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Optimal Inclusion Levels of Cricket and Silkworm as Alternative Ruminant Feed: A Study on Their Impacts on Rumen Fermentation and Gas Production

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Abstract: Due to growing interest in alternative and sustainable high-quality feed sources for the livestock industry, we carried out a study to determine the optimal inclusion levels of two insect species (*Gryllus bimaculatus* and *Bombyx mori*) in a feed for ruminants, as well as evaluating their impacts on rumen fermentation characteristics and methane production. An experiment was performed using an in vitro model for 24 h with a ruminant diet (control group) of 60%:40% grass:concentrate, in order to investigate the effects of insect inclusion into the diet at 10, 20, 30, and 40%, through their substitution into the concentrate mixture. The rumen fermentation parameters indicated that each insect could be included in the diet up to 20% without adverse effects on nutrient digestibility, while increasing the production of ammonia-nitrogen. Increasing the inclusion level beyond 20% led to significant decreases ($p < 0.05$) in the total gas production, nutrient digestibility, and volatile fatty acids production due to the high fat content in these dietary treatments. Therefore, *G. bimaculatus* and *B. mori* could be used as an alternative ruminant feed up to 20%, in order to replace high-quality feed ingredients. Formulating ruminant feed using insects as ingredients should take into consideration their fat content and the total dietary fat content.

Keywords: alternative protein sources; digestibility; global warming; insects; methane; sustainability

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1. Introduction

By 2050, the global human population is expected to increase to approximately 9.5 billion and, hence, the demand for animal-based food production will need to increase by 70% [1]. Although the livestock industry plays an important role in human nutrition, there is severe competition between food and feed producers for natural resources, such as land and water, which is worsening the global situation of food insecurity [2]. The livestock industry uses approximately 70% of the arable land, either for grazing or for crop cultivation used for feed production [3]. The increased cultivation of crops (e.g., soybean or cottonseed meal) used as conventional protein ingredients in livestock diets has put more pressure on non-renewable sources, in addition to their negative impacts on the environment [4]. Additionally, feed prices continue to substantially increase, representing a great threat to the industry, especially for small-scale farmers in the developing world. The feed cost constitutes 60–80% of the total production costs, with protein sources being undoubtedly the most expensive feed ingredients [5]. Considering all these constraints faced by the animal production industry, there is a necessity to find alternative, sustainable, high-quality protein sources with a lower environmental footprint.

At present, insects are considered among the most promising and sustainable alternative protein sources for animals. Insects are characterized by high nutritive value, especially their high protein quantity and quality, which is comparable to that of high-quality and expensive protein sources such as soybean meal [6,7]. Using insects as animal feed has major

environmental advantages over traditional sources, due to their role in waste bioconversion and reduced water and land use, while producing lower greenhouse gas emissions [8,9]. Several scientific research studies in the last decade have demonstrated the effectiveness of insects as an alternative feed for monogastric animals [10–14]. Additionally, according to recent studies, consumers all over the world seem to be easily accepting the consumption of animal products (meat and eggs) from animals fed on insect-based diets [15–18]. Thus, this has encouraged decision-makers in the European Union, in April 2021, to approve the usage of insects to feed pigs and poultry, besides being already approved for aquaculture since July 2017 [19]. Given the ruminant production industry and the fear of mad cow disease transmission, the legislations vary from prohibition to unclear or no specific laws regarding the usage of insects as feed for ruminant [20]; however, due to growing concerns over climate change and the food insecurity situation, the insect production industry and their use as a feed for ruminants has been gaining momentum in both developing and developed countries. To date, there exist scarce data regarding the usage and impacts of insects as alternative feed for ruminants; thus, more scientific research could help to move this topic onto the agendas of policy-makers in Europe and around the world, as well as aiding in the development of a regulatory framework for licensing insects as ruminant feed [21,22].

In a previous trial, we evaluated four kinds of insects—*Acheta domesticus*, *Brachytrupes portentosus*, *Gryllus bimaculatus*, and *Bombyx mori*—as alternative ruminant feed, at an inclusion level of 10% in the diet replacing soybean meal [23]. These insects were found to be rich in fat, with unsaturated fatty acids being of a high proportion. They also had a high protein content and contained the same amino acid profile as soybean meal. These species were efficiently able to partially replace soybean meal in the diet without negative impacts on rumen fermentation or nutrient digestibility. Interestingly, the inclusion of *G. bimaculatus* and *B. mori* led to a reduction in methane (CH_4) yield by 18% and 16%, respectively, thus providing an additional environmental benefit. Therefore, the present study is a step forward to determine the optimal inclusion levels of the latter two promising species. The impacts on rumen fermentation characteristics and CH_4 production were investigated at different inclusion levels, up to a level where they completely replaced the expensive high-quality feed ingredients (a commercial concentrate mixture) in a ruminant diet.

2. Materials and Methods

The Obihiro University of Agriculture and Veterinary Medicine's Animal Care and Ethics Committee approved this study (approval number 21-213). The animals involved in these experiments were cared for by the Field Science Center. Animal management and sampling procedures were performed according to the established guidelines at the Obihiro University of Agriculture and Veterinary Medicine.

2.1. Basal Diets and Insects

A commercial concentrate mixture and kleingrass (*Panicum coloratum*) hay were used as the basal diet. They were ground using the Retsch SM-2000 cutting mill (Retsch GmbH, Haan, Germany) to pass through a 1 mm sieve. Adult field crickets (*G. bimaculatus*) and silkworm pupae (*B. mori*) in fine powder form were purchased from Thailand Unique Co., Udon Thani, Thailand (https://www.thailandunique.com, accessed on 25 November 2022). The insect powder products were 100% natural, without added preservatives, and the insects were raised under clean hygienic conditions and fed a mixed diet of vegetables and grains. The proximate analyses of the concentrate mixture, grass hay, and insects are provided in Table 1. The amino acid and fatty acid profiles of the tested insects have been evaluated and reported previously, by Ahmed et al. [23].

Table 1. Chemical composition (% in dry matter) used for 24 h in vitro incubation.

%	Kleingrass Hay	Concentrate Mixture	*Gryllus bimaculatus*	*Bombyx mori*
Dry matter (% in fresh matter)	90.86	87.95	95.48	97.84
Organic matter	90.74	94.47	95.26	95.08
Crude ash	9.26	5.53	4.74	4.92
Crude protein	14.1	16.3	62.51	62.73
Ether extract	3.0	3.9	21.85	13.35
Neutral detergent fiber	58.1	37.7	32.32	30.46
Acid detergent fiber	33.5	15.7	11.66	9.27
Chitin			10.13	7.38
Ingredients of the Concentrate Mixture (%)				
Corn		41.0		
Wheat		5.0		
Soybean meal		14.0		
Rapeseed meal		11.0		
Corn gluten		17.0		
Dried distiller's grains with solubles		7.0		
Wheat bran		1.0		
Molasses		1.5		
Calcium carbonate		1.5		
Vitamin and mineral complex		1.0		

2.2. Donor Animals and Rumen Fluid Collection

Approximately 1.3 L of rumen fluid was collected from four different locations in the rumen after 3 h of morning feeding from two ruminally cannulated non-lactating Holstein cows approximately 8 years old with an average body weight of 894 kg. At the maintenance level, the cows were fed orchard grass (*Dactylis glomerata*) hay with 98% organic matter (OM), 13% crude protein (CP), 70% neutral detergent fiber (NDF), 35% acid detergent fiber (ADF), 4% acid detergent lignin (ADL), and free access to clean drinking water and mineral blocks. The rumen fluid was collected, strained through four layers of surgical gauze, placed in a thermos flask pre-warmed to 39 °C, and transferred to the laboratory within 15 min.

2.3. In Vitro Experimental Design and Incubation Procedure

The basal diet (control group) was composed of 300 mg grass hay + 200 mg concentrate mixture. In the eight experimental treatments (four for each insect species), the concentrate mixture was replaced with inclusion levels at 10, 20, 30, and 40% in the basal diet as follows: 300 mg grass hay + 150 mg concentrate + 50 mg insect (10%), 300 mg grass hay + 100 mg concentrate + 100 mg insect (20%), 300 mg grass hay + 50 mg concentrate + 150 mg insect (30%), and 300 mg grass hay + 0 mg concentrate + 200 mg insect (40%). There were nine experimental groups in total, each with four replicates. This experimental design was repeated in five runs in different weeks. Two blanks without substrate (buffered rumen fluid only) were included in each run. The substrate (500 mg) was added to pre-weighed nylon bags with pore sizes of 53 ± 10 μm (BG1020, Sanshin Industrial Co., Ltd., Kanagawa, Japan), heat-sealed, and placed in 120 mL glass bottles. These bags were used in the first two experimental runs, and were replaced by ANKOM filter bags (F57, ANKOM Technology, Macedon, NY, USA) for the remaining three runs, as we observed a loss of the insect powder out of the nylon bag during incubation and washing, which may have led to inappropriate estimation of the digestibility-related parameters.

Under continuous carbon dioxide (CO_2) flushing, each fermentation bottle received 40 mL of fresh buffer solution at pH 6.8 [24] and 20 mL of the collected rumen fluid were added to each fermentation bottle. After that, the bottles were CO_2 flushed before being sealed with butyl rubber stoppers and aluminum caps. All bottles were incubated for 24 h at 39 °C. The 24 h incubation time is enough time for in vitro studies to discriminate

the differences between experimental treatments and allow for reliable interpretation of the results [25].

2.4. Incubation Media Sampling

The total gas production was measured using a calibrated syringe at the end of the incubation, and a sample of the headspace gas was collected from each bottle and stored in a vacutainer tube (BD Vacutainer, Becton Drive, NJ, USA) until further CH_4 and CO_2 determination [26]. The pH was immediately measured, and 1 mL of fermentation media was collected in Eppendorf tubes and centrifuged at 16,000× *g* for 5 min at 4 °C. The supernatant was used to estimate the concentrations of volatile fatty acids (VFA) and ammonia-nitrogen (NH_3-N). The substrate-containing bags were rinsed with tap water until the effluent became clear, then dried for 48 h at 60 °C and weighed to determine the in vitro dry matter digestibility (IVDMD). The IVDMD was calculated as the DM that disappeared from the initial DM weight input into the bag [27].

2.5. CH_4 and CO_2 Production

Gas chromatography (GC-8A, Shimadzu Corp., Kyoto, Japan) was used to determine the concentrations of CH_4 and CO_2 by injecting 1 mL of each sample using a gastight syringe (Hamilton Company, Reno, NV, USA). Further details on the GC conditions have been reported previously [28]. The total production (mL) of CH_4 and CO_2 was estimated by multiplying the concentration by the total gas production.

2.6. Volatile Fatty Acids and Ammonia-Nitrogen Analysis

The concentration of VFA was determined by high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan), as processed and described previously [29]. Samples were diluted 100 times with 0.1 M phosphate buffer (pH 5.5) and analyzed using a LabAssay^{TM} Ammonia kit (LABNH3-M1, Fujifilm Wako, Osaka, Japan) to determine the concentration of NH_3-N [30].

2.7. Chemical Analysis

The chemical compositions of grass hay, concentrate, and insects were conducted following the AOAC standard procedures [31]. The DM content was estimated using an oven for 2 h at 135 °C (method 930.15). The OM was measured in a muffle furnace for 3 h at 500 °C (method 942.05). The ether extract (EE) content was estimated using method 920.39. The nitrogen content was determined using the Kjeldahl method (method 984.13), and the CP was calculated as nitrogen × 6.25. The NDF, ADF, and ADL were determined and expressed as inclusive residual ash using an ANKOM200 fiber analyzer (ANKOM Technology Corp., Macedon, NY, USA). The NDF was measured using sodium sulfite without heat-stable α-amylase. The chitin content was calculated according to the formula: chitin = ADF−ADL [32]. The chemical composition of the experimental treatments with different inclusion levels of insects is provided in Table 2.

2.8. Statistical Analysis

Data were analyzed using the PROC MIXED module of the SAS statistical software (version 9.4; SAS Institute Inc., Cary, NC, USA). The statistical model included the treatments as a fixed effect, while the five experimental runs were considered random effects. Orthogonal polynomial contrasts were used to determine the significance of linear or quadratic models describing the response in the variables to the increasing levels of insects in the diet. The values are represented as means with pooled standard errors. Tukey's test was used to estimate mean differences between experimental groups. At $p < 0.05$, differences were considered statistically significant.

Table 2. Chemical composition (% in dry matter) of the experimental treatments with different inclusion levels of insects in the basal diet.

	Control	*Gryllus bimaculatus*				*Bombyx mori*			
Parameter	**0%**	**10%**	**20%**	**30%**	**40%**	**10%**	**20%**	**30%**	**40%**
Dry matter (% in fresh matter)	89.70	90.45	91.20	91.96	92.71	90.69	91.67	92.66	93.65
Organic matter	92.23	92.31	92.39	92.47	92.55	92.29	92.35	92.42	92.48
Crude ash	7.77	7.69	7.61	7.53	7.45	7.71	7.65	7.59	7.52
Crude protein	14.98	19.60	24.22	28.84	33.46	19.62	24.27	28.91	33.55
Ether extract	3.36	5.16	6.95	8.75	10.54	4.31	5.25	6.20	7.14
Neutral detergent fiber	49.94	49.40	48.86	48.33	47.79	49.22	48.49	47.77	47.04
Acid detergent fiber	26.38	25.98	25.57	25.17	24.76	25.74	25.09	24.45	23.81
Acid detergent lignin	3.60	3.54	3.49	3.43	3.37	3.58	3.56	3.54	3.52
Chitin		1.01	2.03	3.04	4.05	0.74	1.48	2.21	2.95

3. Results

3.1. Gas Production and Composition

The inclusion of *G. bimaculatus* led to a linear reduction ($p < 0.01$) in the total gas production yield (mL/g) of digestible DM (D.DM), when compared with the control group (Table 3). The same finding was observed with the inclusion of *B. mori*. The proportion of CH_4 and CO_2 was not affected by the inclusion level of *G. bimaculatus* or *B. mori*. The CH_4 yield (mL/g) of DM decreased significantly, by 11% ($p = 0.03$) and 19.7% ($p < 0.01$), under 30 and 40% inclusion of *G. bimaculatus* in the basal diet, respectively, compared with the control diet (Table 3). Inclusion of *B. mori* indicated a significantly stronger reduction potential at lower inclusion levels. The reduction potential of *B. mori* was 9.9, 9.9, 12.7, and 23.9% at inclusion levels of 10, 20, 30, and 40%, respectively. However, this reduction potential was not observed ($p > 0.05$) when CH_4 yield was corrected by the D.DM for both insect species. The total CO_2 yield (mL/g) of D.DM significantly decreased with the inclusion of *G. bimaculatus* in the basal diet over 20%, while it was significantly lower with *B. mori* over 30%, when compared with the control diet without insect supplementation ($p < 0.05$; Table 3).

3.2. Rumen Fermentation Characteristics

The IVDMD decreased linearly beyond the inclusion level of 20% for both *G. bimaculatus* and *B. mori* ($p < 0.01$; Table 4), when compared with the basal diet. Similarly, inclusion of *G. bimaculatus* and *B. mori* led to a linear reduction in the production of total VFA, which was obvious at inclusion levels of 30 and 40% ($p < 0.01$; Table 4). In a linear manner, substituting the concentrate mixture with *G. bimaculatus* and *B. mori* shifted the rumen fermentation profile toward more acetate and less propionate ($p < 0.01$; Table 4). Inclusion of *G. bimaculatus* and *B. mori* in the diet linearly increased the concentration of NH_3-N when compared with the control group ($p < 0.001$; Table 4).

Table 3. Effect of different inclusion levels of insects in the basal diet on gas production profile from 24 h in vitro incubation (n = 20).

	Control (CT)	***Gryllus bimaculatus* (G. b.)**				***Bombyx mori* (B. m.)**					***p*-Value**		
Parameter	**0%**	**10%**	**20%**	**30%**	**40%**	**10%**	**20%**	**30%**	**40%**	**SEM**	**Trt**	**CT × G. b.**	**CT × B. m.**
Total Gas/DM [1] (mL/g)	110.25	111.60	106.48	94.52 ***	86.16 ***	102.01 ***	100.11 ***	95.32 ***	83.98 ***	1.97	<0.001	L Q	L Q
Total gas/D.DM [2] (mL/g) [#]	275.67	277.21	275.27	258.31 *	248.19 ***	263.18	261.83	263.97	249.85 ***	3.08	<0.001	L Q	L
CH_4 (%)	6.39	6.55	6.65	6.60	6.53	6.20	6.34	6.43	6.34	0.066	0.071	ns	ns
CO_2 (%)	93.61	93.45	93.35	93.40	93.47	93.80	93.66	93.57	93.66	0.066	0.071	ns	ns
CH_4/DM (mL/g)	7.10	7.44	7.21	6.31 *	5.70 ***	6.40 **	6.40 **	6.20 ***	5.40 ***	0.177	<0.001	L Q	L
CH_4/D.DM (mL/g) [#]	17.53	18.10	18.52	17.31	16.77	16.67	17.12	17.65	16.03	0.291	0.002	ns	ns
CO_2/DM (mL/g)	103.15	104.17	99.27	88.21 ***	80.46***	95.61 ***	93.72 ***	89.13 ***	78.59 ***	1.80	<0.001	L Q	L Q
CO_2/D.DM (ml/g) [#]	258.14	259.10	256.75	240.99 *	231.42 ***	246.51	244.71	246.32	233.83 ***	2.85	<0.001	L Q	L

[1] DM, dry matter. [2] D.DM, digestible dry matter. [#] n = 12. SEM: standard error of the mean. Asterisks mean a significant difference between this inclusion level and the control group (0%), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). L: linear. Q: quadratic.

Table 4. Effect of different inclusion levels of insects in the basal diet on rumen fermentation characteristics from 24 h in vitro incubation (n = 20).

	Control (CT)	***Gryllus bimaculatus* (G. b.)**				***Bombyx mori* (B. m.)**					***p*-Value**		
Parameter	**0%**	**10%**	**20%**	**30%**	**40%**	**10%**	**20%**	**30%**	**40%**	**SEM**	**Trt**	**CT × G. b.**	**CT × B. m.**
pH	6.70	6.74 ***	6.76 ***	6.77 ***	6.76 ***	6.69	6.73 *	6.73 **	6.74 ***	0.003	<0.001	L Q	L
IVDMD [1] (%) [#]	36.81	35.62	34.66	33.79 *	31.05 ***	36.57	36.28	34.79 *	30.91 ***	0.413	<0.001	L	L Q
Acetate (mmol/L)	67.54	67.65	67.15	66.25 *	64.57 ***	67.19	67.92	66.97	65.79	0.844	<0.001	L Q	L Q
Propionate (mmol/L)	22.72	21.94 *	21.08 ***	19.70 ***	18.24 ***	21.80 **	20.98 ***	19.56 ***	18.05 ***	0.323	<0.001	L Q	L Q
Butyrate (mmol/L)	11.07	11.20	10.64	9.82 ***	9.27 ***	10.52	9.87 *	9.59 ***	9.12 ***	0.234	<0.001	L Q	L
Total VFA [2] (mmol/L)	101.32	100.79	98.86 *	95.77 ***	92.09 ***	99.50	98.78 *	96.12 ***	92.96 ***	1.33	<0.001	L Q	L
Acetate (mol/100 mol)	66.31	66.77	67.69 ***	69.02 ***	69.98 ***	67.30 *	68.63 ***	69.58 ***	70.74 ***	0.209	<0.001	L	L
Propionate (mol/100 mol)	22.54	21.87 ***	21.41 ***	20.67 ***	19.89 ***	22.03 **	21.40 ***	20.50 ***	19.49 ***	0.098	<0.001	L	L Q
Butyrate (mol/100 mol)	11.15	11.37	10.9	10.31 *	10.13 *	10.67	9.97 *	9.93 **	9.76 ***	0.178	<0.001	L	L
NH_3-N (mg/dL)	11.23	15.11 **	20.30 ***	17.38 ***	17.89 ***	11.79	15.09 **	17.36 ***	18.67 ***	0.49	<0.001	L Q	L

[1] IVDMD: in vitro dry matter digestibility. [2] VFA: volatile fatty acids. [#] n = 12. SEM: standard error of the mean. Asterisks mean a significant difference between this inclusion level and the control group (0%), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). L: linear. Q: quadratic.

4. Discussion

Concerns about rising natural resource demands and climate change have prompted an increase in initiatives to improve production sustainability and efficiency. One such sustainable solution involves the use of insects as an alternative ruminant feed, substituting for traditional feed ingredients. While this area of research is gaining great attention, this topic is still in its infancy. In 2022, the global insect protein market was estimated to be USD 428.12 million, which is expected to rise by 26.5% to USD 1386.5 million by 2027 [33]. There are no economic evaluation studies on the use of insects as a protein source for ruminants instead of other feed ingredients such as soybean meal so far; however, with increasing venture investments in finding alternative and sustainable feed strategies and feed technology innovation at a commercial scale, it is expected that the cost of insect protein as a feed source will be lower than other conventional feed sources when considering their environmental impact and role in waste bioconversion [34]. Our previous trial was one of the very few studies evaluating insect species as ruminant feed [23]. This study indicated the efficiency of *G. bimaculatus* and *B. mori* at an inclusion level of 10% to substitute for soybean meal without adverse impacts on rumen fermentation. Therefore, the current study was conducted to determine the optimal levels of both species, as well as their impacts on rumen fermentation and CH_4 production.

The proximate analysis conducted in the present study confirmed the previous findings reported in several studies regarding the high nutritive value of insects, in terms of their high protein and fat contents [6,35]. It is worth mentioning that the nutrient profile of insects depends mainly on the quality of the rearing substrate, life stage, and species [36,37]. Additionally, the variations in the analytical procedures for the proximate analysis of insects between studies should be considered [38]. The exoskeletons of insects are characterized by the presence of 8–9% of chitin, which is a long-chain polymer of N-acetylglucosamine [39]. This component is regarded as a fiber which is well known to be hardly digested, which may disturb nutrient digestibility and absorption [40,41]. Therefore, it has been recommended that the removal of chitin content from insect products may increase nutrient availability and digestibility [42].

Due to the high fat content of the tested insects, the IVDMD decreased under the higher inclusion levels, especially those over 20% in the basal diet. This finding confirms the observation made in our previous trial, where the inclusion of both insect species at 10% in the diet did not have adverse effects on the IVDMD [23]. In this line, it has been recommended that the total fat content in ruminant diets should not exceed 6–7%; otherwise, it may have detrimental effects on digestibility and fermentation [43,44]. It is well known that high fat content negatively affects ruminal fibrolytic bacteria such as *Fibrobacter succinogenes*, as well as decreasing the number of rumen protozoa which are involved in carbohydrate digestion [45]. This theory has recently been supported by the finding of Thirumalaisamy et al. [46], who have reported that increasing the level of silkworm pupae oil reduced the IVDMD and the total number of protozoa. Therefore, de-fatted insects might be a suitable option as a protein source, in the case where they are included at higher levels. De-fatted silkworm pupae meal has been incorporated into the diet of cattle (70%:30% forage:soybean) up to 30%, replacing the soybean meal, without affecting rumen fermentation characteristics or nutrient utilization [47]. Surprisingly, and in contrast to our finding, Phesatcha et al. [48] have found that by increasing the inclusion level of *G. bimaculatus* up to a level where it completely replaced soybean meal in the diet (the total dietary fat content was 11%), the IVDMD increased. Another factor that might have played a role in the lower IVDMD with increasing inclusion levels of insects in the current study could be the chitin content; however, when the chitin content of *G. assimilis* was removed, either manually or chemically, there were no differences in the in vitro OM digestibility [41]. In a recent in vitro trial, it has been reported that, when *G. bimaculatus* was used as a feed at different inclusion levels to replace soybean meal, the digestibility was also affected by the forage:concentrate ratio of the diet, revealing the great complexity of factors and interactions involved, which should be considered in further studies [48].

As expected, the concentration of NH_3-N increased with increasing level of inclusion of the tested species, which might be related to their high degradable protein content, as has been previously observed [23]. It is well established that rumen microorganisms can use ammonia as a nitrogen source and a precursor of amino acids and microbial protein synthesis [49]. Interestingly, the rumen fermentation profile was shifted toward more acetate and less propionate with increasing inclusion of insects, which might be related to the less-fermentable nutrients or the increased chitin content, which has a similar chemical structure to cellulose [39]. Higher acetate is usually associated with the presence of fibrous carbohydrates [50]. A contradictory finding was observed in the study of Phesatcha et al. [48], in which the authors found that the fermentation was directed toward more propionate and less acetate, which was associated with increased IVDMD, when increasing the inclusion level of *G. bimaculatus* in the diet. Therefore, this divergence warrants the importance of microbiome analyses in future studies, in order to understand the alterations in the microbial community following the inclusion of insects into the diet.

As a consequence of the lower digestibility and inhibited fermentation rate, the total gas yield decreased. Fats inhibit methanogenesis through four mechanisms: Reducing nutrient degradation and fermentation, a toxic effect on cellulolytic bacteria and protozoa, decreasing the activity of methanogens, and biohydrogenation of unsaturated fatty acids [51,52]. The variation in CH_4 reduction potential is strongly dependent on the composition, form, and concentrations of the fatty acids [44,53]; however, in the current study, the inclusion of insects could not have decreased the CH_4 production when correlated to the degraded DM. Furthermore, the proportion of CH_4 was not affected by the inclusion of insects at all levels, which supports the previously reported fact that lipids do not have a direct anti-methanogenic efficiency; instead, their role in decreasing CH_4 production is a consequence of their lower digestibility and, thus, less substrates being available for methanogenesis [54]. In our previous trial, we observed a reduction in CH_4 yield by up to 16–18% under 10% inclusion of *G. bimaculatus* and *B. mori*, which was not reproduced in the current trial. We attribute this discrepancy to the different diet offered to the donor animals in this experiment, compared with the one in the previous trial. Moreover, in the present trial we used a concentrate mixture, while previously it was soybean meal. Thus, further trials with different feed ingredients and different forage-to-concentrate ratios must be conducted, in order to better understand the factors affecting the response in rumen fermentation and CH_4 mitigation.

5. Conclusions

For the present study, we evaluated the effects of different inclusion levels of *G. bimaculatus* and *B. mori* as an alternative feed to substitute for a commercial concentrate mixture in a ruminant diet on rumen fermentation characteristics and gas production. Inclusion of the tested insect species up to 20% had no adverse effect on nutrient digestibility. Beyond this level, increasing inclusion level adversely affected rumen fermentation parameters. Therefore, the use of insects in ruminant diets as a feed should be considered carefully, based on their fat content. Otherwise, the use of de-fatted insects might provide a suitable strategy for higher inclusion levels. Further trials with different forage:concentrate ratios are required, in order to better understand the optimal inclusion levels. In vivo trials are also required to confirm these findings.

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