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Preliminary Study on the Impact of Ruminal Ciliate Inoculation in Fauna-Free Conditions on the Ruminal Fermentation and Ciliate–Prokaryote Association In Vitro

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Abstract: Ruminants rely on the rumen for the anaerobic fermentation of fibrous plant materials, facilitated by a complex microbial community of bacteria, archaea, fungi, and ciliates. Among them, ruminal ciliates significantly influence ruminal fermentation, methane production, nitrogen utilization efficiency, and microbial interactions. This study examined the impact of ciliate inoculation on ruminal fermentation, microbial composition, and functional profiles in fauna-free conditions. Six treatments were tested: control (no ciliates), small entodinia, *Epidinium* spp., isotrichids, *Ophryoscolex* spp., and a mixed inoculum. Using QIIME2 to analyze 16S rRNA gene sequences, the study revealed group-specific effects on methane production, volatile fatty acids (VFAs), and microbial diversity. Small entodinia inoculation increased *Streptococcus* abundance, while isotrichids enriched *Megasphaera*, enhancing butyrate production. Alpha diversity indices indicated reduced richness in the ciliate-inoculated groups, reflecting predation on prokaryotes. Beta diversity showed distinct microbial and functional profiles among the treatments. Functional analysis highlighted elevated glycerolipid metabolism in isotrichid groups, associated with *Bacteroides* and *Megasphaera*, suggesting roles in lipid metabolism and oxidative stress resistance. Despite limited ciliate cell counts, the study emphasizes ciliate-specific interactions and the role of lactic acid-associated bacteria in shaping ruminal fermentation.

Keywords: ruminal ciliates; ruminal microbiome; ciliate–prokaryote association; fauna-free

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1. Introduction

Ruminants are distinguished from monogastric animals by their four-compartment stomachs: the rumen, reticulum, omasum, and abomasum [1]. Among them, the rumen serves as the primary site for the anaerobic fermentation of lignocellulose, which is fibrous plant materials and starches ingested through the diet, which the host animal cannot directly degrade [2,3]. The rumen is home to ruminal microorganisms that produce lignocellulolytic enzymes capable of breaking down plant lignocellulose [4]. These microorganisms include bacteria, ciliated protozoa (hereafter referred to as ciliates), archaea (including methanogens), and fungi. Together, they interact to perform essential functions in ruminal fermentation [5]. Among them, ruminal ciliates are particularly significant due to their biomass, which can account for up to 50% of the total microbial mass in the rumen [6]. Their presence has a substantial impact on ruminal fermentation and function.

To understand the roles and characteristics of ruminal ciliates, numerous in vitro and in vivo studies have utilized defaunated ruminants [7–9]. A meta-analysis conducted in 2015 synthesized these studies to explore how defaunation affects ruminal function,

fermentation profiles, and interactions with other ruminal microorganisms, particularly cellulolytic bacteria [10]. The key findings from this meta-analysis include the following effects of defaunation:

(1) A decrease in overall digestibility, including organic matter digestibility (OMD), neutral detergent fiber digestibility (NDFD), acid detergent fiber digestibility (ADFD), and volatile fatty acid (VFA) concentrations, particularly butyrate.
(2) A reduction in methanogen abundance, methane emissions, and ammonia concentrations, alongside increases in duodenal nitrogen flow and microbial protein synthesis efficiency.
(3) Significant decreases in the abundance of anaerobic fungi and cellulolytic bacteria such as *Ruminococcus albus* and *Ruminococcus flavefaciens*.
(4) Reduced dry matter intake (DMI) by the host ruminant, while average daily gain (ADG) increased.

In summary, ruminal ciliates increase VFA concentrations, fiber digestibility, and methane production in the rumen, thereby enhancing host productivity but reducing nitrogen utilization efficiency.

Despite their significant roles and interactions with other microorganisms, the precise functions of ruminal ciliates remain poorly understood, largely due to limitations in cultivation techniques, including the inability to establish axenic cultures [11]. This challenge is further compounded by the limited research on ciliate-associated bacteria and methanogens, which are likely to cohabit with ciliates and significantly influence their functional roles. Addressing these gaps is essential for enhancing our understanding of ruminal ciliates.

Ruminal ciliates encompass various genera with distinct functions and characteristics. These include cellulolytic ciliates (e.g., *Epidinium* and *Ophryoscolex*), soluble sugar-lovers (e.g., isotrichids, such as *Dasytricha* and *Isotricha*), bacterivores (e.g., small entodinia, such as *Entodinium* and *Diplodinium*), and starch utilizers (e.g., *Entodinium*). Many studies using mono- or mero-faunated ruminants have investigated how these ciliates influence fermentation profiles and other microorganisms in the rumen [12–15]. Among them, three studies measured ruminal fermentation parameters but did not employ molecular-based methods [12–14]. Another study utilized real-time quantitative polymerase chain reaction (real-time qPCR), a molecular-based method, but its focus was limited to specific microbial species and a small number of fermentation parameters [15]. In summary, research on fauna inoculation that simultaneously explores ruminal fermentation profiles and ciliate–prokaryote associations remains scarce.

To overcome these limitations, this study aimed to investigate how the inoculation of various ruminal ciliate groups (i.e., small entodinia, isotrichids, *Epidinium* spp., *Ophryoscolex* spp.) into a fauna-free environment affects in vitro fermentation parameters and ruminal microbiota composition using a 16S rRNA gene-based approach. We hypothesize that certain ciliate–bacteria association patterns, specifically with inoculated ciliates, will be consistent or reconstructed after inoculation. Furthermore, we expect that each ruminal ciliate group will be able to impact in vitro fermentation parameters, including NDFD, ADFD, NH_3-N, CH_4, and VFAs.

2. Materials and Methods

2.1. Experimental Design

A completely randomized design was used for a 24 h in vitro fermentation experiment to evaluate the effects of ruminal ciliates. Each treatment was conducted in triplicate. The six treatment groups included CON (control, no ciliates inoculated), ENTO (small entodinia inoculated), EPI (*Epidinium* spp. inoculated), ISO (isotrichids inoculated), OPH

(*Ophryoscolex* spp. inoculated), and MIX (all cultured ciliates inoculated). The experimental feeds included concentrate pellets (particle size between 0.425 mm and 1 mm) and oat hay, as detailed in Table S1.

2.2. Preparation of Inoculated In Vitro Cultures of Each Ruminal Microbe

The ruminal ciliate groups used in this study were selected based on their potential to influence fiber digestibility (NDFD and ADFD), ammoniagenesis, ruminal microbiome, and methane production. The cellulolytic ciliates (*Epidinium* and *Ophryoscolex*) were included for their effects on fiber digestibility, while bacterivory ciliates (small entodinia, including *Entodinium* and *Diplodinium*, and potentially *Epidinium*) were chosen for their effects on nitrogen utilization efficiency by their predatory behavior and impact on microbial interactions. Isotrichids (*Isotricha* and *Dasytricha*) were selected due to their potential impact on methane production.

The cultures of each ciliate group were established using two donor cows with distinct protozoal populations (types A and B; reference). *Epidinium* was isolated from a cannulated Holstein cow, while *Ophryoscolex* and small entodinia were isolated from a Hanwoo cow using stomach tubing. All entodiniomorphid ciliate cultures were maintained as mono- or meroxenic cultures for at least 3 months prior to inoculation. Small entodinia and *Ophryoscolex* were cultured in SP medium, while *Epidinium* was cultured in M medium, following the procedures outlined by Dehority [16]:

(1) Rumen fluid used for isolation was filtered through two layers of cheesecloth, sedimented in a separatory funnel at 39 °C for 45 min, and the white pellet layer enriched with ciliate cells was collected.
(2) The white pellet layer was further filtered through a 100 μm pluristrainer (J.One LifeScience, Seoul, Korea) to remove feed particles and large ciliates (>100 μm).
(3) For the *Epidinium* and *Ophryoscolex* cultures, filtrates were resuspended in sterile M or SP medium after sequential filtration through a 70 μm pluristrainer. The single-cell isolation method was performed using microscopy to obtain meroxenic cultures [17].
(4) For the small entodinia culture, the filtrate collected below a 30 μm pluristrainer was resuspended in a sterile SP medium to establish the culture.

The isotrichid culture was prepared in *Dasytricha ruminantium* medium (DRM) following these steps [18]:

(1) Rumen fluid filtered through two layers of cheesecloth was passed through a 100 μm pluristrainer to remove impurities and ciliates larger than 100 μm.
(2) The filtrate (300 mL) was transferred to a separatory funnel, supplemented with 1.5 g of glucose, and incubated at 39 °C for 30 min to sediment isotrichid cells into a white layer [19].
(3) The collected white layer was sequentially filtered through 100, 85, 70, 50, and 40 μm pluristrainers, with microscopy used to identify fractions rich in *Isotricha* (50–70 μm) and *Isotricha* plus *Dasytricha* (40–50 μm). These fractions were cultured in DRM for seven days to remove the remaining entodiniomorphid ciliates.
(4) After confirming the absence of entodiniomorphids under a microscope, viable isotrichid cells were used for inoculation in this experiment.

Before the in vitro experiment, all ciliate cultures were fasted for 24 h to remove residual feed from the medium. Ciliate cells were then prepared by filtering through pluristrainers of appropriate sizes and resuspending in fresh media (i.e., SP, M, or salt solution for DRM), ensuring all cultures had the same medium conditions.

A portion of the bacterial fraction was inoculated into rumen bacteria medium (330: rumen bacteria medium, DSMZ) and cultured to make feed for ruminal ciliates. Subsequently, 1 mL of the cultured bacterial fraction was inoculated into each bottle.

2.3. Preparation of In Vitro Ruminal Inoculums and Animal Donors

Rumen fluid for the in vitro fermentation experiment was collected via stomach tubing from three Hanwoo cows maintained at the Nonghyup Co., Ltd. research farm in Anseong, South Korea. The fluid was transferred to pre-warmed CO_2-flushed thermos bottles and delivered to the laboratory within 30 min. Upon arrival, the rumen fluid was filtered through two layers of cheesecloth, and the bacterial fraction was isolated. To minimize ciliate contamination, low-speed centrifugation (500× *g* for 15 min) was performed, and the supernatant was collected. This supernatant was sequentially filtered through 30, 15, and 10 µm pluristrainers, and the filtrate was used as the final bacterial fraction for the experiment. The bacterial fraction was mixed with an in vitro buffer solution that had been flushed with 99.999% CO_2 gas for 1 h. The mixture was further flushed with CO_2 for an additional 20 min to maintain anaerobic conditions.

2.4. In Vitro Digestibility Experiment

After 20 min of CO_2 flushing, 0.2 g of feed (0.1 g each of forage and concentrate) was preloaded into 60 mL serum bottles containing media (SP, M, or salt solution for DRM) and inoculum. Each treatment bottle was inoculated with the corresponding ciliate culture and ruminal bacteria, as detailed in Table 1.

Table 1. Experimental design and the composition of culture components.

Sample		CON	ENTO	EPI	ISO	OPH	MIX
Bacterial fraction (mL)		15	15	15	15	15	15
Ruminal bacteria for feed (mL)		1	1	1	1	1	1
Culture medium (mL)	SP *	2	1	2	2	1	1.5
	M *	1	1	0	1	1	0.75
	SP salt solution of DRM *	1	1	1	0	1	0.75
Ciliate culture (mL)	ENTO	0	1	0	0	0	0.25
	EPI	0	0	1	0	0	0.25
	ISO	0	0	0	1	0	0.25
	OPH	0	0	0	0	1	0.25

* SP and M medium, and SP salt solution of DRM compositions were based on previously established protocols as detailed in [16,18]. Approximate counts of each inoculated ciliate culture are as follows: (1) 6225 cells/mL of small entodinia; (2) 1200 cells/mL of *Epidinium* spp.; (3) 900 cells/mL of isotirichids; (4) 210 cells/mL of *Ophryoscolex* spp. CON, control group without ciliate added; ENTO, small entodinia inoculated; EPI, *Epidinium* spp. inoculated; ISO, isotrichids inoculated; OPH, *Ophryoscolex* spp. inoculated; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. inoculated; DRM, *Dasytricha ruminantium* medium.

The bottles were quickly sealed with blue butyl rubber stoppers and crimped with aluminum caps. The sealed bottles were incubated in a shaking incubator at 39 °C and 60 rpm for 24 h. After 24 h of incubation, each sample (1.6 mL) was transferred into a 2-mL Eppendorf tube and centrifuged at 17,278× *g* for 15 min at 4 °C. From the resulting supernatant, 1 mL was combined with 0.2 mL of 25% metaphosphoric acid and stored at −20 °C for subsequent volatile fatty acid (VFA) analysis using gas chromatography (7890B GC, Agilent Technologies, Santa Clara, CA, USA). Additionally, 0.1 mL of the supernatant was mixed with 0.02 mL of 0.2 M H_2SO_4 and stored at 4 °C for ammonia nitrogen (NH_3-N) analysis. The remaining pellet was stored at −80 °C to be used for metagenomic DNA extraction. The gas pressure in the headspace of the fermentation bottles was measured using a pressure transducer (L20000DCV3, Laurel Electronics, Inc., Costa Mesa, CA, USA) attached to a rubber stopper via a 3-way cock and a 22-gauge needle. Gas production was recorded at 3, 6, 12, and 24 h during the incubation period, while maintaining headspace gas pressure at levels that would not interfere with microbial fermentation [20,21]. The

collected gas was transferred into a gas bag using the 3-way cock [22]. To convert pressure values (mbar) into volume (mL), a standard curve was created by injecting a known volume of gas into a similarly sized serum bottle containing 20 mL of distilled water [22]. Methane (CH_4) production, expressed as mL or mL per gram of degraded dry matter (methane yield), was determined via gas chromatography (YL6500, YOUNGIN Chromass, Anyang, Korea). Following subsampling, the remaining inoculum was transferred to a sterile 50 mL conical tube for pH measurement using a pH meter (MW150, Milwaukee Instruments, Inc., Rocky Mount, NC, USA). The remainder of the inoculum was then placed into pre-weighed nylon bags (pore size 50 μm, R510, Ankom Technology, Fairport, NY, USA) for dry matter digestibility (DMD) analysis. To ensure that no particles were left behind, the serum bottle was rinsed thoroughly with distilled water. Both pH measurements and the handling of dry matter (DM) samples were conducted on ice to minimize further microbial fermentation. The collected particles were squeezed 2–3 times to remove excess liquid and then dried completely in a 65 °C oven for 72 h. Once the DM content was measured, the samples were transferred to pre-weighed fiber bags (pore size 25 μm, F57, Ankom Technology, USA) for the determination of neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents using an A220 fiber analyzer (Ankom Technology, USA) following the methodology outlined in a previous study [23].

2.5. Metagenomic DNA Extraction and Microbiome Analysis

Metagenomic DNA was extracted from the pellets of all samples using the RBB + C method described by Yu and Morrison [24]. The concentration and purity of the extracted DNA were assessed using a Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Amplicons targeting the V3-V4 region of the 16S rRNA gene were amplified using the primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′)/805R (5′-GACTACHVGGGTATCTAATCC-3′) as reported by Herlemann et al. [25]. These amplicons were sequenced on the MiSeq platform (Illumina, San Diego, CA, USA). The initial microbiome analysis was conducted using Quantitative Insights into Microbial Ecology Version 2 (QIIME2) [26], which processed 16S rRNA sequences. Cutadapt [27] was employed to remove primer sequences, followed by quality control ($Q \geq 25$), denoising, merging, and chimera removal using the DADA2 plugin [28]. Taxonomic classification of the resulting amplicon sequence variants (ASVs) was performed with the weighted Silva-138 99% classifier [29,30], which had been pre-trained using the Naïve Bayes algorithm [31]. Any ASVs that were unassigned or associated with mitochondria, chloroplasts, or archaea were excluded. The ASV table in BIOM format and the corresponding representative sequences were then utilized for subsequent analyses. In this study, only taxa present in all samples from at least one treatment group were included. Alpha diversity was evaluated using various metrics, including richness (e.g., observed ASVs and Chao1 estimates), evenness, Faith's phylogenetic diversity (Faith's PD), Shannon's index, and Simpson's index. These metrics were calculated using averaged rarefied ASV tables with 1000 iterations of random sampling, each at a depth of 31,119 sequences [32]. Microbial metabolic functions were inferred from the ASV sequences using PICRUSt2 [33]. The functional dissimilarity among the ciliate groups was evaluated using Bray–Curtis dissimilarity, with the KEGG ortholog profiles [34] serving as a representation of microbial functions. Nonmetric multidimensional scaling (NMDS) was conducted to visualize the difference of overall microbiota and its functional profile, and the NMDS plots were created using the 'ggplot2' R package [35].

2.6. Quantitative Real-Time PCR

The abundance of total bacteria, ciliate protozoa, and methanogenic archaea was determined by quantifying bacterial and archaeal 16S rRNA gene copies and ciliate 18S rRNA gene copies, respectively, for each sample. Quantitative real-time PCR (qPCR) was conducted using universal primers specific to each microbial group: (1) 340F (5′-TCCTACGGGAGGCAGCAGT-3′) and 806R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) for bacteria [36], (2) uniMet1-F (5′-CCGGAGATGGAACCTGAGAC-3′) and uniMet1-R (5′-CGGTCTTGCCCAGCTCTTATTC-3′) for methanogens [37], and (3) 316F (5′-GCTTTC-GWTGGTAGTGTATT-3′) and 539R (5′-CTTGCCCTCYAATC-GTWCT-3′) for ciliates [38]. The NanoDrop One Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, NC, USA) was used to measure the concentration of nucleic acids in the purified PCR products, allowing for the calculation of copy numbers per mL for standard curve generation. These prepared standards were stored at −20 °C for future use. Microbial quantification was performed using the QuantStudio 1 system (Thermo Fisher Scientific, Wilmington, NC, USA). Each qPCR reaction included 1 µL of template DNA combined with 15 µL of a reaction mixture containing 0.075 µL of each primer (forward and reverse, each at 100 µM), 7.5 µL PowerUp SYBR Master Mix (2X), and 6.35 µL ultra-pure water. For total ciliate quantification, 2 µL of template DNA was added. The qPCR amplification followed protocols optimized for each primer set to ensure precise and reproducible results.

2.7. Statistical Analysis

Data on fermentation parameters and copy numbers of total bacteria, ciliates, and methanogens were analyzed using the PROC GLIMMIX procedure in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) to evaluate the effects of inoculated ciliates. Alpha-diversity indices and the number of microbial functional profiles (represented by KEGG orthologs and KEGG pathways) were also analyzed with inoculated ciliates treated as fixed effects using the same procedure. Differences between group means were identified using Duncan's test. Statistical analysis of the relative abundance (RA) of bacterial taxa and functional profiles, considering the effects of inoculated ciliates, was conducted using the 'MaAsLin2' R package (v1.14.1) with a significance threshold of $q \leq 0.05$ [39]. The RA of the bacterial taxa and KEGG pathways [40] was normalized using the centered log-ratio (CLR) transformation and analyzed without additional data transformations, as implemented in MaAsLin2. To evaluate differences in bacterial communities (represented by phyla and genera levels) and their functional profiles (represented by KEGG orthologs and KEGG pathways), permutational multivariate analysis of variance (PERMANOVA) was performed using the Bray–Curtis dissimilarity metric and the 'vegan' R package (Adonis function with 9999 random permutations). This analysis treated inoculated ciliates as fixed effects. Pairwise comparisons were adjusted using the Benjamini–Hochberg procedure to control for multiple testing. Spearman correlation coefficients between the RA of differentially abundant bacterial genera and in vitro rumen fermentation parameters were calculated using PROC CORR in SAS 9.4. Only correlations that were medium or strong ($|r| \geq 0.4$) and statistically significant ($p \leq 0.05$) were visualized using the 'ggplot2' R package [35]. A p-value of less than 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Effect of Inoculated Ciliates on In Vitro Fermentation Parameters

Inoculation of different rumen ciliate groups had a significant effect only on the A:P ratio among in vitro fermentation parameters (Table 2). Dry matter digestibility (DMD) did not differ significantly among treatments ($p = 0.791$). Neutral detergent fiber digestibility (NDFD) and acid detergent fiber digestibility (ADFD) did not show significant differences

among groups (NDFD, $p = 0.808$; ADFD, $p = 0.526$). The pH values across treatments remained stable, with no significant differences observed among the groups ($p = 0.664$). Similarly, ammonia nitrogen (NH_3-N) concentrations and total gas production showed no significant variation among treatments (NH_3-N, $p = 0.414$; total gas production, $p = 0.838$). Methane production tended to be higher in the ISO, OPH, and MIX groups compared to the CON group. Methane yield [CH_4 (mL/g of degraded dry matter)] followed a similar pattern, with the highest values observed in the MIX group, though the differences were not statistically significant ($p = 0.130$). The total volatile fatty acid (VFA) concentrations did not differ from any of the ciliate group inoculations ($p = 0.496$), but the acetate-to-propionate (A:P) ratio showed significant differences among treatments ($p < 0.001$). The MIX group exhibited the highest A:P ratio, which was significantly higher than that of all the other groups. Additionally, the ISO group had an intermediate level of A:P ratio, and it was significantly higher than that of the other remaining groups.

Table 2. In vitro fermentation parameters after 24 h of incubation.

Item	CON	ENTO	EPI	ISO	OPH	MIX	*p*-Value	Pooled SEM
DMD (%)	71.26	70.51	70.78	72.04	72.40	72.67	0.791	3.17
NDFD (%)	51.61	49.95	50.13	52.24	52.89	51.61	0.808	4.72
ADFD (%)	37.33	36.23	36.23	40.14	41.88	37.33	0.526	7.53
pH	6.47	6.46	6.49	6.48	6.49	6.48	0.664	0.03
NH_3-N (mg/dL)	4.49	4.73	4.74	4.42	4.78	4.44	0.414	0.39
Total Gas (mL)	39.06	38.28	38.50	38.73	38.70	39.98	0.838	2.31
CH_4 (mL)	0.98 ^{b}	1.27 ^{a}	1.15 ^{ab}	1.33 ^{a}	1.38 ^{a}	1.39 ^{a}	0.096	0.25
CH_4 (mL/g dDM)	6.86	9.19	8.15	9.22	9.56	9.58	0.130	1.78
Total VFA (mM)	38.16	34.28	29.22	33.31	24.95	41.13	0.496	14.90
A:P ratio	1.66 ^{C}	1.63 ^{C}	1.64 ^{C}	1.72 ^{B}	1.66 ^{C}	1.78 ^{A}	<0.001	7.95

$^{A–C}$, Means ($n = 3$) within a row followed by different superscripts indicate significant differences among treatments ($p \leq 0.05$). ^{a,b}, Means ($n = 3$) within a row followed by different superscripts indicate tendency among treatments ($0.05 < p \leq 0.10$). CON, control group without ciliate added; ENTO, small entodinia inoculated; EPI, *Epidinium* spp. inoculated; ISO, isotrichids inoculated; OPH, *Ophryoscolex* spp. inoculated; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. inoculated; DMD, dry matter digestibility; NDFD, neutral detergent fiber digestibility; NH_3-N, ammonia nitrogen; CH_4 (mL/g dDM), mL of methane production per degraded gram of dry matter; total VFA (mM), total volatile fatty acid concentration; A:P ratio, acetate to propionate ratio; Pooled SEM, pooled standard error of the mean.

The addition of different ruminal ciliates significantly influenced each VFA concentration and profile after 24 h of incubation (Table 3). The molar proportion of acetate was higher in the MIX group than in all other groups ($p = 0.001$). The ENTO group exhibited the highest molar proportion of propionate, and it was significantly higher than the ISO, OPH, and MIX groups ($p < 0.001$). The molar proportion of butyrate exhibited the highest level in the ISO group, showing a significantly higher value than all other groups ($p = 0.012$). The ENTO, EPI, ISO, and OPH groups had significantly higher molar proportions of isovalerate compared to the CON and MIX groups ($p = 0.005$). The molar proportion of valerate was significantly higher in the ISO group compared to all other groups ($p < 0.001$). However, the molar proportions of isovalerate showed no significant differences among treatments (molar proportion of isovalerate, $p = 0.111$).

Table 3. Inoculated ciliates' effects on volatile fatty acid (VFA) profiles after 24 h of incubation.

Item	CON	ENTO	EPI	ISO	OPH	MIX	*p*-Value	Pooled SEM
Total VFA (mM)	38.16	34.28	29.22	33.31	24.95	41.13	0.496	14.90
A:P ratio	1.66 ^{C}	1.63 ^{C}	1.64 ^{C}	1.72 ^{B}	1.66 ^{C}	1.78 ^{A}	<0.001	7.95
Acetate (%)	53.27 ^{B}	52.51 ^{B}	52.52 ^{B}	52.34 ^{B}	52.72 ^{B}	54.77 ^{A}	0.001	4.82

Table 3. *Cont.*

Item	CON	ENTO	EPI	ISO	OPH	MIX	*p*-Value	Pooled SEM
Propionate (%)	32.03 ^{AB}	32.28 ^{A}	32.00 ^{AB}	30.40 ^{C}	31.79 ^{B}	30.78 ^{C}	<0.001	0.03
Isobutyrate (%)	0.69	0.62	0.58	0.68	0.57	0.52	0.111	0.11
Butyrate (%)	11.96 ^{B}	12.38 ^{B}	12.60 ^{B}	13.56 ^{A}	12.59 ^{B}	11.75 ^{B}	0.012	1.75
Isovalerate (%)	0.88 ^{B}	1.06 ^{A}	1.09 ^{A}	1.06 ^{A}	1.07 ^{A}	0.91 ^{B}	0.005	0.15
Valerate (%)	1.17 ^{B}	1.16 ^{B}	1.20 ^{B}	1.96 ^{A}	1.27 ^{B}	1.28 ^{B}	<0.001	0.16
Total VFA (mM)	38.16	34.28	29.22	33.31	24.95	41.13	0.496	14.90
A:P ratio	1.66 ^{C}	1.63 ^{C}	1.64 ^{C}	1.72 ^{B}	1.66 ^{C}	1.78 ^{A}	<0.001	7.95

$^{A–C}$, Means ($n = 3$) within a row followed by different superscripts indicate significant differences among treatments ($p \leq 0.05$). CON, control group without ciliate added; ENTO, small entodinia inoculated; EPI, *Epidinium* spp. inoculated; ISO, isotrichids inoculated; OPH, *Ophryoscolex* spp. inoculated; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. inoculated; Pooled SEM, pooled standard error of the mean.

3.2. Impasct of Inoculated Ciliates on Alpha and Beta Diversity of Ruminal Microbiota

After processing the raw sequences, an average of 34,576 high-quality amplicon sequence variants (ASVs) were obtained across 18 samples derived from in vitro batch cultures (Table S2). Alpha- and beta-diversity analyses were performed using a repeatedly rarefied BIOM table standardized to 31,199 ASVs per sample. Good's coverage for all samples was calculated to be 99.97%.

Inoculated ciliates significantly altered the alpha-diversity metrics in the ruminal microbiota (Table 4). The observed ASVs were highest in the ENTO group and significantly decreased in the EPI, MIX, and OPH groups. Among them, the OPH group showed the lowest observed ASVs. The results for the Chao1 estimate were completely identical to the observed ASV, except for the figures for the ENTO group. The Shannon diversity and Simpson diversity indices were highest in the CON group and significantly reduced in groups inoculated with specific ciliates such as ENTO, EPI, and OPH groups, particularly in the OPH group. Faith's phylogenetic diversity (PD) was significantly lower in the EPI, OPH, and MIX groups compared to the other treatments.

Table 4. Alpha-diversity measurements for inoculated ciliates effects.

Item	CON	ENTO	EPI	ISO	OPH	MIX	*p*-Value	Pooled SEM
Observed ASVs	777 ^{A}	783 ^{A}	727 ^{B}	744 ^{AB}	674 ^{C}	709 ^{BC}	0.003	38.160
Evenness	0.816 ^{A}	0.804 ^{AB}	0.796 ^{BC}	0.813 ^{A}	0.789 ^{C}	0.816 ^{A}	0.008	0.012
Faith's PD	57.209 ^{AB}	59.345 ^{A}	55.137 ^{BC}	57.246 ^{AB}	52.061 ^{C}	52.671 ^{C}	0.033	3.696
Chao1	777 ^{A}	784 ^{A}	727 ^{B}	744 ^{AB}	674 ^{C}	709 ^{BC}	0.003	38.161
Shannon	7.835 ^{A}	7.733 ^{A}	7.563 ^{B}	7.756 ^{A}	7.415 ^{C}	7.721 ^{A}	<0.001	0.117
Simpson	0.986 ^{A}	0.983 ^{B}	0.979 ^{C}	0.985 ^{AB}	0.978 ^{C}	0.985 ^{AB}	<0.001	0.002

$^{A–C}$, Means ($n = 3$) within a row followed by different superscripts indicate significant differences among treatments ($p \leq 0.05$). CON, control group without ciliate added; ENTO, small entodinia inoculated; EPI, *Epidinium* spp. inoculated; ISO, isotrichids inoculated; OPH, *Ophryoscolex* spp. inoculated; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. inoculated; Pooled SEM, pooled standard error of the mean.

The evenness of microbial communities was highest in the CON and MIX groups, whereas it was significantly lower in the OPH group. In addition, the EPI and OPH groups showed lower evenness values than the other treatments. These results indicate that specific ciliate inoculation reduces microbial richness and diversity, with OPH having the most pronounced impact.

3.3. Predicted Microbial Functional Changes Induced by Inoculated Ciliates

Bacterial functional profiles were inferred from 16S rRNA gene sequencing data using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2), aiming to detect changes in bacterial metabolic activities in response to the inoculation of ciliates. Predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs were grouped into KEGG pathways. The inoculation of ciliates did not significantly affect the total number of KEGG orthologs or pathways (KEGG orthologs, $p = 0.185$; KEGG pathways, $p = 0.713$; Table S3). On the other hand, inoculated ciliates had a significant quantitative effect on the overall microbiota (Phyla, $p = 0.018$; Genera, $p = 0.0155$; Figure 1A,B), although pairwise comparisons among the six groups showed no statistically significant differences (Benjamini–Hochberg corrected p-value, $Q > 0.05$). Similarly, while inoculated ciliates significantly influenced the KEGG ortholog ($p < 0.001$, Figure 1D), there was no significant effect on the KEGG pathway ($p = 0.7413$; Figure 1D). Pairwise comparisons between the six groups also revealed no significant differences ($q > 0.05$).

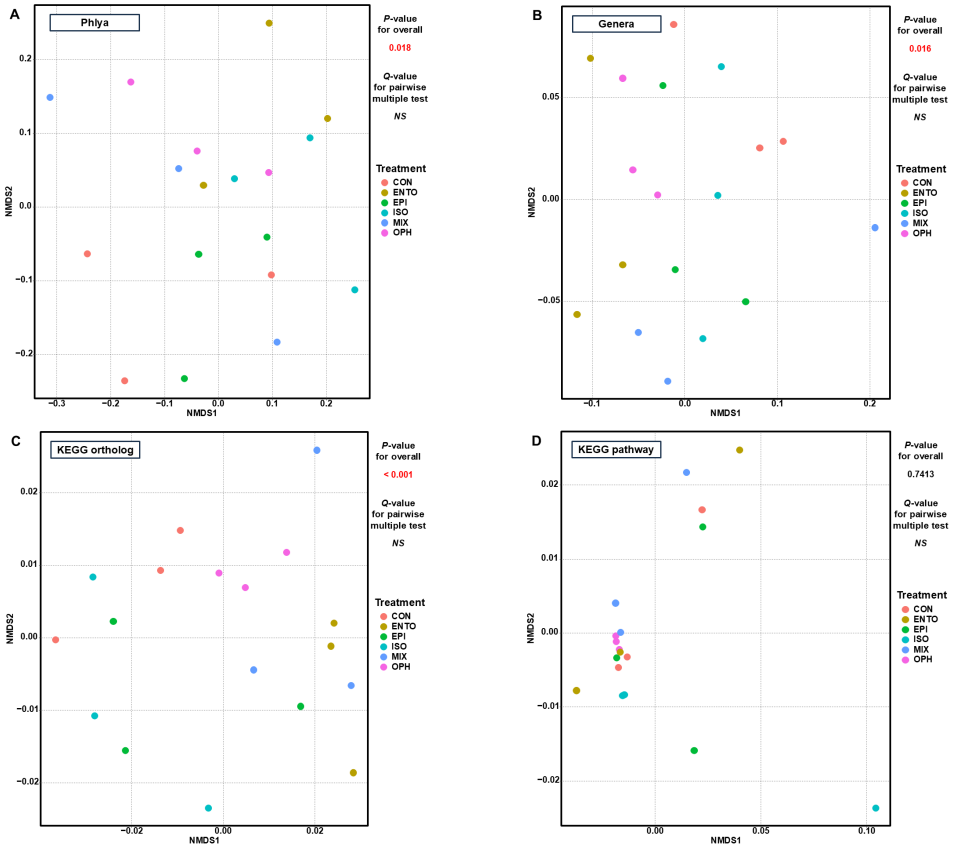

Figure 1. NMDS plots for overall microbiota at different taxonomic levels (phyla, (**A**); genera, (**B**), respectively), and prokaryotic functional profile (KEGG orthologs, (**C**); KEGG pathways, (**D**), respectively) based on the Bray–Curtis dissimilarity. Red font indicates statistically significant differences ($p \leq 0.05$). CON, control group without ciliate added; ENTO, small entodinia inoculated; EPI, *Epidinium* spp. inoculated; ISO, isotrichids inoculated; OPH, *Ophryoscolex* spp. inoculated; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. inoculated; *NS*, no significant difference between treatment groups.

3.4. Alterations in the Ruminal Microbial Composition by Inoculated Ciliates

The differential abundance test results, conducted using MaAsLin2, revealed distinct bacterial genera across the treatment groups ($q < 0.05$). Regarding the relative abundance of bacterial genera, *Streptococcus* was the most abundant in the MIX group and the second most abundant in the EPI group, while no significant differences were observed between

the ENTO group and these two groups. In contrast, the CON and ISO groups showed significantly lower abundances compared to the EPI and MIX groups, but significantly higher abundances compared to the OPH group. Ruminococcaceae unclassified was significantly more abundant in the ENTO and EPI groups than in the other groups. *Lachnoclostridium* exhibited the highest abundance in the EPI group, while its abundance significantly decreased in the ENTO, OPH, and MIX groups. It was nearly absent in the ISO group and completely absent in the CON group. *Basfia*, *Megasphaera*, *Parabacteroides*, and *Bacteroides* were predominantly detected in the ISO group, followed consistently by the MIX group. *Basfia* and *Megasphaera* were significantly less abundant in the MIX group than in the ISO group, but significantly more abundant than in the other groups (Table 5). *Fusobacterium* was significantly more abundant in the ISO group compared to all the other groups, while the MIX group displayed intermediate levels. No significant differences were found between the MIX and ENTO groups, but the MIX group had significantly higher abundances than the remaining groups (Table 5).

Table 5. Differentially abundant bacterial genera and the KEGG pathway by inoculated ciliates analyzed using MaAsLin2 ($q \leq 0.05$).

Bacterial Genera							
Genus	**CON**	**ENTO**	**EPI**	**ISO**	**OPH**	**MIX**	**Pooled SEM**
Streptococcus	5.047 ^{BC}	7.846 ^{AB}	9.477 ^{A}	5.863 ^{BC}	9.629 ^{A}	4.500 ^{C}	1.265
Ruminococcaceae unclassified	0.068 ^{B}	0.147 ^{A}	0.134 ^{A}	0.068 ^{B}	0.076 ^{B}	0.096 ^{B}	0.029
Lachnoclostridium	0 ^{C}	0.127 ^{B}	0.206 ^{A}	0.004 ^{C}	0.132 ^{B}	0.109 ^{B}	0.032
Basfia	0.004 ^{C}	0 ^{C}	0 ^{C}	0.196 ^{A}	0 ^{C}	0.065 ^{B}	0.012
Megasphaera	0 ^{C}	0 ^{C}	0 ^{C}	0.410 ^{A}	0 ^{C}	0.205 ^{B}	0.007
Parabacteroides	0 ^{B}	0 ^{B}	0 ^{B}	0.020 ^{A}	0 ^{B}	0.001 ^{B}	0.003
Fusobacterium	0.019 ^{C}	0.054 ^{BC}	0.023 ^{C}	0.209 ^{A}	0.013 ^{C}	0.077 ^{B}	0.024
Bacteroides	0 ^{C}	0 ^{C}	0 ^{C}	0.477 ^{A}	0 ^{C}	0.102 ^{B}	0.019
KEGG pathways							
Pathway	**CON**	**ENTO**	**EPI**	**ISO**	**OPH**	**MIX**	**Pooled SEM**
ko00561 (Glycerolipid metabolism)	0 ^{B}	0 ^{B}	0 ^{B}	0.497 ^{A}	0 ^{B}	0.491 ^{A}	0.012

$^{A–C}$: Means (n = 3) within a row followed by different superscripts indicate significant differences among treatments ($q \leq 0.05$). CON, control group with ciliate added; ENTO, small entodinia added group; EPI, *Epidinium* spp. added group; ISO, isotrichids added group; OPH, *Ophryoscolex* spp. added group; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. added; Pooled SEM, pooled standard error of the mean.

In terms of metabolic pathways, the glycerolipid metabolism pathway (ko00561) was significantly enriched in the ISO and MIX groups, whereas it was not detected in other groups (Table 5).

3.5. Effect of Inoculated Ciliates on the Correlations Between In Vitro Fermentation Parameters and Microbial Composition

A total of 32 significant correlations ($|r| \geq 0.4$, $p \leq 0.05$) were identified between the RA of differentially abundant bacterial genera, total bacteria copy number, total methanogen copy number, total ciliate copy number, and in vitro ruminal fermentation parameters (Figure 2).

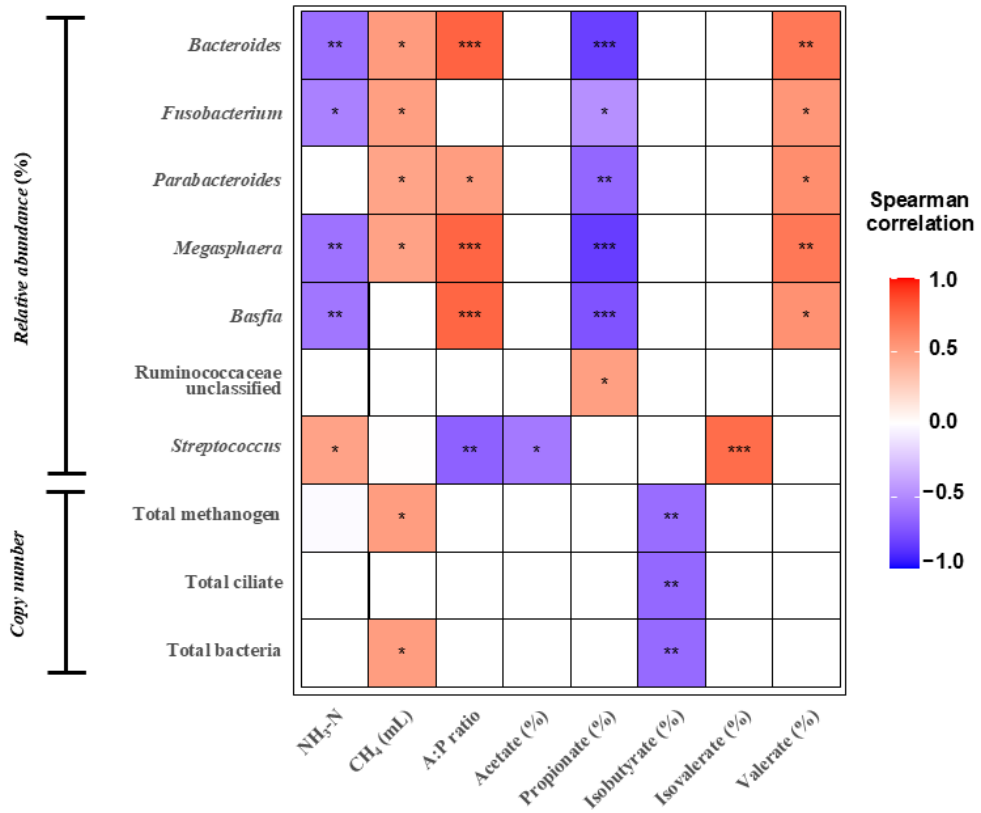

Figure 2. Heatmap of Spearman's correlation coefficients between the in vitro rumen fermentation parameters and relative abundance of differentially abundant bacterial genera, as well as log copy number total methanogen, total ciliates, and total bacteria after 24 h of incubation ($|r| \geq 0.4$, *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$). NH_3-N, ammonia nitrogen; A:P ratio, acetate-to-propionate ratio.

For RA of microbial genera, *Bacteroides* and *Megasphaera*, which were highly abundant in the ISO group, exhibited positive correlations with the CH_4 production, A:P ratio, and molar proportions of valerate, whereas they exhibited negative correlations with NH_3-N concentration and molar proportion of propionate. Similarly, *Basfia*, abundant in both ISO and MIX groups, had the same trend of correlation as *Bacteroides* and *Megasphaera* except for methane production. *Fusobacterium* showed positive correlations with methane production and the molar proportion of valerate, whereas it was negatively correlated with NH_3-N concentration and the molar proportion of propionate. *Parabacteroides*, which was also associated with the ISO group, exhibited a significant positive correlation with CH_4 production, A:P ratio, and molar proportion of valerate, while it exhibited a negative correlation with molar proportion of propionate. Additionally, Ruminococcaceae unclassified, which was highly abundant in the ENTO and EPI groups, was positively correlated with the molar proportion of propionate. The genus *Streptococcus*, highly abundant in the EPI and MIX groups, exhibited a positive correlation with NH_3-N concentration and molar proportion of isovalerate, while it was negatively correlated with the A:P ratio and acetate molar proportion.

The copy number of total methanogen and bacteria was positively correlated with CH_4 production but negatively correlated with the molar proportion of isobutyrate. Similarly, the total ciliate copy number exhibited negative correlations with isobutyrate molar proportion.

3.6. Quantitative Analysis of Ruminal Microbes Using Real-Time qPCR

Absolute quantification using qPCR revealed significant differences in microbial populations among treatments (Table 6). Total bacterial abundance was significantly higher in the OPH and MIX groups compared to the other groups ($p = 0.006$). Similarly, total ciliate

abundance was highest in the MIX group, followed by the EPI group, while the ISO group exhibited the lowest values. In addition, the ENTO and OPH groups exhibited intermediate levels in total ciliate abundance ($p < 0.001$). Similarly, methanogen populations were significantly elevated in the OPH and MIX groups, with the significantly lower abundance observed in the CON, ENTO, and EPI groups ($p < 0.001$).

Table 6. Quantitative real-time polymerase chain reaction analysis for absolute quantification of ruminal bacteria, ciliates, and methanogen.

Sample	CON	ENTO	EPI	ISO	OPH	MIX	*p*-Value	Pooled SEM
Total bacteria	10.00 ^{B}	10.11 ^{B}	10.01 ^{B}	10.02 ^{B}	10.74 ^{A}	10.86 ^{A}	0.006	0.41
Total ciliates	-	6.14 ^{C}	7.02 ^{B}	5.76 ^{D}	6.29 ^{C}	7.45 ^{A}	<0.001	0.19
Total methanogens	6.73 ^{B}	6.73 ^{B}	6.82 ^{B}	6.92 ^{B}	7.43 ^{A}	7.39 ^{A}	<0.001	0.23

$^{A–D}$, Means ($n = 3$) within a row followed by different superscripts indicate significant differences among treatments ($p \leq 0.05$). -, Data were not measured. CON, control group without ciliate added; ENTO, small entodinia inoculated; EPI, *Epidinium* spp. inoculated; ISO, isotrichids inoculated; OPH, *Ophryoscolex* spp. inoculated; MIX, mixed group with small entodinia, *Epidinium* spp., isotrichids, and *Ophryoscolex* spp. inoculated; Pooled SEM, pooled standard error of the mean.

4. Discussion

This study aimed to investigate the effects of various ruminal ciliates on ruminal fermentation and microbiota, particularly focusing on prokaryotic microorganisms. To address the scarcity of studies on symbiotic or preferentially associated bacteria of ruminal ciliates, this research included microbiome analysis based on the 16S rRNA gene. This molecular approach strengthens the study compared to previous defaunation studies, which often lacked molecular insights. However, the limited biomass and cell counts of the inoculated ciliates in this study may pose a limitation in fully replicating the ciliate ecosystem within the rumen.

4.1. Impact of Inoculated Ciliates on Ruminal Fermentation and Total Copy Number

The trend of increased methane production in the OPH and MIX groups is associated with the total methanogen copy numbers in these groups. Considering the significant increase in total ciliate copy numbers in the EPI and MIX groups, as well as previous studies showing positive correlations between ruminal ciliates and methane production [10,41], significant methane production was expected in these groups. However, the total methanogen copy number did not increase in the EPI group, aligning with earlier findings that *Epidinium* has a relatively weak association with methanogens [42,43]. The RA of *Streptococcus*, a lactate-producing bacterium, increased in the EPI and OPH groups compared to the other groups, likely influencing the VFA profile. According to Hayirli et al. [44], lactic acid abundance reduces acetate while increasing propionate and butyrate. This explains the significantly higher acetate proportion and lower propionate proportion observed in the MIX group, where *Streptococcus* RA was the lowest. The significant increase in total methanogen copy numbers in the MIX group, combined with the use of acetate by methanogens [45], further supports the observed acetate proportion in this group. Additionally, *Megasphaera*, which was not detected in the other groups, was identified in the ISO and MIX groups. The numerically higher abundance of *Streptococcus*, a lactate-producing bacteria [46], in the ISO group, along with the significantly higher abundance of *Megasphaera*, a lactate-utilizing bacteria, in the same group, explains the significantly increased butyrate proportion observed in the ISO group [46]. This finding is consistent with previous research showing enhanced butyrate production following *Megasphaera* inoculation [47] and a study indicating that

butyrate is efficiently converted by acid-utilizing bacteria like *Megasphaera* under lactic acid accumulation [48].

4.2. Implications of Inoculated Ciliates on Alpha and Beta Diversity of the Ruminal Microbiota

The alpha diversity indices, including richness (observed ASVs and Chao1 estimates), significantly decreased in the EPI, OPH, and MIX groups compared to the CON group. Additionally, Faith's PD values significantly decreased in the OPH and MIX groups, correlating with the total ciliate copy numbers in these groups. The higher total ciliate copy numbers in these groups suggest the establishment of a ciliate consortium due to inoculation, potentially reducing prokaryotic diversity through predation. This is consistent with previous findings showing a 50–90% increase in bacterial populations in defaunated animals compared to faunated animals [6].

Beta diversity analysis based on Bray–Curtis dissimilarity revealed significant differences at the phylum, genus, and KEGG ortholog levels among treatments. This indicates that the diversity and functional diversity of prokaryotes associated with different ciliates vary in fauna-free environments. However, the lack of significant differences in KEGG pathways aligns with previous findings, suggesting that microbial communities in the rumen maintain functional stability despite compositional changes [49].

4.3. Functional Predictions of Ruminal Microbiota in Response to Inoculated Ciliates

Significant differences in the predicted microbial functions due to inoculated ciliates were observed only in the ko00561 pathway (glycerolipid metabolism). This pathway significantly increased only in the ISO and MIX groups, where isotrichids were presumably inoculated, suggesting a potential association between isotrichids and this pathway. Additionally, the bacteria uniquely identified in these groups (*Basfia*, *Megasphaera*, *Parabacteroides*, and *Bacteroides*) may be linked to this pathway. In particular, *Bacteroides*, which is known to produce glycerolipids, such as plasmalogens, has been reported to exhibit species-specific lipid profiles [50]. Furthermore, *Megasphaera elsdenii* was found to produce plasmalogens via the plasmalogen synthase MeHAD [51]. The antioxidative properties of plasmalogens, which reduce oxidative stress, may be connected to the greater oxygen tolerance of isotrichids compared to entodinia [52,53].

4.4. Metabolic Relationship Between Ruminal Fermentation Parameters and Microbial Genera

Previous studies have highlighted the association between *Entodinium caudatum* and amylolytic bacteria [43], and its relationship with lactic acid [44,54]. The present study corroborates these findings, as all groups inoculated with entodinia showed a significantly higher RA of *Streptococcus* compared to the ISO and CON groups. This suggests that entodinia, beyond *E. caudatum*, may influence *Streptococcus* abundance and lactic acid levels.

Furthermore, *Streptococcus lutetiensis* was predominantly identified in this study, and its significantly higher abundance in cellulolytic ciliate-associated groups (EPI and OPH) compared to ENTO and MIX groups implies a potential antagonistic interaction with *E. caudatum*. The significantly higher A:P ratio in the MIX group is attributed to the significant increase in acetate and decrease in propionate, driven by the successfully established ciliate consortium in this group, consistent with one study [10].

5. Conclusions

This study investigated the impact of ruminal ciliate inoculation in a fauna-free environment on (1) in vitro fermentation parameters and (2) ruminal prokaryotic community composition and functional changes. While no significant changes were observed in fermentation parameters, such as NDFD, ADFD, and NH_3-N, likely due to insufficient ciliate cell counts, the findings in this study underscore the critical role of ruminal ciliates in

shaping microbial community dynamics and metabolic processes. Specifically, *Streptococcus* and *Megasphaera* were associated with fermentation byproducts such as lactic acid, while *Bacteroides* and *Megasphaera* were linked to broader metabolic pathways, including lipid metabolism and oxidative stress tolerance. These findings suggest that ruminal ciliates may adapt to the anaerobic environment of the rumen through interactions with associated prokaryotes and simultaneously influence metabolic processes within the rumen. Furthermore, these findings highlight the need for further research on the relationship between lactic acid-associated bacteria and ruminal ciliates, as well as the oxygen tolerance and plasmalogen metabolism of isotrichids. Future studies should aim to overcome the limitations of this study by inoculating ciliates with sufficient biomass to establish a robust ruminal consortium.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation11010028/s1: Table S1: Diet composition used in the in vitro digestibility experiment (% of dry matter, DM); Table S2: Sample information and sequencing statistics of 16S rRNA gene amplicon sequencing variants; Table S3: Number of KEGG orthologs and pathways among each group after 24 h of incubation.

Author Contributions: T.P. and G.K. conceptualized and designed the study. G.K. and W.L. were responsible for collecting rumen fluid samples, developing experimental protocols, and carrying out the experiments. Microbiome data analysis was conducted by G.K. and T.P., while fermentation data analysis was handled by G.K. The initial draft of the manuscript was written by G.K., with T.P. providing additional contributions to the writing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University (protocol code 202401030032, approved on 1 April 2024). All animal-related procedures in this study were conducted in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and relevant regulations.

Data Availability Statement: The datasets produced in this study are accessible through online repositories. The repository details and associated accession numbers are available in the NCBI database at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1196625 (accessed on 11 December 2024).

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

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