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Research article

Potency of Indian gooseberry peel supplementation for suppressing rumen methane production via alteration of rumen microbiota: Batch culture evaluations

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Abstract

Importance of the work: There is an urgent need to find feed additive candidates for mitigating enteric methane in ruminants for sustainable animal production.

Objectives: To evaluate Indian gooseberry peel (IGP) as one of the new additive candidates for rumen methane mitigation.

Materials & Methods: The methane-suppressing potency of IGP was assessed by measuring gas production in two *in vitro* studies using cattle rumen fluid. Studies were performed using different supplementation levels of IGP (0–20% in total substrate; experiment 1), and dietary substrate with different hay-to-concentrate ratios (33:67 and 67:33; experiment 2). The rumen microbial response to IGP supplementation was monitored based on MiSeq amplicon sequencing analysis.

Results: IGP supplementation at ≥5% suppressed methane production without affecting hydrogen gas and total short-chain fatty acid production. These changes were consistent, irrespective of differences in the hay-to-concentrate ratio, while the degree of methane suppression was in the range 10–14%. A simultaneous increase in lactate concentration was observed with IGP supplementation, with minimal changes in short chain fatty acid proportions. The MiSeq analysis revealed specific changes in rumen microbiota associated with IGP supplementation, with a decrease in Christensenellaceae R7 involved in hydrogen production and an increase in Lachnospiraceae NK3A20 related to lactate production being notable for bacteria, whereas a decrease in *Methanobrevibacter* and increases in *Methanosphaera* and *Methanomicrobium* were observed for methanogenic archaea.

Main finding: IGP could be a candidate feed to mitigate rumen methane production without depressing rumen digestive capability at 5–15% supplementation in the diet, suggesting microbial and metabolic changes.

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Introduction

Ruminant animals emit methane gas by belching, which accounts for 24% of global methane emissions (Jackson et al., 2020). Considerable efforts have been made to lower the emission of methane from the rumen, especially through the development of functional feed additives that act by manipulating rumen microbes and their fermentation patterns (Kobayashi et al., 2016). Chemicals such as 3-nitrooxypropanol (3-NOP; Hristov et al., 2015) and natural products, including marine red algae (Roque et al., 2019) and cashew nutshell liquid (Shinkai et al., 2012), are representative materials showing methane-inhibiting potential in the rumen, with efforts underway to apply these products in ruminant livestock production. For worldwide application, such additive materials must be satisfactorily supplied over a number of years. Furthermore, it is useful to have multiple additives to ensure a sustainable strategy for rumen methane mitigation, especially considering problems regarding the functional persistence of each additive for rumen methane mitigation over a long period of time and also considering seasonal limitations in supply, if the materials are natural products. When natural products are locally available, transportation costs must also be considered for a wider range of applications (Kobayashi et al., 2016).

The most important factor in the application of additive candidates is determining the mode of action. Farmers can readily accept an additive candidate once they understand how it works within the rumen. Therefore, candidate materials should be thoroughly assessed in terms of rumen metabolism, as was done for the ionophore antibiotic monensin, which was a popular additive in the 1980s (Bergen and Bates, 1984). Generally, rumen methane mitigation occurs either through direct inhibition of methanogens or via an indirect mode of inhibition of the bacteria involved in the generation of hydrogen and formate, which are substrates for methane synthesis (Russell and Strobel, 1989). Of the above listed additives, 3-NOP and marine red algae exhibit the former mode (direct inhibition; Hristov et al., 2015), whereas cashew byproduct exhibits the latter mode (indirect inhibition; Watanabe et al., 2010). By changing the rumen metabolism using these additives, propionate generally increases as a hydrogen sink alternative to methane (Ungerfeld, 2020).

To identify new additive candidates, other methane-suppressing agents have been targeted, including tropical fruits, especially their residues, which are known to contain plant secondary compounds, such as phenolics (Kamra et al., 2006), many of which have shown methane-decreasing potential (Kobayashi et al., 2016). Residues or byproducts of such fruits have been evaluated in both *in vitro* and *in vivo* studies, mainly in South and Southeast Asian countries. For example, rambutan peel and mangosteen peel (Paengkoum *et al*., 2015) were found to exhibit methane-suppressing ability, whereas Indian gooseberry (IG) has received recent attention for use of its pomace (a byproduct in the juice-making process) to suppress methane production in feeding studies using local buffaloes (Singla et al., 2021). IG pomace is thought to decrease ruminal methane by its componential phenolic acids, tannins, flavonoids and saponins (Variya et al., 2016). However, the details are unclear regarding the mode of action of IG in rumen metabolism and its effectiveness for use in other cattle besides local Indian breeds. In particular, the action of IG against rumen microbes is completely unknown. The present study focused on IG peel (IGP), another by product of IG, as a candidate additive material, expecting the accumulation of bioactive compounds soluble in the ethanol and methanol in the peel. In fact, enrichment of tannins and flavonoids have been reported in the peel of fruit (Wannes and Marzouk, 2012).

The objectives of the present study were to clarify the efficacy of IGP in suppressing rumen methane production and to elucidate the mode of action based on rumen fermentation and microbial analyses. The resulting data should drive further feeding trials to demonstrate the functionality of IG and its on-farm applications in the future.

Materials and Methods

Animals, feeds, sampling, and incubation

All procedures related to animal management and sampling described below were approved by the Animal Care and Welfare Committee of Hokkaido University.

Tested feed candidate IGP was separated by hand from Indian gooseberry (*Phyllanthus emblica*) purchased in a general market in Mueangm, Rachaburi, Thailand. The IGP was oven-dried (at 60°C) overnight, air-dried, ground-milled and used for incubation studies. The tested IGP consisted of 10.5% crude protein (CP) and 47.3% neutral detergent fiber (NDF) in dry matter (DM), as detailed in Table 1.

Table 1 Chemical composition of experimental diets

Component	Concentrate	Hay	Indian gooseberry peel
Crude ash	6.3	7.6	6.2
Crude protein	20.2	10.1	10.5
Ether extract	3.7	2.8	6.2
Neutral detergent fiber	19.2	64.8	47.3
Acid detergent fiber	10.7	39.7	22.0

All values are % as dry matter

The value of CP was lower, whereas that of NDF was higher compared to IG fruit pomace (32.0% CP, 41.9% NDF), according to Singla et al. (2021).

For batch culture, the rumen contents were collected before morning feeding from two rumen-fistulated Holstein dry cows (622 and 589 kg in body weight) fed 90% corn silage and 10% Timothy hay at the Experimental Farm, Field Science Center, Hokkaido University, Sapporo, Japan. The total diet consisted of 7.8–8.2% CP and 56.8–57.7% NDF (depending on the intake of each animal determined from daily allowance and refusal data). The collected rumen content from each animal was filtered through four layers of surgical gauze, equally mixed and then used as an inoculum after two-fold dilution with McDougal's buffer (Camacho et al., 2019).

Two *in vitro* experiments were performed using IGP for different purposes. Experiment 1 was conducted in a single factor design, using supplementation level as an independent variable, to determine the range of the supplementation level necessary to decrease methane production. Six different levels were examined (0%, 2.5%, 5%, 10%, 15% or 20% of IGP supplementation in the total feed substrate in DM). Then, the substrate consisted of a hay-to-concentrate ratio of 33:67 on a dry matter (DM) basis. Experiment 2 was conducted to assess the effect of substrate on methane suppression by IGP. Two substrates differing in their hay-to-concentrate ratios (67:33 and 33:67) were used to reflect different dietary feed conditions (a high-hay diet and a high-concentrate diet, respectively). The supplementation level of IGP was set at 0%, 5% or 10%. Therefore, the experiment was carried out in a two factorial design (2 substrates × 3 supplementation levels).

The substrate used in both *in vitro* experiments consisted of a mixture of Timothy hay-to-commercial concentrate (Shin-Yogyu Green; ZEN-NOH; Tokyo, Japan) at a ratio of 33:67 or 67:33, which was ground and passed through a 2.0 mm sieve. The chemical compositions of these feeds are shown in Table 1. The substrate and IGP (0.2 g in total as DM) was added to a screw-capped test tube and then the inoculum (10 ml) was added into each tube. The amount of IGP added was in the range 0–40mg/tube, based on the supplementation level (0–20% as DM). Then, the headspace of each tube was flushed with N_2 gas, and the tubes were fitted with a butyl rubber stopper and plastic cap. The tubes were incubated at 39°C for 24 hr, with each treatment tested in quadruplicate (n = 4) for experiment 1 and in quintuplicate (n = 5) for experiment 2.

After 24 hr incubation, gas and culture samples were used to determine the gas composition and fermentation and microbial profiles, respectively (see below).

Chemical and microbial analyses

The major nutrients of the experimental substrates were determined according to Association of Official Analytical Chemists (2016) for proximate components and Van Soest et al. (1991) for fiber components. Total gas production was determined using a pressure gauge with a needle (Aφ60B; GL Sciences; Tokyo, Japan), with the gas composition (H_2, CH_4 and CO_2) being analyzed as described by Watanabe et al. (2010) using a GC-8A gas chromatograph (Shimadzu; Kyoto, Japan) equipped with parallel Porapak Q columns (Waters; Milford, MA, USA), a molecular sieve 13X (Restek; Bellefonte, PA, USA) and a thermal conductivity detector. Short-chain fatty acids (SCFAs) were analyzed as described by Oh et al. (2017) using a GC-2025 gas chromatograph (Shimadzu; Kyoto, Japan) equipped with an ULBON HR-20M fused silica capillary column (0.53 mm in diameter, 30 m in length, 3.0 μm film; Shinwa; Kyoto, Japan) and a flame ionization detector. The lactate concentration was determined using a commercial assay kit (Megazyme; Bray, Ireland) according to the manufacturer's instructions. The pH of the cultures was measured using an electrode (pH meter F21; Horiba; Kyoto, Japan).

Microbial analysis was performed using 0 and 10% supplemented samples in experiment 2. Microbial DNA was extracted from frozen (-80°C) culture samples using the repeated bead-beating plus column method (Yu and Morrison, 2004) with a commercial kit (QIAmp DNA Stool Mini Kit; Qiagen; Hilden, Germany). The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA), after which the samples were diluted with Tris– ethylenediaminetetraacetic acid buffer to a final

concentration of 5 ng/μL. The diluted DNA was subjected to amplification of the V3–V4 region of the 16S rRNA gene using two primer sets: S-D-Bact-0341-bS-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-aA-21 (5'-GACTACHVGGGTATCTAATCC-3') for bacterial rRNA genes (Herlemann et al., 2011) and arch349F (5'-GYGCASCAGKCGMGAAW-3') and arch806R (5'-GGACTACVSGGGTATCTAAT-3') for archaeal rRNA genes (Takai and Horikoshi, 2000). The polymerase chain reaction (PCR) mixture consisted of 12.5 μL of 2× KAPA HiFi HotStart ReadyMix (Roche Sequencing; Basel, Switzerland), 5.0 μL of each primer (1.0 μM), and 2.5 μL of DNA. The PCR steps were performed according to the following program: initial denaturation at 95°C for 3 min, 25 cycles (for bacteria) or 30 cycles (for archaea) at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension step at 72°C for 5 min. Amplicons were purified using AMPure XP beads (Beckman-Coulter; Brea, CA, USA) and subjected to 2× 300-bp paired-end sequencing on an Illumina MiSeq platform (Illumina; San Diego, CA, USA) using an MiSeq Reagent Kit v3. The raw sequences were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) under accession number PRJNA972255.

The data obtained from amplicon sequencing using the MiSeq platform were analyzed using the QIIME2 version 2021.8.0 software (Bolyen et al., 2019). Paired-end reads were filtered, dereplicated, merged and chimera-filtered using the q2-dada2 plugin (Callahan et al., 2016) to generate amplicon sequence variants (ASVs). The taxonomic classification of the ASVs was performed at the phylum, class, order, family and genus levels using the SILVA 132 99% operational taxonomic units, full-length, seven-level taxonomy classifier (silva-132-99-nb-classifier.qza). The sequence data were exported to the R program, version 4.2.2 (R Core Team, 2022), for further analyses. The ASV and taxonomy tables generated using QIIME2 were imported into R and merged with the sample metadata using the Phyloseq Bioconductor package (McMurdie and Holmes, 2013). ASVs identified as chloroplasts and mitochondria were excluded. All samples were rarefied to a sampling depth of 15,651 reads for bacteria and 17,104 reads for archaea, which was the smallest number of reads observed per sample in the filtered ASV table for the respective target microbes. Alpha diversity indices (the Chao1 and Shannon indices), were calculated based on the rarefied ASV count table. Principal coordinates analysis was performed to analyze differences in the microbial community structure based on Bray-Curtis dissimilarity matrices at the genus level. The relative abundance of each bacterial or archaeal taxon was calculated by dividing the number of reads assigned to each taxon by the total number of reads. Taxa with an average relative abundance >1% in at least one control or treatment group were used for the analysis. Quantitative PCR assays were performed to monitor rumen protozoa and fungi, as described by Koike et al. (2021).

Statistical analysis

All statistical analyses were performed using the R program. Data from experiment 1 were analyzed based on one way analysis of variance (ANOVA). When a significant effect was found, multiple comparisons among supplementation levels were separated based on the Tukey-Kramer method. Data from experiment 2 (excepting the microbial data) were analyzed based on two-way ANOVA to detect diet, supplementation level and interaction effects. Multiple comparisons were performed as described above. Microbial data (collected only from 0 or 10% IGP supplementation) were compared using the Student t test within each dietary substrate, with p-values <0.05 regarded as significantly different, while those between 0.05 and 0.10 were taken to indicate a trend.

Results

The dose responses of rumen fermentation profiles to IGP supplementation in experiment 1 are shown in Table 2. Although the initial evaluation was made only with the high-concentrate diet, gas production, especially methane production, was suppressed by supplementation with IGP at levels ≥5% ($p < 0.05$), without affecting hydrogen gas production. Proportions of methane gas in the total gas decreased from 22.6% (0% supplementation) to 17.6% (15% supplementation). In addition, the total SCFA concentration was unchanged, even though minimal changes in the proportions of SCFAs were observed (an increase in the proportion of acetate and decreases in the proportions of propionate and butyrate). These changes in fermentation profiles were again assessed under two different dietary conditions in experiment 2, as indicated in Table 3. IGP supplementation at 5–10%

decreased methane production ($p < 0.05$) by 9.3–9.7% and 3.5–10.4% for the high-concentrate and high-hay diets, respectively, without accumulation of hydrogen gas. Proportions of methane gas in total gas decreased from 19.2 to 17.8% and from 17.9 to16.7 % for the high-concentrate and high-hay diets, respectively. The total SCFA concentration was not changed by IGP supplementation, although a higher proportion of acetate and a lower proportion of butyrate ($p < 0.05$) were observed, even though the changes were minimal. A higher level of lactate was observed with IGP supplementation, irrespective of the dietary condition ($p < 0.05$). Diet effect was significant to increase total gas, CO_2 and CH_4, total SCFA production and the butyrate proportion for the high-concentrate diet. No interaction was observed between supplementation level and diet effects.

Table 2 Effect of Indian gooseberry peel supplementation at different levels in batch cultures (24 hr incubation) on rumen gas and short-chain fatty acid (SCFA) profiles

Parameter	Supplementation level of Indian gooseberry peel						SEM	*p* value
	0% (control)	2.5%	5%	10%	15%	20%		
Gas production								
Total gas (mL/tube)	14.01^{a}	13.94^{a}	12.45^{b}	12.36^{b}	11.52^{b}	11.74^{b}	0.221	**0.038**
CO_2 (mL/tube)	10.85^{a}	10.70^{a}	10.08^{b}	9.89^{bc}	9.49^{cd}	9.37^{d}	0.103	**0.043**
CH_4 (mL/tube)	3.16^{a}	3.24^{a}	2.37^{bc}	2.46^{b}	2.03^{c}	2.38^{bc}	0.052	**0.031**
H_2 (mL/tube)	0.0011	0.0010	0.0010	0.0011	0.0008	0.0008	1×10^{-7}	0.545
SCFA								
Total SCFA (mM)	102.25	101.97	103.47	97.63	100.84	95.64	0.872	0.256
Acetate (molar %)	51.00^{d}	50.71^{d}	51.52^{d}	53.57^{c}	55.08^{b}	56.61^{a}	0.212	**0.042**
Propionate (molar %)	31.41^{b}	32.48^{a}	31.68^{b}	29.89^{c}	28.34^{d}	27.38^{c}	0.296	**0.047**
Butyrate (molar %)	14.74^{a}	14.10^{b}	14.14^{ab}	13.98^{b}	14.12^{ab}	13.72^{b}	0.128	**0.049**
Others (molar %)	2.85	2.71	2.66	2.56	2.46	2.29	0.031	0.385

SEM = pooled standard error of mean

Values in same row with different lowercase superscripts differ significantly ($p < 0.05$).

p value in bold indicates statistical significance.

Table 3 Effect of Indian gooseberry peel supplementation in batch cultures (24 hr incubation) on rumen gas, pH, short-chain fatty acid (SCFA) and lactic acid profiles

Parameter	High-concentrate diet			High-hay diet			SEM	*p* value		
	0% (control)	5%	10%	0% (control)	5%	10%		Level (L)	Diet (D)	L×D
Gas production										
Total gas (mL/tube)	12.90^{a}	12.62^{a}	12.31^{b}	11.24^{c}	11.04^{c}	10.75^{c}	0.020	**0.048**	**0.036**	0.380
CO_2 (mL/tube)	10.42^{a}	10.37^{a}	10.06^{a}	9.23^{b}	9.10^{bc}	8.95^{c}	0.111	**0.031**	**0.029**	0.418
CH_4 (mL/tube)	2.48^{a}	2.25^{b}	2.24^{b}	2.01^{c}	1.94^{c}	1.80^{d}	0.001	**0.020**	**0.045**	0.351
H_2 (mL/tube)	0.00180	0.00096	0.00062	0.00038	ND	0.00043	1×10^{-8}	0.311	0.510	0.607
pH	5.90	5.94	5.92	6.07	6.07	6.05	0.023	0.470	**0.030**	0.471
SCFA										
Total SCFA (mM)	101.59^{a}	94.89^{a}	95.24^{a}	88.86^{b}	89.31^{b}	91.72^{b}	0.801	0.415	**0.041**	0.184
Acetate (molar %)	58.54^{f}	59.28^{e}	59.90^{d}	61.00^{c}	61.77^{b}	62.06^{a}	0.315	**0.042**	**0.047**	0.210
Propionate (molar %)	22.26^{a}	21.90^{b}	21.70^{b}	21.60^{b}	21.18^{c}	21.56^{b}	0.212	0.155	0.375	0.381
Butyrate (molar %)	16.48^{a}	16.27^{b}	15.92^{c}	14.80^{d}	14.56^{e}	14.28^{f}	0.177	**0.049**	**0.038**	0.216
Others (molar %)	2.72	2.55	2.48	2.60	2.49	2.10	0.001	0.180	0.466	0.301
Lactate										
L-lactate (mM)	0.46^{b}	0.60^{b}	0.94^{a}	0.49^{b}	0.75^{a}	0.87^{a}	0.019	**0.040**	0.401	0.190
D-lactate (mM)	0.45^{b}	0.58^{b}	0.92^{a}	0.63^{b}	0.83^{a}	0.91^{a}	0.015	**0.032**	0.393	0.248

SEM = pooled standard error of mean; ND = not detected.

Values in same row with different lowercase superscripts differ significantly ($p < 0.05$).

p value in bold indicates statistical significance.

Rumen bacterial and archaeal diversities are shown in Figs. 1 and 2, respectively. Alpha diversity indices were unchanged, whereas the bacterial community with IGP supplementation was clearly separated from the non-supplemented control based on beta diversity indices ($p < 0.05$). This was observed irrespective of the two different diets. By contrast, the archaeal community became more diverse based on the alpha diversity indices, especially with the high-concentrate diet ($p < 0.05$), but no clear differences in the beta diversity indices were observed compared to those of the bacterial community.

The MiSeq analysis revealed the specific changes in the rumen microbiota associated with IGP supplementation (Tables 4 and 5). Following IGP supplementation, a decrease in the Christensenellaceae R7 group and an increase in the Lachnospiraceae NK3A20 group were notable for bacteria ($p < 0.05$), whereas a decrease in *Methanobrevibacter* and increases in *Methanosphaera* and *Methanomicrobium* were observed for methanogenic archaea ($p < 0.05$). These changes were common to both dietary conditions. Other changes, such as a decrease in *Selenomonas* ($p < 0.05$) and increases in *Clostridia* ($p < 0.05$), in Prevotellaceae ($p < 0.05$) and in Methanomethylophilaceae ($p < 0.05$), were dependent on the diet. Protozoal and fungal abundance did not differ between the control and IGP-supplementation treatment, being at 1×10^6 and 1×10^4 (18S rDNA copy number/mL of culture) for protozoa and fungi, respectively. The different dietary conditions did not affect these parameters.

Discussion

In a preliminary *in vitro* study (data not shown) examining 17 residues of tropical fruits in Thailand, IGP was selected as one of the most potent materials for rumen methane suppression; subsequently, it was examined in greater detail in the present study in terms of dose and diet dependence and rumen microbial community shift.

Methane suppression was observed in batch cultures of rumen fluid supplemented with IGP at levels ≥5% in the dietary substrate. This was confirmed with diets that differed in their hay-to-concentrate ratios. IG fruit pomace, another byproduct of IG (mainly consisting of fruit fiber and peel), reportedly decreased methane production in a feeding study using Murrah buffaloes (Singla et al., 2021). In that study, methane suppression was in the range 14.4–25.1%, indicating that the peel or fruit fiber, or both, might exhibit anti-methanogenic activity in the rumen. However, the mechanism remains unclear by which IG fruit pomace suppresses methane production.

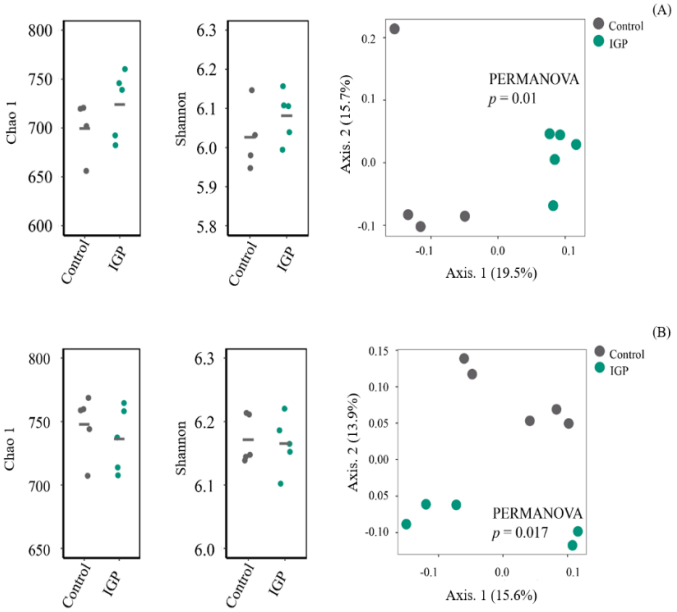

Fig. 1 Effect of Indian gooseberry peel (IGP) supplementation on bacterial alpha and beta diversity indices in batch cultures with: (A) high-concentrate diet; (B) high-hay diet

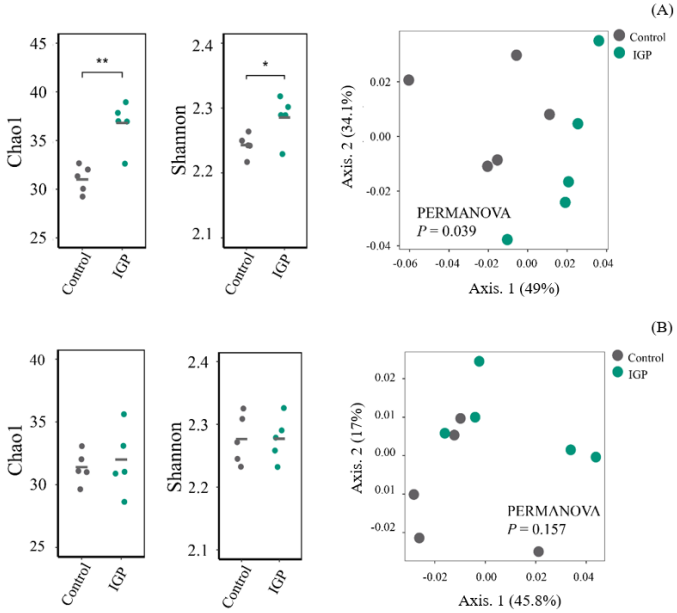

Fig. 2 Effect of Indian gooseberry peel (IGP) supplementation on archaeal alpha and beta diversity indices in batch cultures with: (A) high-concentrate diet; (B) high-hay diet, where * = $p < 0.05$ and ** = $p < 0.01$

Table 4 Effect of Indian gooseberry peel (IGP) supplementation at 10% in batch cultures on relative abundance of rumen bacterial taxa at genus level

High-concentrate diet	Control	IGP	SEM	*p* value	High-hay diet	Control	IGP	SEM	*p* value
g__*Prevotella*	14.22	13.45	0.250	0.445	g__*Prevotella*	12.21	13.01	0.225	0.378
g__*Pseudobutyrivibrio*	7.35	5.87	0.095	0.287	g__Rikenellaceae_RC9	7.68	7.70	0.127	0.605
g__Rikenellaceae_RC9p	7.14	6.57	0.128	0.750	g__Christensenellaceae_R-7	7.44	5.79	0.375	**0.038**
g__Christensenellaceae_R-7	6.39	5.52	0.080	0.085	g__NK4A214_group	5.72	5.45	0.125	0.550
g__NK4A214_group	4.66	4.71	0.091	0.701	g__*Pseudobutyrivibrio*	4.66	4.35	0.686	0.472
g__*Succiniclasticum*	4.10	3.75	0.075	0.495	g__*Succiniclasticum*	4.30	4.09	0.065	0.326
g__*Treponema*	4.01	3.83	0.070	0.420	f__Lachnospiraceae	3.54	3.97	0.068	0.285
f__Lachnospiraceae	3.41	4.94	0.041	**0.040**	g__*Treponema*	2.35	2.04	0.060	0.206
g__*Butyrivibrio*	2.57	3.21	0.038	0.109	g__Prevotellaceae_UCG-001	2.08	2.34	0.031	**0.045**
g__Prevotellaceae_UCG-001	2.46	2.57	0.059	0.665	g__WCHB1-41	1.93	2.28	0.049	0.335
g__Bacteroidales_BS11	1.94	1.66	0.052	0.520	g__Lachnospiraceae_NK3A20	1.93	2.68	0.032	0.066
g__WCHB1-41	1.79	1.98	0.059	0.590	g__*Butyrivibrio*	1.89	2.10	0.057	0.550
g__F082	1.76	1.59	0.050	0.478	g__Bacteroidales_BS11	1.78	1.72	0.052	0.471
g__Lachnospiraceae_NK3A20	1.69	2.47	0.031	**0.031**	g__*Ruminococcus*	1.66	1.38	0.029	0.081
g__*Selenomonas*	1.47	1.04	0.028	**0.043**	g__F082	1.61	1.83	0.033	0.071
g__*Ruminococcus*	1.44	1.54	0.050	0.320	g__UCG-005	1.60	1.65	0.055	0.335
f__Prevotellaceae	1.27	1.13	0.065	0.415	g__Clostridia_UCG-014	1.39	1.42	0.068	0.460
g__UCG-005	1.19	1.33	0.061	0.302	c__Clostridia	1.36	1.04	0.072	0.605
g__Clostridia_UCG-014	1.13	1.160.072	0.078	0.770	g__Absconditabacteriales_(SR1)	1.21	1.13	0.084	0.471
g__vadinBE97	1.12	0.94	0.071	0.681	g__vadinBE97	1.08	1.09	0.074	0.660
g__Absconditabacteriales_(SR1)	1.08	1.09	0.070	0.570	f__Prevotellaceae	1.07	1.19	0.061	0.278
g__Prevotellaceae_UCG-003	1.01	0.84	0.068	0.471	Others	31.53	31.76	0.402	0.452
c__Clostridia	0.81	1.01	0.021	**0.048**					
Others	26.01	27.79	0.301	0.402					

SEM = pooled standard error of mean;
p value in bold indicates statistical significance

Table 5 Effect of Indian gooseberry peel (IGP) supplementation at 10% in batch cultures on relative abundance of rumen archaeal taxa at genus level

High-concentrate diet	Control	IGP	SEM	*p* value	High-hay diet	Control	IGP	SEM	*p* value
g_*Methanobrevibacter*	92.69	90.93	0.755	**0.045**	g_*Methanobrevibacter*	92.31	91.50	0.920	0.084
g__*Methanosphaera*	4.58	5.72	0.051	**0.038**	g__*Methanosphaera*	4.93	5.79	0.095	0.080
f__Methanobacteriaceae	2.20	2.15	0.065	0.512	f__Methanobacteriaceae	2.24	1.94	0.022	**0.044**
g__Methanomethylophilaceae_UCG	0.47	1.03	0.051	**0.029**	g__Methanomethylophilaceae_UCG	0.45	0.68	0.095	0.363
g__*Methanomicrobium*	0.02	0.12	0.009	**0.032**	g__*Methanomicrobium*	0.01	0.06	0.015	0.072
Others	0.04	0.05	0.004	0.475	Others	0.06	0.02	0.003	0.241

SEM = pooled standard error of mean
p value in bold indicate statistical significance

As suggested by their analysis, the IG pomace contained total phenolics and other bioactive compounds at high levels (224g/kg DM for total phenolics), many of which are known to decrease methane generation (Vasta et al., 2019) possibly via a selective inhibition of hydrogen-producing microbes in the rumen. The IGP tested in the present study might have contained these compounds at functional levels.

Often, tropical fruits and their peel contain bioactive and functional substances that are categorized as plant secondary compounds, such as polyphenols, tannins, and flavonoids. It is known that IG contains amblicanin, a hydrolysable tannin (and its constituents of gallic and ellagic acids), all of which exhibit anti-methanogenic effects (Variya et al., 2016). Often these compounds act against specific microbes by which rumen microbes are selected, leading to a shift in the microbial community and fermentation pattern, including methane production (Ungerfeld, 2020). The mechanisms involved in microbial selection by phenolic compounds are not fully clarified due to their variety in structure, though the phenolics bind to microbial cells and their enzymes, leading to functional

inactivation (Vasta et al., 2019). The sensitivity of microbes against the phenolics might be different depending on their cell surface structure and components. The amount of phenolics supplied from the tested IGP is also a key to defining microbial selectivity, judging from the dose-dependency of the methane decrease in the present study.

While IGP had a minimal effect on the SCFA profile in the present study, the lactate concentration consistently increased, irrespective of the dietary substrates. Accordingly, microbial shifts induced by IGP could stimulate the acrylate pathway to convert metabolic hydrogen into lactate and then propionate (Ungerfeld, 2020), although propionate enhancement was not observed in the present study. This suggested that there was not sufficient promotion of the growth of the microbes that produce propionate from lactate. It could take time to fully develop the acrylate pathway in the case of IGP supplementation, especially under *in vitro* conditions, suggesting that short-term incubation studies can provide only limited information.

As indicated by another study, the functionality of tropical fruit byproducts can be first screened through *in vitro* studies and then confirmed in feeding trials (Singla et al., 2021), because *in vitro* results sometimes overestimate the functions of the test materials. However, IG could be a useful feedstuff or additive candidate, or both, for methane suppression, as indicated in the present study and that by Singla et al. (2021) and as reported for rambutan peel and other tropical fruit residues (Paengkoum *et al*., 2015).

The observed changes in the rumen microbial community structure with IGP supplementation partially describe how this material works in the rumen. The decrease in the Christensenellaceae R7 group, which is involved in the production of hydrogen (a primary substrate for methane generation by hydrogenotrophic methanogens), might reasonably explain the methane suppression. Recently, the representative strain R7 of this group was proposed as the new species *Aristaeella hokkaidonensis* (Mahoney-Kurpe et al., 2023), which plays a key role in hydrogen production. The increase in lactate could be attributed to the increase in Lachnospiraceae and the decrease in *Selenomonas* (only for the high-concentrate diet), causing the production of lactate and a lower metabolism of lactate, leading to the accumulation of lactate in the present cultures. As discussed above, for the satisfactory development of microbiota to shift hydrogen metabolism toward propionate production via the acrylate pathway, the increase and activation of lactate-utilizing bacterial species, such as *Selenomonas* and *Megasphaera* (Shinkai, 2023), should be essential in the rumen. The suppression of *Selenomonas* observed by IGP supplementation would cause rumen acidosis, to which attention should be paid for practical uses, especially when a high concentrate diet is fed.

Although the shift in the community of archaea following IGP supplementation was not very notable compared to that of the bacteria, *Methanobrevibacter*, a major genus of methanogens in the rumen, was partially replaced by minor methanogenic groups, leading to increases in the alpha diversity indices of the methanogens. As low-methane-emitting cattle harbor a more diverse methanogenic community (Auffret et al., 2018), the present results (showing a lowering of methane production with a high diversity of methanogens by IGP supplementation) agreed with this Auffret et al. (2018). Indeed, a lower abundance of *Methanobrevibacter*, a major contributor to methane production, and a higher abundance of Methanomethylophilaceae following IGP supplementation might reasonably support methane suppression. Notably, the Methanomethylophilaceae include Methanomassiliicoccales, the abundance of which is negatively correlated with methane production (Martínez-Álvaro et al., 2020) and is present at a higher level in low-methane-emitting cattle (Auffret et al., 2018). Thus, the current detailed microbial data reasonably explained how IGP decreased methane production from the aspect of rumen microbial community changes.

In conclusion, IGP (at 5–15% supplementation) suppressed rumen methane production by altering the rumen bacterial and archaeal community structures, possibly through its antimicrobial substances, such as phenolics and their components. Although there was no clear evidence indicating which microbial group triggered methane suppression, several groups of rumen microbes are thought to be involved. At 5% supplementation, a methane decrease might be more evident in the high-concentrate diet than in the high-hay diet. Therefore, it would be reasonable to recommend using IGP for a high-concentrate diet when the least (but effective) supplementation level (5%) is considered. IGP could serve as a functional feed candidate, possibly a roughage candidate based on its fibrous nature; however, IGP must be examined further in terms of its application potential, such as global availability (biomass) and cost performance (transport and processing carbon footprint).

Conflict of Interest

The author declares that there are no conflicts of interest.

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