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In Vitro Screening of Feed Additives for Maintaining Ruminal Fermentation Levels Under Heat Stress

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Abstract

Heat stress (HS) alters rumen function and may compromise fermentation efficiency in dairy cows. This in vitro study evaluated the effects of six additives—betaine, sodium bicarbonate, bentonite clay, protected fat, *Saccharomyces cerevisiae* yeast, and *Melissa officinalis* (lemon balm)—on ruminal fermentation under increasing incubation temperatures (39.0 °C, 40.5 °C, 41.5 °C). This study was designed as an initial in vitro screening approach aimed at evaluating additive resilience under a conservative, worst-case thermal challenge. The highest incubation temperature (41.5 °C) was not used to reproduce the exact physiological rumen environment but rather served to identify additives capable of maintaining fermentation when exposed to extreme conditions that may occur during severe heat load. These results therefore constitute a preliminary step before in vivo validation under realistic HS scenarios. Fermentation was assessed after 24 h by measuring gas production, pH, redox potential, volatile fatty acids (VFAs), and ammonia (NH_3) and performing protozoa counts. Temperature alone produced limited effects in the control, with numerical increases in gas, acetate, total VFAs, and NH_3 at 41.5 °C. Betaine and yeast maintained overall stable fermentation across all temperatures, preserving gas production, pH, redox potential, and VFA profiles. Sodium bicarbonate increased the pH but reduced gas production. Lemon balm enhanced VFAs and propionate at 39.0 °C but showed reduced activity at high temperature. Protected fat and bentonite clay resulted in lower gas production with minimal influence on other parameters. The protozoa counts were not affected by temperature and showed statistically detectable differences among additives. Overall, betaine and yeast exhibited the highest thermal stability and appear to be suitable candidates for inclusion in feeding strategies aimed at supporting rumen fermentation during periods of HS.

Keywords: nutrition; rumen fermentation; additives; heat stress

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1. Introduction

Extreme heat events have become increasingly frequent, longer, and more intense in temperate regions over recent decades. In fact, meteorological analyses from the Royal Belgian Meteorological Institute report a rise of approximately +0.3 heat waves per decade, combined with increasing duration and intensity (+1 °C/day per decade) [1]. In 2025, a major heat event occurred as early as June, illustrating the acceleration of climatic extremes

in this region. Dairy cows, being homeothermic animals, maintain optimal physiological functions within a narrow thermoneutral zone. Above 26 °C and at 20% relative humidity, cows typically exhibit a reduced heat dissipation capacity and enter heat stress (HS), a condition frequently described using the temperature–humidity index (THI) [2,3]. Physiological disturbances generally appear once THI exceeds 70 [3].

HS induces profound behavioral, physiological, and metabolic changes, including reduced feed intake, increased water consumption, elevated respiratory rate, and hormonal disruptions [4,5]. At the rumen level, HS can alter motility, modify microbial populations, influence fermentation pathways, and in certain cases raise the ruminal temperature [6]. These disruptions underline the necessity of stabilizing rumen fermentation during HS.

Nutritional strategies are among the most effective approaches to mitigate HS. Several feed additives have been proposed to support ruminal function under such conditions. Betaine (or trimethylglycine), an organic osmolyte and methyl donor, can enhance osmoregulation, modulate rumen fermentation, decrease starch fermentation and improve microbial resilience during thermal stress [7–9]. Live yeast (*Saccharomyces cerevisiae*) improves ruminal pH stability, redox environment, fiber digestion, and dry matter intake, effects that are particularly valuable during HS [10–12]. Bentonite clay possesses buffering capacity, absorbs mycotoxins and interacts with ruminal or intestinal environments, potentially modulating fermentation [13,14]. Protected fats provide dense energy with a low fermentative heat increment and are therefore considered "cold nutrients" under HS because fat hydrolysis in the rumen produces less heat [15,16]. Plant extracts exhibit strong antioxidant properties and have shown beneficial effects in several HS models [17–19]. *Melissa officinalis* (lemon balm) has been found to successfully favor conditions for microbial development [20,21].

Despite this diversity of additives, no study has systematically screened multiple additives under controlled in vitro conditions simulating HS, nor compared their isolated effects across increasing ruminal temperatures. Given that the rumen temperature typically ranges from 38.5 to 39.5 °C in thermoneutral conditions, but may exceed 40–41 °C during severe HS [2,3,6,22,23], an in vitro evaluation across these temperatures is relevant; however, such elevations occur in conjunction with complex thermoregulatory processes that cannot be fully reproduced in vitro. For this reason, the present experiment was conceived as an initial, reductionist screening step aimed at evaluating additive functionality under a conservative worst-case temperature challenge rather than as a direct physiological simulation of the in vivo rumen. The upper incubation temperature (41.5 °C) was therefore selected not to mimic normal ruminal conditions, but to impose controlled thermal stress on the fermentative system to identify additives that remain stable when fermentation is challenged beyond typical physiological limits. This approach reduces unnecessary animal experimentation in the early stages of evaluation while providing mechanistic insight that will guide subsequent in vivo studies under realistic HS conditions.

Therefore, the objective of this study was to evaluate the effects of six additives—betaine, yeast, protected fat, sodium bicarbonate, bentonite clay, and lemon balm—on in vitro ruminal fermentation at three temperatures representing thermoneutral (39.0 °C) and HS-like conditions (40.5 °C and 41.5 °C). Fermentation responses were measured in terms of gas production, pH, redox potential, volatile fatty acids (VFAs), ammonia (NH_3), and protozoa counts. This work constitutes the first step of a broader research project aiming to identify additives capable of maintaining ruminal stability under HS before proceeding to in vivo testing in dairy cows.

2. Materials and Methods

2.1. Feed Additives

A total of six different feed additives were evaluated (bentonite clay, sodium bicarbonate, betaine, protected fat, yeast, and lemon balm) based on a literature review that indicated

their market availability, cost, and effectiveness against HS. The protected fat, provided by INVE (Dendermonde, Belgium), was composed of refined and hydrogenated rapeseed fat. Yeasts were purchased from Lesaffre (*Saccharomyces cerevisiae*, Marcq-en-Barœul, France), betaine was from ORFFA (beta key), lemon balm was provided by Salus Provadix (Breda, The Netherlands), sodium bicarbonate from Eti Soda (Istanbul, Turkey) and the bentonite clay came from Lafaure quaries (Mazeyrolles, France). The additives were dosed according to the recommendations provided by their suppliers, food manufacturers, and the literature. Doses were calculated by proportional scaling from recommended in vivo inclusion rates for a cow with a rumen volume of 100 L to the 100 mL flasks used in vitro.

Recommendations have been adapted along with their refences given in Table 1. Quantities tested for each additive are also provided. The minimum quantity for weighing was 0.005 g.

Table 1. Intake requirements by additive and quantity tested in vitro based on rumen flasks volume of 100 mL.

Additives	Recommendations (g/Cow/Day)	Reference	Quantity Tested (g/Flasks)
Bentonite clay	200	[24]	0.2
Betaine	20	[25]	0.02
S. bicarbonate	200	[26]	0.2
Protected fat	250	[27]	0.25
Yeast	5	[28]	0.005
Lemon balm	200	[29]	0.2

2.2. Rumen Collection

Rumen fluid was obtained from three multiparous Holstein cows (~662 ± 150 kg BW) producing ~25 kg milk/day and fed a standard total mixed ration (TMR). This diet was composed of grass from grazed pasture to simulate grazing (41% DM), grass silage (18% DM), maize silage (18% DM), and concentrate (23% DM).

In this experiment, three multiparous Prim'Holstein cows, weighing 662 ± 150 kg, at the same stage of lactation, with an average rate of milk production of 25 kg per day, were used as rumen fluid donors. The cows were offered a total mixed ration (TMR) diet (Table 2). Collection was performed before morning feeding, using an ororuminal sampling device (Genia, St-Hilaire-de-Chaléons, France) connected to a vacuum pump. The rumen fluid was filtered through four layers of cheesecloth into pre-warmed flasks and kept under CO_2 until use [30].

Table 2. Chemical composition of the total mixed ration (TMR).

Composition	Total Mixed Ration
DM (g/kg)	906
Ash (g/kg DM)	107
OM (g/kg DM)	119
CP (g/kg DM)	178
CF (g/kg DM)	218
NDF (g/kg DM)	494
ADF (g/kg DM)	262
Lignin (g/kg DM)	38
Hemicellulose (g/kg DM)	232
WSC (g/kg DM)	137
VEM	907
DVE (g/kg DM)	102

Table 2. *Cont.*

Composition	Total Mixed Ration
Calcium (g/kg DM)	6.2
Phosphorus (g/kg DM)	4.3
Potassium (g/kg DM)	22.2
Sodium (g/kg DM)	2.4
Magnesium (g/kg DM)	2.1

DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; WSC: water-soluble carbohydrate; VEM: Dutch net energy; DVE: Dutch digestible protein.

2.3. In Vitro Gas Production

The technique applied in this study followed the procedures provided in [31]. Here, 100 mL glass vials were used as incubation containers. This method was designed and standardized to simulate rumen conditions by creating "small artificial rumens" to study the gas produced by a tested sample; the evolution of the protozoa count, pH, and redox potential; and the production of VFAs and NH_3.

The quantity of TMR in the flasks was 0.2 g. The Menke buffer was prepared following the methodology in [31], using solutions A–E described in Table 3. For each liter of buffer, 0.5 L of fresh rumen fluid was added immediately before incubation. The Menke buffer was mixed with CO_2 gas to create a saturated environment. The quantity of rumen fluid added to the Menke buffer was determined by the number of samples used during fermentation. In the laboratory, the obtained solution was mixed in one beaker under a continuous stream of CO_2 and maintained in a water bath at 39 °C before being added to the flasks. A volume of 30 mL of this mixture (Menke buffer + rumen fluid) was added to the 100 mL vials containing either just the diet as a control or the additive samples to be tested for the experimental group. Approximately 70 mL of headspace was left for gas accumulation. This liquid-to-headspace ratio is consistent with the in vitro gas production systems described by [32], who incubated 30 mL of inoculum in 100 mL calibrated syringes to prevent gas-limitation artifacts and ensure accurate measurement of fermentation kinetics.

Table 3. Composition of Menke buffer solution (A–E).

Solution	Components	Concentration	Volume for 1 L
A (micro-minerals)	$CaCl_2{\cdot}2H_2O$, $MnCl_2{\cdot}4H_2O$, $CoCl_2{\cdot}6H_2O$, $FeCl_3{\cdot}6H_2O$	13.2 g; 10.0 g; 1.0 g; 0.8 g	0.12 mL
B (micro-buffer)	$NaHCO_3$, NH_4Cl	35.0 g; 2.7 g	237 mL
C (macro-minerals)	Na_2HPO_4, KH_2PO_4, $MgSO_4{\cdot}7H_2O$	5.74 g; 6.20 g; 0.60 g	237 mL
D (indicator)	Resazurin	100 mg	1.22 mL
E (reductive solution)	NaOH 1N, $Na_2S{\cdot}7H_2O$	2 mL; 285 mg	to discoloration

After bubbling with CO_2 for ten seconds, flasks were sealed, flushed with CO_2, and incubated in oscillating water baths (60 rpm) at different temperatures: one close to that of the rumen (39.0 °C) and two others simulating HS conditions (40.5 and 41.5 °C).

The final solution was flushed with CO_2 until color stabilization was reached.

Each treatment × temperature combination underwent 3 independent runs, each with 4 replicates (flasks). Gas production was measured in all flasks until 24 h had elapsed. pH and redox were measured in 2 flasks of each run, and fatty acids, NH_3 content, and protozoa number were measured in 3 flasks of the last run.

2.4. In Vitro Rumen Fermentation Characteristics

The flasks were placed in 39.0, 40.5, and 41.5 °C incubators, and the cumulative gas volume was measured using a needle (0.6 × 25 mm) connected to a barometer (Gems

pressor and sensor, type T443A, Bailey and Mackey, Birmingham, England) through the rubber stopper after 2, 4, 6, 8, 10, 12, 24, 48, and 72 h of incubation. In the last run, after 24 h of incubation, the liquid from three flasks from each treatment plus the control was collected in 3 flasks and analyzed for NH_3 and VFA concentrations according to the protocols provided in [33,34]. A pH meter was used to measure the pH of the incubation solution immediately after collection. The redox potential was measured using a redox probe.

2.5. Protozoa Count

From the same flasks as those used for NH_3 and VFA analyses, after 24 h of fermentation, a 1 mL sample of the incubation liquid was immediately added to 6 mL of 10% formaldehyde. Protozoa counts were conducted in Sedgwick–Rafter counting chambers (S52 glass; Pyser-SGI, Edenbridge, Kent, UK). The numbers of small (up to 40 × 60 µm), medium (up to 100 × 150 µm), and large (larger than 100 × 150 µm) ciliates per mL of rumen fluid were determined by light microscopy at 10× magnification [35]. After exploratory analysis, the data obtained from protozoa counts were transformed to log10(x + 10). This correction avoids undefined values when counts are low and stabilizes residual variance. Protozoal counts displayed very limited numerical variation across treatments because batch cultures do not allow ciliates to multiply; their abundance after 24 h therefore reflects survival rather than population growth, as previously demonstrated for in vitro systems. Fixation in 10% formaldehyde arrests protozoal motility and preserves cell morphology, which further reduces variability across chambers. In addition, Sedgwick–Rafter chambers impose a fixed counting volume and are known to generate narrow concentration ranges when protozoa are not actively proliferating. The log10(x + 10) transformation compresses the remaining variation when absolute values are high and the dynamic range is limited.

2.6. Statistical Analysis

Analyses were performed using SAS 9.4, 2023, SAS Institute Inc., Cary, NC, USA.

A mixed-effects model was applied for the variables gas production, pH, and redox potential at 24 h:

$$Y_{ijk} = \mu + T_i + A_j + (T \times A)_{ij} + R_k + \varepsilon_{ijk}.$$

where *Yijk* = observation for temperature *i*, additive *j*, run *k*; μ = overall mean; T_i = fixed effect of temperature; A_j = fixed effect of additive; $(T \times A)_{ij}$ = interaction between temperature and additive; R_k = random effect of run (intercept); ε_{ijk} = residual error. Models were fitted using restricted maximum likelihood (REML).

Fixed-effects model (two-way ANOVA) for variables measured in the VFA analysis was used for total AGV, C2, C3, C2/C3, C4, iC4, iC5, NH_3, and protozoa:

$$Yij = \mu + Ti + Aj + (T \times A)ij + \varepsilon ij$$

where *Yij* = observation for temperature iii and additive, μ = overall mean, *Ti* = fixed effect of temperature, *Aj* = fixed effect of additive, $(T \times A)ij$ = interaction, εij = residual error.

For both models, least squares means (LSMeans) were estimated for each factor level. Post hoc comparisons were performed using Tukey's Honest Significant Difference (HSD) test at $\alpha = 0.05$. SEM values represent the pooled standard error of LSMeans based on the model residual error term. A significance level of $\alpha = 0.05$ was applied for all tests.

3. Results

In Vitro Gas Production Parameters

The rate of gas production after 24 h is presented in Table 4. Temperature had a significant effect, with higher gas production observed at 41.5 °C compared to 39.0 °C and 40.5 °C ($p < 0.05$). Of all the additives, betaine showed the highest level of gas production

across temperatures, with significantly greater values at 41.5 °C than at 39.0 °C ($p < 0.05$). Conversely, the addition of sodium bicarbonate, bentonite clay, protected fat, and lemon balm resulted in lower gas production compared to the control at all temperatures ($p < 0.05$). Yeast addition led to gas production values comparable to those of the control, without significant effects of temperature ($p > 0.05$).

Table 4. Gas production (ml/g DM) after 24 h of incubation for each additive at each temperature.

Additives	39 °C	40.5 °C	41.5 °C	LSMeans (Additives)	SEM	P > F
Control	178 ^{Aa}	185 ^{Aa}	195 ^{ABb}	187 ^{AB}		
Bentonite clay	85 ^{Ba}	89 ^{Ba}	106 ^{Cb}	92 ^{D}		
Betaine	182 ^{Aa}	191 ^{Aa}	204 ^{Ab}	192 ^{A}		
S. bicarbonate	89 ^{B}	92 ^{B}	88 ^{D}	89 ^{D}	2.50	0.001
Protected fat	79 ^{B}	81 ^{B}	81 ^{D}	80 ^{E}		
Yeast	172 ^{A}	184 ^{A}	179 ^{B}	178 ^{B}		
Lemon balm	129 ^{C}	133 ^{C}	138 ^{E}	133 ^{C}		
LSMeans (T°)	130 ^{a}	136 ^{b}	141 ^{c}	136		
SEM		1.86				
P > F		0.03				

Values are least squares means (LSMeans) estimated from a mixed model for Gas production: Temperature × Additive, with a random intercept for run (REML). Uppercase superscripts (A–E) indicate within-column comparisons among additives. Lowercase superscripts indicate within-line comparisons among temperatures (a–c). LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values represent the pooled standard error of LSMeans based on the model residual error term. Abbreviations: LSMeans = least-squares means; SEM = pooled standard error of the mean.

Table 5 shows the average pH values after 24 h. Incubation temperature did not affect pH in the control or with most of the additives ($p > 0.05$). Sodium bicarbonate consistently increased pH compared to the control and all other additives at all temperatures ($p < 0.05$); in contrast, yeast, betaine, protected fat, bentonite clay, and lemon balm maintained pH values similar to the control, with no significant shifts across temperatures.

Table 5. pH after 24 h of incubation for each additive at each temperature.

Additives	Temperature					
	39 °C	40.5 °C	41.5 °C	LSMeans (Additives)	SEM	P > F
Control	6.43 ^{A}	6.45 ^{A}	6.42 ^{A}	6.43 ^{A}		
Bentonite clay	6.47 ^{A}	6.42 ^{A}	6.43 ^{A}	6.44 ^{A}		
Betaine	6.45 ^{A}	6.43 ^{A}	6.42 ^{A}	6.43 ^{A}	0.068	0.001
S. bicarbonate	6.80 ^{B}	6.80 ^{B}	6.80 ^{B}	6.80 ^{B}		
Protected fat	6.47 ^{A}	6.45 ^{A}	6.42 ^{A}	6.44 ^{A}		
Yeast	6.47 ^{A}	6.41 ^{A}	6.43 ^{A}	6.44 ^{A}		
Lemon balm	6.32 ^{A}	6.30 ^{A}	6.32 ^{A}	6.31 ^{C}		
LSMeans (T°)	6.49	6.46	6.46	6.47		
SEM		0.066				
P > F		0.57				

Values are least squares means (LSMeans) estimated from a mixed model for pH: Temperature × Additive, with a random intercept for run (REML). Uppercase superscripts (A–C) indicate within-column comparisons among additives. LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values represent the pooled standard error of LSMeans based on the model residual error term. Abbreviations: LSMeans = least-squares means; SEM = pooled standard error of the mean.

The redox potential values are presented in Table 6. The control showed no significant differences between different temperatures, and all additives except sodium bicarbonate

exhibited redox potentials comparable to the control ($p > 0.05$). In contrast, sodium bicarbonate significantly decreased the redox potential at all temperatures relative to the control ($p < 0.05$), indicating a more reduced environment.

Table 6. Redox potential (mV) after 24 h of incubation with each additive at each incubation temperature.

Additives	Temperature					
	39 °C	40.5 °C	41.5 °C	LSMeans (Additive)	SEM	P > F
Control	−251 ^{A}	−260 ^{A}	−247 ^{A}	−253 ^{A}		
Bentonite clay	−272 ^{A}	−271 ^{A}	−264 ^{A}	−269 ^{BC}		
Betaine	−256 ^{A}	−251 ^{A}	−246 ^{A}	−251 ^{A}		
S. bicarbonate	−286 ^{B}	−283 ^{B}	−274 ^{B}	−281 ^{C}	3.54	<0.001
Protected fat	−269 ^{A}	−260 ^{A}	−253 ^{A}	−261 ^{AB}		
Yeast	−259 ^{A}	−258 ^{A}	−250 ^{A}	−253 ^{A}		
Lemon balm	−259 ^{A}	−258 ^{A}	−255 ^{A}	−257 ^{AB}		
LSMeans (T°)	−265 ^{a}	−263 ^{b}	−256 ^{b}	−261		
SEM		2.64				
P > F		0.03				

Values are least squares means (LSMeans) estimated from a mixed model for Redox potential: Temperature × Additive, with a random intercept for run (REML). Uppercase superscripts (A–C) indicate within-column comparisons among additives. Lowercase superscripts (a–b) indicate within-line comparisons among temperatures. LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values represent the pooled standard error of LSMeans based on the model residual error term. Abbreviations: LSMeans = least-squares means; SEM = pooled standard error of the mean.

The total VFA concentrations after 24 h are shown in Table 7. Temperature did not significantly affect the total VFAs in the control; in contrast, sodium bicarbonate produced significantly higher VFA concentrations at 40.5 °C than at 39.0 °C ($p < 0.01$), with a tendency for higher values at 41.5 °C. Notably, lemon balm significantly increased VFAs at 39.0 °C and 40.5 °C relative to the control ($p < 0.05$), but not at 41.5 °C.

Table 7. VFA concentration (mg/L) after 24 h of incubation with each additive at each temperature.

Additives	Temperature					
	39 °C	40.5 °C	41.5 °C	LSMeans (Additives)	SEM	P > F
Control	77.0 ^{BCa}	81.5 ^{ABa}	84.1 ^{Aa}	80.9 ^{BCD}		
Bentonite clay	71.6 ^{CDab}	62.9 ^{Bb}	85.5 ^{Aa}	73.3 ^{CD}		
Betaine	93.1 ^{Aba}	80.3 ^{ABa}	91.3 ^{Aa}	88.2 ^{AB}		
S. bicarbonate	64.2 ^{Da}	78.1 ^{Bb}	72.1 ^{Aab}	71.5 ^{D}	3.75	0.001
Protected fat	76.9 ^{BCD}	71.5 ^{B}	81.9 ^{A}	76.8 ^{BCD}		
Yeast	86.1 ^{ABC}	79.4 ^{A}	88.7 ^{A}	84.7 ^{ABC}		
Lemon balm	101.9 ^{A}	100.1 ^{C}	84.9 ^{A}	95.6 ^{A}		
LSMeans (T°)	81.5	79.1	84.1	81.6		
SEM		3.99				
P > F		0.20				

Values are least squares means (LSMeans) estimated from the two-way ANOVA model (Temperature × Additive). Uppercase superscripts (A–D) indicate within-column comparisons among additives. Lowercase superscripts (a–b) indicate within-line comparisons among temperatures. LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values are based on the model error term. Pooled SEM = 6.13. Abbreviations: LSmeans = least-squares means; SEM = standard error of the mean.

The concentrations of acetic, propionic, and butyric acid in the flasks after 24 h of incubation are presented in Table 8. No significant differences were observed among the

control samples across the three temperatures. Nevertheless, the numerical trends showed that the acetate concentrations tended to increase with increasing temperature, whereas the propionate and butyrate concentrations tended to decrease.

Table 8. VFA concentrations (mg/L) after 24 h of incubation with each additive at each temperature.

Additives	C2	C3	C2/C3	C4	iC4	iC5
			39 °C			
Control	43.3 ^{BCa}	18.7 ^{BCa}	2.32 ^{Ab}	11.6 ^{ABCa}	0.56 ^{ABa}	1.12 ^{Ca}
Bentonite clay	41.8 ^{BCa}	16.8 ^{Ca}	2.49 ^{ABa}	10.4 ^{BCa}	0.40 ^{Ba}	0.87 ^{CDb}
Betaine	55.4 ^{Ba}	20.9 ^{Ca}	2.66 ^{Ab}	13.2 ^{Aa}	0.53 ^{ABa}	1.17 ^{ABa}
S. bicarbonate	35.6 ^{Aa}	16.9 ^{Ca}	2.10 ^{Aba}	9.2 ^{Ca}	0.41 ^{Ba}	0.83 ^{Da}
Protected fat	46.5 ^{Ba}	17.1 ^{Ca}	2.72 ^{Aa}	10.4 ^{BCa}	0.44 ^{Ba}	0.94 ^{Ca}
Yeast	45.6 ^{BCa}	22.0 ^{Ba}	2.08 ^{Bb}	13.9 ^{Aa}	0.66 ^{Aa}	1.40 ^{Aa}
Lemon balm	60.0 ^{Aa}	25.7 ^{Aba}	2.33 ^{ABa}	12.7 ^{ABa}	0.50 ^{ABa}	1.03 ^{CDa}
			40.5 °C			
Control	49.4 ^{ABa}	18.1 ^{Ba}	2.72 ^{Aab}	10.7 ^{ABa}	0.47 ^{Aa}	1.08 ^{Aa}
Bentonite clay	37.1 ^{Ba}	14.6 ^{Ba}	2.53 ^{Aa}	8.6 ^{Ba}	0.48 ^{Aa}	0.9 ^{Cb}
Betaine	48.9 ^{ABa}	17.9 ^{Ba}	2.73 ^{Aab}	10.5 ^{ABb}	0.53 ^{Aa}	1.13 ^{ABa}
S. bicarbonate	48.2 ^{ABb}	18.0 ^{Ba}	2.68 ^{Aa}	9.1 ^{Ba}	0.38 ^{Ba}	0.84 ^{Ca}
Protected fat	42.9 ^{Ba}	16.2 ^{Ba}	2.65 ^{Aa}	9.5 ^{ABa}	0.39 ^{Ba}	0.87 ^{BCb}
Yeast	47.8 ^{Aa}	18.0 ^{Bb}	2.66 ^{Aa}	10.5 ^{ABb}	0.54 ^{Ab}	1.15 ^{Ab}
Lemon balm	62.0 ^{ABa}	23.4 ^{Aa}	2.65 ^{Aa}	11.3 ^{Aa}	048 ^{Aa}	1.05 ^{ABa}
			41.5 °C			
Control	53.1 ^{Aa}	17.6 ^{Aa}	3.00 ^{ABa}	10.3 ^{Aa}	0.45 ^{Ab}	1.05 ^{Aa}
Bentonite clay	52.1 ^{Aa}	18.0 ^{Aa}	2.77 ^{ABCa}	11.4 ^{Aa}	0.49 ^{Aa}	1.18 Aa
Betaine	58.3 ^{Aa}	18.5 ^{Aa}	3.14 ^{Aa}	11.1 ^{Ab}	0.45 ^{Aa}	1.13 ^{Aa}
S. bicarbonate	44.3 ^{Ab}	16.6 ^{Aa}	2.68 ^{BCa}	8.6 ^{Ba}	0.36 ^{Aa}	0.79 ^{Ba}
Protected fat	50.4 ^{Aa}	17.1 ^{Aa}	2.87 ^{ABa}	10.7 ^{Aa}	0.45 ^{Aa}	1.12 ^{Aa}
Yeast	55.0 ^{Ab}	19.2 ^{Aa}	2.87 ^{ABa}	11.0 ^{Ab}	0.46 ^{Ab}	1.19 ^{Aa}
Lemon balm	50.6 ^{Aa}	20.6 ^{Ab}	2.45 ^{Ca}	10.3 ^{Ab}	0.44 ^{Aa}	1.00 ^{Aa}
LSMeans (39 °C)	46.9 ^{a}	19.8 ^{a}	2.39 ^{c}	11.0 ^{a}	0.50 ^{a}	1.05 ^{a}
LSMeans (40.5 °C)	48.1 ^{ab}	18.1 ^{a}	2.66 ^{b}	10.3 ^{a}	0.45 ^{a}	0.98 ^{a}
LSMeans (41.5 °C)	52.0 ^{b}	18.4 ^{a}	2.83 ^{a}	10.5 ^{a}	0.43 ^{b}	1.06 ^{a}
SEM	1.36	0.38	0.037	0.21	0.01	0.21
P > F	0.03	0.006	0.001	0.001	0.001	0.024
			LSMeans (Additives)			
Control	48.6 ^{ABC}	18.1 ^{BC}	2.68 ^{ABC}	10.9 ^{ABC}	0.486 ^{A}	1.083
Bentonite clay	43.7 ^{C}	16.7 ^{C}	2.60 ^{BC}	10.1 ^{CD}	0.446 ^{B}	0.978
Betaine	54.2 ^{AB}	19.2 ^{BC}	2.84 ^{A}	11.6 ^{AB}	0.487 ^{A}	1.110
S. bicarbonate	42.7 ^{C}	17.2 ^{C}	2.49 ^{C}	9.0 ^{D}	0.378 ^{C}	0.817
Protected fat	46.6 ^{BC}	17.0 ^{C}	2.74 ^{AB}	10.2 ^{BCD}	0.419 ^{B}	0.968
Yeast	49.6 ^{ABC}	19.7 ^{B}	2.54 ^{BC}	11.8 ^{A}	0.548 ^{A}	1.238
Lemon balm	57.5 ^{A}	23.2 ^{A}	2.48 ^{C}	11.4 ^{ABC}	0.467 ^{B}	1.021
SEM	2.08	0.58	0.056	0.32	0.016	0.032
P > F	0.001	0.001	0.001	0.001	0.001	0.02

Values are least squares means (LSMeans) estimated from the two-way ANOVA model (Temperature × Additive). Uppercase superscripts (A–D) indicate within-column comparisons among additives. Lowercase superscripts (a–c) indicate within-line comparisons among temperatures. Means sharing at least one common letter are not significantly different. LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values are based on the model error term. Abbreviations: LSMmeans = least-squares means; SEM = standard error of the mean.

No significant differences in acetate concentrations ($p > 0.05$) were found between the control and the samples with bentonite clay, betaine, protected fat, and lemon balm additives incubated under HS. However, acetate concentrations were numerically higher

for the samples with lemon balm than for the control at 39.0 °C but were lower at 41.5 °C. For the betaine samples, the acetate concentrations were numerically higher than the control and remained stable at 41.5 °C relative to 39.0 °C. Sodium bicarbonate addition resulted in significantly higher acetate concentrations ($p < 0.005$) at 40.5 °C and 41.5 °C compared with those at 39.0 °C, although the values at 39.0 °C and 41.5 °C were numerically lower than those of the control. Yeast samples exhibited significantly higher acetate concentrations ($p < 0.03$) at 41.5 °C than at the two lower temperatures.

For propionate, no significant differences ($p > 0.05$) were observed at any temperature for the samples with betaine or protected fat; in contrast, lemon balm induced a significant increase in propionate at 39.0 °C compared with the control ($p < 0.05$). Bentonite clay led to lower propionate concentrations at 40.5 °C than the control. Yeast and lemon balm additives both exhibited significantly lower propionate concentrations at high temperatures ($p < 0.05$), suggesting temperature-dependent suppression of propionate formation.

For butyrate, no significant differences ($p > 0.05$) were observed with sodium bicarbonate or protected fat at any temperature. However, yeast addition resulted in significantly higher butyrate concentrations at 39.0 °C compared with the control ($p < 0.05$), whereas bentonite clay addition led to significantly lower values at 40.5 °C ($p < 0.05$). With betaine, yeast, and lemon balm, propionate concentrations showed a decreasing trend with increasing temperature ($p < 0.05$), consistent with the shifts observed in the total VFA profiles.

The NH_3 concentrations are presented in Table 9. In the control, NH_3 significantly increased at 41.5 °C compared to lower temperatures. Bentonite clay, yeast, and sodium bicarbonate addition showed no significant differences from the control across the different temperatures ($p > 0.05$). However, betaine, protected fat, and lemon balm resulted in significantly higher NH_3 concentrations at 41.5 °C ($p < 0.05$), consistent with increased protein degradation or decreased microbial incorporation.

Table 9. Total concentration of NH_3 (mmol/L) after 24 h of incubation for each additive at each incubation temperature.

Additives	Temperature					
	39 °C	40.5 °C	41.5 °C	LSMeans (Additives)	SEM	P > F
Control	10.6 ^{ABa}	12.4 ^{Aa}	16.2 ^{Ab}	13.1 ^{ABC}		
Bentonite clay	10.2 ^{AB}	11.1 ^{A}	13.6 ^{AB}	11.7 ^{BC}		
Betaine	11.1 ^{ABa}	14.1 ^{Aab}	16.1 ^{Ab}	13.8 ^{AB}	0.80	0.002
S. bicarbonate	10.7 ^{AB}	10.7 ^{A}	11.0 ^{B}	10.8 ^{C}		
Protected fat	9.8 ^{ABa}	12.0 ^{Aab}	13.8 ^{ABb}	11.9 ^{ABC}		
Yeast	13.4 ^{A}	14.1 ^{A}	15.7 ^{A}	14.4 ^{A}		
Lemon balm	8.6 ^{Ba}	13.2 ^{Aa}	15.4 ^{ABb}	12.4 ^{ABC}		
LSMeans	10.6 ^{a}	12.5 ^{ab}	14.5 ^{bc}			
SEM		0.755				
P > F		0.001				

Values are least squares means (LSMeans) estimated from the two-way ANOVA model (Temperature × Additive). Uppercase superscripts (A–C) indicate within-column comparisons among additives. Lowercase superscripts (a–c) indicate within-line comparisons among temperatures. Means sharing at least one common letter are not significantly different. LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values are based on the model error term. Abbreviations: LSMeans = least-squares means; SEM = standard error of the mean.

Although the protozoa counts did not differ among the three incubation temperatures (Table 10), the overall additive effect was significant when comparing the LSMeans averaged across temperatures ($p < 0.05$). Specifically, sodium bicarbonate and protected fat addition led to the lowest overall LSMeans; the bentonite clay and lemon balm samples

displayed intermediate values; and the yeast, betaine, and control groups had the highest overall protozoa counts.

Table 10. Number of protozoa ($\times 10^5$/mL) for each additive at each incubation temperature after 24 h of incubation.

Additives	Temperature					
	39 °C	**40.5 °C**	**41.5 °C**	**LSMeans**	**SEM**	**P > F**
Control	3.64 ^{AB}	3.64 ^{AB}	3.64 ^{AB}	3.64 ^{A}		
Bentonite clay	3.61 ^{C}	3.61 ^{C}	3.61 ^{C}	3.61 ^{C}		
Betaine	3.65 ^{A}	3.64 ^{A}	3.64 ^{AB}	3.64 ^{A}		
S. bicarbonate	3.58 ^{E}	3.58 ^{E}	3.58 ^{D}	3.58 ^{E}	0.002	0.001
Protected fat	3.60 ^{D}	3.60 ^{D}	3.59 ^{D}	3.60 ^{D}		
Yeast	3.64 ^{A}	3.64 ^{AB}	3.64 ^{A}	3.64 ^{A}		
Lemon balm	3.63 ^{AB}	3.63 ^{B}	3.63 ^{A}	3.63 ^{B}		
LSMeans	3.62	3.62	3.62			
SEM		0.02				
P > F		0.54				

Values are least squares means (LSMeans) estimated from the two-way ANOVA model (Temperature × Additive). Uppercase superscripts (A–E) indicate within-column comparisons among additives. LSMeans that share at least one letter are not significantly different at $\alpha = 0.05$ according to Tukey's Honest Significant Difference (HSD) test. SEM values are based on the model error term. Abbreviations: LSMeans = least-squares means; SEM = standard error of the mean.

4. Discussion

The objective of this study was to evaluate the effects of six additives on ruminal fermentation under increasing temperatures simulating HS. The present in vitro model was intentionally designed as a reductionist screening tool rather than a direct physiological reproduction of the rumen in HS conditions. Although temperatures above 40 °C have been reported under severe heat load, such elevations occur in vivo together with complex thermoregulatory responses involving changes in blood flow, heat dissipation, feeding behavior, and metabolic adaptation. These processes cannot be fully reproduced in vitro, and the upper incubation temperature used here (41.5 °C) should therefore be interpreted as a conservative worst-case challenge applied to the fermentative system rather than a realistic representation of the ruminal environment. Consequently, the responses observed here must be viewed as preliminary indicators of additive resilience, and their relevance to practical feeding situations will require confirmation through controlled in vivo studies under naturally occurring HS conditions.

The temperature of the water baths was based on the work of Lees et al. [6], who reported that ruminal temperature is linked to rectal temperature (RT). They consider that the ruminal temperature is 1 to 2 °C higher than the rectal temperature; however, the RT of a dairy cow is between 38 and 39 °C [36] and exceeds 39 °C under HS [4]. In the control treatments, temperature alone produced limited but noticeable numerical increases in gas production, acetate, total VFAs, and NH_3 at 41.5 °C. These observations are consistent with reports indicating that HS can alter ruminal fermentation patterns, although, in vivo, the cow's thermoregulation mechanisms usually prevent large increases in rumen temperature [2,3].

The protozoa count remained stable across the temperatures. Differences among additives were statistically detectable only in overall LSMeans. As protozoa do not multiply in batch cultures, their numbers reflect survival rather than true population dynamics [35]. The absence of significant differences suggests that none of the additives had a major detrimental effect on protozoal viability at the doses tested. Across all treatments, the extremely limited variability and the near-identical log-transformed protozoal counts observed in

Table 10 arise from biological and methodological features inherent to batch culture systems. Rumen ciliates do not proliferate under static in vitro conditions; their abundance after 24 h therefore reflects survival rather than population growth, a phenomenon widely documented in protozoal physiology [35,37]. Fixation in 10% formaldehyde stabilizes cell morphology and reduces variability across chambers, and the Sedgwick–Rafter chamber imposes a fixed counting volume that limits the dynamic range when no cell division occurs [38]. Under these conditions, the log10(x + 10) transformation further compresses residual variation when absolute counts fall within a narrow range. Consequently, the small SEM values observed represent a combined biological and analytical floor effect rather than an absence of protozoal response to temperature or additives.

Among the additives, betaine is known to have many effects that may reduce the effects of HS in lactating dairy cows. As an organic osmolyte [39], it has been demonstrated to decrease the susceptibility of some microorganisms to oxidative stress [39]. It can also be used as a powerful nutrient supplement for cows under HS conditions due to its ability to increase milk production [40]. In this study, betaine showed the most stable and favorable fermentation profile across the different temperatures investigated [41]. With this additive, gas production was significantly greater at higher temperatures, while the pH and redox potential remained within normal ranges. These effects may reflect betaine's function as a methyl donor that modulates fermentation and stabilizes the ruminal environment [42]. The authors of [8] observed a linear increase in total ruminal VFA concentrations when animals received different concentrations of betaine. This increase in VFA concentration indicates a greater microbial fermentation rate. Even though our research showed consistent VFA concentrations throughout the experiment, we also noticed a high fermentation rate for the samples incubated with betaine, highlighting this additive as a likely candidate for HS mitigation. Betaine also acts as a thermo- and osmoprotectant for bacterial cells and is a direct substrate for microbial metabolism [43]. Studies by [7,43] showed improved digestibility and microbial fermentation during HS, supporting our findings of high gas production (non-significant) and stable protozoa counts across temperatures.

The yeast additive *Saccharomyces cerevisiae* has been widely used in dairy nutrition. According to references [9,11,44], supplementation helps maintain ruminal pH and redox balance during hot seasons, facilitating microbial development. Additional benefits such as increased rumination and dry matter intake have also been reported by the authors of [10]. This positive impact can be advantageous during the HS period, when cows typically intake less dry matter, consequently affecting the quality and quantity of milk produced. Yeast cultures also contain growth factors that enhance rumen digestibility and lactation performance [45]. During HS, yeast supplementation has been associated with increased milk production [46]; for example, a diet containing 30 g/cow/day of *Saccharomyces cerevisiae* yeast culture during an HS period resulted in improved lactation performance, with a milk production rate of 43.4 kg/cow/day for supplemented animals compared to 42.2 kg/cow/day for non-supplemented cows. Protein and lactose yields were also higher for the cows receiving yeast. The authors of [47] found that the addition of *Saccharomyces cerevisiae* fermentation products had positive dose-dependent effects. Under the in vitro conditions created in this study, temperature did not significantly affect gas production, pH, redox potential, or NH_3 concentration with the yeast treatment, and the VFA profiles remained similar to those of the control.

The antioxidant effects of *Melissa officinalis* (lemon balm) have been extensively documented [18–20,48]. In poultry, lemon balm supplementation has improved performance under HS and supported immunity and production in laying hens [49]. The effect of lemon balm on HS is therefore well-documented in poultry species but remains relatively poorly studied in dairy cows. In the present study, lemon balm increased the total VFA

and propionate levels at 39 °C, consistent with prior evidence of fermentation-modulating effects [29]. However, these benefits diminished at 41.5 °C, suggesting impaired microbial stimulation at high temperatures. The elevated NH_3 at high temperatures may reflect increased deamination or reduced microbial protein synthesis.

Protected fat is commonly used to increase dietary energy density and has been considered a "cold nutrient," as fat digestion produces less heat [15]. In this study, protected fat did not affect rumen pH, redox potential, or VFA production; only gas production was consistently reduced. This lower gas production across temperatures likely reflects the partial displacement of fermentable substrates rather than direct microbial inhibition. These observations align with those of the authors of [16], who found minimal effects of protected fat on rumen pH and VFA proportions. The authors of [50] also noted that cracked rapeseed increased the proportion of acetate while decreasing propionate when used as a fat source, but no such effects were detected in the present study, likely due to the use of protected (rumen-inert) fat rather than an unprotected lipid source.

The effect of bentonite clay under HS has been investigated little, despite clay being known to act as a buffer. In this study, bentonite clay reduced gas production compared to the control at all temperatures, consistent with observations reported in [51]. No significant effect on pH was observed, in agreement with [14], but in contrast with the findings of [51,52]. Under the conditions tested here, bentonite clay did not appear to be advantageous for improving rumen fermentation during HS.

Sodium bicarbonate is widely used to correct ruminal acidosis [26] as a ruminal buffer. In this study, bicarbonate increased pH, as expected, consistent with [53], and decreased redox potential, as reported by [54]. However, no significant effects were observed on the concentrations of total or individual VFAs (acetate, propionate, butyrate), which contrasts with previous reports from these authors. This discrepancy may be related to the moderate starch content of the diet used in our trial. Regardless of temperature, sodium bicarbonate significantly reduced gas production compared with the control. The protozoa counts also showed slightly lower numerical values with the addition of sodium bicarbonate, but these differences were not statistically significant at any temperature; only the overall LSMeans differed—at a very small magnitude—from those achieved with the other additives. Therefore, the observed reduction in the protozoa count with bicarbonate should be interpreted cautiously and primarily as a numerical tendency rather than a biological effect.

Overall, betaine and yeast emerged as the two additives most capable of maintaining stable fermentation under HS-like conditions. Their documented mechanisms—osmoprotection for betaine and fermentative stabilization for yeast—are consistent with the responses observed in this study and support their potential use in nutritional strategies for dairy cows during hot periods.

5. Conclusions

This in vitro study showed that increasing the incubation temperature had moderate effects on ruminal fermentation in the control group, with increases in certain fermentation products at 41.5 °C. Among the additives tested, betaine and yeast demonstrated the greatest ability to maintain stable ruminal fermentation as temperature increased. Both additives preserved gas production, pH, redox potential, VFA profiles, and protozoal survival at elevated temperatures, indicating their potential value in supporting rumen function during HS. In contrast, lemon balm, protected fat, sodium bicarbonate, and bentonite clay produced more variable or limited effects. These findings identify betaine and yeast as promising candidates for further in vivo investigations, in which their impacts on milk production, metabolism, behavior, and microbial dynamics during natural HS conditions should be evaluated.

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Abbreviations

The following abbreviations are used in this manuscript:

HS	Heat Stress
RT	Rectal Temperature

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