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Effect of Brittleness in Rice Straw on Rumen Fermentation by *In vitro* Gas Production Technique

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Abstract

The objective of this study was to examine the impact of rice straw brittleness on its efficacy as roughage for ruminants, in comparison to a wild-type variety, utilizing an *in vitro* study approach. The experimental design was a complete randomized design (CRD). The experimental treatments included various breeds of rice straw (RS), such as the wild-type (WT, T1), the green brittle bred line 8 and line 13 with brittleness level-3 (GBL8-B3, T2 and GBL13-B3, T3), and the purple brittle bred line 8 with brittleness level-5 (PBL8-B5, T4). The findings revealed that the brittleness RS group had higher levels of crude protein and hemicellulose, and lower levels of cellulose compared to the wild-type group. Brittleness RS varieties showed a significantly greater gas production accumulation ($p = 0.001$) and IVDMD ($p = 0.003$) compared to the WT group. At 8 h post-incubation, the brittleness RS group showed significantly higher ($p = 0.004$) total of volatile fatty acids concentration compared to the WT group. After 1 h of post-incubation, GBL8-B3 exhibited the highest ($p = 0.032$) proportion of propionate and the lowest (0.009) C2:C3 ratio. Additionally, the brittleness RS did not have any effect on the population of ruminal microorganisms. Based on the brittleness of RS, it can be deduced that it has a greater potential as a roughage source when compared to the wild-type variety, consequently enhancing the quality of RS for roughage intake by ruminants.

Keywords: Brittleness rice straw, *In vitro* study, Rumen fermentation, Wild-type rice

Introduction

RS, while abundant and inexpensive, presents several disadvantages as livestock feed due to its low nutritional value and digestibility. Primarily, RS is low in protein, typically containing less than 3 % crude protein, which is insufficient to meet the nutritional requirements of high-producing livestock such as dairy cattle [1]. The high fiber content and the presence of anti-nutritional factors like lignin, silicates, and oxalates further reduce its digestibility and nutritional value, making it challenging for ruminants to derive adequate nutrients from it [2,3]. Additionally, the high silica content in RS contributes to poor nutrient digestibility, with dry matter and protein digestibility often falling below 50 % [4].

Enhancing the nutritional value and effectiveness of RS for ruminant feed requires various techniques such as biological, chemical treatment, and gene modification. For example, is Bio-fermentation, which boosts feed intake, weight gain, and meat quality in sheep, while also improving gas production and fiber degradation by enhancing the microbial community on

RS particles [5]. Ammonia and Basidiomycete white-rot fungi are capable of increasing crude protein content and enhancing digestibility [6]. Mixing microecological (Lactobacillus, Cellulase, Xylanase and β-glucanase) agents with molasses transforms the structure of RS and enhances fermentation [7]. The addition of mineral, protein-energy supplements, and fibrolytic enzymes (Xylanase) can also improve digestibility and fermentation kinetics [8]. Mixed with agro-industrial by-product such as monosodium glutamate by-product could improve quantity and quality of RS [9]. In recent years, genetic technology has been used in rice breeding to improve the nutritive value of RS through the production of brittle rice varieties [10].

Brittleness in RS is a significant trait that has garnered attention due to its implications for both agricultural practices and potential industrial applications. Brittleness RS varieties have been bred from mutants of Indica rice Var. IR64 (Wild-type, WT) treated with sodium azide (NaN_3), with a focus on enhancing agronomic traits such as brittleness, bacterial blight disease resistance, antioxidant property [11]. Brittle rice mutants, such as AZ1803 and Bc19, have been studied extensively to understand the genetic and biochemical underpinnings of this trait. The AZ1803 mutant, derived from the IR64 mutant pool, exhibits a 25 % reduction in cellulose content due to a mutation in the OsCesA7 gene, which is crucial for cellulose synthesis in the secondary cell wall. This mutation not only makes the straw more brittle but also enhances its digestibility for livestock feed, thereby increasing farmer income and reducing environmental pollution from straw burning [12]. Similarly, the Bc19 mutant, identified through chemical mutagenesis, shows a semi-dominant brittle phenotype due to a P507S missense mutation in the OsCESA4 gene, affecting cellulose synthesis in a dosage-dependent manner. This mutant maintains normal grain yield and morphology, making it suitable for dual-purpose hybrid rice breeding [13]. Overall, the study of brittle rice mutants not only advances our understanding of cell wall biosynthesis and mechanical strength but also opens up new avenues for agricultural and industrial applications, from improved livestock feed to biofuel production.

Therefore, the objective of this study is to examine the impact of RS brittleness on its efficacy as roughage for ruminants, in comparison to a wild-type variety, utilizing an *in vitro* study approach.

Materials and methods

Experimental design and fermentation technique

This study was conducted using an *in vitro gas* production technique at various incubation time intervals. The experimental design was a CRD with 3 replications per treatment. The experimental treatments included various breeds of RS, such as the wild-type RS (WT), the green brittle bred line 8 with brittleness level-3 (GBL8-B3), the green brittle bred line 13 with brittleness level-3 (GBL13-B3), and the purple brittle bred line 8 with brittleness level-5 (PBL8-B5).

The 3 genotypes of rice used in this study were manually harvested from the paddy field of the Beigou experimental farm, National Chung Hsing University, Wufeng district, Taichung, Taiwan. Fresh RS from Var. IR64, wild-type, GBL8-B3, GBL13-B3 and PBL8-B5 were collected at the yellow stage of maturity, and their grains were subsequently removed. After wilting, RSs were chopped (1 - 2 cm) for the experiment. All RS were dried in the oven at 60 °C for 48 h to constant weight then ground to pass through a 1-mm screen (30 mesh) using a Wiley mill to determine nutrient content. All experimental RS were analyzed by proximate analysis. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were measured following Soest *et al.* [14]. Chemical compositions are shown in **Table 1**.

For the *in vitro* gas production study. The rumen fluid was collected from the slaughterhouse. Three, Thai crossbred beef cattle (Kamphaengsaen: *Bos indicus*) were used as rumen fluid donor. The 1000 mL rumen fluid was obtained from each of the animal. The rumen fluid was filtered through 4 layers of cheesecloth into prewarmed thermos flasks. Preparation of artificial saliva was done according to Menke and Steingass [15].

Artificial saliva was prepared, and rumen fluid was mixed in a 2:1 ratio to prepare fermentation solution. The serum bottles with the mixture of substrate treatments were pre-warmed in a water bath at 39 °C for 1 h before filling with 30 mL of rumen inoculum's mixture. During the incubation, the gas production was recorded at 0, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h.

Cumulative gas production data were fitted to the model of Orskov and McDonal [16].

Determination of fermentation parameters

Inoculum's ruminal fluid was collected at 1, 4 and 8 h post inoculations. Rumen fluid samples were then filtered through 4 layers of cheesecloth. Samples were divided into 2 portions; the 1st portion was used for NH_3–N analysis. The sample was centrifuged at 16,000×g for 15 min, and the supernatant was stored at −20 °C before NH_3–N analysis according to Chaney and Marbach [17] and VFAs analysis using gas chromatography.

The final portion was stored at −20 °C for DNA the extraction [18]. At 48 h post inoculation a set of samples was determined *in vitro* true digestibility (IVDMD) according to Soest and Robertson [19]. In brief, the content of the bottle was transferred quantitatively to a spoutless beaker by repeated washing with 100 mL neutral detergent solution. The content was refluxed for 1 h and filtered through pre-weighed Gooch crucibles. The DM of the residue was weighed and IVDMD of feed was calculated as follows:

IVDMD (%) = [(DM of feed taken for incubation – DM residue)×100]/DM of feed taken for incubation

Quantitative analysis of microbial populations

Community DNA was extracted from 1.0 mL aliquots of each sample by the RBB+C method [18], which was shown to substantially increase DNA yields. The quality and quantity of these DNA samples were also determined by agarose gel electrophoresis and spectrophotometry. In total, 36 samples belonging to 4 treatments, 3 incubation times (1, 4 and 8) and 3 replicates were extracted for genomic DNA. The primers used for the real-time PCR are as follows: Primers for General bacteria primers, F (5′-CGG CAA CGA GCG CAA CCC-3′) and R (5′-CCA TTG TAG CAC GTG TGT AGC C-3′) (130-bp product). General anaerobic fungi primers, F (5′-GAG GAA GTA AAA GTC GTA ACA AGG TTT C-3′) and R (5′-CCA TTG TAG CAC GTG TGT AGC C-3′) (120-bp product). General protozoa primers, F (5′-GCT TTC GWT GGT AGT GTA TT-3′) R (5′-CTT GCC CTC YAA TCG TWC T-3′) (223-bp product) *Fibrobactor succinogenes*, F (5-′GTT CGG AAT TAC TGG GCG TAA A-3′) and R (5′-CGC CTG CCC CTG AAC TAT C-3) (121-bp product). *Ruminococcus flavefaciens* primers, F (5′-CGA ACG GAG ATA ATT TGA TTT ACT TAG G-3′) and R (5′-CGG TCT CTG TAT GTT ATG AGG TAT TAC C-3′) (132-bp product) (20). *Ruminococcus albus* primers, Ra1281f (5′-CCC TAA AAG CAG TCT TAG TTC G-3′) and Ra1439r (5′-CCT CCT TGC GGT TAG AAC A3′) (175-bp product) [21].

Four sample-derived standards were prepared from treatment pool set of community DNA. The regular PCR was used to generate sample-derived DNA standards for each real-time PCR assay. Then the PCR product was purified using a QIA quick PCR purification kit (QIAGEN, Inc., Valencia, CA) and quantified using a spectrophotometer. For each sample-derived standard, copy number concentration was calculated based on the length of the PCR product and the mass concentration. Tenfold serial dilution was made in Tri-EDTA prior to real-time PCR [22]. In total, 6 real-time PCR standards were prepared. The conditions of the real-time PCR assays of target genes were the same as those of the regular PCR described above. Biotools QuantiMix EASY SYG KIT (B&M Labs, S. A., Spain) was used for real-time PCR amplification. All PCRs were performed in duplicate.

Statistical analysis

All data were analyzed by using the General Linear Models (GLM) procedures (SAS Inst. Inc., Cary, NC). Data were analyzed using the model $Yij = \mu + Ti + \varepsilon ij$ where Yij, observation from treatment i, j, the replication; μ, the overall mean, Ti, the mean of treatment and εij, the residual effect. Multiple comparisons among treatment means were performed by Duncan's New Multiple Range Test (DMRT) [23]. The contrast among groups and treatments was perform by orthogonal contrasts.

Results and discussion

The analysis of the nutritional composition of the 4 types of roughage indicated that the levels of dry matter (DM) were within a comparable range, specifically ranging from 93.41 to 93.90 %. Notably, the green brittleness RS group (T2, T3) exhibited a greater proportion of organic matter (OM) in comparison to both the wild-type (T1) and the purple brittleness RS (T4). The percentage of crude protein (CP) was

determined to be higher in the brittleness RS group across all 3 treatments (T2, T3, T4) with proportions of 8.43, 9.42 and 11.82 %, respectively, compared to the wild-type (T1) at 7.02 %. Additionally, it was observed that the purple brittleness RS exhibited the highest CP proportion.

Table 1 Chemical composition of experimental roughages, wild type, and brittleness RS.

Items	Wild type (T1)	GBL8-B3 (T2)	GBL13-B3 (T3)	PBL8-B5 (T4)
Dry matter, (DM)	93.90	93.41	93.87	93.85
	---------- (Dry matter basis, %) ----------			
Organic matter, (OM)	87.87	89.24	88.51	86.60
Crude protein, (CP)	7.02	8.43	9.42	11.82
Ether extract, (EE)	0.43	0.79	0.96	0.60
Ash	12.13	10.76	11.49	13.40
Neutral detergent fiber, (NDF)	62.18	57.86	60.10	62.30
Acid detergent fiber, (ADF)	40.65	33.08	34.71	38.49
Acid detergent lignin, (ADL)	5.29	6.18	5.85	6.14
Hemicellulose	21.53	24.78	25.39	23.81
Cellulose	35.36	26.90	28.86	32.35

T1 = wild-type RS (WT), T2 = green brittle bred line 8, brittleness level-3 (GBL8-B3), T3 = green brittle bred line 13, brittleness level-3 (GBL13-B3) and T4 = purple brittle bred line 8, brittleness level-5 (GBL8-B5).

Table 2 Effects of the brittleness in RS on kinetic gas value, gas production, IVDMD and ammonia nitrogen in *in vitro* study.

Items	Wild type (T1)	GBL8-B3 (T2)	GBL13-B3 (T3)	PBL8-B5 (T4)	*p*-value	SEM	Orthogonal contrast		
							T1 vs (T2, T3, T4)	(T2, T3) vs T4	T2 vs T3
Kinetic gas value*									
a	2.09	1.80	0.77	0.09	0.815	0.313	0.223	0.254	0.795
b	9.39^{b}	27.26^{a}	28.99^{a}	23.32^{a}	0.009	6.039	0.002	0.015	0.004
c	0.026^{ab}	0.03^{a}	0.024^{c}	0.01^{d}	0.004	0.006	0.162	0.065	0.051
d	11.48^{b}	29.07^{a}	29.76^{a}	23.41^{a}	0.006	5.707	0.002	0.013	0.002
Gas (96 h, mL)	11.2^{b}	28.1^{a}	26.7^{a}	15.7^{b}	0.002	5.526	0.001	0.015	0.001
IVDMD (%)	46.5^{c}	55.1^{a}	50.7^{b}	49.0^{bc}	0.003	2.097	0.003	0.954	0.005
NH_3N (mg/dL)									
1 h	10.5	11.0	10.2	10.8	0.314	0.313	0.574	0.185	0.212
4 h	10.6	10.9	11.7	12.0	0.248	0.548	0.158	0.166	0.721
8 h	10.7	10.9	10.2	11.9	0.175	0.560	0.533	0.394	0.724
Mean	10.6^{b}	10.9^{ab}	10.7^{ab}	11.6^{a}	0.030	0.246	0.975	0.324	0.034

$^{a, b, c}$ values on the same row with different superscripts differed ($p < 0.05$), SEM = Standard error of the mean. *a = the gas production from soluble fractions (mL), b = the gas production from insoluble fraction (mL), c = the rate constants of gas production for the insoluble fraction (mL), and d = the potential extent of gas production (mL), T1 = wild-type RS (WT), T2 = green brittle bred line 8, brittleness level-3 (GBL8-B3), T3 = green brittle bred line 13, brittleness level-3 (GBL13-B3) and T4 = purple brittle bred line 8, brittleness level-5 (GBL8-B5).

The green brittleness RS group exhibited decreased proportions of NDF and ADF compared to the purple brittleness RS group. In contrast, the wild-type (T1) demonstrated the highest proportion of ADF relative to the other groups. When comparing the proportion of ADL, it was found that the brittleness RS group (T2, T3, T4) and the wild-type (T1) were found to be different, with the wild-type (T1) having the least proportion of ADL, as shown in **Table 1**. The brittleness RS group (T2, T3, T4) showed that the cellulose content ranged from 26.90 - 32.35 %, lower than the value observed in the wild-type (T1) (35.36 %). Analysis of the brittleness levels revealed that brittleness level 3 (T2, T3) exhibited a lower cellulose proportion compared to level 5 (T4). The proportion of hemicellulose detected revealed that the brittleness RS group (T2, T3, T4) exhibited a proportion ranging from 23.81 - 25.39 %, which was higher than that of the wild-type (T1) at 21.53 %. Analysis of the brittleness levels revealed that level 5 (T4) contained a lower percentage of hemicellulose when compared with level 3 (T2, T3).

From **Table 2**, the data illustrates the quantity of gas accumulated after 96 h, IVDMD, and the level of ammonia nitrogen (NH_3-N). The analysis revealed that the brittleness RS varieties (T2, T3, T4) exhibited a higher accumulation of gas compared to the WT (T1) (p = 0.001). The green brittleness RS group exhibited a greater amount of accumulated gas compared to the purple brittleness RS group (p = 0.015), while the GBL8-B3 (T2) group demonstrated the highest level of accumulated gas (p = 0.001). From the kinetic gas value, it was determined that the values of b, c, and d exhibited significant differences ($p < 0.05$). The examination of b value or gas production from the insoluble fraction revealed that the brittleness RS group demonstrated a notably higher value compared to the WT group (p = 0.002). Furthermore, the green brittleness RS group exhibited a higher value than the purple brittleness RS group (p = 0.015), and the GBL13-B3 group was observed to possess higher values than GBL8-B3 (p = 0.004), respectively. While the constants of gas production for the insoluble fraction or c value was found to be the highest in the GBL8-B3 group (p = 0.004), the potential extent of gas production or d value had a similar trend to the b value.

In terms of *in vitro* dry matter digestibility (IVDMD), the brittleness of RS (T2, T3, T4) exhibited a higher level compared to the wild-type (T1) with a statistically significant difference (p = 0.003). Within the category of brittleness, there was no significant difference observed between green brittleness RS and purple brittleness RS (p = 0.954). Nevertheless, IVDMD of GBL8-B3 was the highest with statistical significance (p = 0.003). The concentration of NH_3-N at 1, 4, 8 h post-incubation was found to be not significantly different among treatment ($p > 0.05$). But for the mean value, it was found that there was a statistically significant difference (p = 0.030). It was found that purple brittleness RS (T4) had the highest value (p = 0.030).

Table 3 Effects of the brittleness in RS on volatile fatty acid production and C2:C3 ratio.

Items	Wild type (T1)	GBL8-B3 (T2)	GBL13-B3 (T3)	PBL8-B5 (T4)	*p*-value	SEM	Orthogonal contrast		
							T1 vs (T2, T3, T4)	T2, T3 vs T4	T2 vs T3
Total VFAs, (mmol/L)									
1 h	58.83[a]	33.95[d]	42.46[c]	47.98[b]	< 0.001	5.579	< 0.001	0.054	0.001
4 h	70.39[a]	70.55[a]	63.51[ab]	58.22[b]	0.043	3.953	0.092	0.082	0.971
8 h	62.57[b]	77.28[a]	75.85[a]	71.82[a]	0.019	4.157	0.004	0.111	0.005
Mean	63.93	60.59	60.60	59.34	0.985	7.878	0.731	0.886	0.802
Acetic acid, (mol/100 mol total VFAs)									
1 h	69.15	69.77	70.56	68.53	0.942	2.143	0.871	0.724	0.861
4 h	68.05	67.62	67.74	71.39	0.564	2.033	0.733	0.970	0.888

Items	Wild type (T1)	GBL8-B3 (T2)	GBL13-B3 (T3)	PBL8-B5 (T4)	*p*-value	SEM	Orthogonal contrast		
							T1 vs (T2, T3, T4)	T2, T3 vs T4	T2 vs T3
8 h	66.81	67.87	67.25	67.25	0.918	0.941	0.618	0.945	0.507
Mean	68.00	68.42	68.51	69.05	0.882	0.826	0.556	0.796	0.760
Propionate, (mol/100 mol total VFAs)									
1 h	12.18[b]	20.54[a]	14.97[b]	13.55[b]	0.032	2.380	0.061	0.510	0.007
4 h	11.02	11.08	11.79	12.49	0.376	0.653	0.324	0.367	0.943
8 h	11.67	10.95	10.86	10.54	0.208	0.387	0.056	0.323	0.175
Mean	11.62	14.19	12.54	12.19	0.764	1.615	0.527	0.869	0.335
Butyric acid, (mol/100 mol total VFAs)									
1 h	18.67	9.68	14.47	17.92	0.104	2.974	0.135	0.923	0.030
4 h	20.93	21.30	20.47	16.12	0.526	2.620	0.614	0.849	0.924
8 h	21.52	21.19	21.89	22.21	0.943	0.549	0.871	0.736	0.857
Mean	20.37	17.39	18.94	18.75	0.861	2.185	0.498	0.983	0.415
C2:C3 ratio									
1 h	5.68[a]	3.59[b]	4.71[a]	5.06[a]	0.009	0.531	0.009	0.843	0.001
4 h	6.17	6.11	5.76	5.75	0.225	0.186	0.157	0.098	0.786
8 h	5.75	6.20	6.19	6.38	0.129	0.198	0.031	0.326	0.094
Mean	5.87	5.30	5.55	5.73	0.879	0.464	0.586	0.961	0.463

[a, b, c, d] values on the same row with different superscripts differed ($p < 0.05$), SEM = Standard error of the mean, T1 = wild-type RS (WT), T2 = green brittle bred line 8, brittleness level-3 (GBL8-B3), T3 = green brittle bred line 13, brittleness level-3 (GBL13-B3) and T4 = purple brittle bred line 8, brittleness level-5 (GBL8-B5).

From **Table 3**, it was found that the concentrations of total VFAs at the 1, 4, and 8 h post-incubation were significantly different ($p < 0.05$). In the 1 h post incubation, the brittleness RS group (T2, T3, T4) was significantly lower than WT (T1) ($p < 0.001$), while at the 8 h post-incubation, the brittleness RS group (T2, T3, T4) was significantly greater than WT (T1) ($p = 0.004$), and found that GBL8-B3 (T2) had the highest value ($p = 0.005$).

For the proportion of propionate (C3), it was found that in the 1 h post-incubation there was a statistically significant difference ($p = 0.032$). The GBL8-B3 (T2) was found to have the highest value ($p = 0.007$). While at the 4, 8 h and mean, no differences were found between the treatments. The C2:C3 ratio was found that in the 1 h post-incubation, the brittleness RS group had a value that was significantly lower than that of WT (T1) ($p = 0.009$) and GBL8-B3 (T2) had the lowest value ($p = 0.009$). While at the 8 h post-incubation, it was found that the brittleness RS group had a value that was significantly higher than WT (T1) ($p = 0.031$).

The investigation into the impact of brittleness RS on the alteration of microbial population within the rumen through the assessment of cycle threshold or ct-value revealed not significantly different among treatment in total bacteria, total protozoa, total fungi, and predominant cellulolytic bacteria population (**Table 4**).

Table 4 Effect of the brittleness in RS on rumen microorganisms (Ct-value, cycle threshold).

Items	Wild type (T1)	GBL8-B3 (T2)	GBL13-B3 (T3)	PBL8-B5 (T4)	*p*-value	SEM	Orthogonal contrast		
							T1 vs T2, T3, T4	T2, T3 vs T4	T2 vs T3
Total bacteria, (cycle threshold)									
1 h	34.18	33.54	34.33	34.93	0.231	0.471	0.858	0.396	0.326
4 h	35.13	34.59	34.13	34.65	0.263	0.346	0.109	0.102	0.271
8 h	31.57	31.24	32.42	31.49	0.361	0.475	0.791	0.112	0.626
Mean	33.62	33.12	33.62	33.69	0.971	0.831	0.898	0.836	0.721
Total protozoa, (cycle threshold)									
1 h	37.20	37.50	37.32	36.55	0.458	0.358	0.896	0.936	0.605
4 h	36.31	35.97	34.84	36.26	0.904	1.407	0.747	0.526	0.884
8 h	35.05	36.39	36.80	37.26	0.374	0.895	0.120	0.340	0.310
Mean	36.19	36.63	36.39	37.02	0.781	0.536	0.493	0.978	0.612
Total fungi, (cycle threshold)									
1 h	36.86	36.09	36.01	35.62	0.564	0.583	0.211	0.548	0.395
4 h	35.68	36.67	36.07	37.21	0.426	0.664	0.239	0.896	0.320
8 h	34.47	34.39	35.06	33.36	0.269	0.606	0.761	0.387	0.916
Mean	35.67	35.72	35.71	35.39	0.988	0.651	0.946	0.983	0.966
Ruminococcus albus, (cycle threshold)									
1 h	36.42	38.29	36.55	36.60	0.295	0.772	0.402	0.390	0.106
4 h	38.08	36.62	36.87	38.90	0.238	0.886	0.531	0.645	0.239
8 h	34.69	36.00	35.75	35.69	0.861	1.040	0.427	0.784	0.449
Mean	36.40	36.97	36.39	37.08	0.878	0.693	0.655	0.767	0.617
Ruminococcus flavefaciens, (cycle threshold)									
1 h	29.66	30.14	30.12	29.89	0.282	0.196	0.101	0.362	0.099
4 h	29.99	29.60	30.03	29.99	0.219	0.166	0.531	0.232	0.104
8 h	27.53	28.20	28.84	27.96	0.224	0.448	0.126	0.086	0.279
Mean	29.06	29.31	29.66	29.28	0.919	0.545	0.627	0.549	0.777
Fibrobacter succinogenes, (cycle threshold)									
1 h	36.84	36.09	37.07	36.34	0.866	0.818	0.754	0.607	0.579

Items	Wild type (T1)	GBL8-B3 (T2)	GBL13-B3 (T3)	PBL8-B5 (T4)	*p*-value	SEM	Orthogonal contrast		
							T1 vs T2, T3, T4	T2, T3 vs T4	T2 vs T3
4 h	36.35	36.75	36.93	36.81	0.940	0.609	0.570	0.669	0.696
8 h	36.36	35.90	35.40	36.16	0.758	0.598	0.496	0.392	0.632
Mean	36.52	36.25	36.47	36.43	0.937	0.281	0.725	0.835	0.570

T1 = wild-type RS (WT), T2 = green brittle bred line 8, brittleness level-3 (GBL8-B3), T3 = green brittle bred line 13, brittleness level-3 (GBL13-B3) and T4 = purple brittle bred line 8, brittleness level-5 (GBL8-B5).

Discussion

The nutritional composition analysis of brittle RS revealed that the brittleness group exhibited a reduced proportion of NDF, ADF, and cellulose, while it show higher proportion of hemicellulose and crude protein content compared to the WT group, due to selective breeding practices targeting agronomic characteristics like brittleness, resistance to bacterial diseases, and antioxidant properties. In the present study, the rice varieties exhibiting brittleness were developed through the breeding of mutants derived from the Indica rice variety IR64, following treatment with sodium azide (NaN_3).

Brittleness in RS refers to a genetic mutation that results in rice plants with easily breakable tissues, including the culm, leaf, sheath, and node, due to reduced cell wall thickness and altered cell wall composition, particularly in cellulose content. This mutation is often associated with genes such as OsCesA4, OsCesA7, and OsCesA9, which encode cellulose synthase catalytic subunits responsible for cellulose synthesis in the cell wall [12]. The brittle culm mutants exhibit a higher proportion of hemicellulose and crude protein, but lower cellulose content compared to their wild-type counterparts, enhancing the digestibility of the RS for ruminants [10].

Brittleness in RS, particularly in brittle culm mutants, is associated with higher protein content due to several factors related to the genetic and structural modifications in the plant. The genetic modifications leading to brittleness do not significantly affect the lignin levels but do result in higher hemicellulose content, which is beneficial for the digestibility and nutritional profile of the RS [24]. The brittle culm mutants, such as those derived from the indica variety Shuang-Ke-Zao, exhibit a higher proportion of stem and lower proportion of leaf blade, which contributes to the overall increase in crude protein content [25].

The present study revealed a significant association between the quantity of gas accumulated by the 96 h and IVDMD. In addition, an observation was made suggesting that brittleness RS showed higher levels of accumulated gas and digestibility when compared to the WT variety. The heightened gas accumulation and enhanced digestibility of RS brittleness could potentially be attributed to modifications in the composition of sclerenchyma cells and the vascular bundles distance [10,26].

Sclerenchyma cells are a type of plant cell characterized by their thick, lignified secondary cell walls, which provide structural support and strength to various plant tissues [27]. The secondary cell walls of sclerenchyma cells are rich in lignin, a complex organic polymer that adds rigidity and resistance to decay. The lignin content in sclerenchyma cells increases with the age of the plant, contributing to the cell wall's mechanical strength and durability [28]. These mutants or brittleness RS also show fewer sclerenchyma cells and thinner sclerenchyma cell walls, which contribute to their brittleness and improved in situ digestibility [10]. *In vivo* studies with lucerne and Italian ryegrass fed to cattle demonstrated that thick-walled cells, including sclerenchyma, were more prevalent in faeces, indicating lower digestibility compared to thin-walled cells [29]. The lignin and its binding with cellulose in thick-walled cells like sclerenchyma limit degradation, and pretreatments that reduce wall thickness improve digestibility [30].

The anatomical changes in brittle culm mutants include increased vascular bundle distance and reduced

culm wall thickness, which are linked to variations in cell wall components such as pectins, lignin, suberin, and cellulose [26]. Increased vascular bundle distance in forage plants can significantly impact rumen degradability, as it influences the structural composition and accessibility of nutrients to rumen microbes. For instance, studies on brown midrib (bmr) sorghum-sudangrass hybrids, which typically have altered vascular bundle structures, show higher ruminal degradability of dry matter (DM), neutral detergent fiber (NDF), and crude protein (CP) compared to conventional hybrids. This is attributed to the reduced lignin content and modified vascular bundle arrangement in bmr plants, facilitating better microbial access and faster degradation rates [31]. Similarly, the degradability of tropical legumes like *Mucuna pruriens* and *Canavalia ensiformis*, which have different vascular bundle arrangements, was found to be high, indicating that structural differences can enhance rumen degradability [32]. Despite their fragility, brittle culm mutants maintain normal growth and development, with some exhibiting shorter plant height and stronger resistance to lodging, making them valuable for energy crop breeding due to their higher biomass yield and enzymatic digestibility [24].

The elevated level of ammonia nitrogen found in the purple brittleness RS group may be attributed to the greater proportion of crude protein (CP) in comparison to the other groups, despite having a similar digestibility level as the green brittleness RS group and the WT group. Consequently, the ammonia nitrogen concentration is the highest in this group. Increased concentrations of rumen ammonia nitrogen were linked to enhanced rumen protein degradability and improved efficiency of rumen microbial activity.

The key chemical differences between purple brittleness RS and regular RS can be discerned through various studies that highlight their distinct compositions and properties. Purple RS, particularly from varieties like KDK and K4, is noted for its high concentrations of anthocyanins and phenols, which contribute to its significant antioxidant capacity [33]. This is in contrast to regular RS, which generally lacks these high levels of bioactive compounds.

Anthocyanins, such as those found in natural grape extract and red cabbage extract, exhibit antioxidant properties that can benefit ruminant health and performance. However, their rapid degradation in the rumen can limit their effectiveness, necessitating protective measures like rumen-by-pass formulas to maintain their biological activity [34]. However, the effects of phenolic compounds on rumen degradation can be variable, with some compounds like vanillin and cinnamic acid inhibiting the digestibility of cellulose and xylan by affecting the attachment of fibrolytic microorganisms to fiber particles [35]. Despite these inhibitory effects, other phenolic compounds such as syringic acid and p-hydroxybenzoic acid have been shown to stimulate microbial growth and fiber degradation [36.]

At the 8-hour post-incubation period, the concentration of total volatile fatty acids exhibited a comparable trend to both the gas volume accumulated by the 96 h and the IVDMD values. The brittleness RS group demonstrated elevated levels of total VFAs in contrast to the WT group, a difference linked to variations in chemical composition containing in each RS varieties. These distinctions among groups correlate with the trends observed in kinetic gas values, as well as parameters b and d value. In the 1-hour post-incubation period, the GBL8-B3 group demonstrated superior yield efficiency compared to the C3:C2 group, a result attributed to factors such as cumulative gas volume and IVDMD digestibility.

The brittleness of RS significantly influences its degradation rate in the rumen and the subsequent production of volatile fatty acids (VFAs). Brittle RS, such as the AZ1803 mutant with a 25 % reduction in cellulose content due to a mutation in the OsCesA7 gene, shows improved digestibility and reduced bacterial lesion length, making it more suitable for livestock feed [12]. The BC15 gene mutation, which decreases the fibrosis of the cell wall, enhances early-stage rumen fermentation, leading to higher dry matter (DM) and neutral detergent fiber (NDF) degradation, and shifts fermentation patterns from acetate to propionate and butyrate production, ultimately reducing methane (CH4) production [37]. Compaction of RS also affects its anaerobic biodegradability, with moderate compaction (555 Pa) significantly increasing biogas production compared to non-compacted straw, indicating that physical changes can enhance microbial access and degradation [38]. The degradation pattern of RS in the rumen shows that significant degradation

occurs between 6 and 24 h, with tightly attached bacteria, particularly Firmicutes, playing a crucial role in this process [39]. Overall, the brittleness of RS, influenced by genetic mutations and various pretreatment methods, enhances its degradation rate in the rumen, leading to increased VFA production and improved feed efficiency for ruminants.

Conclusions

Brittleness RS exhibited elevated levels of CP, high hemicellulose content, and lower cellulose levels compared to the WT group. Moreover, the brittleness RS group displayed a rapid degradation rate impacting the total VFAs concentration and C2:C3 ratio, with the GBL8-B3 group demonstrating superior utilization efficiency. Consequently, the brittleness of RS is indicative of a higher potential as a roughage source when compared to the wild-type variety, thereby improving the quality of RS for roughage consumption by ruminants.

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