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Assessing the effects of live yeast supplementation to diets containing high starch of dairy cows on rumen fermentation and methane production *in vitro*

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ABSTRACT

This study was conducted to evaluate the effects of live *Saccharomyces cerevisiae* yeast (LY) supplementation on high-starch diets for dairy cows. Using a 2 × 2 factorial design, four treatment groups were examined: adequate starch without LY (AS-control), AS with LY (AS-LY), high starch without LY (HS-control), and HS with LY (HS-LY). LY was added at 2.9×10^6 CFU/200 mg DM or without yeast. In vitro gas and methane (CH_4) production, organic matter (OM) digestibility, rumen fermentation, and metabolizable energy (ME) were analyzed. The Hohenheim Gas Test apparatus was used, and total gas volumes were measured at various incubation hours. Results showed no significant effects of LY or dietary starch ratio on gas kinetics, total gas, or CH_4 production. Similarly, OM digestibility, fermentation kinetics, and ME were unaffected. In conclusion, LY supplementation did not alter in vitro gas, CH_4 production, OM digestibility, rumen fermentation, or ME. Further research could investigate the underlying mechanisms, such as rumen microbial population influences.

Keywords: dairy cow, high starch, in vitro fermentation, live yeast, methane production

INTRODUCTION

It is a common procedure to provide dairy cows with diets containing substantial amounts of starch derived from cereal grains to mitigate postpartum negative energy balance and enhance milk production (Lara et al., 2018). Diets with higher starch (HS) content for lactating dairy cattle contain 26% to 33% starch of diet dry matter (DM) (Boerman et al., 2015). When

dairy cattle are fed high starch diets for an extended period of time, they become predispose to subacute rumen acidosis (SARA), a common metabolic disorder (Amin and Mao, 2021). The intake of excessive levels of fermentable concentrates has been shown to alter the function of the rumen by elevating the ruminal concentration of short chain fatty acids (SCFA) (Li et al., 2012). Furthermore, excessive amounts of fermentable concentrates have been reported to have a deleterious impact on the rumen environment by diminishing salivary secretions and the rumination process, lowering the ruminal pH, and causing rumen epithelial damage (Wanapat et al., 2014). Its consequences are diverse and complex, which include feed intake depression, fluctuations in feed intake, reduced diet digestibility, reduced milk yield, reduced milk fat percent, liver abscesses, and lameness (Abdela, 2016).

A practice to prevention of the risk of developing SARA in feeding dairy cows starch-rich concentrate feedstuffs is the supplementation of *Saccharomyces cerevisiae* which is the most common active dry yeast (Baker et al., 2022). Active dry yeasts are dried fermentable living yeasts and contain at least 15×10^9 active live yeast (LY) cells per gram (Alugongo et al., 2017). LY are facultative anaerobes, unlike rumen-beneficial microorganisms, which are strictly anaerobes (Chaucheyras-Durand et al., 2008). Consequently, *S. cerevisiae* possesses the capability to scavenge the oxygen entering the rumen via rumination and salivation on a daily basis, thereby maintaining the anaerobic environment in rumen (Barrera et al., 2019). *S. cerevisiae* is also able to stabilize rumen pH due to limiting the lactate concentration produced in the rumen by competing for the utilization of sugars with *Streptococcus bovis* (Dias et al., 2018). Studies have established that yeasts have the capability to enhance the metabolic activity of particular species of lactic acid consuming bacteria, namely *Selenomonas ruminantum* and *Megasphaera elsdenii* in the rumen (Barrera et al., 2019). These activities create a rumen ecosystem optimized for cellulolytic bacteria, improving fiber degradation (Moya et al., 2018). Although the aforementioned benefits of dietary supplementation of *S. cerevisiae* have been documented, the responses to such supplementation in rumen microbiota (Bayat et al., 2015) and fermentation parameters (Dias et al., 2018; Barrera et al., 2019; Sun et al., 2021) are inconsistent, potentially due to variations in yeast strain, dosage, and research methodology (Baker et al., 2022). Therefore, research is ongoing focusing on continuous monitoring and comparison of the variables of *S. cerevisiae* contribution in stabilizing ruminal pH (Baker et al., 2022).

Despite substantial research on *S. cerevisiae* in the dairy sector, there is little data on how LY affects high-starch diets. This study aimed to explore the potential of live *S. cerevisiae* to decrease CH_4 production and enhance the efficiency of fermentation and digestibility in a corn-based high-starch diet for dairy cows. The hypothesis being tested was that adding *S. cerevisiae* would

lead to enhanced in vitro fermentation and OM digestibility and decreased CH_4 production in such diets

MATERIALS AND METHODS

Experimental design, diets and live yeast product

Current investigation employed a 2 × 2 factorial experimental design, factors included two different starch (23% and 33% of diet DM) and two different doses of live *S. cerevisiae* yeast (2.9×10^6 CFU [without addition of yeast] and 2.9×10^6 CFU/200 mg substrate DM), resulting in the examination of four distinct treatment groups. Treatments were 1) adequate starch (AS) (AS-control); (2) AS and LY (AS-LY); (3) high starch (HS) (HS-control) and (4) HS and LY (HS-LY).

Two experimental total mixed rations, containing 23% (AS) and 33% (HS) of starch, were formulated (Table 1) to fulfil the nutritional requirements of lactating Holstein cows (body weight of 650 kg, DM intake target of 17 kg/d, a daily milk production target of 40 kg/d, with a composition of 3.5% fat, 3.0% protein, and 4.8% lactose) (NRC, 2001). By substituting a portion of the forage with finely ground corn, the starch concentration of the HS diet was increased (Table 1). Diets were prepared isonitrogenically and isocalorically. Diet samples were dried in a forced ventilation oven for 48 h at 55 °C, grounded to pass the 1-mm screen of a Wiley mill, and then stored in plastic bags for compositional analysis and in vitro gas production. *S. cerevisiae* containing 1×10^{10} CFU/g (VISTACELL; Nutrivet, İzmir Turkey) was supplemented at 0.06 mg/200 mg of diet DM, which was expected to have 2.9×10^6 CFU/200 mg of diet DM (manufacturers' recommendations).

Table 1. Feed ingredient and chemical composition of the experimental diets (% DM)

Ingredient	**Diets**	
	Adequate starch	**High starch**
Corn silage	35.40	27.80
Alfalfa silage	12.80	7.30
Alfalfa hay	12.60	7.60
Ground corn	17.30	34.30
Wheat bran	2.90	1.40
Soybean meal, solvent extracted	11.00	15.30
Sunflower meal	1.50	4.00
Full fat soybean	5.00	1.10
Salt	0.28	0.28
Limestone	0.56	0.56
Vitamin - mineral premix[1]	0.56	0.56
Nutrients		

OM	84.27	85.41
CP	17.00	17.10
EE	4.0	4.30
Ash	5.70	4.60
ADF	20.20	15.70
NDF	34.10	28.70
NFC	38.70	45.30
Starch	23.20	33.20
ME[2], MJ/Kg KM	11.73	11.75

[1]10.000 gr'da vita A 2.500.00, Vit D3 500.00 IU, Vit E 2.500 mg, Niyasin 10.00 mg, Fe 6.000 mg, I 240 mg, C0 40 mg, Cu 3.00 mg, Mn 6.00 mg, Zn 20.000 mg, Se 40 mg. OM: organic matter; DM: dry matter; CP: crude protein; EE: ether extract, ADF: acid detergent fiber; NDF: neutral detergent fiber; NFC: non-fiber carbohydrate: 100- (NDF% + CP% + EE% + Ash%);
[2]ME: Calculated based on tabular value of NRC (2001)

In vitro incubation

The Hohenheim Gas Test apparatus incorporating modifications to the pistons was utilized for the incubation process, as detailed by Menke and Steingass, (1988). In brief, ruminal fluid samples as an inoculum source was collected from two beef cattle (Simmental, ~ 600 kg live weight) for production purposes slaughtered in a commercial slaughterhouse, after being fed for 3 weeks a basal diet (commercial concentrate 50%, corn silage 25%, alfalfa hay 10% and grass hay 5%). The ruminal contents were swiftly transported to the laboratory using a thermos flask that had been preheated, subjected to filtration through four layers of cheesecloth, and subsequently mixed with a Menke buffer mixture (Menke and Steingass, 1988) at 10:20 mL to preparation of the incubation media. The substrate, weighing 200 mg, was weighed into a 100 mL graduated glass syringe. The determined dose of *S. cerevisiae* was then added and the syringe was filled with 30 mL of the incubation medium. All the procedures were performed under a continuous stream of CO_2. The filled syringes were promptly sealed with rubber stoppers, subjected to agitation, and incubated in a 39° C water bath. Four blanks syringes, containing only rumen fluid/buffer solution, were also simultaneously incubated. Each treatment was incubated with 4 replicates. During the experimental procedure, the syringes containing the samples were subjected to gentle agitation every two hours to ensure proper mixing of the substrates. The in vitro total gas volumes were measured manually at 3, 6, 9, 12, and 24 h of incubation. The in vitro incubation was concluded after a duration of 24 h. The medium pH in each syringe was determined through direct measurement using a portable pH meter. In vitro CH_4 emissions were measured with an infrared analyzer (Sensor Europa GmbH, Erkrath, Germany) (Goel et al., 2008).

Chemical analyses and calculations: The procedures described in the AOAC were used to analyze the diets for DM, crude protein (CP), ether extract

(EE), and ash (1990). The Ankom 200 Fibre Analyzer (Ankom Technology, Macedon, NY, USA) was used to measure the neutral detergent fiber (NDF) and acid detergent fiber (ADF) values according to the Van Soest et al., (1991) technique.

The overall net gas production was determined through the subtraction of the gas values acquired from the control experiment. The computation of the gas production profiles was carried out using the computer software NEWAY, based on the "a + b (1 – e^{-ct})" mathematical model developed by Ørskov and McDonald (1979), where a, the gas volume of the rapidly degradable fraction; b, the gas volume of the potentially degradable fraction; c, gas production rate of the b fraction and t, incubation time.

The methodology proposed by Goel et al., (2008) was applied to calculate the quantity of methane produced, which is represented by the formula CH_4 production (mL) = Total gas production (mL) × CH_4 (%). The OM digestibility and ME of the samples were estimated by applying the method of Menke and Steingass, (1988) as:

OM Digestibility (%) = 4.88 + 0.8893 GP + 0.4484 CP + 0.651 Ash

ME,(MJ/kg DM)=1.06 + 0.157 GP + 0.084 CP + 0.22 EE- 0.081 Ash

SCFA for diets was calculated using equations of Getachew et al., (2002) as:

SCFA (mmol/200 mg DM)=0.0222 GP- 0.00425

where GP (mL/200 mg DM) represents the 24 h net gas production; CP and EE are crude protein, crude fat and crude ash (% DM), respectively.

Statistical analysis

Data analysis was performed using a 2 × 2 factorial design, with starch and yeast levels being the main sources of variability. The GLM procedure of the SPSS software was employed, and the results were presented as means. A p-value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

In vitro gas production and organic matter digestion

The results of the study, presented in Table 2 and Figure, reveal the findings regarding the gas production kinetics, GP, and OM digestibility for the AS-Control, AS-LY, HS-Control, and HS-LY treatments. The examination of Table 2 indicates the clear absence of any impact on the gas kinetic parameters, GP, and OM digestibility by the factors of starch level and yeast addition as well as their interaction.

Studies have established a strong correlation between OM digestion and GP during the microbial degradation of the substrate (Tan et al., 2011). In this study, gas production and OM digestion results were consistent and found to

be similar in AS and HS-containing substrates with and without LY. Moreover, the lack of significant variation in organic matter digestibility reinforces our conclusion that there were no alterations in the pattern of fermentation. In this study, the reason for no effect of the starch level of the substrates and *S. cerevisiae* supplemented on GP and OM digestibility is not clear. However, it is well known that the ruminal degradation of corn starch is slower and less extensive than that of other cereals due to the starch-protein matrix (McAllister and Cheng, 1996). This conclusion aligns with the results of Ovinge et al., (2018), who established that the addition of *S. cerevisiae* did not affect the digestibility of starch in steam-flaked corn-based diets for beef cattle. In the present study, the previously suggested mechanism of action for *S. cerevisiae* and the outcomes reported in most prior studies (Elghandour et al., 2014; Elghandour et al., 2017; Cagle et al., 2019; Phesatcha et al., 2021; Jasmine Rani et al., 2022) were not substantiated, as a noticeable enhancement in GP and OM digestibility due to *S. cerevisiae* was not detected. These findings are consistent with the results of several in vivo and in vitro studies that found that LY supplementation had no effect on OM digestibility (Maamouri et al., 2016; Garcia Diaz et al., 2018; Moya et al., 2018; Song et al., 2021) and GP (Amanzougarene et al., 2020) when different substrates (varying forage to concentrate ratios) were fermented. The inconsistencies in the results might be due to a range of factors, including dietary type, fiber composition, concentrate proportion, yeast strain, and dose utilized (Yaun et al., 2015). A possible explanation for this result may be the lack of effective on microbial growth and activity of *S. cerevisiae* due to different mechanisms (Phesatcha et al., 2020). The first of these mechanisms may be that the single dose addition of yeast does not lead to short-term changes in the in vitro fermentation pattern. For example, Opsi et al., (2012) showed that long-term adaptation and interaction with yeasts are required for changes in the rumen. Desnoyers et al., (2009) also reported in a meta-analysis of *S. cerevisiae* supplementation in ruminants that an enhancement in OM digestibility was positively correlated with the amount of yeast added. Second, due to the single dose addition of *S. cerevisiae* to the in vitro medium, it may not have shown optimal metabolic activity in the fermentation zone. Since the optimum growth conditions of yeasts (facultative anaerobes, 30 - 35º C, pH 4.4 - 5.6; Nayak, 2011) are different from normal rumen conditions (strictly anaerobic, 39 - 40º C, pH 5.8 - 6.8), it is recommended to constantly add them to the diet, since they cannot survive for a long time in the rumen (Jouany, 2006). As reported by Jiao et al., (2018), also it may be associated with measuring ambient pH as 6.44-6.45 above the threshold value in all groups. Jiao et al., (2018) conducted a study that demonstrated that yeast supplementation resulted in higher GP and DM digestion with a pH of 5.8, as compared to a pH of 6.5. This study showed that the response of microbial digestion to yeast supplementation was influenced by the pH of the incubation medium when

the substrate consisted of a mixture of silage (10%) and concentrate (90%) that was incubated with yeast at two different pH levels (5.8 and 6.5) for 24 h. Third, yeast is likely to be subject to competitive exclusion for substrates by the local microbiota at pH above the threshold (Freilich et al., 2011). Finally, the lack of beneficial effects of *S. cerevisiae* in this study may be due to the composition of the diets. Several studies have reported that the addition of *S. cerevisiae* to diets high in forage improved substrate digestibility (Tang et al., 2015; Monroy Salazar et al., 2016), while other studies have reported no significant impact on digestibility when *S. cerevisiae* was added to high-concentrate diets (Opsi et al., 2012). In this study, both TMRs were prepared isonitrogenous and isocaloric, and the forage source of NDF was obtained from forage of the same quality and had similar chemical composition and almost the same fiber fraction (Table 1).

Table 2. The effects of treatments on in vitro gas kinetics, gas production and organic matter digestion

Treatments	Yeast dose, 200 mg/g DM	GP Kinetics		In vitro GP, mL/200 gm DM					OMD, %
		a + b, mL	c, mL/h	GP_3	GP_6	GP_9	GP_{12}	GP_{24}	
AS-Control	0	52.43	0.045	23.71	34.52	40.68	46.08	55.54	75.60
AS-LY	0.06	51.98	0.050	23.96	34.38	40.44	46.35	54.83	74.97
HS-Control	0	52.20	0.052	23.17	32.28	38.58	45.04	55.34	74.75
HS-LY	0.06	50.37	0,047	23.39	34.39	41.36	47.55	59.01	78.02
SEM		0.497	0.002	0.188	0.462	0.609	0.657	0.919	0.797
P - value									
Starch		0.440	0.440	0.167	0.228	0.639	0.952	0.295	0.510
Yeast		0.434	0.434	0.550	0.286	0.321	0.332	0.431	0.431
Starch × Yeast		0.231	0.231	0.969	0.227	0.244	0.430	0.251	0.252

Notes: AS- Control: adequate starch without *S. cerevisiae*; ASLY: AS with *S. cerevisiae*; HS-Control: high starch without *S. cerevisiae*; HSLY: HS with *S. cerevisiae*; DM: dry matter; OMS: organic matter digestibility; GP: gas production; a: the gas volume of the rapidly degradable fraction; b: the gas volume of the potentially degradable fraction; c: gas production rate of the b fraction; t: incubation time; SEM: standart error mean;

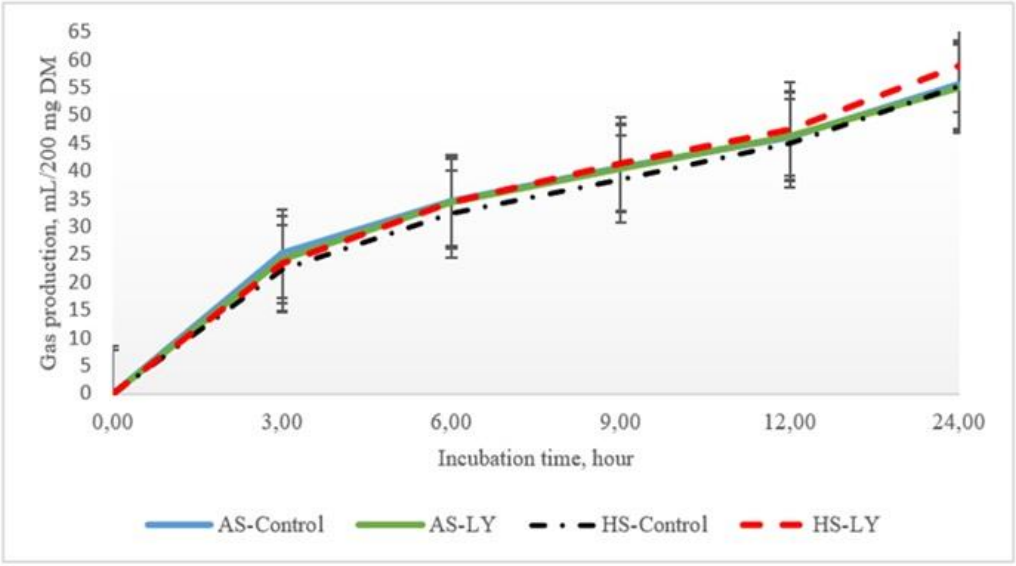

Figure 1. Volume of gas accumulated at different incubation times

In vitro ruminal fermentation parameters and methane production

The study findings depicted that the level of starch in the diet, the integration of LY, and their interaction did not significantly affect the fermentation parameters (pH and SCFA), ME, and CH_4 production (Table 3) of the diets. The stability of ruminal pH is considered a key indicator of the rumen environment's internal homeostasis, and thus, maintaining its stability is imperative for ensuring the continued health and productivity of the host. It is known that increasing the dietary starch amounts lowers ruminal pH (Knollinger et al., 2022). The present experiment's HS diet included 34.30% ground corn to challenge the fermentation medium. However, the same trends were not observed in the HS diet compared to AS diet, and ruminal pH was measured within the physiological range. Additionaly, the supplementation of LY did not alter the pH of the fermentation medium in the present experiment. The low rate of ruminal degradation of corn starch may be an explanation for this, as reported by Lynch and Martin, (2002) in a study using ground corn as a fermentation substrate. Also, our result is confirmed by the results of the study using ground corn grain as the main starch source by Knollinger et al., (2022), who observed that pH values of both the low starch (22.8%) and high starch diet (31.2%) remained within physiological limits (6.38 vs 6.10), and no difference by time-points. In the present study, the inability to detect pH fluctuations at time points during incubation is a limitation. An alternative

explanation for the results obtained could be that the buffering capacity of the fermentation medium which possesses a substantial ability to regulate pH has not yet been depleted, as noted by Getachew et al., (1998) and Chai et al., (2004). Getachew et al., (1998) documented that when the volume of gas surpassed 90 mL or 130 mL (55.34 to 59.1 mL in this study), the standard buffer was exhausted. Likewise, the results of an in vitro study conducted by Chai et al., (2004) support the conclusions of the current study, as they found that the pH was maintained 12 hours after incubation when the medium was highly buffered at pH values of 6.50, 6.25, and 6.00. Conversely, in media with low buffering capacity, represented by pH values of 5.75 and 5.50, a substantial decline in pH (to final values of 5.51 and 5.31) was observed after only 10 h of incubation, due to the depletion of the buffering capacity (Chai et al., (2004). Present findings are in agreement with previous studies by Pulido et al., (2015) and Xiao et al., (2016), which also concluded that the inclusion of *S. cerevisiae* did not result in a significant alteration in the pH levels. In contrast to the present findings, Garcia Diaz et al., (2018) reported that the inclusion of *S. cerevisiae* in the diet of cattle fed 90.6% ground corn resulted in an increase in ruminal pH. Additionally, some researchers found that adding *S. cerevisiae* increased ruminal pH by stimulating the activity of *Selenomonas ruminantum* and *Megasphaera elsdenii*, consume lactate (Crossland et al., 2019; Cagle et al., 2020; Direkvandi et al., 2021; Phesatcha et al., 2022). Inconsistent results among studies may be due to differences in experimental conditions (Moya et al., 2009).

Table 3. The effects of treatments on in vitro fermentation kinetics and methane emissions

Treatments	Yeast dose, 200 mg/g DM	pH	SCFA, mmol/200 mg KM	Methane, %	Methane, mL	ME, Mj/kg DM
AS-Control	0	6.45	12.32	21.90	3.64	11.73
AS-LY	0.06	6.44	12.16	21.22	3.11	11.62
HS-Control	0	6.44	12.28	21.22	3.26	11.75
HS-LY	0.06	6.45	13.09	21.65	4.25	12.33
SEM		0.003	0.203	0.341	0.301	0.146
P - value						
Starch		0.865	0.294	0.870	0.551	0.224
Yeast		0.865	0.433	0.870	0.713	0.429
Starch × Yeast		0.080	0.250	0.474	0.245	0.252

Notes: AS-Control: adequate starch without *S. cerevisiae*; ASLY: AS with *S. cerevisiae*; HS: hıgh starch without *S. cerevisiae*; HSLY: HS with *S. cerevisiae*; DM: dry matter; SCFA: s*hort-chain fatty acids;* SEM: standart error mean

Increasing the dietary content of fermentable carbohydrates generally increases SCFA concentrations and lowers rumen pH (Agle et al., 2010). However, in the present study, starch amount and LY addition had no interaction effect on SCFA. This was not surprising given that gas production, OM digestibility, and pH were unaffected as well. Our findings indicated a lack of increase in the concentration of SCFA with LY supplementation, a result that concurs with previous studies that utilized various substrates in their experiments (Jeyanathan et al., 2016; Kumprechtova et al., 2019; Cagle et al., 2020). Different yeast strains and types of diets may have an impact on this discrepancy (Cherdthong et al., 2019). The lack of impact of *S. cerevisiae* on the total SCFA quantity in the present study could be attributed to its inability to improve the microbial fermentation of carbohydrates, as discussed in relation to the results on gas production and OM digestion.

The increased generation of gas can make a significant contribution to energy provision through SCFA, as noted by Remesy et al., (1995). However, in the present study, no variance in the ME levels was observed due to the similarity of the GP of the substrates. The findings of this study are at odds with prior research that documented an increase in SCFA and ME as a result of the enhanced activity of rumen microbial populations due to the presence of *S. cerevisiae* (Dias et al., 2018; Phesatcha et al., 2022; Mora et al., 2023).

In this study, LY was included in both the AS and HS diets; however, its presence did not affect CH_4 production. According to several in vitro and in vivo research results, *S. cerevisiae* may mitigate CH_4 emissions by inducing the utilization of hydrogen by acetogens and enhancing acetogenesis (Elghandour et al., 2014; Pulido et al., 2015). Several in vitro studies have previously suggested that supplementation of yeast has the potential to reduce CH_4 production (Tristant and Moran, 2015; Wang et al., 2016; Phesatcha et al., 2020). However, a recent meta-analysis by Darabighane et al., (2019) has challenged these findings, as the study did not observe a significant effect of yeast supplementation on CH_4 concentrations in the subjects investigated. Our findings are in agreement with those of other studies that have shown that yeast supplementation has no meaningful impact on CH_4 emissions (Bayat et al., 2015; Li et al., 2021). The discrepancies in the outcomes regarding CH_4 production are likely attributed to the diversity in yeast strains used and dietary regimes applied in the studies (Yaun et al., 2015).

CONCLUSION

In conclusion, the supplementation of LY to diets containing high starch in dairy cows did not significantly alter in vitro gas and CH_4 production, OM digestibility, rumen fermentation, or ME. Both the presence of LY and the dietary starch ratio did not have a discernible effect on these parameters.

Further studies could focus on the mechanisms underlying the lack of response observed in this study, such as the influence of rumen microbial populations.

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