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Reduction of enteric methane emission using methanotroph-based probiotics in Hanwoo steers

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Abstract

Background Methane emission from enteric rumen fermentation is a main source of greenhouse gas (GHG) emission and a major concern for global warming.

Results In this study, we isolated methanotroph-methylotroph consortium NC52PC from the rumen after a series of sub-culture and repetitive streaking on an agar plate and polycarbonate membrane filter. The NC52PC comprises methanotroph species (*Methylocystis* sp.) and methylotroph species (*Methylobacterium* sp.), forming a consortium capable of growing solely on methane as a carbon source. Their morphology, growth, and genome sequence were characterized. We assessed its effectiveness in mitigating methane emissions through both in vitro and in vivo experiments. During the in vitro trial, the introduction of NC52PC (at a concentration of 5.1×10^7 CFUs/ml) demonstrated a reduction in methane production exceeding 40% and 50% after 12 and 24 h, respectively. Also, NC52PC did not significantly alter other aspects of the in vitro rumen fermentation parameters such as pH, total gas production, and digestibility. Further investigation involved testing NC52PC as a dietary supplement in 12 young Hanwoo steers over three 30-day test periods. The steers received a diet comprising 70.8% concentrate and 29.2% bluegrass on a dry matter basis, with variations including 3×10^7 CFUs/ml of NC52PC (**LOW**) and 3×10^8 CFUs/ml (**HIGH**) of NC52PC, and without NC52PC as a control (**CON**). Steers administered with **HIGH** and **LOW** concentrations of NC52PC exhibited reduced enteric methane emission (g/day) by 14.4% and 12.0%, respectively.

Conclusion Feeding methanotroph-methylotroph consortium NC52PC significantly reduced methane emissions in Korean beef cattle without any adverse effects on animal health. These findings suggest that this probiotic could serve as a promising feed additive to effectively mitigate methane emissions from ruminants. However, further research is needed to evaluate the long-term effects of NC52PC on animal health, and on meat and milk quality.

Keywords Methane emission, Hanwoo steer, Methanotroph, Rumen fermentation, Probiotics, VFA

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Background

Ruminants contribute significantly to global methane emissions, accounting for approximately 16% of the total [1]. As countries strive for carbon neutrality, there is growing pressure on the livestock industry to reduce methane emissions [2, 3]. Methane, a potent greenhouse gas, has experienced a steady increase in atmospheric concentrations, reaching over 1,930 parts per billion in January 2024 [4, 5]. Its short lifespan and high warming potential make it a prime target for climate mitigation efforts [6, 7].

Within the rumen, methane is produced as a by-product of microbial fermentation by methanogens [8, 9]. This by-product formation also translates to a significant loss of gross energy, ranging from 2 to 12%, energy that could otherwise be utilized to enhance animal productivity [10]. UN projections indicate that the global population will surge to 9.8 billion by 2050 and 11.2 billion by 2100, driving a substantial increase in food demand [11]. This includes a projected rise in milk consumption to 1.04 million tons and meat consumption to 465 million tons by 2100 [12]. This escalating demand for ruminant livestock is anticipated to intensify methane production, thereby exacerbating global warming [13]. To align with the Paris Agreement's 1.5 °C target, it is essential to implement strategies that mitigate enteric methane emissions from ruminants [2]. Such efforts not only support climate stability but also present opportunities to enhance animal productivity and ensure the long-term sustainability of agricultural systems [14, 15].

Various strategies, including dietary adjustments and supplementation of chemical and biological additives, have been employed to mitigate or inhibit methane emissions from ruminants. Several chemical additives have successfully reduced methane emissions in various cattle by directly inhibiting the growth of methanogens, thereby reducing methanogenesis from its source [16]. To date, very few researchers have tried to investigate the presence of methanotrophs and their potential to mitigate methane emissions in ruminants [17–19]. In this study, we aim to introduce a novel use of methanotrophs as Direct-Fed Microbials (DFM) and evaluate the efficacy of methanotroph-based probiotics in both in vitro and in vivo conditions.

Results

Isolation and characterizations of NC52PC

Three potential isolates (NC75PC, NC77PC, and NC52PC) obtained from polycarbonate membranes were then tested for growth at 30 °C and at a rumen temperature of 39 °C. The isolates NC75PC, NC77PC, and NC52PC grew to an OD_{600} value above 2 for 36 h at 30 °C with a specific growth rate of 0.1164 h^{-1}, 0.1172 h^{-1}, and 0.0915 h^{-1}, respectively (Fig. 1A). However, the growth rate of isolates NC75PC and NC77PC significantly reduced to 0.0308 h^{-1} and 0.0275 h^{-1}, respectively, when cultured at 39 °C (Fig. 1B). On the other hand, NC52PC grew relatively similar at both temperature as the specific growth rate remained stable (0.1075 h^{-1}) (Table 1).

Morphological analysis

Scanning electron microscopy examination of NC52PC unveiled two morphologically distinct cell types (Fig. 2). One type of cells appeared as smooth-surfaced rod-shaped bacilli, measuring approximately 2.4–2.9 × 0.8-1 micron. The second type presented

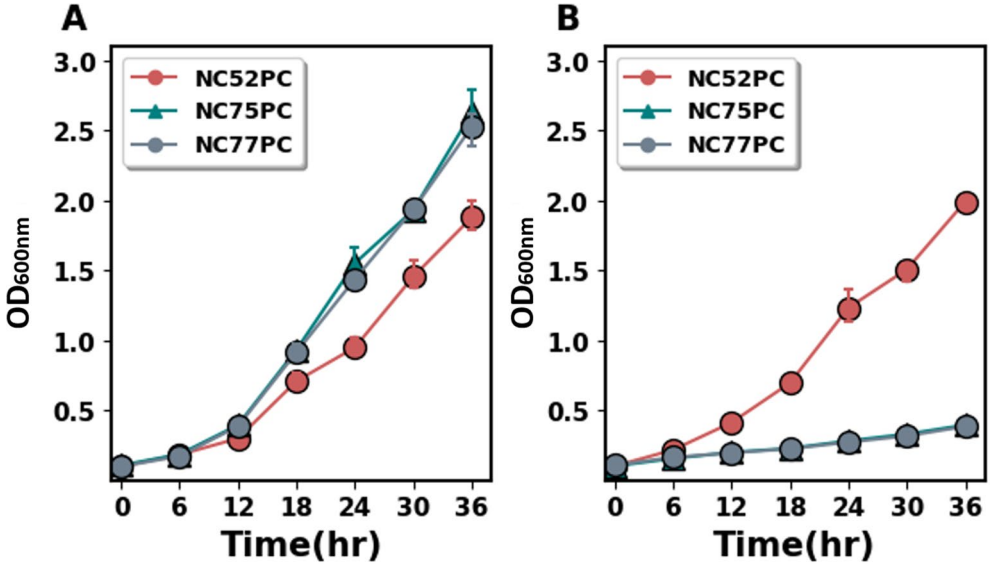

Fig. 1 Growth test of three isolates NC52PC, NC75PC, and NC77PC. Cell growth (OD_{600}) from 0 h to 36 h for three isolates at 30 °C (**A**) and 39 °C (**B**)

Table 1 Growth rate of the three rumen isolates

Isolate	Temperature (°C)	μmax (hr^{-1})	Td (h)
NC52PC	30	0.0915 ± 0.0030	7.6 ± 0.25
	39	0.1075 ± 0.0094	6.5 ± 0.58
NC75PC	30	0.1164 ± 0.0023	6.0 ± 0.12
	39	0.0308 ± 0.0016	22.6 ± 1.18
NC77PC	30	0.1172 ± 0.0014	5.9 ± 0.07
	39	0.0275 ± 0.0046	25.7 ± 4.50

Data are expressed as mean ± standard deviation (SD). μmax, Maximum growth rate. Td, doubling time

as curved coccobacilli with a rough surface, measuring approximately 1.3–1.5 × 0.8-1 microns. Moreover, genome sequencing detected two genomic DNA, one of which is closely related to the methanotroph species *Methylocystis echinoides*, and the other similar to the methylotroph species *Methylobacterium organophilum*. Hence, we are assuming smooth-surfaced rod-shaped bacilli to be a *Methylobacterium* species [20] and the other curved coccobacilli with a rough surface to be a *Methylocystis* species [21] based on the existing literature.

Genomic analysis

The genome of isolate NC52PC was sequenced using hybrid Long-read & Short-read sequencing, generating a total of 4 contigs, two circular chromosomes, and two circular plasmids. Based on the BlastX analysis, a larger chromosome with 5.1 Mbp in size showed similarity to the genus of *Methylobacterium*. The other chromosome, with 3.95 Mbp in size and two plasmids, belong to the genus of *Methylocystis*. The sizes of the two plasmids were 167 kb and 165 kb. Genomic features such as GC content, number of tRNAs, rRNA, genes, and proteins were calculated using Prokka [22] (Table 2).

The genome of each species in NC52PC was visualized along with their closely related species, as shown in Fig. 3 [23].

The genome-based comparisons of both *Methylocystis* species and *Methylobacterium* species present in NC52PC with closely related species were performed to calculate the average nucleotide identity [24], in silico DNA–DNA hybridization [25] and the average amino acid identity [26]. The ANI, AAI, and DDH

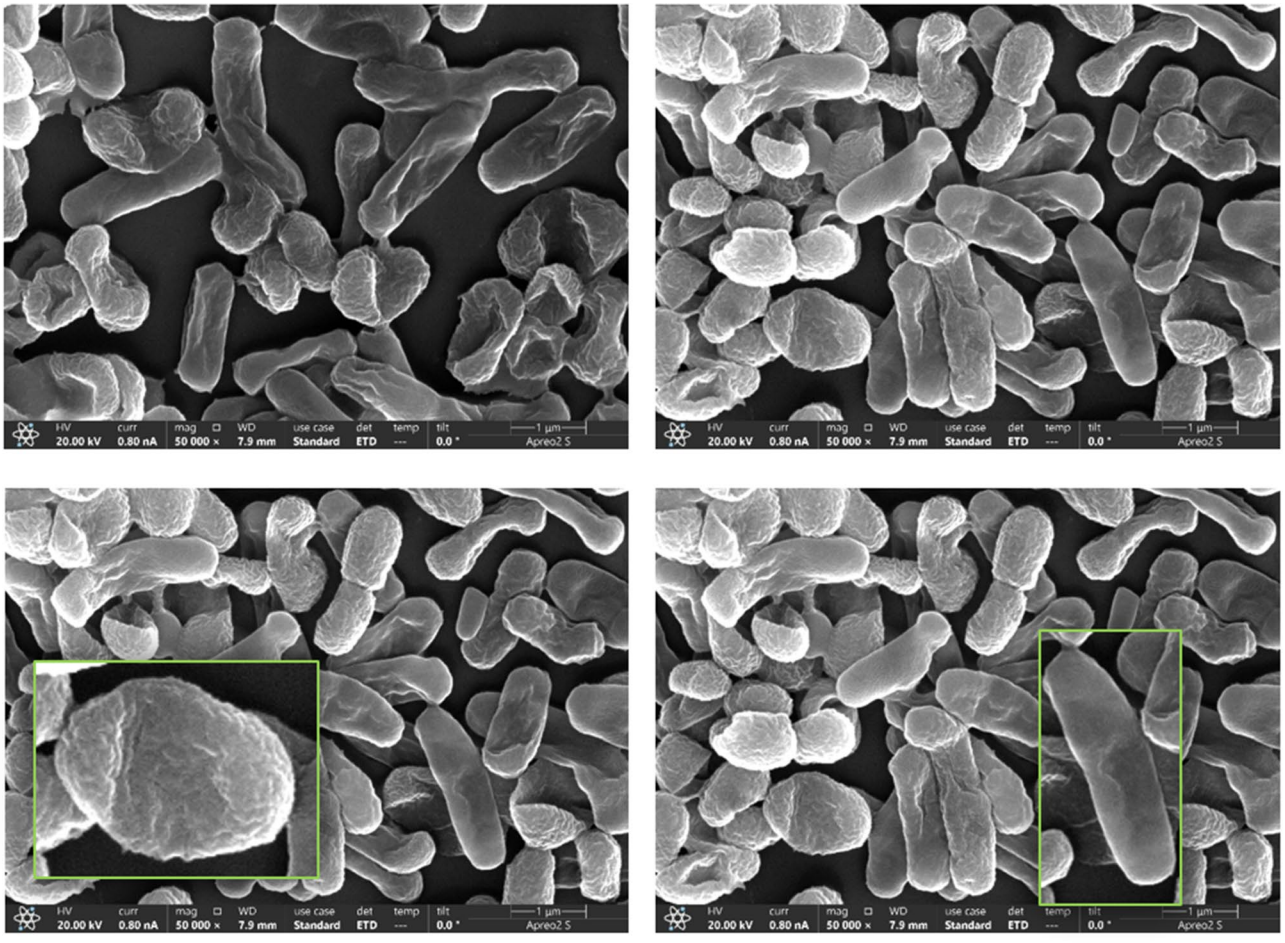

Fig. 2 SEM image of NC52PC cells showing two morphologically distinct cell types

Table 2 Genome features of two circular chromosomes in NC52PC

Traits	Methylobacterium sp.	Methylocystis sp.
Genome size	5.07 Mbp	3.9 Mbp
Contigs	1	3
Circular	Yes	Yes
GC%	69.98	64.46
tRNA	150	150
rRNA	24	12
CDS	4683	3884
16 S	4	2
*pmoA*s	-	2
Integrated plasmids	-	2
Genbank Accession No	CP168955	CP170127

values between the *Methylocystis* strain and its closest relative, *Methylocystis echinoides* LMG27198, were 81.07%, 81.35%, and 25.76%, respectively (Table 3), which were lower than the threshold values (95% for ANI or AAI and 70% for DDH [27–29]). Hence, we propose that the strain *Methylocystis* species in NC52PC represent a novel species of *Methylocystis* genus within the family *Methylocystaceae*. Similarly, the genome of *Methylobacterium* species in NC52PC was also compared with closely related *Methylobacterium* species and shared the highest similarity with *Methylobacterium organophilum* WPA_B with ANI, AAI, DDH values of 98.59%, 98.79%, and 88.5%, respectively (Table 3). Therefore, the *Methylobacterium* species in the NC52PC consortium is most likely *Methylobacterium organophilum*. Further streaking of the NC52PC consortium to obtain pure methanotroph significantly affects its growth rate. As a faster growth rate often translates to optimum methane consumption [30], a consortium of *Methylocystis* sp. and *Methylobacterium* sp. was utilized instead to achieve maximum methane reduction.

Nucleotide sequence accession numbers

The complete genome sequences of both strains have been deposited in the GenBank databases under accession numbers CP170127 (chromosome *Methylocystis* sp. NC52PC), CP170125 (*Methylocystis* sp. plasmid pNC52PC-1), CP170126 (*Methylocystis* sp. plasmid pNC52PC-2), and CP168955 (chromosome *Methylobacterium organophilum* NC52PC).

In vitro rumen fermentation parameters

During in vitro rumen fermentation, no significant differences were observed in pH, total gas production, and digestibility except methane production between control and NC52PC-inoculated samples across all sampling points ($p > 0.05$). pH levels dropped from 6.5 to 5.8 after 24 h in both samples (Fig. 4A), likely due to the synthesis of various organic acids during rumen fermentation. After 24 h, the total headspace volume in the serum bottle increased from 130 ml to over 160 ml (Fig. 4B). Dry matter degradation continued to rise from 25% at 12 h to over 32% at 24 h (Fig. 4C), indicating an active rumen fermentation process throughout the period. However, methane production significantly declined by 41.7% and 53.6% under anaerobic conditions at 12 and 24 h, respectively, when inoculated with NC52PC ($p < 0.05$) (Fig. 4D). The slow increase in methane production from 12 h to 24 h in the treatment group shows persistent methanotrophic activity (Fig. 4D). Moreover, methane consumption may have been sustained by *Methylocystis* sp. as its population remains relatively stable throughout the 24-hour period (Fig. 4E).

Effects of NC52PC on the composition of rumen microorganisms in vitro

We extracted total genomic DNA from three technical replicates of 24-hour samples and one replicate of 0-hour sample from both in vitro rumen fermentation of control and NC52PC. 16S rRNA (V3-V4) gene sequencing analysis was performed to identify differences in composition, richness, and diversity of the rumen microbiota between the control and NC52PC samples after 24 h of in vitro fermentation. Overall, 18 bacterial phyla and 276 bacterial genera were detected in the combined experimental samples. *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were the dominant phyla, accounting for up to 80% of the total bacterial ASVs (Fig. 5A and B). In control, *Prevotella* is the most predominant genus (16.56%), followed by *Intestinimonas* (4.21%), *Aristaeella* (4.20%), *Succiniclasticum* (2.51%), *Ruminococcus* (2.41%), *Sodaliphilus* (2.17%), *Lentimicrobium* (1.63%), *Bifidobacterium* (1.41%), *Gehongia* (1.39%), and *Paludibacter* (1.32%). Meanwhile, *Methylocystis* is the most dominant genus (28.7%) in NC52PC inoculated samples, followed by *Prevotella* (9.5%), *Aristaeella* (3.2%), *Sodaliphilus* (3.2%), *Intestinimonas* (3.16%), *Methylobacterium* (2.2%), *Ruminococcus* (1.74%), *Segatella* (1.61%), *Succiniclasticum* (1.16%), *and Bifidobacterium* (1.1%). Since *Methylocystis* is the predominant genus in NC52PC samples, the relative abundance of other dominant bacterial genera, including *Prevotella*, *Aristaeella*, *Intestinimonas*, *Rumminococcus*, and *Lentimicrobium* were significantly lower ($p < 0.05$). Further analysis revealed that NC52PC supplementation did impact the microbial community beyond simply increasing the abundance of the introduced genera. By excluding the genera comprising the NC52PC consortium revealed that five genera, which together constitute approximately 10% of the total microbial community, were significantly affected by the addition of NC52PC. Specifically, we observed a reduction in the relative abundance of *Lentimicrobium* from 2.51 to 1.27% ($p < 0.05$). Conversely, the relative abundance of *Aristaella*, *Sodaliphilus*, *Segatella*, and *Bifidobacterium* slightly increased following NC52PC inoculation ($p < 0.05$).

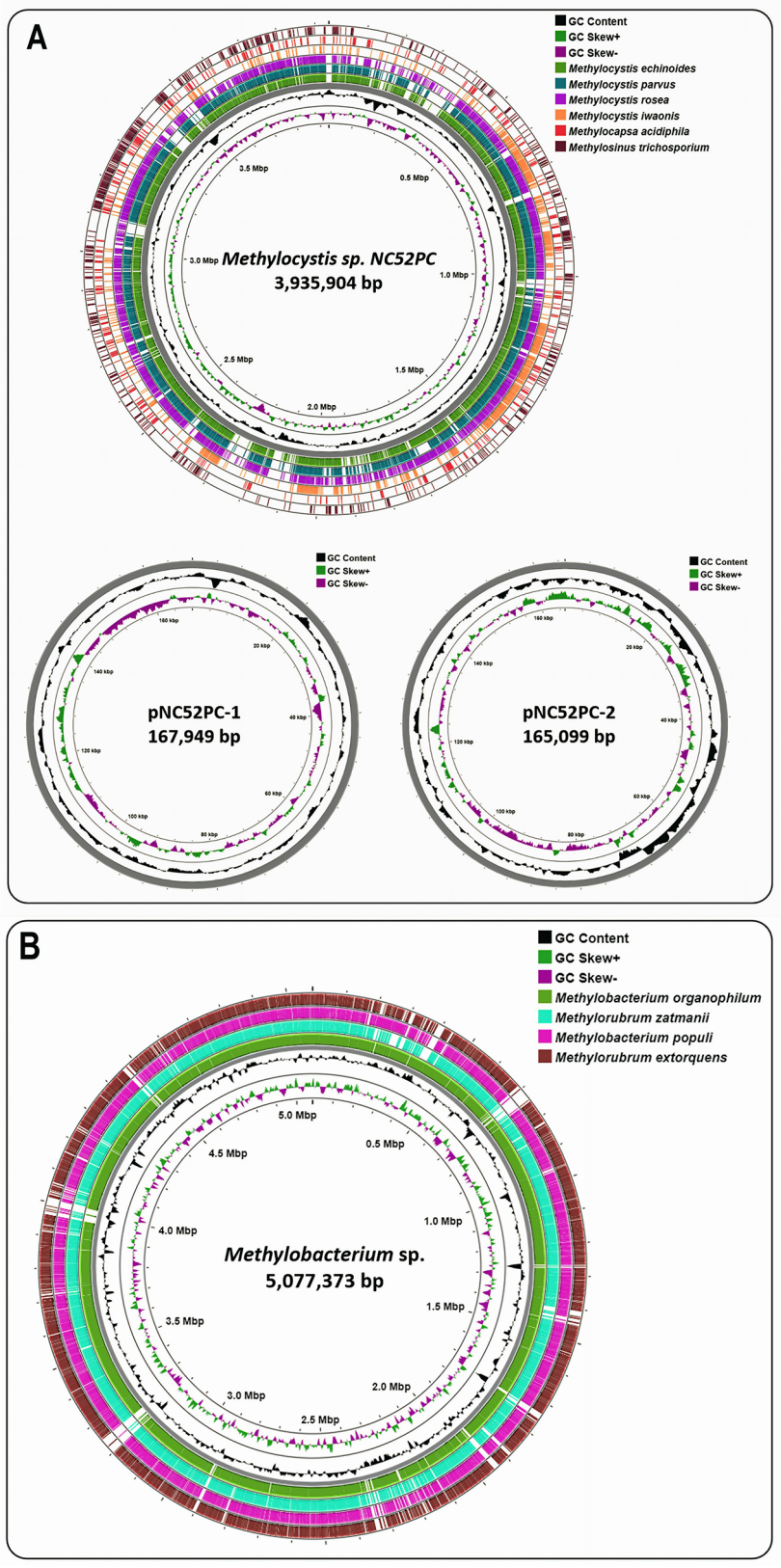

Fig. 3 (**A**) Circular chromosome map of the complete genome of *Methylocystis* sp. and its two plasmids, including genome comparison with its closely related species. The innermost rings show GC skew (green -, purple +) and GC content (black). The rings and colors in the legend represent the closely related strains used for comparison with *Methylocystis* sp. from NC52PC. (**B**) Circular chromosome map of the complete genome of *Methylobacterium* sp. from NC52PC compared against its closely related species

Table 3 Genome comparison between *Methylocystis* sp. and *Methylobacterium* sp. from NC52PC with other closely related species. IsDDH: in Silico DNA-DNA hybridization, ANI: average nucleotide identity, and AAI: average amino-acid identity

NC52PC	Closely related members*	isDDH (%)	ANI(%)	AAI(%)
Methylobacterium sp.	*Methylobacterium organophilum* WPA^T	88.5 [87.6–90.3]	98.59	98.79
	Methylorubrum populi BJ001^T	26.6 [25.4–28.0]	81.68	78.16
	Methylorubrum extorquens AM1^T	24.5 [23.9–25.1]	80.77	77.58
	Methylorubrum zatmanii LMG 6087^T	28.2 [27.7–29.2]	80.75	77.48
Methylocystis sp.	*Methylocystis echinoides* LMG 27198^T	25.76 [24.6–27.2]	81.07	81.35
	Methylocystis iwaonis JCM 34278^T	23.66 [22.8–24.4]	80.39	80.42
	Methylocystis parvus OBBP^T	23.9 [23.4–24.7]	80.02	80.46
	Methylocystis rosea SV98^T	19.2 [17.8–21.9]	77.22	74.67
	Methylosinus trichosporium OB3b^T	18.5 [15.8–21.8]	75.97	69.36

*^{T}, Type strain

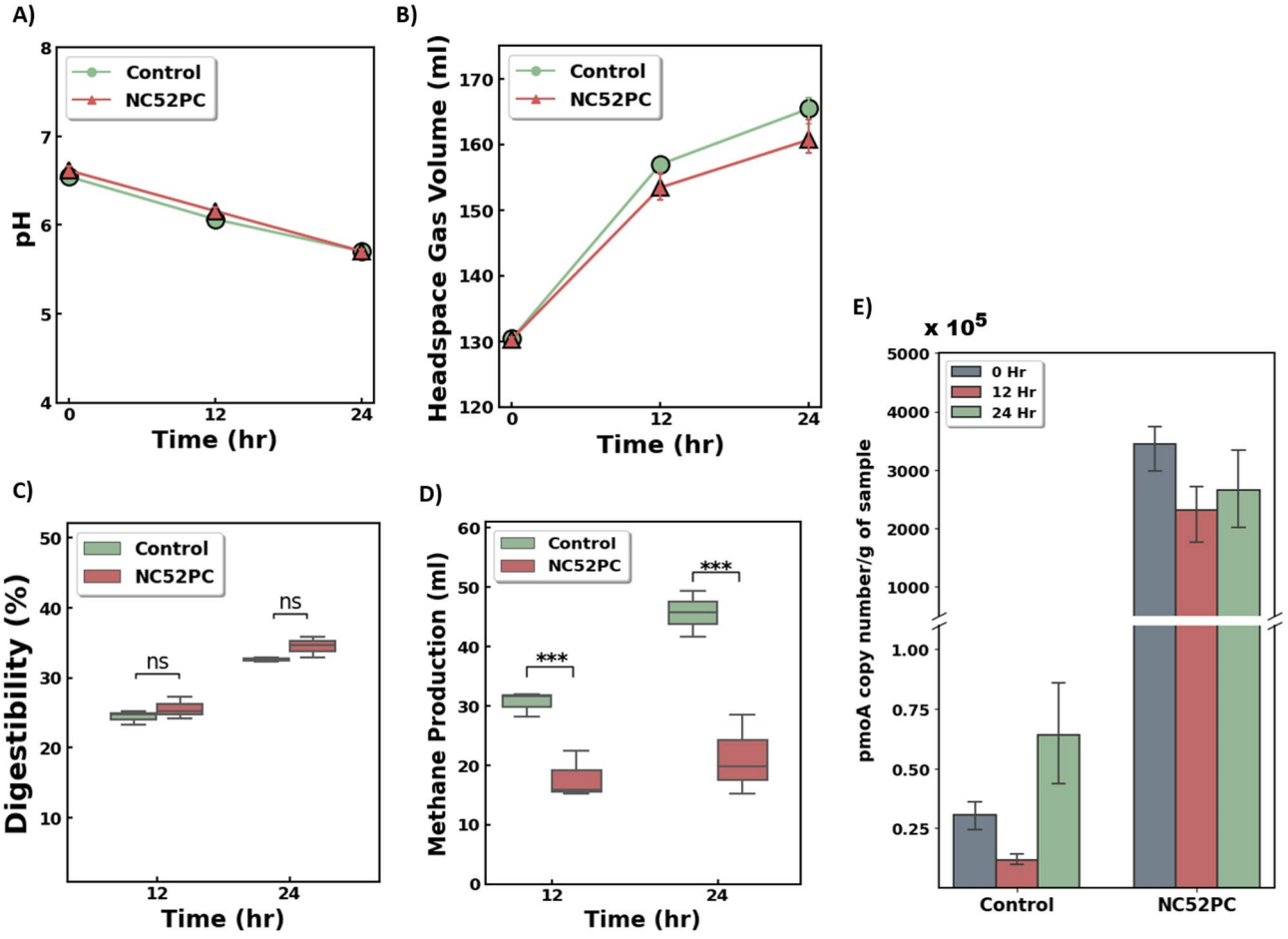

Fig. 4 The pH, and headspace gas volume in control and NC52PC inoculated sample at 0, 12 and 24 hours (**A** and **B**). Digestibility and methane production between control and NC52PC inoculated samples after 12 and 24 hours (**C** and **D**). *pmoA* copy number for methanotroph population between control and NC52PC samples (**E**). "ns" indicates not significant ($p > 0.05$), whereas the asterisk indicates significant difference ($p < 0.05$)

Archaeal community in the 24-hour samples for both control and NC52PC were dominated mainly by the *Methanobrevibacter* genus (78% in Control and 86% in NC52PC), followed by *Methanomassiliicoccus* and *Methanosphaera* (Fig. 5C and D). The addition of NC52PC did not negatively impact the methanogenic community as *Methanobrevibacter*, the dominant methanogenic archaea in the rumen, increased in relative abundance after 24 h. For the fungal community, three phyla, *Neocallimastigomycota*, *Ascomycota*, *and Basidiomycota*, *were dominant*, contributing over 80% of all fungal ASVs in both control and NC52PC samples (Fig. 5E). In particular, *Neocallimastigomycota*, which hosts an array of enzymes involved in lignocellulosic degradation

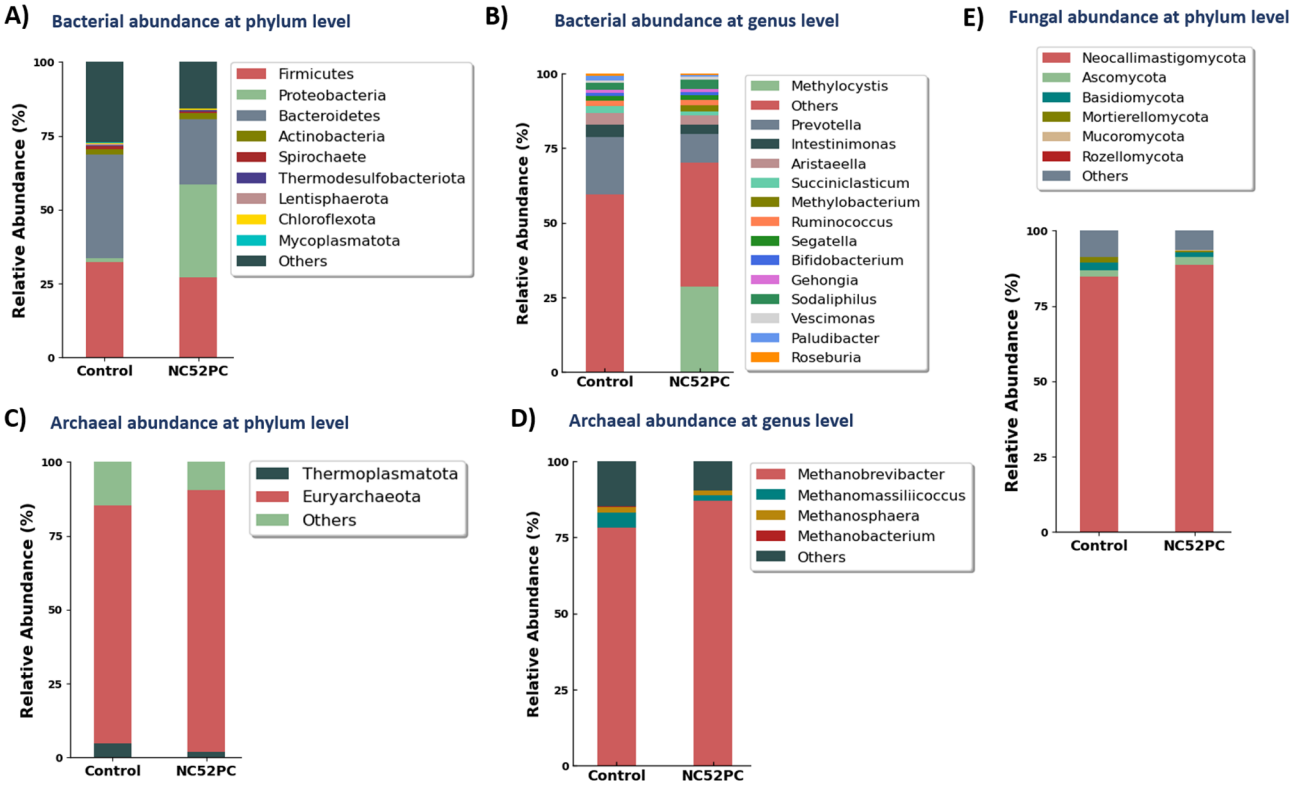

Fig. 5 Taxonomy analysis of in vitro rumen fermentation samples. Relative abundance at phylum level for bacteria (**A**), archaea (**B**), and fungi (**C**). Relative abundance at genus level for bacteria (**D**) and archaea (**E**)

[31], slightly increased, which could indicate a slight boost in feed digestion.

Analysis of the alpha diversity indicated that bacterial species diversity (Shannon index) was significantly higher for the control sample than NC52PC in both 0 and 24-hour samples (Fig. 6A), while the species richness (Chao 1 index) was not significantly impacted (Fig. 6D). Moreover, NC52PC significantly affected archaeal species richness as the Chao 1 index was substantially lower in NC52PC than in control samples (Fig. 6E) while not affecting archaeal diversity (Fig. 6B). However, there were no significant changes in both bacterial and archaeal populations in the NC52PC inoculated 0-hour sample and 24-hour sample (Fig. 6), indicating the differences existed from the beginning of the experiment. Also, the differences in the diversity and richness of the fungal species remained non-significant (Fig. 6C F) ($p > 0.05$).

In vivo rumen fermentation

The rumen fermentation characteristics, such as pH, ammonia-nitrogen, and volatile fatty acid production, are provided in Table 4.

Production of acetate, propionate, butyrate, and total volatile fatty acid (VFA) for the CON was slightly lower compared to the LOW and HIGH treatments ($p > 0.05$). This may have resulted in lower ruminal pH for the NC52PC treated LOW (6.30) and HIGH (6.29) than CON (6.55). Both LOW and HIGH had no significant impact on ammonia content and A:P ratio. Overall, total weight gain (kg), average daily gain (ADG), total DMI, and feed efficiency did not differ significantly among the treatment groups ($p > 0.05$) (Table 5).

Most importantly, CH_4 emission (g/d), CH_4 yield (g/ kg DMI), and CH_4 intensity (g/kg $BW^{0.75}$) were significantly higher in CON than in LOW and HIGH NC52PC treated steers ($p < 0.05$) (Fig. 7A and B, C). This methane emission over a duration of three test periods is consistent with the *pmoA* copy number in both rumen fluid as well as fecal samples, as the *pmoA* gene copy number was significantly higher in LOW and HIGH-treated groups as compared to the CON group (Fig. 7D). Total methane emissions were reduced by 12% in the LOW group and by 14.4% in the HIGH group compared to the CON group.

Additionally, levels of other greenhouse gases, such as CO_2, remained consistent across all treated cows (Table 6).

Discussion

Ruminal microorganisms play an important role in the metabolic processes of ruminants by breaking down complex feedstuffs into volatile fatty acids, which

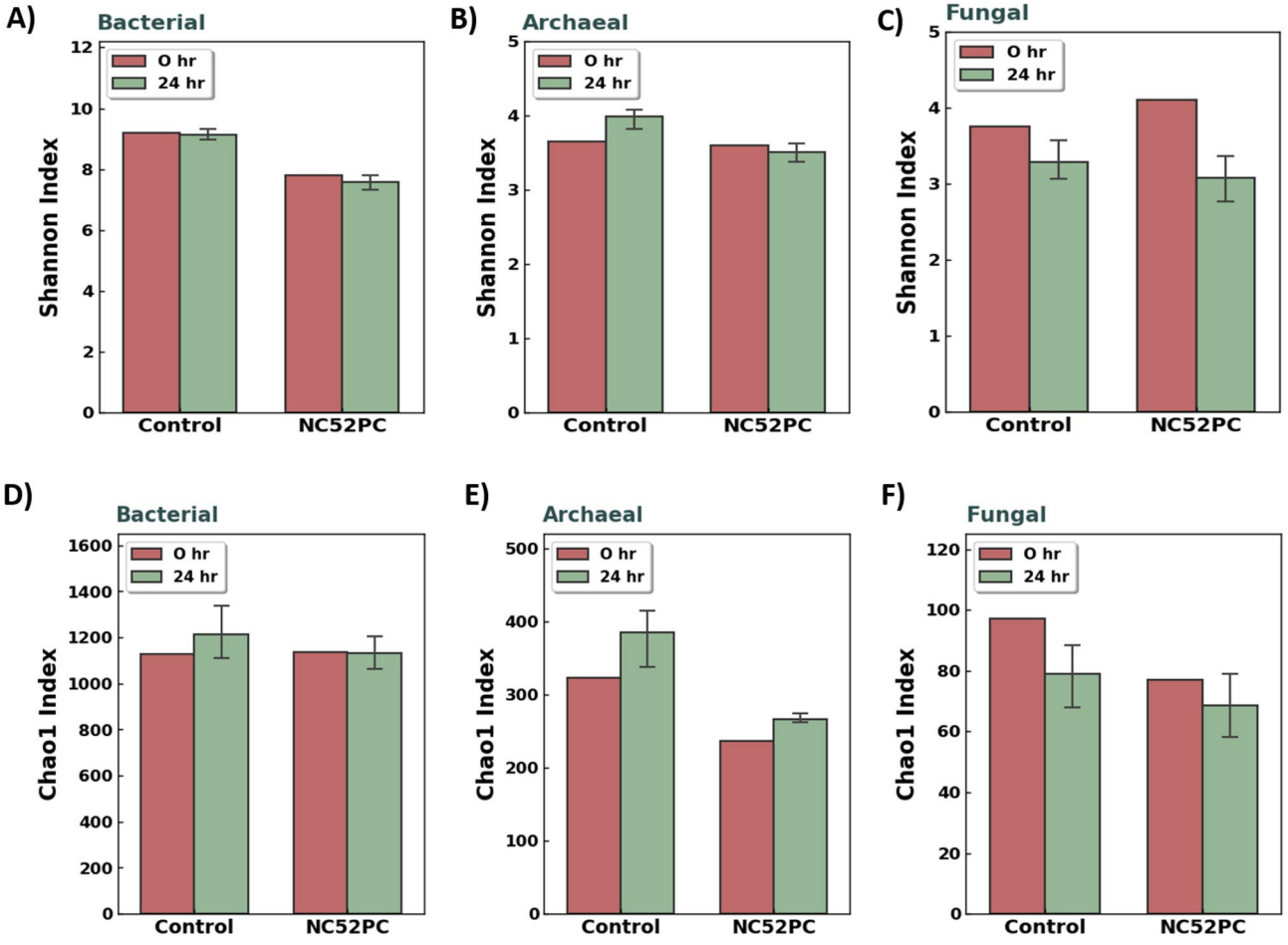

Fig. 6 Alpha-diversity for in vitro rumen fermentation samples. Shannon indices for bacteria (**A**), archaea (**B**), and fungi (**C**). Chao 1 indices for bacteria (**D**), archaea (**E**), and fungi (**F**)

Table 4 Effects of methanotroph-based probiotic on pH, ammonia-nitrogen, and volatile fatty acid production

Item	Treatments[1]			SEM[2]	*p* value
	CON	LOW	HIGH		
pH	6.55[a] ± 0.22	6.30[b] ± 0.23	6.29[b] ± 0.24	0.067	0.0157
NH_3-N (mg/dL)	3.89 ± 2.53	3.65 ± 2.14	3.32 ± 2.32	0.673	0.8388
Acetate (mmol/L)	57.93 ± 10.68	62.35 ± 7.66	65.75 ± 8.05	2.539	0.1110
Propionate (mmol/L)	18.74 ± 4.65	22.10 ± 6.21	22.48 ± 5.07	1.533	0.1848
Butyrate (mmol/L)	34.70 ± 20.20	41.13 ± 19.03	49.32 ± 29.52	6.616	0.3207
Total VFA (mmol/L)	111.37 ± 33.12	125.88 ± 22.14	137.55 ± 33.04	8.496	0.1150
A: P[3]	3.17 ± 0.59	2.93 ± 0.44	3.03 ± 0.61	0.157	0.5830

Data are expressed as mean ± standard deviation (SD). [a, b]Means ($n = 12$) within a row with different superscripts differ ($p < 0.05$). [1]Treatments: CON, basal diet (0.2% wheat bran); LOW, basal diet with 0.2% NC52PC (3×10^7 CFUs/ml); HIGH, basal diet with 0.2% NC52PC (3×10^8 CFUs/ml). [2]SEM, standard error of the mean. [3]A:P, acetic to propionate ratio

provide up to 70% of the ruminant's energy requirements [32]. Methane is generated as a byproduct of this microbial fermentation process that not only contribute to anthropogenic greenhouse gas emissions and enlarge the carbon footprint of dairy or beef production but also deplete nutritional energy [8, 33]. Various strategies have been explored to reduce enteric methane emission. Here, we exploited the potential of methane metabolizing microbes to mitigate methane emission in ruminants. Methanotrophs are ubiquitous in either anoxic or aerobic environments and have been previously enriched but were never applied in vitro or in vivo rumen fermentation systems. The cannulated Holstein Friesian cows were used in the in vitro setting of this study, as rumen cannulation is widely recognized as the reference method

Table 5 Effects of methanotrophic bacteria supplementation on the growth performance of Hanwoo steers

Item	Treatments[1]			SEM[2]	*p* value
	CON	LOW	HIGH		
Initial body weight (kg)	462.67 ± 47.78	464.75 ± 47.27	461.92 ± 40.98	13.09	0.9876
Final body weight (kg)	478.83 ± 48.23	480.83 ± 50.25	477.83 ± 41.92	13.51	0.9874
Weight gain (kg)	16.17 ± 2.52	16.08 ± 4.19	15.92 ± 3.09	0.94	0.9827
ADG (kg)[3]	0.66 ± 0.10	0.66 ± 0.18	0.65 ± 0.13	0.04	0.9779
Total DMI (kg/d)[4]	9.91 ± 0.48	10.03 ± 0.21	10.09 ± 0.38	0.10	0.5032
Feed efficiency[5]	0.07 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.00	0.9833

Data are expressed as mean ± standard deviation (SD). [1]Treatments: CON, basal diet (0.2% wheat bran); LOW, basal diet with 0.2% NC52PC (3×10^7 CFUs/ml); HIGH, basal diet with 0.2% NC52PC (3×10^8 CFUs/ml). [2]SEM, standard error of the mean. [3]ADG, average daily gain. [4]DMI, dry matter intake. [5]Feed efficiency was calculated as ADG divided by average DMI

for obtaining representative samples of rumen digesta from donor animals [34, 35]. For the in vivo experiments, the oral stomach tube technique was employed on Hanwoo steers, as this method was suitable for collecting liquid fractions only, whereas sampling via rumen cannula allows for the collection of both solid and liquid digesta fractions [36]. In this study, aerobic methanotrophs were isolated from a rumen sample. The aerobic methanotrophs are likely present due to oxygen entering the rumen via diffusion across the epithelium [37]. After a series of sub-culture and repetitive re-streaking on agar plates, colonies were transferred to a polycarbonate membrane to ensure purity and minimize heterotrophic contamination. Among three isolates, NC52PC robustly grew at 39 °C, making it the best candidate for further characterization and testing under in vitro and in vivo rumen fermentation setup.

Further morphological and genomic analysis revealed that NC52PC consisted of two bacterial strains; one belonging to methanotrophic group (*Methylocystis* sp. NC52PC) and other belonging to methylotrophic group (*Methylobacterium organophilum* NC52PC). Methanotrophs and methylotrophs have often coexist in nature [38]. Methylotrophs can metabolize the excess methanol formed from the methane oxidation of methanotrophs, thereby reducing methanol toxicity and enhancing the growth of methanotrophs [39]. There is also a possibility of essential nutrient exchange between *Methylocystis* and *Methylobacterium* species that can drive the overall growth performance of this consortium [40].

Under anaerobic in vitro rumen fermentation, NC52PC decreased methane production by approximately 50% after 24 h of incubation. This substantial suppression of generated methane in vitro makes NC52PC a potential candidate for in vivo testing. However, since NC52PC primarily requires oxygen for growth and methane oxidation, methane reduction was tested in NMS-Cu using 20% methane under anaerobic conditions to confirm further NC52PC's ability to grow and consume methane without oxygen. Similar to the in vitro fermentation test, we inoculated NC52PC in NMS-Cu media with the final concentration of 5×10^7 CFUs/mL. Results show that methane concentration reduced from 20 to 18% after 48 h, indicating NC52PC's ability to oxidize approximately 2% (equivalent to 20,000 ppm) methane in 48 h under anaerobic conditions (Figure S4). This highlights the versatility of aerobic methanotrophic NC52PC, such as its ability to oxidize methane despite the steady lack of oxygen supply. Oxidation of methane by aerobic methanotrophs under an anaerobic environment is possible by exploiting other alternative electron acceptors in the rumen content. Members of the *Methylomonadaceae* and *Methylocystaceae* family have been shown to utilize nitrate/nitrite- or mineral oxide-dependent methane oxidation under oxygen limitation [41–44]. NC52PC may have evolved to utilize denitrification or mineral reduction processes in an anoxic environment such as rumen. Finally, we assessed the efficacy of NC52PC in reducing methane emission in Hanwoo steers for a 3-cycle 30-day period. NC52PC when fed as a methanotroph-based probiotic at a concentration of 3×10^8 CFUs/ml significantly lowered methane emission by 14.4% compared to the control group without negatively impacting animal growth. Although methane reduction exceeded 50% during in vitro rumen fermentation, the in vivo experiment showed only about a 14% reduction. This discrepancy may be due to the amount of methanotrophs supplied in vivo, which was roughly 1,000 times less, considering the rumen size and the final methanotroph concentration. The *pmoA* gene copy number observed in vitro (Fig. 4E) compared to in vivo rumen fluid samples (Fig. 7D) further highlights the significant difference in methanotroph concentration. We hypothesize that matching in vivo concentrations to in vitro levels could significantly boost methane consumption. Future studies will focus on optimizing delivery methods and dosages to achieve these higher in vivo concentrations and investigate the kinetics of NC52PC in the complex rumen environment, including factors such as passage

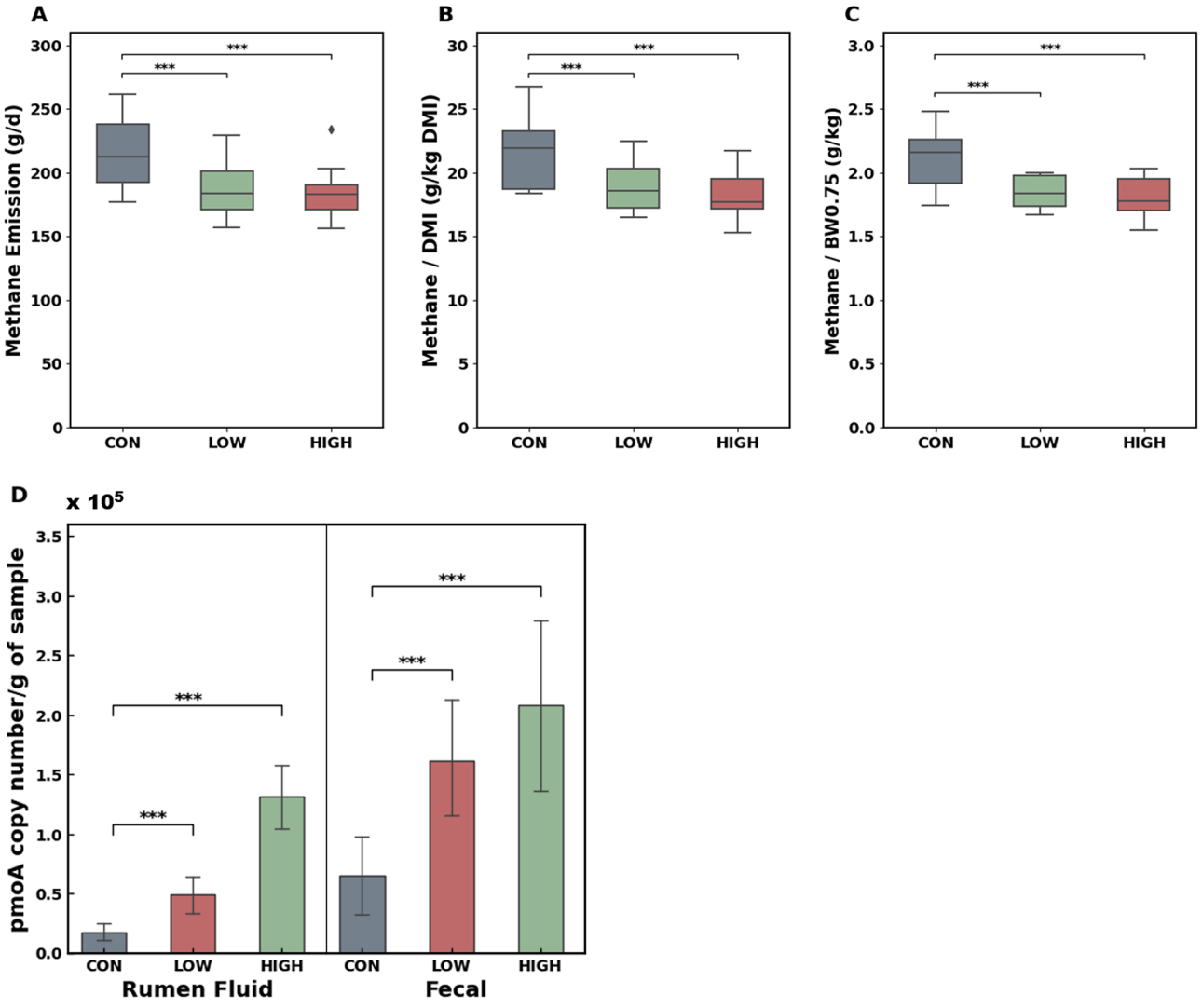

Fig. 7 Effect of low and high concentrations of methanotroph-based probiotics (NC52PC) on methane emission over three *in vivo* test periods. (**A**) Methane emission (g/d), (**B**) Methane/DMI (g/kg DMI), and (**C**) Methane /$BW^{0.75}$(g/kg), (**D**) Methanotroph population (*pmoA* gene copy number) in rumen fluid and fecal samples

Table 6 Effects of methanotrophic bacteria supplementation on the greenhouse gas emission

Item	Treatments[1]			SEM[2]	*p* value
	CON	LOW	HIGH		
DMI (kg/d)[3]	9.97 ± 0.40	9.98 ± 0.33	10.19 ± 0.52	0.121	0.3606
CH_4 (g/d)	215.13 ± 29.75[a]	189.23 ± 23.49[b]	184.19 ± 21.44[b]	7.186	0.0106*
CH_4/DMI (g/kg DMI)	21.59 ± 2.88[a]	18.93 ± 2.07[b]	18.06 ± 1.81[b]	0.651	0.0019*
CH_4/$BW^{0.75}$ (g/kg)	2.11 ± 0.24[a]	1.85 ± 0.13[b]	1.81 ± 0.04[b]	0.05	0.0006*
CO_2 (g/d)	9,437 ± 1,523	9,263 ± 1,772	9,056 ± 1,162	429	0.8250

Data are expressed as mean ± standard deviation (SD). [a, b]Means ($n = 12$) within a row with different superscripts differ ($p < 0.05$). [1]Treatments: CON, basal diet (0.2% wheat bran); LOW, basal diet with 0.2% NC52PC (3×10^7 CFUs/ml); HIGH, basal diet with 0.2% NC52PC (3×10^8 CFUs/ml). [2]SEM, standard error of the mean. [3]DMI, dry matter intake

rate and competition with other microbial populations. This methanotroph-based probiotic holds immense potential as a sustainable feed additive to effectively reduce methane emissions from ruminants. However, the evaluation of long-term effects of NC52PC on animal health and productivity will be our future goal.

Conclusion

This research aims to provide a novel approach by utilizing methanotrophs as potential probiotics to primarily reduce enteric methane emissions without negatively impacting the ruminal ecosystem. Our results show that methane emission was reduced by over 14% when 12 Hanwoo steers were administered with 3×10^8 CFUs/mL of methanotroph-based probiotics for two weeks without adversely impacting overall animal health. To the best of our knowledge, this is the world's first study on the isolation of methanotrophs from the rumen, and the successful application of methanotroph-based probiotics to reduce methane emission in cattle. The methanotroph-based probiotics hold tremendous potential to mitigate methane emissions from ruminants and could serve as a promising feed additive to combat climate change. Despite a significant methane reduction, further study is required to evaluate the long-term effect of methanotroph-based probiotics on methane emission and overall animal productivity.

Methods

Enrichment and isolation of methanotrophic consortia

A rumen sample was collected from an adult (*Bos taurus*) Hanwoo steer and then immediately added into a sterile nitrate mineral salts media (ATCC medium: 1306) supplemented with 10mM $CuCl_2$ (NMS-Cu), and incubated for 1 week inside a serum bottle at 30 °C with a headspace of 20:80 methane/air mixture. After 1 week, the enrichment was diluted by a 1:10 ratio of fresh NMS-Cu media and incubated using the same conditions mentioned above for another week. This procedure has been repeated for 8 weeks to ensure the enrichment of methanotrophs while reducing the possibility of heterotrophs from growing.

DNA extraction and quantitative PCR analysis

To monitor the presence of methanotrophs, DNA was extracted weekly from the liquid culture during the enrichment process and was screened by PCR using the *pmoA* gene and methanotroph 16S rRNA-specific primers (Table S1) [45–47]. All DNA extraction was performed using FastDNA spin kit for soil (MP Biomedicals, USA). Primers A189f and mb661R were used for quantitative PCR assay, according to Sabrekov et al., using the protocol directed by QuantiNova SYBR Green PCR Kit (Qiagen, Germany) [48].

Growth characterization

Growth experiments were conducted in 120 ml serum bottles containing 30 ml of medium NMS-Cu. Vials were capped air-tight with butyl rubber stoppers, and 20% (v/v) CH_4 and 80% air (v/v) were added. The same methane: air mixture was used in all growth experiments. Cultures were incubated on a rotary shaker at 180 rpm. The growth rate and doubling time of three isolates (NC52PC, NC75PC, NC77PC) were determined under a rumen temperature of 39°C. Growth was observed by absorbance (OD_{600}) on the Genesys 150 spectrophotometer (Thermo Scientific). All tests were performed in triplicate.

Morphological characterization

Bacterial culture was fixed and processed to observe under Scanning Electron Microscopy (SEM) (Thermo Apreo S LoVac SEM) [49, 50].

Genomic characterization

High molecular weight genomic DNA was extracted from NC5PC using Promega's Wizard® HMW DNA extraction kit according to the manufacturer's instructions. Bacterial genome sequencing was performed using a combination of Oxford Nanopore Technologies long reads (ONT) and Illumina short reads sequencing technology (NovaSeq6000) for enhanced accuracy and completeness. Assembled genomes were annotated using Prokka version- 1.14.6 [22]. A circular chromosome map of the two complete genomes and two circular plasmids was generated using the Proksee tool [23]. To further confirm their taxonomic position, in silico DNA-DNA hybridization (isDDH), average nucleotide identity (ANI), and average amino acid identity (AAI) were calculated. The isDDH, ANI, and AAI values were also calculated against closely related species using the Type (Strain) Genome Server [25], OrthoANIu algorithm [24], and EzAAI tool from EZBioCloud [26], respectively.

In vitro rumen fermentation

Two ruminal cannulated Holstein-Friesian cows (874 ± 69 kg body weight, 8 years old) were used to supply ruminal fluid for in vitro rumen fermentation. Ruminal contents were collected in a thermal bottle before morning feeding and transported immediately to the lab. It is then squeezed and strained through four layers of surgical gauze and pooled in an amber bottle. Subsequently, nitrogen purging was performed directly for 30 min. which was then capped and stored to maintain temperature at 39 °C.

Filtered rumen fluid was then mixed with the Asanuma buffer at a ratio of 1:3 (v/v) while maintaining an anaerobic environment [51]. Thirty milliliters of the buffered rumen fluid mixture was dispensed into a 160mL serum bottle under a stream of pure nitrogen gas. Each serum bottle contains 0.3 g of a substrate composed of 80% bluegrass and 20% concentrate feed that were milled to pass through a 1 mm sieve; substrates were then placed in a nylon bag, which was later heat-sealed. This in vitro fermentation was performed using a batch technique consisting of two experimental sets performed simultaneously, wherein one set was inoculated with 5×10^7 CFUs/mL NC52PC and the control set without NC52PC inoculation. Each set consists of 3 replicates per time point at 0, 12, and 24 h of incubation. After combining buffered rumen fluid, substrate, and NC52PC inoculum (w/o inoculum in the control set), the serum bottles were further bubbled with nitrogen for 15 min, which were subsequently capped with a butyl rubber, then hermetically sealed and placed in a shaking incubator at 100 rpm and 39 °C condition.

Total gas production in each bottle during 0, 12, and 24 h of incubation was recorded using the pressure transducer technique [52]. Headspace gas (10mL) was collected from each bottle using a syringe equipped with a 2-way stopcock and moved into air-evacuated gas vials. Methane concentration was determined by gas chromatography equipped with a flame ionization detector (YL Instrument 6500GC System, Korea). A 10mL liquid culture sample was collected from 0, 12, and 24 h of incubations, then immediately frozen at -80 °C for DNA extraction and microbial community analysis later. The pH was measured using a pH meter (LaquaTwin, Horiba, UK). After the incubation periods, the nylon bags containing residual feed were rinsed with cold tap water and placed to dry in a forced-air oven at 80°C for 48 h. Once the bags were dried, they were cooled to room temperature and weighed. Dry matter digestibility was calculated by subtracting the weight after drying the nylon bags from the initial weight [53].

Microbial community analysis

During in vitro fermentation, total genomic DNA was extracted from liquid samples using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA), following the manufacturer's protocol. The integrity and concentration of the extracted DNA were assessed using 1% agarose gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Amplicons of the V3-V4 and V5-V6 regions of the 16S rRNA gene were selected for bacterial and archaeal community analysis, while the ITS2 region was chosen for fungal community analysis. Following purification and quantification, amplicons from all samples were pooled in equimolar concentrations and sequenced using an Illumina MiSeq platform at Macrogen Co., Ltd (Seoul, South Korea).

Animal experimental design

The study was conducted at the Sunchon National University (SCNU) Smart Farm and Greenhouse Gas Research Demo Farm, with approval from the Institutional Animal Care and Use Committee (approval number: SCNU-IACUC-2022-06). Twelve Hanwoo steers (*Bos taurus*; 15 months old, initial body weight 448 ± 43 kg) were used in a four-replicate 3×3 Latin square design with three 29-day experimental periods. The experimental design followed a cyclical pattern consisting of four distinct phases: a 17-day feeding period (including a 3-day measurement period for methane and carbon dioxide); a 1-day rumen fluid collection and weighing period, and an 11-day washout period (Fig. 8). During the washout period, steers received the same diet without any feed supplements to ensure the complete elimination of any residual microbes from the previous cycle.

Animals were initially grouped by body weight and then randomly allocated to one of the three treatments with varying feed supplements: control (**CON**), 20 g of wheat bran mixed with 20 ml of NMS-Cu medium only (without NC52PC cells); low concentration of NC52PC (**LOW**), 20 g of wheat bran mixed with 20 ml of NC52PC at final concentration of 3×10^7 CFUs/ml; and higher concentration of NC52PC (**HIGH**), 20 g of wheat bran mixed with 20 ml of NC52PC at final concentration of 3×10^8 CFUs/ml. Steers were fed a consistent forage-to-concentrate ratio of 70:30 for 17 days per period, with each animal receiving a total of 12.0 kg of feed, with bluegrass serving as the forage (Figure S1). The detailed nutritional composition is provided in Table S2. All treatment mixes were prepared weekly, refrigerated, and thoroughly mixed into the daily feed rations. Feeding occurred four times a day (05:00, 09:00, 13:00, 18:00 h), with the supplement additive provided at 09:00 am. Dry matter intake, total weight gain, average daily gain, and feed efficiency were calculated to analyze animal performances. Feed efficiency was calculated by dividing the average daily gain by the average DMI.

Measuring CH4 emissions

Enteric methane (CH_4) emissions were assessed using a GreenFeed (GF) unit (C-Lock, Rapid City, SD, USA), following the methodology outlined by Hristov with minor adjustments (Figure S2) [54]. Before the commencement of the experiment, all steers underwent training to acclimate themselves to the GF unit, minimizing potential

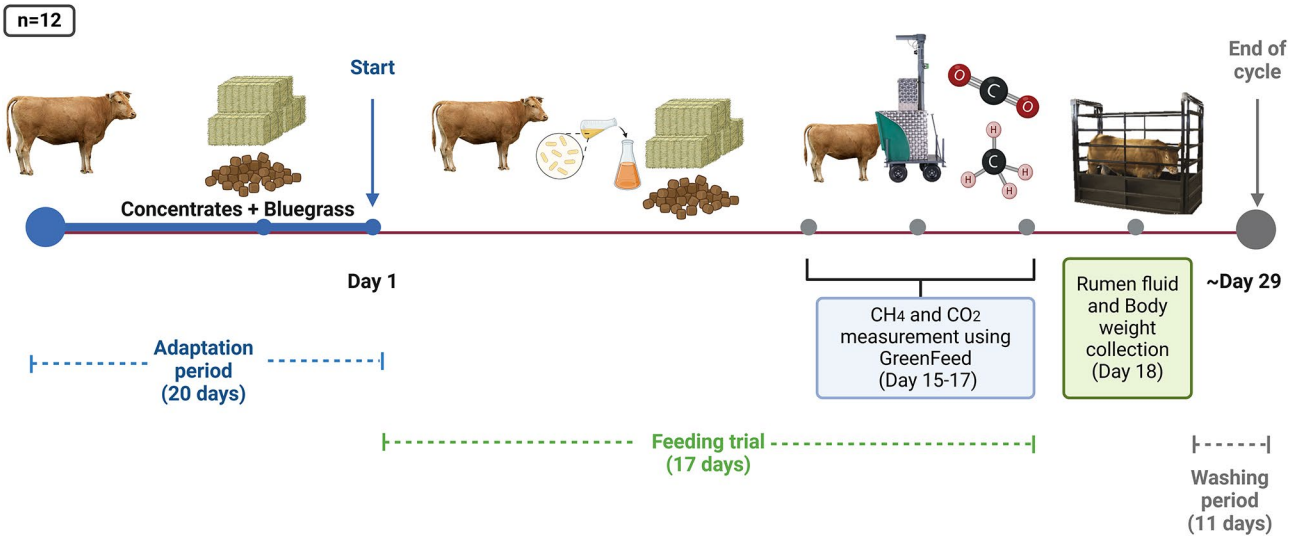

Fig. 8 The systematic representation of the study design

psychological stress. CH_4 and CO_2 emissions were monitored at eight different intervals (Hours: 0 before feeding time and 2, 4, 6, 9, 12, 15, and 21 after feeding time) over three consecutive days during each measurement period. The GF unit was in a separate pen where the steers measured one at a time, sequentially moving from their pens to the GF unit. To encourage the steers to approach the GF unit, concentrated pellets (250–300 g/visit) were used as bait, and the correct head-down position within the hood was ensured for accurate measurements. All relevant data were transmitted to C-Lock, including the times of animal entry and exit times from the GF unit, standard gas calibration details, CO_2 recovery timing, and gas release measurements. CH_4 and CO_2 production (g/d) data were computed using a web-based data management system [55]. CH_4 yield (g/kg DMI) and CH_4 intensity (g/d/kg $BW^{0.75}$) were also determined. Methane intensity was calculated by dividing methane emission (g) by metabolic body weight $BW^{0.75}$ (kg).

Rumen fluid sample collection and rectal temperature measurement

During each period, rumen fluid was obtained from each steer using an oral stomach tube before the 11-day washout. The initial 300 mL of rumen fluid was discarded to prevent contamination from saliva, and 50 ml of fresh rumen fluid from each animal was retained (Figure S3). Immediately following collection, ruminal pH was assessed using a pH meter (SevenCompactTM pH/Ion meter S220, Mettler Toledo, Greifensee, Switzerland). Subsequently, three separate aliquots were prepared from each rumen fluid sample and transported to the laboratory with dry ice. For subsequent analysis of parameters including ammonia nitrogen (NH_3-N), volatile fatty acids (VFA), and rumen microbiota, these aliquots were stored at -80°C. Additionally, the steers' rectal temperature (RT) was measured on the same day as the rumen fluid collection using a digital thermometer (WPT-1, CAS, Yangju, Korea).

Analyses of ruminal NH3-N and volatile fatty acid concentrations

NH_3-N concentration was measured using a UV-visible spectrophotometer (Genesys 180, Thermo Fisher Scientific Inc.) according to the protocol described by Chaney and Marbach [56]. VFA concentration was measured using high-performance liquid chromatography (HPLC; Agilent Technologies 1200 series, Agilent Technologies, Waldbronn, Germany) according to the protocol described by Han et al. [57]. To perform HPLC, a UV detector (set at 210 nm and 220 nm), METACARB87H column (Varian, Palo Alto, CA, USA), and buffered solvent (0.85% N H_2SO_4; at a flow rate of 0.6 mL/min) were used.

Calculations and statistical analyses

All data for animal growth performances, methane and carbon dioxide emissions, and rumen fluid parameters such as volatile fatty acids (VFA) were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC, USA). This procedure accounts for both fixed and random effects in the model. The model used for the analysis was expressed as:

All data for animal growth performances, methane and carbon dioxide emissions, and rumen fluid parameters such as volatile fatty acids (VFA) were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC, USA). This procedure accounts for both fixed and random effects in the model [58, 59]. The model used for the analysis was expressed as:

$$Y_{ijk} = \mu + T_i + \beta_i + \gamma_k + \epsilon_{ijk}$$

Where:

γ_{ijk} was the observed response for the *k*-th observation in the *j*-th treatment group for the *i*-th fixed effect (ex: growth parameters, emissions, or VFA). μ is the overall mean response. τ_i represents the fixed effect of the *i*-th treatment (ex: the effect of different treatments). βj is the random effect associated with the *j*-th factor (ex: animal), accounting for animal-specific variation. γ_k represents fixed effects for other covariates (ex: period, time, or other factors influencing the response). ϵ_{ijk} is the residual error term in the observations.

Post-hoc comparisons between treatment means were performed using Duncan's multiple range test (DMRT) to assess the significance of differences between the groups at a 5% significance level.

Abbreviations

DFM	Direct Fed Microbials
CH_4	Methane
CO_2	Carbon dioxide
NH_3	N–Ammonia nitrogen
RT	Rectal temperature
VFA	Volatile fatty acids
NMS	Cu–Nitrate mineral salts + 10mM Copper chloride
DNA	Deoxyribonucleic acid
OD	Optical density
SEM	Scanning electron microscope
IsDDH	in silico DNA–DNA hybridization
ANI	average nucleotide identity
AAI	average amino acid identity
GF	GreenFeed
PCR	Polymerase Chain Reaction
GC	Gas chromatography
HPLC	High Performance Liquid Chromatography
DMI	Dry matter intake
DWG	Daily weight gain
$BW^{0.75}$	Metabolic weight
CFU	Colony Forming Unit
pmoA	particulate monooxygenase subunit A

Supplementary Information

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Supplementary Material 1

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Author contributions
T.T., R.A.S., S.S.L., and S.W.K. designed research; T.T., R.A.S., J.W.S., K.S.B., and J.I.B. performed research; S.H.K., S.H.Y., M.K.K., M.K, S.S.L., and S.W.K. analyzed data; and T.T., R.A.S., S.S.L., and S.W.K. wrote the paper.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The animal study was conducted at the Sunchon National University (SCNU) Smart Farm and Greenhouse Gas Research Demo Farm, with approval from the Institutional Animal Care and Use Committee (approval number: SCNU-IACUC-2022-06).

Consent for publication
Not applicable.

Competing interests
Authors declare that they have competing financial interests as the strains described in this paper are covered by patents in an application.

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