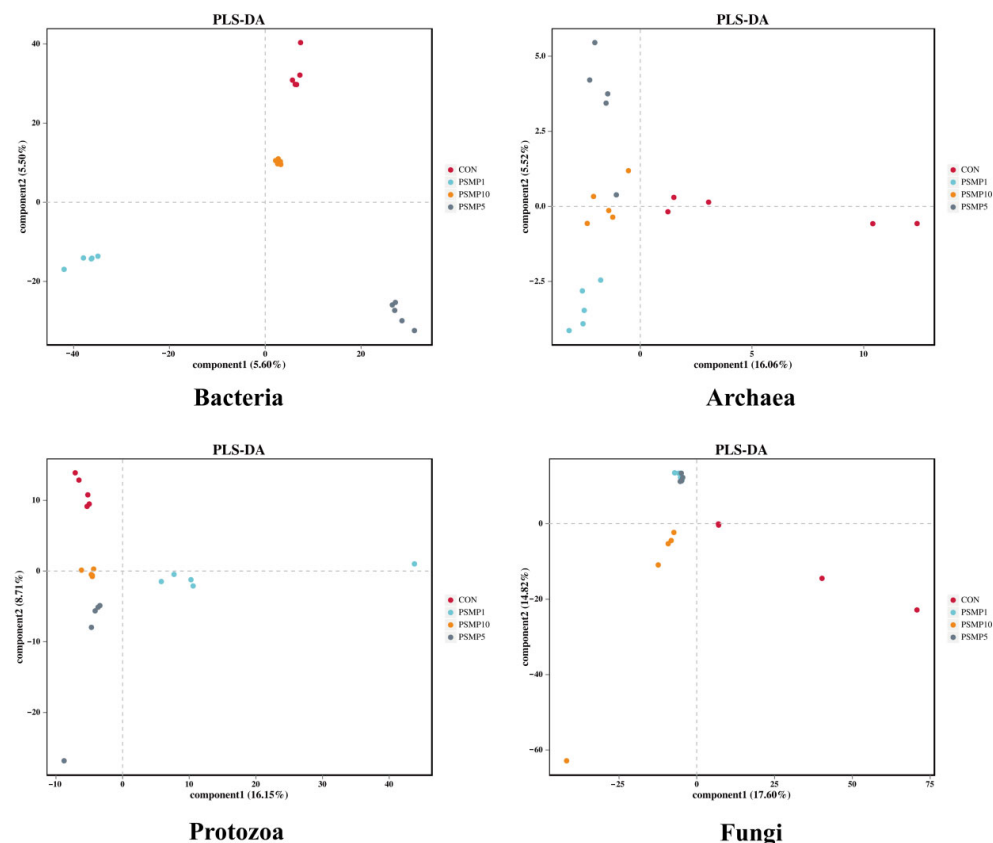


Figure 3. Partial least squares discriminant analysis (PLS-DA) of rumen microbiome profiles in rumen fluid after 24 h exposure to polystyrene microplastics. Treatments were defined as follows: CON, control (no PS-MPs); PSMP1, 1 μm PS-MPs; PSMP5, 5 μm PS-MPs; PSMP10, 10 μm PS-MPs. PLS-DA score plots are shown for bacteria, archaea, protozoa, and fungi. PS-MPs, polystyrene microplastics; PLS-DA, partial least squares discriminant analysis; μm , micrometer.

3.3. Changes in the Concentrations of Polystyrene in Rumen Samples at 0 h and After 24 h Incubation

To determine whether rumen microbial fermentation affects the biodegradation of PS-MPs, the Py-GC/MS method was used to detect the content of PS-MPs in rumen fluid before fermentation and 24 h after fermentation. Figure S1 illustrates the Py-GCMS chromatograms, providing visual evidence for the degradation of PS-MPs in rumen fluid. The chromatograms compare the profiles across different groups: A (0 h control), B (TMR substrate), C-E (0 h rumen fluid for 1 μ m, 5 μ m, and 10 μ m PS-MPs), and F-H (24 h rumen fluid post-incubation for corresponding sizes). A marked reduction in peak intensities in F-H relative to C-E corroborates the significant decrease in PS-MP concentration quantified in this study, highlighting the biodegradation effect of rumen microbial fermentation. According to the result (Table 5), the PS-MP content with different particle sizes was significantly lowered after 24 h fermentation ($p < 0.05$).

Table 5. Concentrations of PS-MPs by pyrolysis–gas chromatography–mass spectrometry in rumen samples at 0 h and after 24 h incubation.

Polystyrene	Exposure Time		SEM	<i>p</i> -Value
	0 h	24 h		
1 μ m, mg/kg	2.02	0.88	0.149	0.0003
5 μ m, mg/kg	2.05	0.84	0.075	<0.0001
10 μ m, mg/kg	2.01	0.68	0.057	<0.0001

PS-MPs, polystyrene microplastics; SEM, standard error of mean.

3.4. PS-MP Exposure Affects Metabolites and KEGG Pathway

In this study, a total of 2414 metabolites were identified, of which 533 were filtered between the control group and the 1 μ m, 50 mg/L group; 1123 metabolites were filtered between the control and the 5 μ m, 50 mg/L group; and 803 metabolites were filtered between the control and the 10 μ m, 50 mg/L group. The differences were annotated using the KEGG database. In PCA analysis, the greater the distance between sample points, the lower the similarity between metabolites. The PCA analysis showed significant differences in metabolites between the control and the 1 μ m, 5 μ m, and 10 μ m groups at 50 mg/L. OPLS-DA was employed to distinguish metabolic profiles between the control (CON) group and three microplastic exposure groups (1, 5, and 10 μ m). Model validation parameters demonstrated high reliability across groups (1 μ m: $R^2Y = 0.999$, $Q^2Y = 0.935$; 5 μ m: $R^2Y = 0.998$, $Q^2Y = 0.955$; 10 μ m: $R^2Y = 0.998$, $Q^2Y = 0.919$), with values approaching unity indicating robust model fit. Permutation testing (Figure 4) further confirmed model validity, as evidenced by a negative slope in the Q^2Y regression line and the majority of permuted R^2Y values (blue) falling below the original Q^2Y values (red). Clear separation in OPLS-DA score plots revealed significant divergence in ruminal fluid metabolic profiles between all microplastic-exposed groups and controls throughout the experimental period.

As shown in Figure 5, volcano plot maps reveal the difference between the two sets of metabolites. In the volcano plot maps, differentially abundant metabolites are color-coded as follows: red dots denote significantly up-regulated metabolites, blue dots represent significantly down-regulated metabolites, and gray dots indicate metabolites detected but not meeting statistical significance thresholds. Figure 6 shows KEGG enrichment analysis between the CON group and the 1 μ m, 5 μ m, and 10 μ m groups, which displayed enrichment in 20 pathways.

Among the many different metabolic pathways, a subset of metabolic pathways and metabolites significantly associated with PS-MP degradation and rumen fermentation were selected (Tables 6 and 7). Styrene and ethylbenzene degradation were identified by comparing the CON group and the 1 μ m, 5 μ m, and 10 μ m groups. Compared with the control

group, styrene degradation in the 1 μm and 10 μm groups and ethylbenzene degradation in the 10 μm group significantly differed ($p < 0.05$). Ethylbenzene degradation of 1 μm PS-MPs and styrene degradation of 5 μm PS-MPs tended to differ from the CON group ($0.05 < p < 0.1$), but no differences between CON and 5 μm groups ($p = 0.21$) in ethylbenzene degradation were observed. In addition, porphyrin metabolism and retinol metabolism were identified in the 1 μm group, porphyrin metabolism and tryptophan metabolism in the 5 μm group and phenylpropanoid biosynthesis and flavonoid biosynthesis in the 10 μm group differed significantly from the CON group ($p < 0.05$). In Table 6, several metabolites associated with rumen fermentation parameters and degradation pathways of PS-MPs which are annotated in the KEGG pathway are selected. 2-Phenylacetamide, phenylacetoneitrile, styrene, and acetophenone of styrene degradation and ethylbenzene degradation in the 1 μm , 5 μm , and 10 μm groups differed significantly from the CON group ($p < 0.05$). The following all differed significantly from the CON group: retinol of retinol metabolism in the 1 μm group; protoporphyrinogen IX, coproporphyrinogen I, porphobilinogen of porphyrin metabolism in the 1 μm and 5 μm groups; Indole-3-ethanol, L-Tryptophan, 3-methylindole of tryptophan metabolism in the 5 μm group; chlorogenate, p-coumaroyl quinic acid, coniferyl aldehyde, 4-vinyl phenol, spermidine of phenylpropanoid biosynthesis and flavonoid biosynthesis in the 10 μm group ($p < 0.05$).

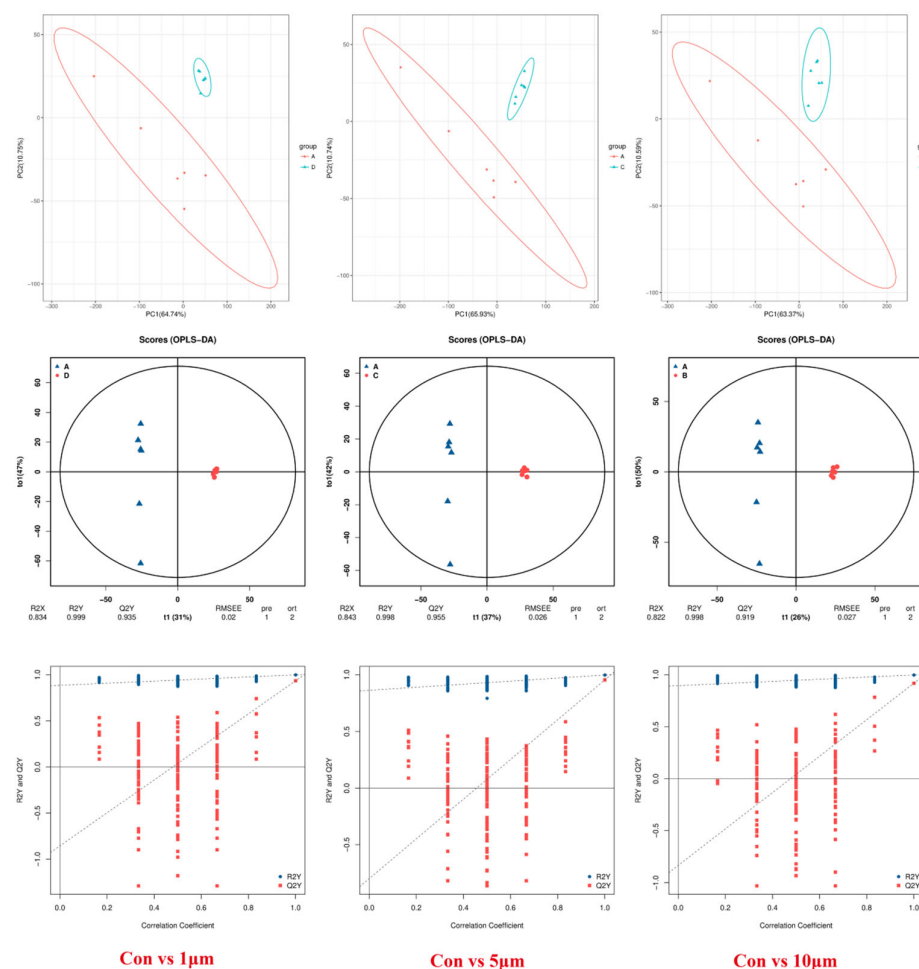


Figure 4. Principal component analysis (PCA), orthogonal projections to latent structures discriminant analysis (OPLS-DA) plot, and permutation test of OPLS-DA in vitro among different groups. R^2Y (cum) and Q^2Y (cum) represent the interpretability and predictability of models, respectively. A: CON; B: 10 μm polystyrene microplastics; C: 5 μm polystyrene microplastics; D: 1 μm polystyrene microplastics.

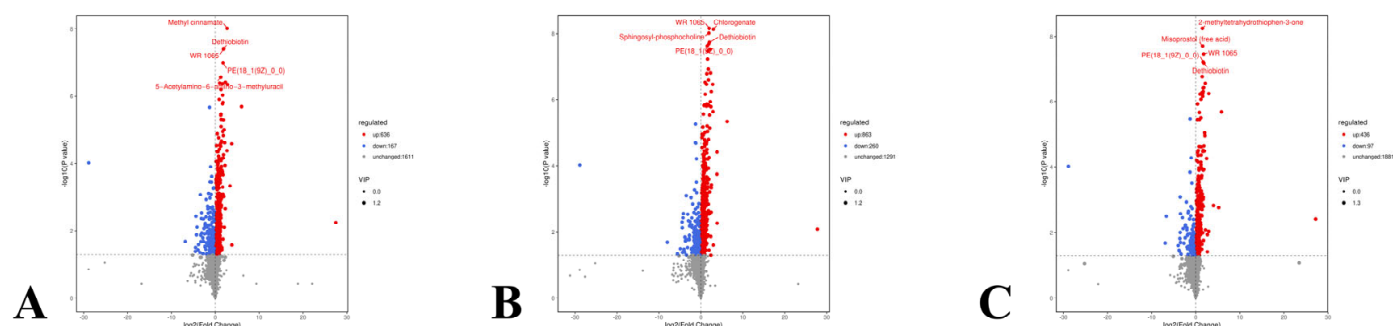


Figure 5. The volcano plots of different metabolites between the control group and the 1 μm , 5 μm and 10 μm groups ((A): CON vs. 1 μm ; (B): CON vs. 5 μm ; (C): CON vs. 10 μm).

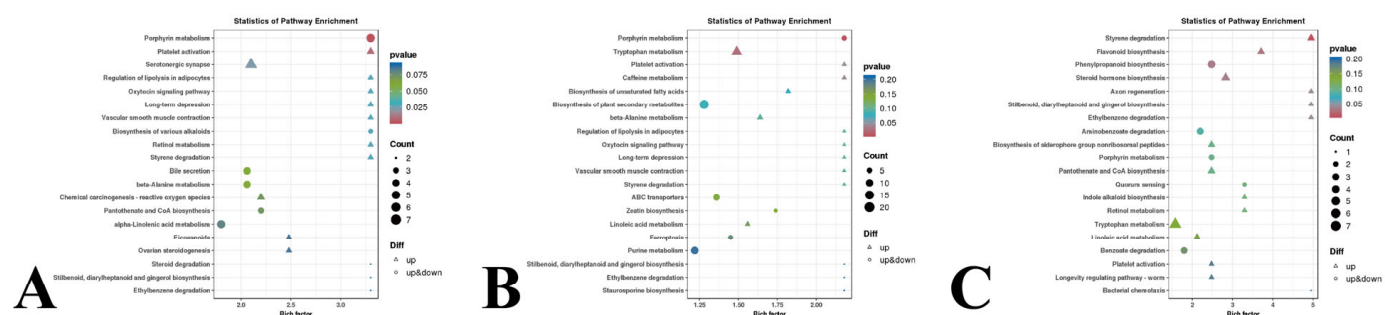


Figure 6. KEGG enrichment analysis between the control group and the 1 μm , 5 μm and 10 μm groups ((A): CON vs. 1 μm ; (B): CON vs. 5 μm ; (C): CON vs. 10 μm). The degree of enrichment was analyzed by the enrichment factor, the p -value and the number of enriched metabolites in each pathway. Different sizes of circles represent different amounts of metabolites, and the color of circles indicates the p -value of pathways).

Table 6. KEGG pathway enrich analysis identified from the significantly different metabolites between the CON and 1 μm groups, the CON and 5 μm groups, and the CON and 10 μm groups.

ID	Description	Metabolite Ratio ¹	Bg Ratio ²	Enrich_Factor ³	p -Value
CON vs. 1 μm					
ko00860	Porphyrin metabolism	4%	1.21%	3.3	<0.001
ko00830	Retinol metabolism	2%	0.61%	3.3	0.027
ko00643	Styrene degradation #	2%	0.61%	3.3	0.027
ko00642	Ethylbenzene degradation #	1.33%	0.40%	3.3	0.091
CON vs. 5 μm					
ko00860	Porphyrin metabolism	2.64%	1.21%	2.18	0.009
ko00380	Tryptophan metabolism	6.61%	4.44%	1.49	0.027
ko00643	Styrene degradation #	1.32%	0.61%	2.18	0.096
ko00642	Ethylbenzene degradation #	0.88%	0.40%	2.18	0.21
CON vs. 10 μm					
ko00940	Phenylpropanoid biosynthesis	5%	2.02%	2.48	0.033
ko00941	Flavonoid biosynthesis	3%	0.81%	3.71	0.027
ko00643	Styrene degradation #	3%	0.61%	4.95	0.008
ko00642	Ethylbenzene degradation #	2%	0.40%	4.95	0.040

#, KEGG pathways marked by # all existed in the comparisons between CON and 1 μm , 5 μm , 10 μm groups. ¹ Metabolite Ratio = the number of the differential metabolites associated with this pathway/the number of the differential metabolites annotated by KEGG. ² Bg Ratio = the ratio of the number of all metabolites associated with this pathway to the number of all metabolites annotated by KEGG. ³ Enrich factor = Metabolite Ratio/Bg Ratio.

Table 7. Identification of significantly different metabolites in rumen fluid in vitro between the CON and 1 µm groups, the CON and 5 µm groups, and the CON and 10 µm groups.

Metabolite ID	Metabolite Name	FC	p-Value	VIP	Con vs. PS-MPs
CON vs. 1 µm					
neg_2442	Protoporphyrinogen IX	0.638	0.015	1.339	down
pos_1633	Coproporphyrinogen I	1.733	0.019	1.312	up
pos_1975	Porphobilinogen	1.358	0.042	1.157	up
neg_3180	Retinol	1.477	0.006	1.455	up
pos_2562	2-Phenylacetamide #	1.259	0.013	1.312	up
neg_1674	Phenylacetone nitrile #	1.449	0.014	1.319	up
pos_2653	Styrene *,#	1.313	<0.001	1.600	up
pos_2270	Acetophenone *,#	1.694	<0.001	1.716	up
CON vs. 5 µm					
neg_2442	Protoporphyrinogen IX	0.613	0.01	1.207	down
pos_1633	Coproporphyrinogen I	2.057	0.004	1.381	up
pos_1975	Porphobilinogen	1.450	0.018	1.186	up
neg_1617	Indole-3-ethanol	1.582	0.027	1.112	up
neg_494	L-Tryptophan	1.539	0.024	1.137	up
pos_2236	3-Methylindole	1.588	0.011	1.249	up
pos_2562	2-Phenylacetamide #	1.295	0.007	1.235	up
neg_1674	Phenylacetone nitrile #	1.506	0.009	1.284	up
pos_2653	Styrene *,#	1.371	<0.001	1.424	up
pos_2270	Acetophenone *,#	1.808	<0.001	1.600	up
CON vs. 10 µm					
neg_1507	Chlorogenate	4.212	<0.001	1.891	up
pos_2179	p-Coumaroyl quinic acid	1.368	0.045	1.222	up
neg_1698	Coniferyl aldehyde	0.010	0.045	1.249	down
neg_1715	4-Vinylphenol	1.355	0.018	1.424	up
pos_336	Spermidine	2.060	0.009	1.520	up
pos_2562	2-Phenylacetamide #	1.238	0.024	1.284	up
neg_1674	Phenylacetone nitrile #	1.346	0.039	1.279	up
pos_2653	Styrene *,#	1.417	<0.001	1.712	up
pos_2270	Acetophenone *,#	1.788	<0.001	1.879	up

CON = control group; FC = fold change; PS-MPs = polystyrene microplastics. VIP = variable importance projection; up = up-regulated; down = down-regulated. *, styrene and acetophenone were annotated in both styrene degradation (ko00643) and ethylbenzene degradation (ko00642) pathways. #, the content of metabolites marked by # existed in the comparison between CON and the 1 µm, 5 µm, 10 µm groups.

4. Discussion

As an increasingly critical environmental pollutant, microplastics widely exist in soil, atmosphere and water. They have the characteristics of persistence and easy decomposition that other pollutants do not have and can cycle and accumulate in the natural environment. It is well known that plastic mulching [40], sewage irrigation and composting all contribute to microplastic buildup in the soil [41]. Furthermore, when microplastics combine and interact with absorbed pollutants, there will be an impact on the health and function of soil, and this can even lead to migration in the food chain [40]. Moreover, the continuous migration of microplastics through aquifers can lead to more serious pollution due to leaching or percolation of microplastics into the groundwater environment from soil pores [42,43]. Collectively, these factors may increase microplastic exposure in dairy production systems. High-yield dairy cows can consume more than ten types of feed and over 30 kg of dry matter (approximately 60 kg on a fresh-weight basis), drink more than 100 kg of water, and produce more than 50 kg of milk per day [44,45]. Dairy cow feed is highly exposed to microplastics during cultivation, processing, and transportation, making their presence in diets difficult to avoid. Compared with other domestic animals, dairy cows are also more vulnerable to the negative impact of microplastics due to their huge intake of food and water.

Because this study used a 24 h batch culture with end-point gas measurements, our results reflect 24 h fermentation responses rather than full gas production kinetics. The diets without PS-MPs and with lower content of other microplastics were selected as the fermentation substrate in vitro. In this experiment, the influence of PS-MPs on rumen

gas production and fermentation parameters was preliminarily investigated through an *in vitro* rumen simulation test. Rumen microorganisms consume nutrients in fermentation substrates to produce gases and metabolites, such as methane, hydrogen, carbon dioxide, and VFAs. Therefore, the degree of substrate utilization by rumen microorganisms can be revealed through *in vitro* gas production [46]. The mechanism by which PS-MPs change rumen gas production and gas composition is closely related to their effect on rumen microorganisms and metabolites (Tables 6 and 7). One limitation of the current study is the absence of a detailed dose–response analysis for the microbiome and metabolic profiles. Microbiome and metabolomics profiling was performed only for the 50 mg/L treatments (for each particle size) along with the corresponding control; therefore, these omics results should be interpreted as 24 h end-point responses under a defined challenge level, rather than as evidence of dose–response mechanisms. Exploring a broader range of PS-MP concentrations and particle sizes in future research would help clarify how these variables influence microbial community composition and fermentation processes. Additionally, our study focused solely on the highest concentration, which limits our understanding of the impacts of lower doses on microbial dynamics. It is also important to note that SEM analysis has certain limitations in this context. Due to the small size of the PS-MPs and the difficulty in recovering them from rumen fluid, capturing all surface alterations accurately may not be feasible. This study found that when the concentration and particle size of PS-MPs increased, the total rumen gas production *in vitro*, the production and level of methane, hydrogen and carbon dioxide decreased. At the same time, PS-MP exposure caused a negative effect on the relative abundance of some rumen microorganisms. Consequently, it is postulated that PS-MPs may have resulted in a reduction in the number of certain rumen microorganisms, which resulted in a negative impact on rumen fermentation, thereby reducing the total gas production and carbon dioxide production. It is well known that polystyrene is formed by the polymerization of styrene monomers through the addition of radical carbon–carbon double bonds [47]. Research has shown that biodegradation of polystyrene results in main-chain cleavage to produce products such as toluene, ethylbenzene and styrene or side-chain cleavage to break the phenyl ring [48]. Both cleavage methods require the presence of hydrogen, which leads to competition for hydrogen in rumen fluid, which may be one of the reasons for the decrease in hydrogen production. In this experiment, it was also surprising to find that rumen fermentation had a biodegradation effect on PS-MPs (Table 4). This phenomenon is similar to rumen propionate fermentation competing for hydrogen and thus inhibiting methane synthesis. The degradation of PS-MPs in the rumen may reduce the amount of hydrogen and carbon dioxide used by methanogens for methane synthesis [49], which ultimately leads to the reduction in rumen methane production.

Metabolites (ammonia nitrogen and VFAs) produced by rumen microbes to decompose nutrients in feed are major nutrient sources and important intermediates for dairy cows, which contribute significantly to ruminant productivity. However, it is very interesting that the impact of PS-MPs on rumen gas production is significantly associated with the particle size and concentration of microplastics. The larger the particle size and concentration of PS-MPs, the greater the negative impact on rumen gas production. To our surprise, the influence of PS-MP particle size on rumen gas production was opposite to that of VFAs *in vitro*. The precise mechanism underlying this phenomenon remains elusive. Therefore, for a given quantity of PS-MP addition, the smaller the particle size of polystyrene, the higher the concentration of polystyrene deducted. A large number of microplastics with small particle sizes were more likely to affect rumen VFA concentration. In the large-particle-size group, fewer microplastics had less effect on rumen fermentation. Moreover, due to their large size, 10 µm polystyrene microplastics are more likely to be attached and

degraded by microorganisms, and the metabolites (2-phenylacetamide, phenylacetone, styrene and acetophenone) generated by their degradation are more likely to cause toxicity to microorganisms [50–52] or other effects on rumen fermentation, thus affecting rumen gas production. However, the mechanism by which PS-MP exposure negatively affects rumen fermentation parameters and gas production in different trends remains to be further investigated. Rumen pH was closely related to volatile fatty acid production and microbial abundance and diversity [53]. At the community level, alpha diversity did not change uniformly across particle sizes. The 5 μm group showed a clearer shift in within-sample diversity relative to the control, whereas the 1 μm and 10 μm groups appeared more comparable to CON. This pattern suggests that microbial diversity may not respond to PS-MPs in a monotonic manner as particle size increases, which is consistent with reports that polystyrene microplastics can exert size-dependent effects on microbial communities, with different particle sizes inducing distinct community responses in other experimental systems [54]. One possible explanation is that particle size influences the extent of contact and interaction between PS-MPs and rumen microorganisms, such that the 5 μm particles may impose more noticeable ecological disturbance under our exposure condition. Importantly, shifts in specific taxa and fermentation outputs can still occur even when overall community-level diversity changes are modest. The genus *Succinivibrionaceae* ferments succinate and converts it into propionate [55]. *Thermoascus* and *Isotricha* can secrete intracellular or extracellular enzymes to decompose plant cellulose or lignin [56,57] to produce acetate. In this study, PS-MP exposure reduced the abundance of microorganisms that degraded some nutrients, thus affecting the yield of acetate, propionate and butyrate in the rumen. However, the decrease in volatile fatty acid content will inevitably lead to an increase in rumen pH. In this experiment, PS-MP exposure caused a decreasing trend in the relative abundance of hydrogenotrophic methanogens (*Methanosarcina* and *Methanococcus*) [58], which may also be one of the reasons why PS-MPs reduced the methane content in the rumen. Interestingly, this study found that PS-MPs had an increasing trend in the relative abundance of *Butyrivibrio* and *unclassified_Colpodida*, which was different from the decrease in the relative abundance of other rumen microorganisms. *Butyrivibrio* is one of the main cellulose- and hemicellulose-degrading bacteria [59], has a very wide enzyme library and different enzyme activities [60] and can produce β -glucosidase used for the degradation of dense lignin structure [61]. At the same time, our findings indicate that with the increase in the concentration and particle size of PS-MPs, the activities of carboxymethyl cellulase and xylanase in rumen fluid significantly increased. Enhancement in relative abundance of *Butyrivibrio* and *unclassified_Colpodida* may induce β -glycosidase activity at low doses when PS exposure has not dropped significantly. Additionally, studies have found that rumen microorganisms have good biodegradation of PET [62], which provides an important reference for the degradation of PS-MPs by rumen microorganisms. In this study, our findings indicated that the concentration of PS-MPs was significantly decreased after 24 h rumen microbial action. The rumen microorganisms in enormous quantities and of diverse microbial species make biodegradation of PS-MPs possible, but the mechanism of action may be very complex and requires further study.

Microplastics' microbial toxicity mostly focuses on microbial composition, such as changes in community structure or adverse effects of microbial function. It was found that both polystyrene nanoplastics and polyethylene microplastics could reduce methane emissions during anaerobic digestion of sludge or wastewater [58,63,64]. In addition, PS-MPs with different sizes are also toxic to *Escherichia coli* and *Bacillus cereus* [65]. The release of additives in the plastic itself or the charges of modified groups has certain effects on microorganisms. Certain chemical additives are added during plastic production, and these additives will be gradually released into the environment over time, which will also have

an impact on microorganisms [66,67]. In terms of the effects of microplastics on ruminant microflora, further research is needed to reveal the mechanism. The mechanism of microplastics affecting rumen microflora needs further study. At least, our findings indicated that PS-MPs have an impact on the rumen microbiome. In addition, the differences in KEGG metabolic pathways were also predicted, thereby showing differences in functional gene metabolic pathways in microbial communities. This indicates that PS-MP exposure affects rumen fermentation parameters by affecting metabolic pathways and products.

The objective of this study was to elucidate the metabolic pathways and products that are significantly related to the degradation of PS-MPs or rumen fermentation. PS-MPs with different particle sizes had different effects on the rumen microbiome and subsequently affected the levels of different metabolites. PS-MPs can produce different levels of toxic effects on both Gram-negative and Gram-positive bacteria [65]. It was also found that bacteria could degrade PS-MPs, but at a slower degradation rate [68]. Analyzing the relationships between metabolites in vivo using metabolic pathway analysis is a useful technique for illuminating the function of various metabolites in animal metabolism [69]. Styrene, 2-phenylacetamide, phenylacetonitrile and acetophenone are present in the ethylbenzene degradation (ko00642) and styrene degradation (ko00643) pathways, respectively. The multiples of ethylbenzene degradation and styrene degradation were not significant in the 1 μm treatment group, the styrene degradation pathway was enriched in the 5 μm treatment group, and the ethylbenzene degradation and styrene degradation pathway were enriched in the 10 μm treatment group. However, the levels of styrene in the three PS-MP treatment groups were significantly increased, which also confirmed that rumen microorganisms could degrade PS-MPs. It has been found that some enzymes can hydrolyze the C-C bond among PS-MPs [48]. It may be that enzymes secreted by rumen microorganisms can act on PS-MPs for degradation, but the mechanism is not clear and needs further study. It was also found that the levels of 2-phenylacetamide, phenylacetonitrile and acetophenone were significantly elevated in the three PS-MP treatment groups. It was postulated that it might be because microorganisms degraded the PS-MPs, and not all of them were converted into styrene but reacted with other chemical components of rumen fluid to form some derivatives with similar structures. Furthermore, 2-phenylacetamide may be converted into phenylacetylglutamine through the fermentation of the aromatic amino acid phenylalanine by bacteria. It has been demonstrated that phenylacetylglutamine can increase the risk of thrombosis and coronary heart disease [70]. Phenylacetonitrile has been demonstrated to have a lethal effect on mammals and an antibacterial effect following administration at a certain dosage [71,72]. Acetophenone is typically investigated as a metabolite of animal epidermal microorganisms and a pheromone that attracts insects [73]. However, studies have reported that it exerts inhibitory effects on HeLa cells and *Mycobacterium* [49]. Consequently, 2-phenylacetamide, phenylacetonitrile and acetophenone are considered to have the potential to be toxic to ruminants and may affect the rate of digestion by altering the composition of bacterial communities.

Our findings indicate that the retinol metabolism pathway was enriched in the 1 μm treatment group when an enrichment analysis of the KEGG pathway was conducted. Rumen microorganisms can degrade retinol [74]. In this study, the retinol level was increased significantly, which may be due to the degradation of PS-MPs to produce styrene or its derivatives that affect the function of rumen microorganisms [51,52], thereby inhibiting the degradation of retinol. In addition, phenylpropanoid biosynthesis and flavonoid biosynthesis were enriched in the 10 μm treatment group. Coniferyl aldehyde is present in the xylem [75] and causes significant inhibition of cellulolytic enzymes and microbial fermentation [76,77]. In this study, the significant decrease in coniferyl aldehyde level may also be the reason for the increase in activity of xylanase, carboxymethyl cellulose, and

β -glycosidase. In addition, it has been reported that 4-Vinylphenol is the product of the fracture of lignin macromolecule β -O-4 or α -O-4 linkages [78], and the increase in its level also reflects the increase in lignin-degrading enzyme activity. The increase in spermidine level may be due to the increase in amino acid decarboxylase activity of rumen bacteria stimulated [79] by the addition of 10 μ m PS-MPs. In conclusion, it was postulated that the addition of PS-MPs can improve the activity of some enzymes in the rumen. Previous studies found that flavonoids added to rumen fluid can significantly reduce rumen methane production [80]. In this study, the enrichment of flavonoid biosynthesis and the increase in the annotated metabolite level may also be the reason for the significant reduction in methane production in the 10 μ m treatment group. Tryptophan metabolism was enriched in the 5 μ m treatment group, and the metabolites with significantly increased levels of Indole-3-ethanol, L-Tryptophan, and 3-methylindole were annotated. Indole and methylindole are formed from tryptophan. Studies have shown that the transformation rate of tryptophan into 3-methylindole is the highest in rumen fluid at pH 6.5 to 7.0, and the increase in 3-methylindole concentration in the rumen is related to acute interstitial pneumonia (AIP) in ruminants [81]. It can be seen that a high dose of PS-MPs may alter rumen metabolites and cause disease in ruminants. Compared with the control group, porphyrin metabolism was enriched in the 1 μ m and 5 μ m treatment groups, in which coproporphyrinogen and porphobilinogen were significantly increased, while protoporphyrinogen IX was significantly reduced. Some studies have shown that the decrease in the activity of certain enzymes in the porphyrin pathway leads to the accumulation of some metabolites and causes diseases [82], which may also be one of the injuries caused by PS-MPs entering ruminants.

5. Conclusions

In this study, PS-MPs reduced rumen gas production and VFA production in a 24 h *in vitro* batch culture system, and the inhibitory effects at higher concentrations were stronger than those observed at lower concentrations within the same particle size. At the maximum concentration (50 mg/L), PS-MPs were associated with less favorable fermentation profiles accompanied by shifts in the rumen microbiome and metabolite patterns. Notably, the measured PS-MP content in the incubation system decreased after 24 h. In addition, under exposure to PS-MPs of different particle sizes at 50 mg/L, pathways and metabolites annotated to styrene- and ethylbenzene-related degradation were detected, which is consistent with a possible microbial contribution to PS-MP transformation and the formation of styrene-related products. Taken together, these results indicate that PS-MPs can perturb *in vitro* rumen fermentation and provide mechanistic clues for microplastic–rumen interactions. Further *in vivo* work is needed to determine whether similar responses occur under practical feeding conditions and whether they translate into animal-level outcomes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ani16050749/s1>, Figure S1. Py-GCMS Chromatograms of polystyrene (A: 0 h rumen fluid of control group; B: TMR for test; C: 0 h rumen fluid of 1 μ m; D: 0 h rumen fluid of 5 μ m; E: 0 h rumen fluid of 10 μ m; F: 24 h rumen fluid of 1 μ m; G: 24 h rumen fluid of 5 μ m; H: 24 h rumen fluid of 10 μ m).

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Abbreviations

The following abbreviations are used in this manuscript:

PS-MPs	Polystyrene microplastics
KEGG	Kyoto Encyclopedia of Genes and Genomes
VIP	Variable importance projection
FC	Fold change
PS	Polystyrene
Py-GC/MS	Pyrolysis–gas chromatography–mass spectrometry
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
OTUs	Operational taxonomic units
PLS-DA	Partial least squares discriminant analysis
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
SEM	Standard error of the mean
SE	Secondary electron
EI	Electron ionization
DNA	Deoxyribonucleic acid
NH ₃ -N	Ammonia nitrogen
LDPE	Low-density polyethylene
PCoA	Principal coordinates analysis
TMR	Total mixed ration
VFA	Volatile fatty acid

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