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Effects of *Entodinium caudatum* monocultures in an acidotic environment on *in vitro* rumen fermentation

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ABSTRACT. The study evaluated the effect of *Entodinium caudatum* on the prevention of subacute ruminal acidosis (SARA) *in vitro*. Different proportions of wheat and corn [100% wheat (W); 75% wheat and 25% corn (W75); 50% wheat and 50% corn (WC); 75% corn and 25% wheat (C75); and 100% corn (C)] were used to create an *in vitro* acidotic environment. The activity of *E. caudatum* was determined by adding defaunated rumen fluid and protozoan monocultures to the substrates. The effect of *E. caudatum* monoculture on pH was insignificant, while a significant influence of grain type was observed on ammonia (NH_3-N) formation. *E. caudatum* inoculation decreased lactic acid concentration throughout the incubation period and shortened the fermentation start time (lag time). The specific fermentation rate (SFR), which increased with the wheat ratio, was reduced by *E. caudatum culture*. The total gas production varied depending on the substrate and was increased by *E. caudatum*. Protozoan monoculture decreased propionic acid levels, while it increased methane production. As a result, *E. caudatum* stimulated earlier fermentation, but decreased lactic acid production by reducing SFR. It is believed that the instantenuous phagocytosis of *E. caudatum* (iodophilic storage) to digest starch particles prevents rapid bacterial fermentation in the rumen fluid, and thus limit lactic acid production. The results of this work may support future *in vitro* studies requiring *E. caudatum* monoculture, as well as *in vivo* studies investigating the effect of *E. caudatum* on the inhibition of SARA formation.

Introduction

The rumen fauna includes more than 200 resident protozoan species. Protozoa in the rumen are microorganisms that affect dry matter content of the rumen, retention time, volume, type and number of bacteria, concentration and ratio of volatile fatty acids (VFAs) produced, rumen pH, and ammonia level (Williams and Coleman, 1997). Of these 200 species, ciliated protozoa constitute an important part of the total microbial population in the rumen ecosystem (Zhang et al., 2007). Ciliata, which play an essential role in regulating lactic acid and VFA production in the rumen, cannot survive at pH above 7.8 or below 5.0. Sub-acute ruminal acidosis (SARA) is the most common disorder in ruminants and a deviation from normal ruminal fermentation processes. In addition, SARA is characterized by episodes of lower daily pH levels of the rumen content ranging between 5.5 and 5.0 (Krause and Oetzel, 2006).

In order to meet the nutrient and energy needs of particularly high-yielding dairy cows, the proportion of concentrated feed is generally uncontrollably and rapidly increased. This leads to the formation of acute or subacute acidosis, which disrupts the rumen environment and causes significant economic losses (Rabaza et al., 2020). Goad et al. (1998) found

that when SARA was initiated in animals adapted to highly concentrated feed or high roughage, the reduction in the number of protozoa was greater in herdmates adapted to roughage.

In cases of high grain content in rations, a particularly important role in maintaining rumen pH play *Entodiniomorphid* protozoa due to their influence on starch and lactic acid metabolism (Dehority, 2005; Firkins et al., 2020). *Entodinium caudatum,* which tolerates low rumen pH, can prevent bacteria from producing excessive amounts of lactic acid by absorbing starch grains into its cells, thereby preventing the accumulation of lactic acid in the rumen due to starch fermentation. *Entodinium spp. (E. caudatum* and *E. simplex)* initially ingest grains very rapidly and this process subsequently slows down (about 3% of the initial rate) (Bełżecki et al., 2012; Yuste et al., 2019). *Entodinium* spp. have also been found to reduce the risk of clinical and subclinical acidosis by utilising lactic acid and consuming starch, which results in a reduced digestion rate (Park and Yu, 2018a; Park et al., 2019). Despite the available studies, information on the effect of protozoa on rumen fermentation is limited due to their specific development conditions and complex morphology.

This study aimed to determine the usability of *E. caudatum* as an alternative natural product to prevent the observed disorders and yield losses, especially in dairy cows, on high-grain ration without applying an acclimatisation period. For this purpose, the study investigated the effect of *E. caudatum* monocultures on fermentation parameters in an *in vitro* anaerobic acidotic prepared from wheat and corn.

Material and methods

Animal material

The experimental protocol (Project no.: 2013/049) was approved by the Selcuk University Ethics Committee on Animal Experimentation (Konya, Turkey). In this study, the rumen fluid used to provide an *in vitro* environment was obtained from two rumen cannulated heifers weighing approximately 450 kg. The two ruminally cannulated Holstein heifers (aged 18 ± 0.2 months) were housed in individual pens. Thirty days prior to rumen fluid sampling, they were fed exclusively dry alfalfa at a required maintenance level (NRC, 2001) in two equal parts at 8:00 and 17:00. Fresh water was also available *ad libitum*.

Production of *E. caudatum* monocultures from rumen fluid

E. caudatum was captured under a light microscope in the collected rumen fluid. *E. caudatum* protozoa were cultured and multiplied in an incubator in 250 ml pyrex bottles at 39 ℃ in anaerobic dilution fluid (Dehority, 1984). Subsequently, *E. caudatum* cultures were frozen in a freezer with a controlled freezing rate (Ice Cube 14S, Sy-Lab, Neu Purkersdorf, Austria) after the addition of 5% dimethylsulfoxide (v/v) as a cryoprotectant (Nsabimana et al., 2003). Cultures were kept in a liquid nitrogen storage tank (Taylor Wharton, Theodore, AL, USA) at −196 ℃ for approximately two years before being used in the study.

In the experiment, frozen *E. caudatum* monoculture in cryotubes was thawed at 39 °C for 5 min and the viability and motility of protozoa were verified under a microscope. They were then propagated in 250 ml culture bottles in anaerobic conditions. Further, wheat flour (1.5%) and alfalfa (1.0%) were ground through a 0.425-mm sieve (SM100 Comfort, Retsch GmbH, Haan, Germany) and added to the daily cultures as a substrate to feed the protozoa. In order to eliminate the negative effect of fermentation products formed in the environment on the propagation of protozoa, after dividing all pyrex bottles into two every three days, fresh medium solution (Table 1) was added to half of the total liquid volume, obtaining the desired number of protozoa (Dehority, 1998).

Table 1. Composition of medium solution

Composition	% (v/v)
Mineral mixture M[a]	50.0
Sodium acetate, 1.5%	5.0
Rumen fluid (supernatant)[b]	10
Sodium bicarbonate, 6%	8.33
Distilled water	26
Cystein HCL, 3%	0.67
Carbon dioxide	continuously

[a] mineral mixture M: g: NaCl 6.0 , $MgSO_4$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.26, KH_2PO_4/l 2.0; [b] supernatant rumen liquid is obtained by centrifugation at 1 000 g for 10 min after filtration through double layers of cheesecloth

Establishment of *in vitro* acidotic environment and determination of the efficiency of *E. caudatum* monocultures by gas production technique

The rumen fluid required for *in vitro* gas production was sampled from different parts of the rumen of cannulated heifers approximately four hours after the morning feeding. To ensure an anaerobic environment, rumen fluid samples were

flushed with a continuous CO_2 stream in an anaerobic gassing station for at least 30 min and dispensed to 50 ml conical falcon centrifuge tubes. The rumen fluid used *in vitro* was defaunated in order to determine the effect of *E. caudatum* monoculture alone on rumen fermentation. For defaunation, protozoa from rumen fluid for *in vitro* use were centrifuged twice at 1 000 *g* for 10 min (Allegra 64R Centrifuge, Beckman Coulter, Fullerton, CA, USA). After the last centrifugation, the supernatant was collected and examined under a light microscope with a 10 × 10 objective to confirm the absence of protozoa (Gülşen et al., 2018).

The *in vitro* conditions and the medium for determining the amount of gas production in the samples were prepared according to the method reported by Menke and Steingass (1988). Wheat and corn were used in the experiment due to their different starch structures, which affect the fermentation time in the rumen environment. Wheat and corn used in the experiment were ground in a 1-mm sieve, and wheat (100%; W) and corn (100%; C) and their 25, 50, and 75% mixtures were prepared for the *in vitro* experiment (Table 2). The bottles were placed in an incubator at 39 °C for 24 h, and then the pH was measured (HI 8314, Hanna Instruments, Porto, Portugal), and lactic acid levels were determined. Evaluating the pH and lactic acid results of replicates from different days using the method described by Sung et al. (2004), 17 g/l was determined to be the most appropriate wheat and corn ratio required to create a subacute acidosis environment. This quantity was weighed into bottles in the form of different feed groups as substrates providing an adequate acidotic environment in a total volume of 30 ml *in vitro*. The groups were: 100% wheat (W), 75% wheat and 25% corn (W75), 50% wheat and 50% corn (WC), 75% corn and 25% wheat (C75), and 100% corn (C). The test system was prepared using 40 ml of incubation medium and 20 ml of protozoa-free rumen fluid.

Table 2. Chemical composition of alfalfa hay, wheat, corn and grain mixtures used in the experiment (% DM)

Ingredients	DM	CP	CA	CF	NDF	ADF
W	92.12	11.55	1.87	1.7	20.21	3.65
W75	91.91	10.82	1.86	2.29	18.53	3.75
WC	91.7	10.09	1.85	2.88	16.85	3.85
C75	91.48	9.36	1.84	3.47	15.16	3.94
C	91.27	8.63	1.83	4.06	13.48	4.04
Alfalfa hay*	90.89	14.88	9.95	1.12	45.85	34.62

W – 100% wheat, W75 – 75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C75 – 25% wheat + 75% corn; C – 100% corn; DM – dry matter, CP – crude protein, CA – crude ash, CF – crude fat, NDF – neutral detergent insoluble cell wall components, ADF – acid detergent insoluble cell wall components; * – alfalfa hay was used in the daily diet of *Entodinium caudatum* monoculture

The chemical composition of alfalfa hay, wheat, corn, and mixtures with different proportions of wheat and corn used in the experiments are given in Table 2.

Protozoa were counted by collecting 1/10 of the sediment portion of *E. caudatum* monoculture, transferring it into a separating funnel, and allowing to settle for approximately 1 h. The protozoan count in the sediment was 5×10^5/ml, and 5 ml was added to the bottles of the experimental group to provide 10^3–10^4/ml of medium fluid. The remaining part of the sediment was used to fill 50 ml falcon tubes and centrifuged twice at 1 000 *g* for 10 min (Allegra 64R, Beckman Coulter, Fullerton, CA, USA). After centrifugation, the supernatant was examined with a 10 × 10 objective under a light microscope. After examination, the supernatant was confirmed to be free of protozoa and was added to the bottles of the control group, similarly to the experimental group. The bottles were then incubated at 39 °C for 48 h. The procedure was repeated on three different days with two replicates each time. The incubation medium of the rumen fluid supernatant defaunated of protozoa and 5 ml of *E. caudatum* culture were placed in bottles without substrate and used as a blank sample. A total of 36 bottles were prepared, including three replicates per treatment, 15 controls without *E. caudatum,* and 6 blanks without substrate. The amount of gas produced in the blank samples was subtracted from the amount produced by the samples.

Gas pressures were measured at 2, 4, 6, 8, 12, 24, and 48 h of incubation using a digital manometer (Keller Leo 1, Winterthur, Switzerland) with 0.2% sensitivity. The volumes of gas produced during incubation time to allow fermentation were calculated indirectly (López et al., 2007). A two-pool logistic model with independent fermentation characteristics and the theory that gas production is proportional to the amount of substrate, as reported by Schofield et al. (1994), was applied to calculate the gas kinetics. The first of these pools was soluble and easily fermentable, while the other represented the insoluble but fermentable fractions. Gas kinetics was determined using the "curve subtraction" technique (Schofield et al., 1994; Johnston and Tricarico, 2007) to determine the amounts of gas and kinetics of the two pools (fast- and slow-fermenting content) in terms of the degradation and gas formation properties of wheat and corn.

The following equation was used for the nonlinear fit of gas production in a two-pool logistic model:

$$V = V_{1F}(1 + \exp(2 + 4S(\lambda_1 - t))) + V_{2F}(1 + \exp(2 + 4S(\lambda_2 - t))),$$

where: V_{1F} and V_{2F} – the highest gas volume from both pools (ml), Q – specific rate (highest rate/ highest gas produced), t – incubation time (h), λ – lag time (λ1 fast-fermenting and λ2 slow-fermenting fraction).

During each calculation period of gas production, two bottles were used for each sample and sediment culture of *E. caudatum*, and pH was determined with a digital pH meter (HI 8314, Hanna Instruments, Amorim, Portugal). Ammonia (NH_3-N) levels (Weatherburn, 1967) and protozoan counts (Dehority, 1984) were determined in the samples collected after 6, 12, 24, and 48 h of incubation. To determine the levels of volatile fatty acids (VFAs) and lactic acid, 1 ml of fluid was taken from each sample and transferred to a centrifuge tube. Subsequently, 200 µl of 25% meta-phosphoric acid was added, mixed, and left for 30 min. It was then centrifuged at 5 000 *g* for 10 min.

Chemical analyses

Dry matter (DM), crude protein (CP), crude fat (CF), and crude ash (CA) of the feeds were determined according to AOAC International (2005). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) levels in the feeds were determined using an Ankom 200™ Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to Goering and Van Soest (1970). A flame ionization detector (FID) using gas chromatography (model 6890N GC, Agilent Technologies, Santa Clara, CA, USA) and DB-FFAP capillary column (30 m × 320 µm × 1.00 µm; J&W GC column, Agilent, Santa Clara, CA, USA) were applied for the determination of volatile fatty acid and lactic acid levels. The column temperature was maintained isothermally at 60 °C for 5 min and then increased by 5 °C per minute to 140 °C; the injector temperature was 270 °C, and the detector temperature was set to 300 °C (Cobellis et al., 2015). Methane production was calculated stoichiometrically. (Blümmel et al., 1999).

Statistical analysis

Following incubations, relative changes in gas production values, gas kinetic parameters, pH, lactic acid, VFAs and NH_3-N were assessed using a complete randomized design in a 5 × 2 factorial design. Data were evaluated using a two-way analysis of variance (2-way ANOVA). A general linear model was also used, and repeated measurements versus time were subplotted (version 23, SPSS Inc., Chicago, IL, USA):

$$Y_{ijk} = \mu + (TYK)_i + (CS)_j + (TYK*CS)_{ij} + e_{ijk},$$

where: Y_{ijk} – response variable, µ – population mean, TYK_i – i^{th} level of grain mixture, CS_j – j^{th} *E. caudatum* count, $(TYK*CH)_{ij}$ – i^{th} level of grain mixture and j^{th} *E. caudatum* count interaction, e_{ijk} – experimental error.

Results

Neither different types and proportions of grain feeds nor *E. caudatum* monoculture exerted a significant statistical effect on pH (Tables 3 and 4).

Table 3. Effect of *Entodinium caudatum* monoculture on pH, NH_3-N and lactic acid levels of different grain feed types and proportions

Items	*E. caudatum*	pH[1]	NH_3-N[2], mmol/l	Lactic acid[3], mmol/l
W	-	6.17	7.58	0.20
	+	6.22	7.66	0.10
W75	-	6.16	6.28	0.13
	+	6.18	6.83	0.13
WC	-	6.15	5.63	0.22
	+	6.17	6.16	0.13
C	-	6.18	4.08	0.17
	+	6.22	4.60	0.08
C75	-	6.14	5.20	0.23
	+	6.16	4.70	0.12
SEM		0.01	0.12	0.06
ANOVA				
TG		0.735	0.000	0.430
E. caudatum		0.137	0.123	0.001
TG x *E. caudatum*		0.956	0.674	0.566

W – 100% wheat, W75 –75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C – 100% corn, C75 – 25% wheat + 75% corn; TG – type of grain feed, SEM – standard error of the mean, + – *E. caudatum* addition, - – lack of *E. caudatum;* [1] effect of incubation time $P < 0.0001$; [2] effect of incubation time and substrate $P < 0.0001$; [3] effect of incubation time $P < 0.0001$; interaction of *E. caudatum* x incubation time $P < 0.038$; incubation time x substrate interaction $P < 0.008$

Table 4. Effect of *Entodinium caudatum* monoculture on pH values at different incubation times*

Items	Inoculation	6 h	12 h	24 h	48 h
W	*E.caudatum* (-)	6.64	6.14	5.74	5.40
	E.caudatum (+)	6.63	6.25	5.77	5.59
W75	*E.caudatum* (-)	6.67	6.20	5.62	5.50
	E.caudatum (+)	6.63	6.24	5.67	5.57
WC	*E.caudatum* (-)	6.67	6.18	5.60	5.49
	E.caudatum (+)	6.63	6.24	5.65	5.56
C	*E.caudatum* (-)	6.81	6.19	5.54	5.62
	E.caudatum (+)	6.74	6.28	5.65	5.62
C75	*E.caudatum* (-)	6.73	6.19	5.51	5.58
	E.caudatum (+)	6.66	6.23	5.59	5.60

W – 100% wheat, W75 – 75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C – 100% corn, C75 – 25% wheat + 75% corn; + – *E. caudatum* addition, - – lack of *E. caudatum*, * – interaction of *E. caudatum* x incubation time $P < 0.038$

While the type of grain feed markedly influenced ammonia ($P < 0.0001$), *E. caudatum* did not. The experiment determined that ammonia levels were greater at a high wheat level, while its formation decreased with increasing corn proportion (Table 3). No effect of grain feed type on lactic acid (mmol/l) levels was observed (Table 3); however, it was found that inoculation with *E. caudatum* decreased lactic acid concentrations throughout the incubation period ($P < 0.001$; Table 3). Although lactic acid levels increased with incubation time ($P < 0.0001$), *E. caudatum* inoculation reduced lactic acid concentration as a function of incubation time ($P < 0.038$; Figure 1).

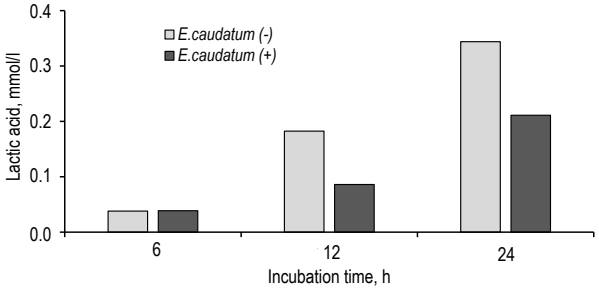

Figure 1. Effect of *Entodinium caudatum* (*E. caudatum* x incubation time interaction) inoculation on lactic acid level in all experimental groups depending on the incubation time ($P < 0.038$); + – *E. caudatum* addition, − – lack of *E. caudatum*

Lactic acid production values differed significantly in the samples collected after 6, 12, and 24 h of incubation depending on the type of substrate ($P < 0.008$). More lactic acid was produced in wheat-based substrates in the first 12 h of incubation, while corn-based substrates produced more lactic acid in the 12–24 h incubation period (Figure 2).

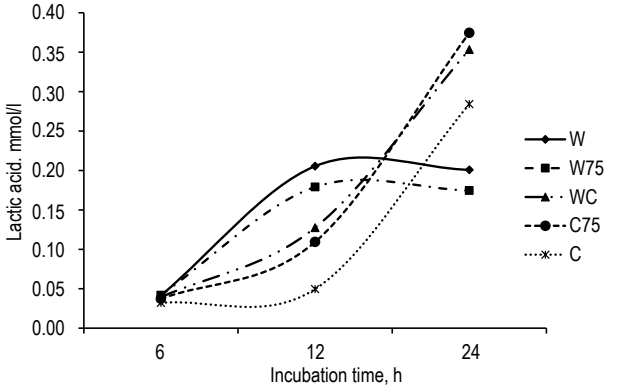

Figure 2. Effect of substrate diversity on lactic acid production depending on the incubation time ($P < 0.008$); W – 100% wheat, W75 – 75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C75 – 25% wheat + 75% corn, C – 100% corn

The influence of different types and proportions of grains and *E. caudatum* monoculture on *in vitro* VFA production is presented in Table 5. The type of feed did not affect the production of TVFA (total volatile fatty acids) and acetic acid, but it did affect propionic acid levels ($P < 0.0001$). Furthermore, the differences acetic:propionic acid ratios ($P < 0.0001$) were statistically significant (Table 5).

Table 5. Effect of different grain feed types and proportions and *Entodinium caudatum* monoculture on *in vitro* fermentation parameters

Ingredients	*E. caudatum*	TVFA, mM	Acetic acid, %	Propionic acid, %	Butyric acid[1], %	Ac:Pr
W	-	0.90	62.21	26.88	9.16	2.43
	+	0.93	62.44	25.08	10.64	2.58
W75	-	0.85	62.26	26.58	9.43	2.44
	+	0.96	62.24	24.89	11.04	2.58
WC	-	0.85	62.48	26.30	9.49	2.47
	+	0.89	62.39	24.40	11.36	2.64
C	-	0.84	63.19	25.69	9.40	2.58
	+	0.92	62.96	23.30	11.92	2.80
C75	-	0.83	62.80	25.91	9.53	2.53
	+	0.91	62.49	24.07	11.59	2.68
SEM		0.03	0.55	0.47	0.18	0.07
ANOVA						
TG		0.880	0.133	0.000	0.064	0.000
E. caudatum		0.052	0.707	0.000	0.000	0.000
T		0.000	0.000	0.000	0.000	0.000
TG x *E. caudatum*		0.945	0.948	0.865	0.377	0.877
E. caudatum x T		0.885	0.004	0.000	0.125	0.090

W – 100% wheat, W75 – 75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C – 100% corn, C75 – 25% wheat + 75% corn; TG – type of grain feed, + – *E. caudatum* addition, - – lack of *E. caudatum*, T – time, TVFA – total volatile fatty acids, Ac:Pr – acetic to propionic acid ratio, SEM – standard error of the mean; [1] grain feed type x time interaction $P < 0.025$

Inoculation of *E. caudatum* monoculture decreased the propionic acid percentage and increased butyric acid content ($P < 0.0001$). As the incubation period progressed, it was observed that inoculation of *E. caudatum* decreased the formation of propionic acid and increased of butyric acid ($P < 0.0001$). Total gas production (TGP), fermentation start time (lag time; h), and specific fermentation rate (SFR, kd; highest gas production rate/total gas production amount) of *E. caudatum* monoculture added to the *in vitro* environment with different types of feed and grain proportion are given in Table 6; Figure 3 presents the properties of fermentation kinetics.

Table 6. Effect of substrate diversity and *Entodinium caudatum* monoculture inoculation on fermentation kinetic parameters and total gas production

Items	*E. caudatum*	SFR, time^{-1}	Lag time, h	TGP, ml	pCH_4, ml/g
W	-	0.12	3.87	180.21	5.16
	+	0.08	3.33	211.67	6.17
W75	-	0.11	3.89	177.09	5.05
	+	0.11	3.68	195.64	6.6
WC	-	0.11	3.87	177.42	5.16
	+	0.07	2.92	241.15	6.3
C	-	0.08	4.20	205.83	5.27
	+	0.07	3.77	228.44	6.98
C75	-	0.08	3.71	205.34	5.14
	+	0.08	3.59	216.56	6.57
SEM		0.00	0.09	3.39	0.12
ANOVA					
TG		0.000	0.350	0.011	0.768
E. caudatum		0.003	0.012	0.000	0.000
TG x *E. caudatum*		0.184	0.696	0.288	0.874

W – 100% wheat, W75 – 75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C – 100% corn, C75 – 25% wheat + 75% corn; TG – type of grain feed, + – *E. caudatum* addition, - – lack of *E. caudatum,* SFR – highest gas production rate constant, Lag time – time to start gas production, TGP – total gas production, pCH_4 – estimated methane production, SEM – standard error of the mean

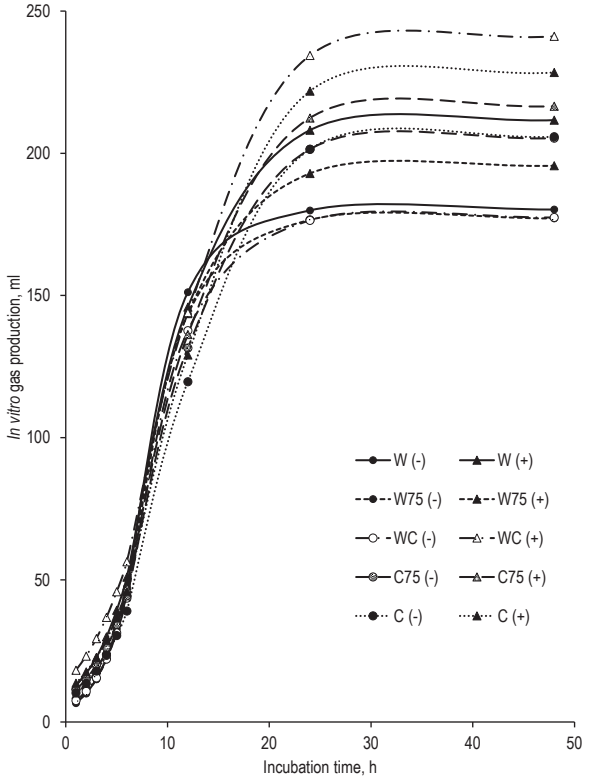

Figure 3. *In vitro* gas production kinetics of different feed types by the effect of *Entodinium caudatum*; W – 100% wheat, W75 – 75% wheat + 25% corn, WC – 50% wheat + 50 corn%, C75 – 25% wheat + 75% corn, C – 100% corn

While no effect of grain feed type on lag time was observed, *E. caudatum* culture shortened the fermentation start time ($P < 0.01$). SFR, which increased with increasing wheat proportion ($P < 0.0001$), decreased when *E. caudatum* culture was added to the substrate ($P < 0.003$). TGP was differentiated by substrate ($P < 0.01$), and the addition of *E. caudatum* increased TGP ($P < 0.0001$; Table 6).

The effect of different types of feed and grain proportion, and *E. caudatum* monoculture on methane (CH_4) production is shown in Table 6. While the type of grain feed did not affect methane production, inoculation of *E. caudatum* monoculture increased it ($P < 0.0001$).

No protozoa were found in the control group, in which *E. caudatum* was not incubated. An increase in *E. caudatum* count was observed up to 12 h of the experiment. The number of *E. caudatum* gradually decreased due to the declining pH over time and the inability to remove the resulting fermentation products. At the 48th h, when the pH of the environment decreased to 5.55, a significant reduction in movement was observed, and *E. caudatum* abundance declined (Table 4, Figure 4).

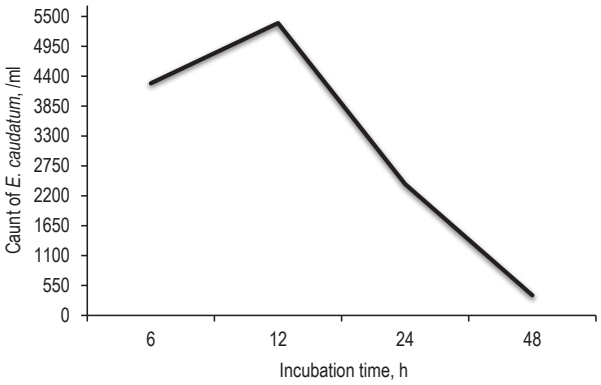

Figure 4. Effect of incubation time on the number of *Entodinium caudatum* in the experimental groups

E. caudatum cells were transparent during the first inoculation. In contrast, as the incubation progressed, the protozoan cells became darker in colour and contained black vesicles (Figure 5).

Discussion

Changes in ruminal pH. An increase in the level of carbohydrates in ruminant rations results in higher acid production by rumen microorganisms,

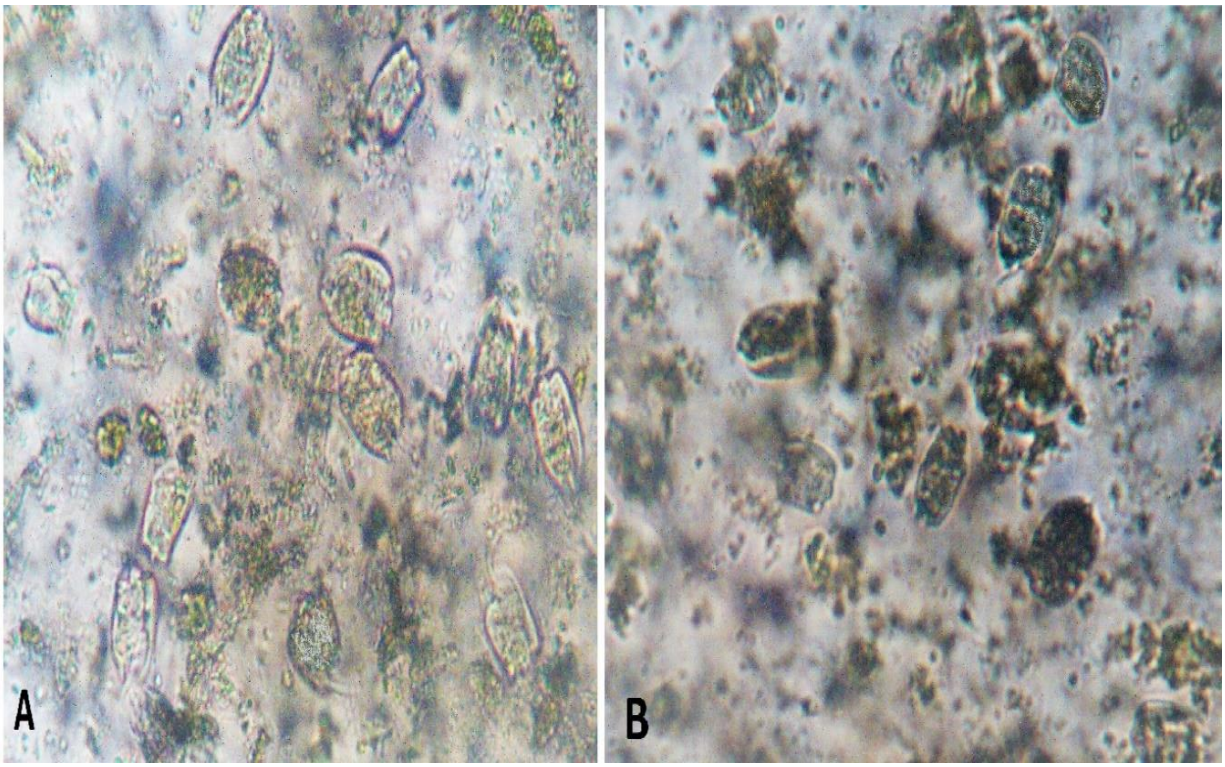

Figure 5. A) Empty *Entodinium caudatum* monocultures at the beginning of incubation, B) *E. caudatum* monocultures filled with intracellular starch granules late in incubation

leading to a lower ruminal pH (McCaughern et al., 2020). A rumen pH between 5.0–5.5 (Enemark et al., 2002) or a pH below 5.8 is considered a prominent feature of SARA (Nordlund and Garrett, 1994). It was observed that the grain feed mixtures used in this study decreased the pH of rumen fluid to a level that would form SARA after 48 h of fermentation (Table 3). As corn starch is covered with a slowly degrading protein matrix, it delays grain degradation time compared to wheat (Kang et al., 2021). For this reason, it was determined that the pH of the fermentation liquid in the wheat-containing group was lower (W; pH: 5.50) than in the maize-containing group (C; pH: 5.61) (Table 3). Similarly to the present *in vitro* study, the pH of the culture solution from wheat was previously shown to be relatively lower than that of corn (Qin et al., 2012).

Protozoa in the rumen play an essential role in regulating rumen conditions, such as pH and redox potential (Williams and Coleman, 1997). Franzolin and Dehority (2010) found that mean rumen pH of defaunated animals was lower compared to non-defaunated counterparts. As in this study, Qin et al. (2012) reported that defaunation significantly decreased pH after 12 h of rumen fluid incubation with grains in *in vitro* conditions. However, contrary to these *in vivo* studies, the fact that *E. caudatum* monoculture inoculated *in vitro* in the present study did not statistically affect the pH of the culture solution (Table 3) could be due to the inability to remove fermentation products from the environment (Menke et al., 1979).

Effects on ammonia production. A significant effect of grain feed type on ammonia production was observed due to the fact that wheat contained higher amount of CP compared to corn (Odle and Schaefer, 1987). More ammonia was produced as a result of protein deamination by microorganisms in the fermentation fluid with a substrate containing wheat with higher CP content than corn (Table 2 and 3). Although it has been previously suggested that rumen protozoa could increase ammonia production (Williams and Coleman, 1997), it was determined that ammonia concentration was not affected in the group with *E. caudatum* monoculture (Park et al., 2019). Factors such as rumen dilution and passage rate, which did not occur in an *in vitro* fermentation environment, could lead to different results (Menke et al., 1979).

Effects on lactic acid production. Although it has been previously suggested that lactic acid levels in the rumen environment tend to increase more in wheat diets compared to feeds based on corn (Moate et al., 2018), no differences were observed in terms of lactic acid production between wheat and corn in the present study (Table 3).

It is believed that lactic acid production can be reduced by limiting the uptake of easily fermentable carbohydrate sources by *E. caudatum* into the cells (iodophilic storage), thereby preventing rapid fermentation (Elghandour et al., 2020). Entodiniomorphid protozoa digest starch grains and ferment them into H_2, CO_2, acetic acid, butyric acid, and glycerol. *Entodinium* spp. (*E. caudatum* and *E. simplex*) ingest the grains rapidly in the early stages and then continue their digestion processes at a slower rate (about 3% of the initial rate) (Yuste et al., 2019). The microscopic examination of *E. caudatum* in the fluid collected during the *in vitro* trials revealed that the protozoan cells did not contain starch granules at the beginning of the incubation. In contrast, the cells appeared filled as incubation progressed (Figure 5), which was consistent with the literature data (Williams and Coleman, 1997; Elghandour et al., 2020). It has been suggested that lactic acid is utilised selectively and acidosis can be prevented by the presence of *E. caudatum* in the environment (Williams and Coleman, 1997). In this study, a decrease in lactic acid production or an increase in its use was determined by inoculating a monoculture of *E. caudatum* into the rumen fluid (Figure 1).

Effect on volatile fatty acids. As the aim of the study was to create an acidotic environment, the substrates used in the trials were prepared exclusively from grain feeds and their mixtures. Consistent with the overall changes in VFA levels reported in the literature, the present study also determined that the proportion of acetic acid decreased (Liu et al., 2015), while that of butyric acid increased with decreasing pH, starting from the first hours of incubation (Steele et al., 2009). In addition, it was observed that TVFA levels were not affected (Moate et al., 2018). Therefore, it is thought that lowering the rumen pH during the fermentation of grain feed and their mixtures (Table 4) causes a decrease in the number of fibrolytic bacteria, thereby reducing cellulose digestion and acetic acid production (Kim et al., 2018).

In this study, it was observed that *E. caudatum* monoculture had no effect on TVFA and acetic acid production, but it increased butyric acid content (Williams and Coleman, 1997; Brossard et al., 2004; Park et al., 2021); however, other studies have reported that *E. caudatum* does not affect butyric acid production. Such differences in results are frequently encountered in this type of monoculture studies (Zeitz et al., 2013). In addition, it was found that an increase in the number of protozoa in the rumen reduced the accumulation of lactic acid and stimulated butyric acid production (Brossard et al., 2004; Park et al., 2021), and the proportion of butyric acid decreased with declining protozoan counts (Eugène et al., 2004).

It was previously reported that the levels of lactic acid and propionic acid increased in the rumen fluid of defaunated animals (Nagaraja and Titgemeyer, 2007). Therefore, the decrease in propionic acid content was expected in the groups inoculated with *E. caudatum* compared to the control group without protozoa.

Effect on methane production. Methanogenesis is a crucial metabolic activity of the rumen microflora. Methane is produced by methanogenic archaea, which account for only a small fraction (3–4%) of the ruminal microbial community (Lyu et al., 2018). Rumen bacteria in the majority of entodiniomorphid protozoa have been found to attach to the outer surface of the protozoan membrane and most of them belong to methanogens (Xia et al., 2014; Malmuthuge and Guan, 2017). Many ruminal protozoa have a hydrogen-producing organelle (hydrogenosome) that attracts methanogens, such as endosymbionts (Patra et al., 2017). While hydrogenosomes have been described in the genera Epidinium, Isotricha, and Dasytricha, they have not been found in the species *E. caudatum* and *Diploplastron affine* (Ellis et al., 1994). In view of this information, although there is a low number of methanogenic archaea in *E. caudatum* monocultures (Park and Yu, 2018b), methanogens associated with ciliata are responsible for 9–25% of methanogenesis in the rumen fluid, and methane production decreases with defaunation (Moss et al., 2000). Another study reported that methane emission decreased by 30–45% after the removal of ciliata protozoa from the rumen, but this effect was transient (Pei et al., 2010). In this *in vitro* study, no differences were observed between methane levels in wheat, corn, and their mixtures in different proportions. However, inoculation of *E. caudatum* increased methane production (Table 6) (Morgavi et al., 2010; Zeitz et al., 2013).

Effects on *in vitro* gas production. The experiment determined that more gas (TGP) was produced as a result of increased starch fermentation when inoculated with *E. caudatum* (Coleman et al., 1976), which is the most dominant starch-preferring species in the rumen (Zeitz et al., 2013). The reduced lag time in the inoculated groups also indicated that

E. caudatum accelerated fermentation (Table 6). Although *E. caudatum* did shorten the lag time, it also reduced the highest gas production rate constant (SFR). It was particularly effective in the decomposition of feed materials such as wheat, which is easily degradable and poses a risk of SARA over a longer period of time. It has been reported that *Entodinium* species ingest starch particles very quickly and ferment very slowly (Elghandour et al., 2020). Rumen protozoa transfer the digestion of starch to the intestines by changing the amount of starch degradation in the rumen (Mendoza et al., 1993). Due to these properties of *Entodinium spp*., it is believed that they can be used to reduce the risk of clinical and subclinical acidosis (Williams and Coleman, 1997).

***E. caudatum* distribution.** Rumen pH decreases with the increase in the proportion of concentrated feed in the ration. It has also been reported that *Entodinium* species are quite resistant to a decrease in rumen pH, and their numbers raise with increasing concentrate feed consumption (Coleman et al., 1976; Brossard et al., 2004). As the proportion of grains containing easily fermentable carbohydrates increases, the number of protozoa in the rumen fluid raises logarithmically (Brown et al., 2006; Hook et al., 2011). This was confirmed in the present study by the increase in protozoa abundance at the beginning of incubation and their starch ingestion. However, as the incubation progressed, the reduced pH resulted in the SARA formation, and the number (Hook et al., 2011) and motility (Dehority, 2005) of *E. caudatum* decreased under microscopic examination (Figure 4).

Conclusions

E. caudatum is believed to prevent rapid bacterial fermentation in the rumen fluid thanks to its phagocytosis activity, thereby limiting lactic acid production. The results of this study may support future *in vitro* monoculture and *in vivo* studies investigating the effect of *E. caudatum* on the inhibition of SARA formation. The efficacy of *E. caudatum* should be supported by *in vivo* studies.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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