RUMINAL PROTOZOA VIABILITY UNDER DIVERSE ADDITIVES USING TRYPAN BLUE EXCLUSION METHOD AS AN ALTERNATIVE COUNTING METHODOLOGY.

*Cuervo W., Gomez-Lopez C., Fernandez-Marenchino I., Maderal A., Vargas J., Tarnonsky F., Fernandez A., Schulmeister T., Podversich F, and DiLorenzo N.**

Outline

- Introduction
- Materials and Methods
- Results & Discussion
- Conclusions
- Acknowledgments

Introduction

Evidence of changing concentrations of atmospheric CO_2, N_2O and CH_4 from air bubbles in Antarctic ice

G. I. Pearman*, D. Etheridge†, F. de Silva* & P. J. Fraser*

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Indirect chemical effects of methane on climate warming

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Max-Planck-Institute for Chemistry, PO Box 3060, D-6500 Mainz, Germany

Nature · VOL 355 · 23 January 1992

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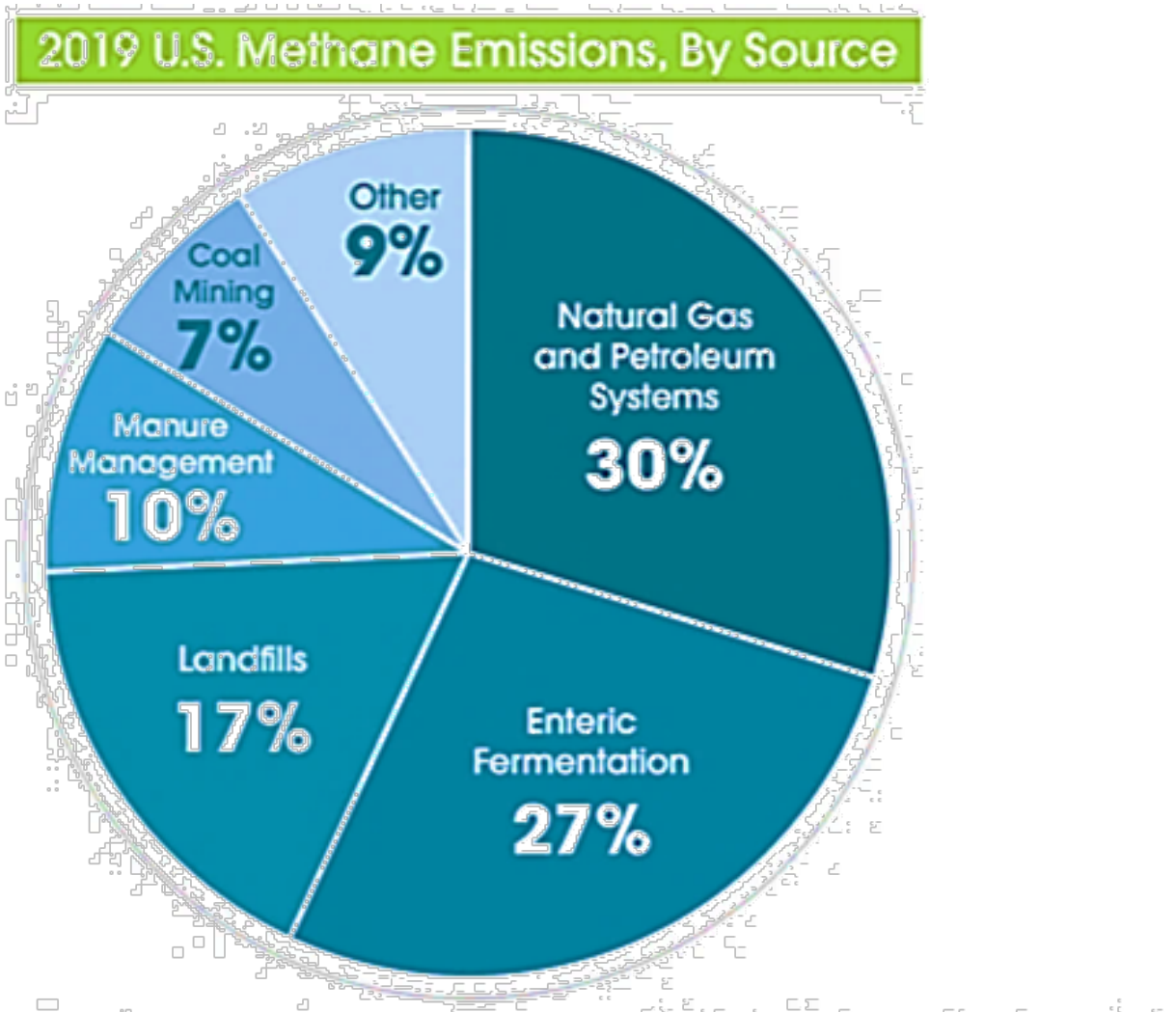

U.S. Environmental Protection Agency (2021). Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2019

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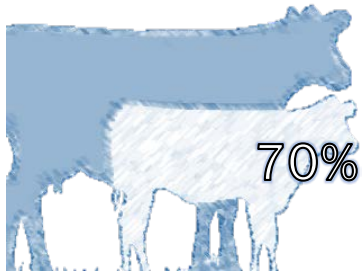

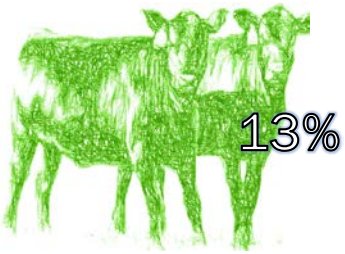

23%

Brocks 2020; USDA - NASS. 2020

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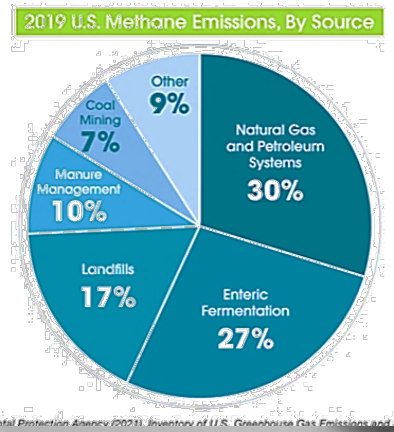

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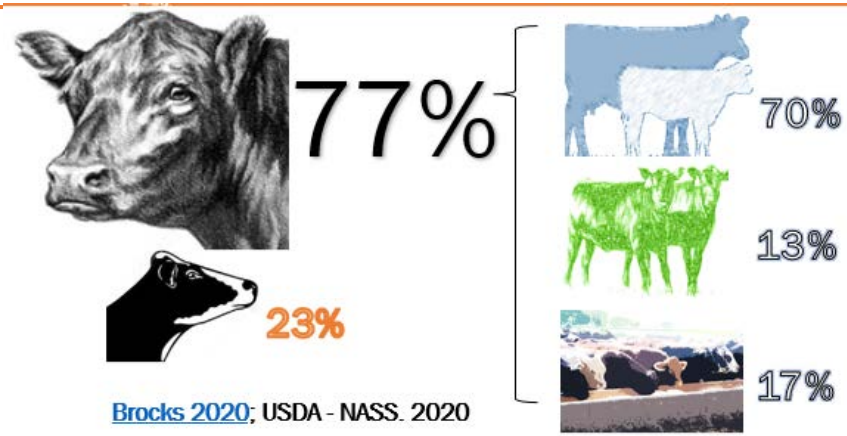

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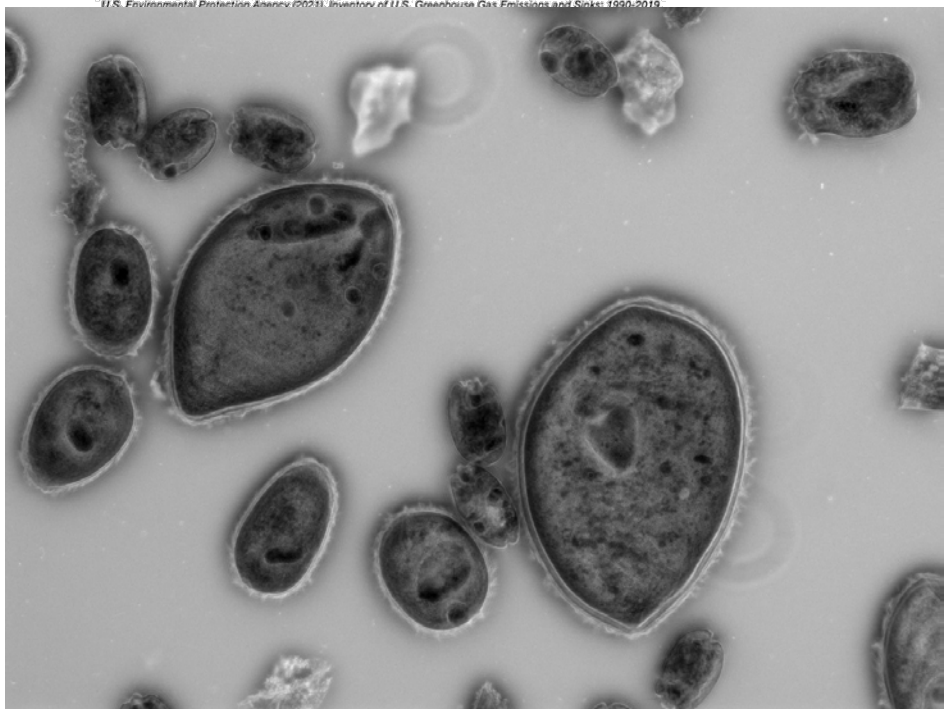
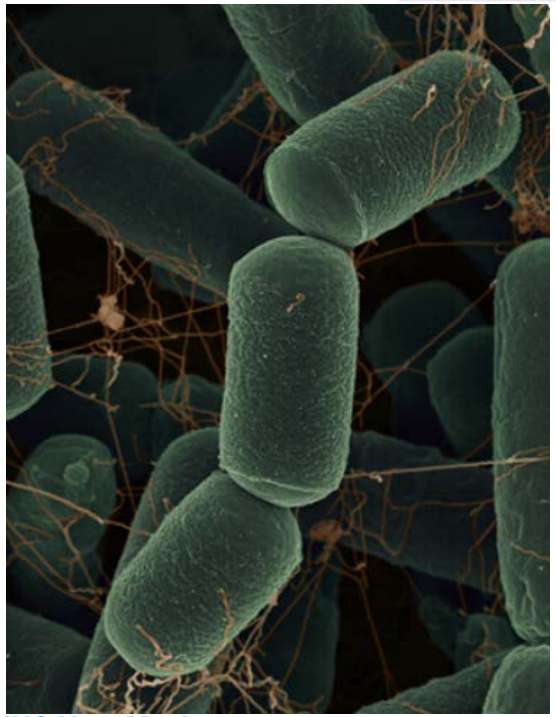


Hackmann 2023



MNHS New Mexico

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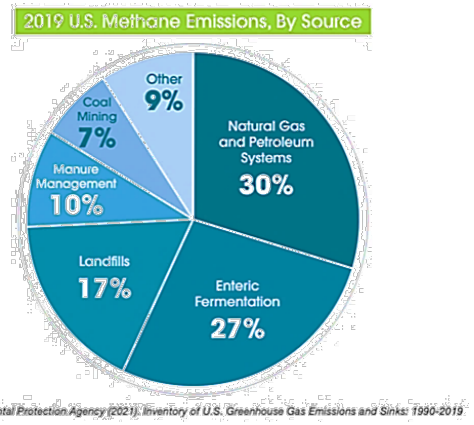

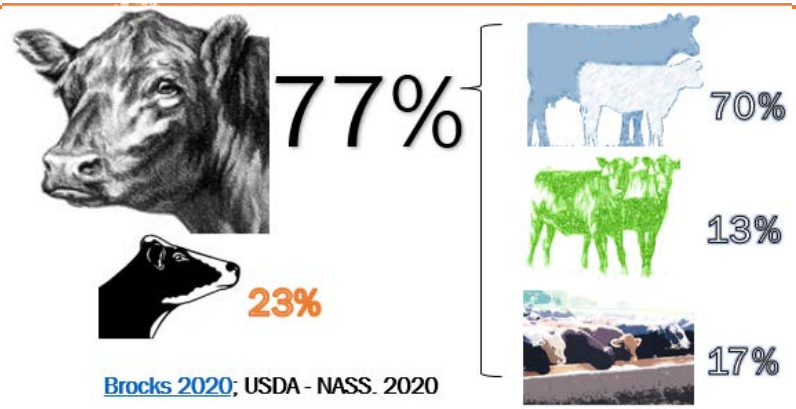

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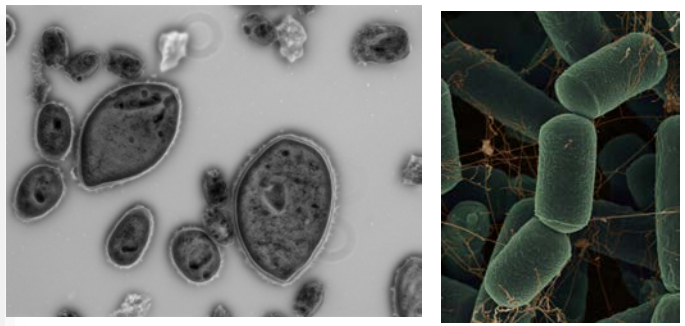


Inside protozoa

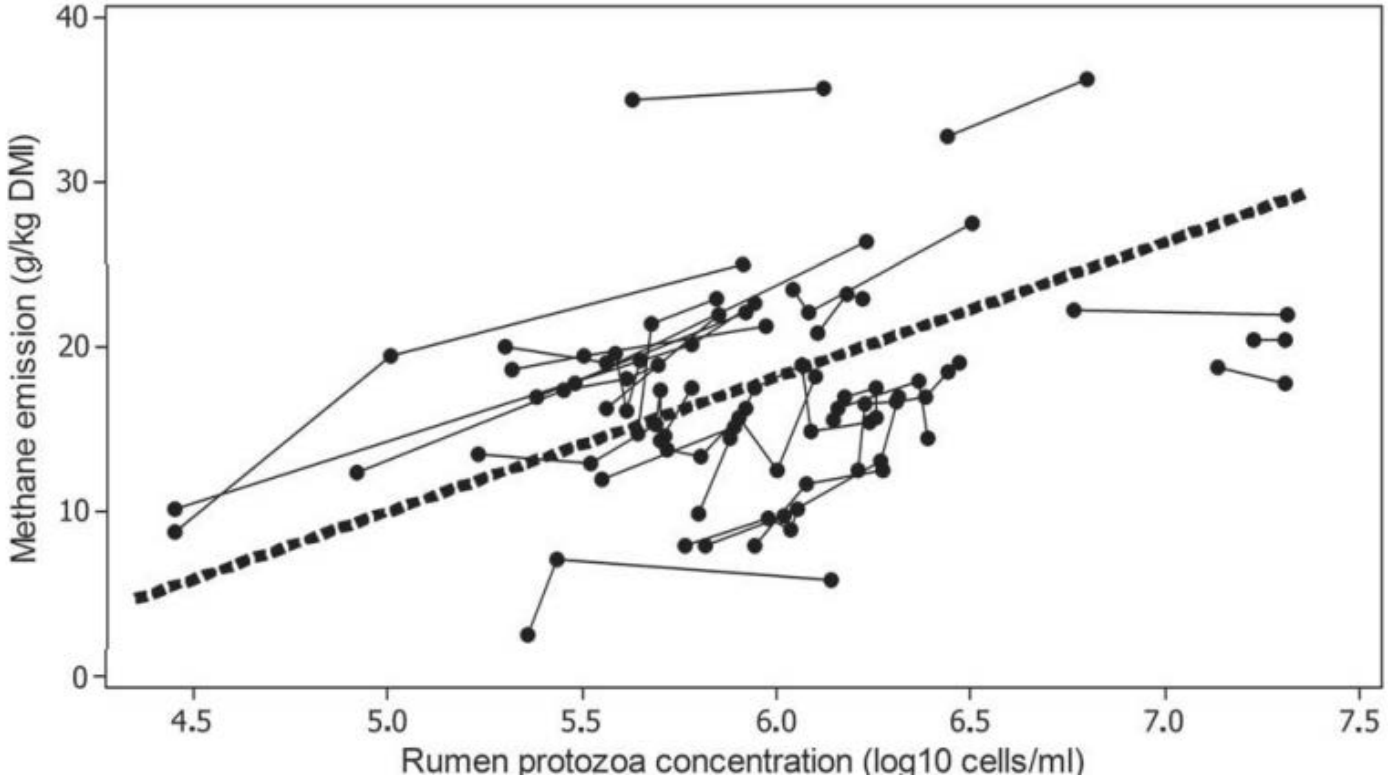
(37% - HIGHER methanogenic)

Ruminal fluid

(~70% – Lower methanogenic activity)

Anim. Feed Sci. Technol, 293, 115471 ; JAST 8(1), 1-11. ; Glab088, Vol 11, 2022

Introduction



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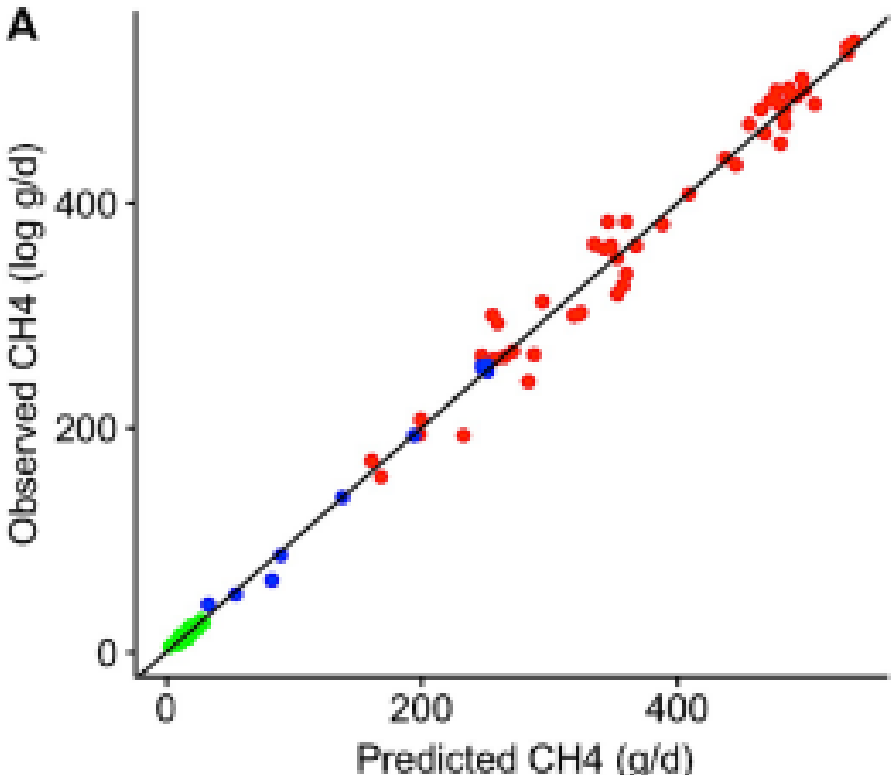
Guayder et al., 2014



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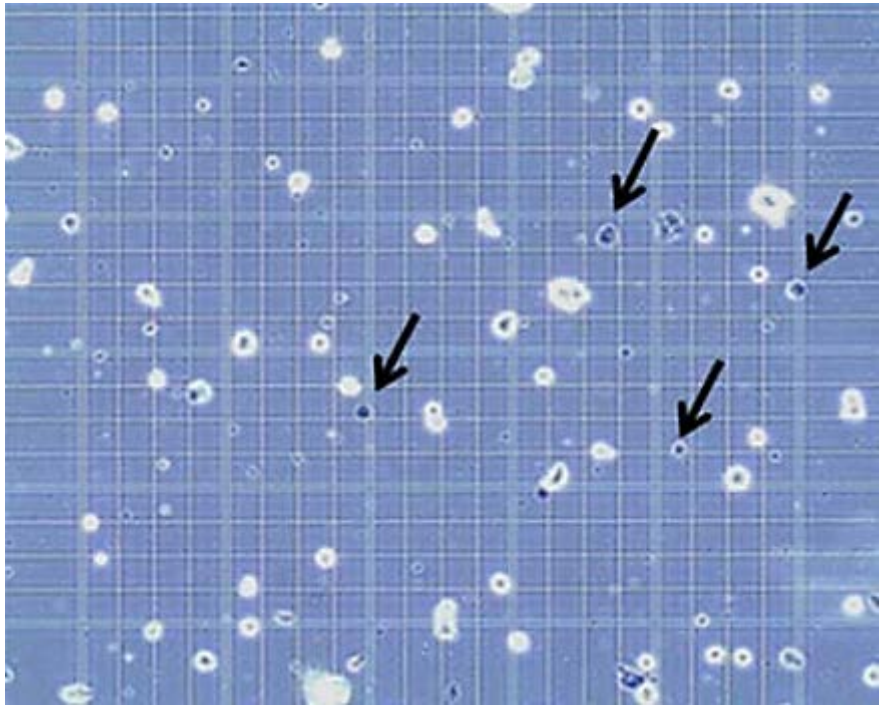
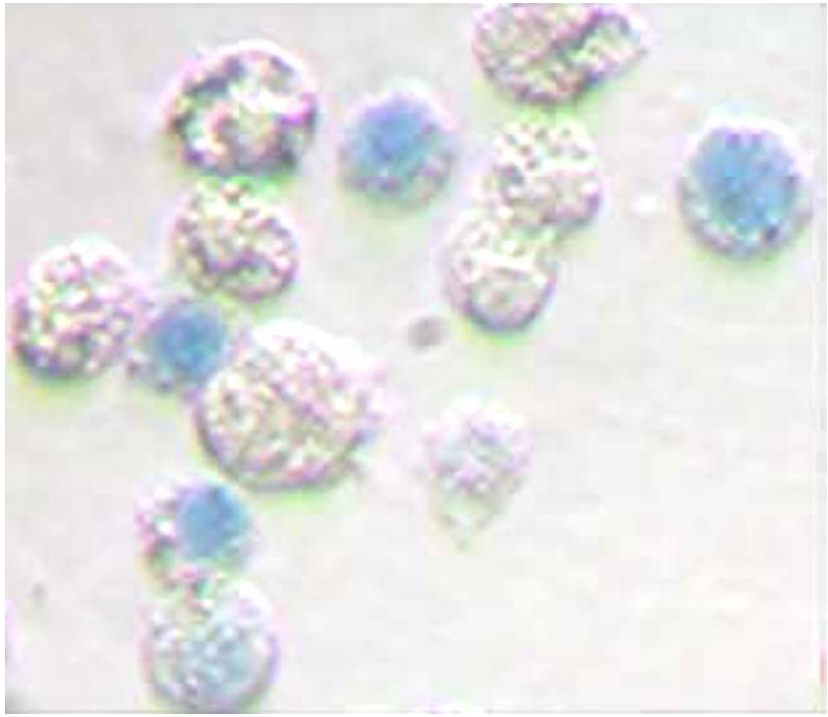
Dai et al., 2021

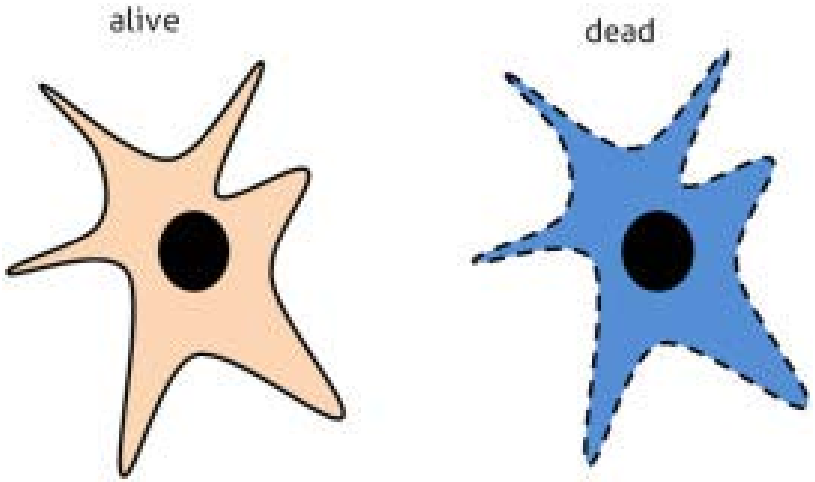
Introduction



Murine colon carcinoma (CT-26)
Hong et al., 2011



Murine Macrophages
Hong et al., 2011



Menna-Barreto (2019). ***Cell Death and Disease (2019) 10:93***

Objective & Hypothesis

- ✓Establish culture conditions to use the TBEM for PROT and VIAB under different diets and feed additives

- ✓Determine the effect of yeast derivatives, essential oils, saponins and Tannins on protozoa viability, ruminal fermentation, in vitro CH4 production and in vitro organic matter digestibility (IVOMD).

"TBEM differentiate alive and dead protozoa and can be used to evaluate the effect of methane mitigation strategies via protozoa reduction, complementing ruminal fermentation data"

Outline

- Introduction
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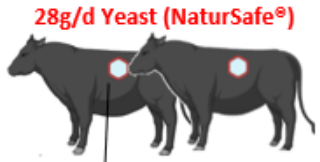
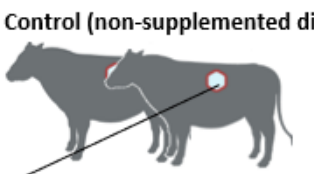
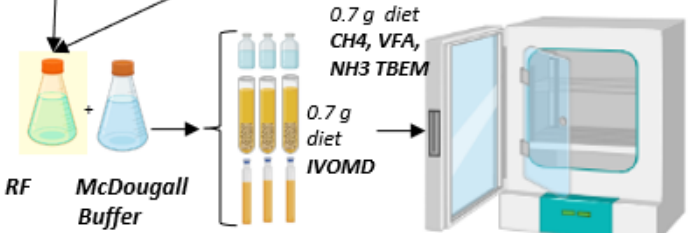
Methodology

Experiment 1 – Culture conditions, Effect of yeast

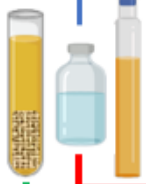
Ingredient	(% of DM)
Cracked corn	65
DDGS	10
Cottonseed hulls	4.9
Bermuda hay	15
Vit&Min Premix	5.1

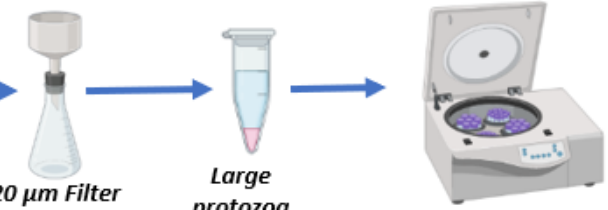


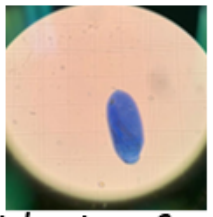


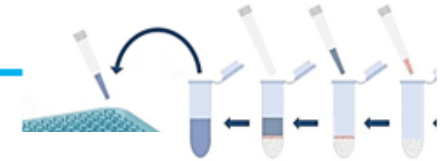


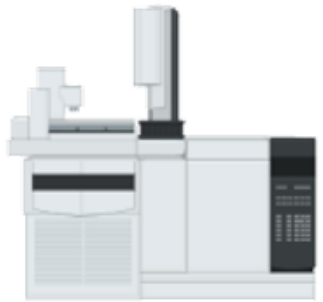
Batch culture set up









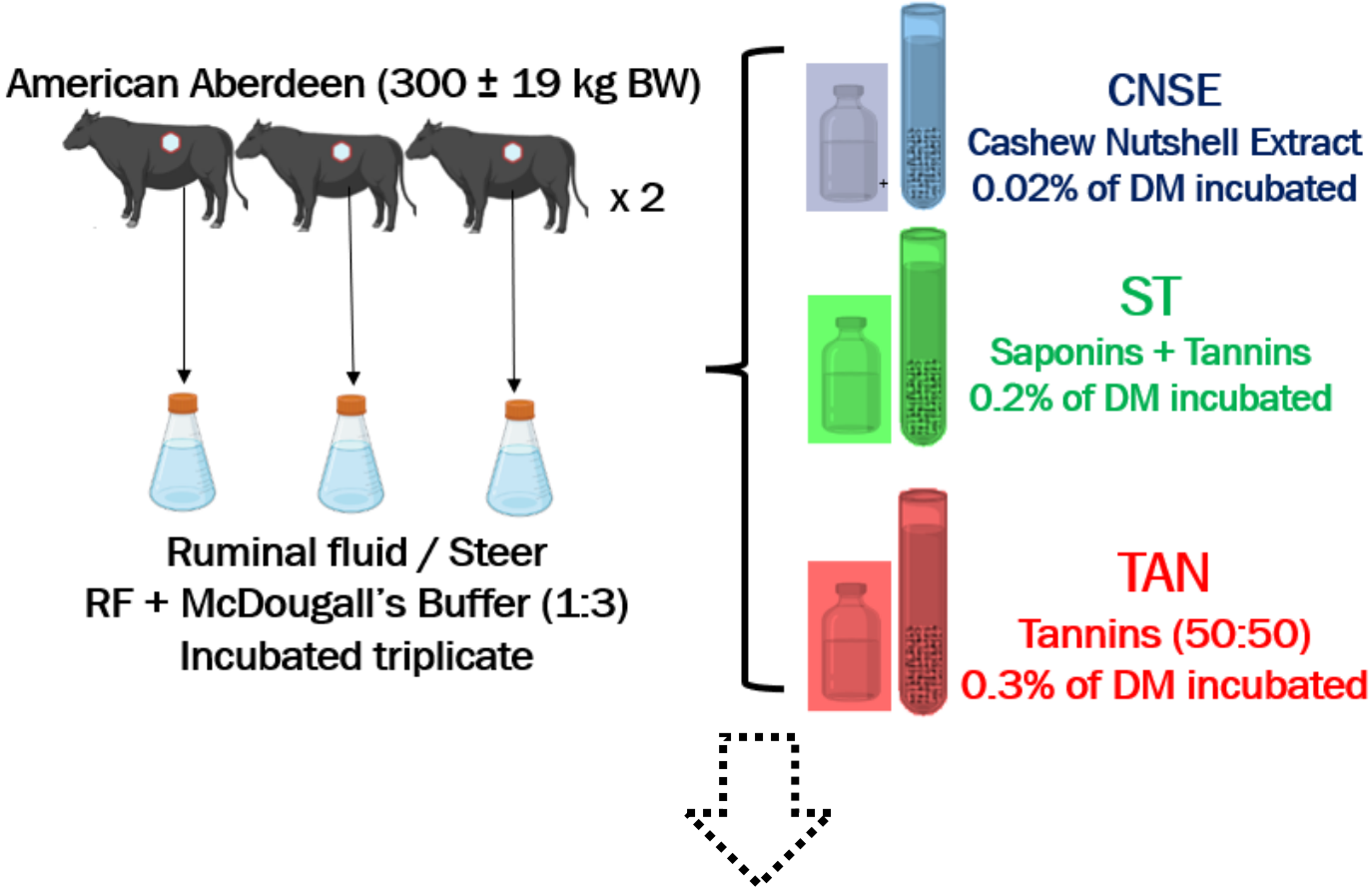


Methodology

Experiment 2 – Effect of feed additives on methane and protozoa viability.

ITEM	Inclusion % DM basis
Corn silage	34
Bermudagrass Hay	22
Cottonseed meal	19
Cracked Corn grain	22
Vit & min Premix	3
Chemical composition[1]	
CP (% of DM)	14.2
OM (% of DM)	90.7
NDF (% of DM)	35.6
ADF (% of DM)	18.8
Lignin[2] (% of DM)	4.23
Starch[2] (% of DM)	27.2
TDN[2] (% of DM)	68.0
NEm[2](Mcal/kg DM)	1.29
NEg[2] (Mcal/kg DM)	0.82

[1] Analyzed by a commercial laboratory using a wet chemistry package (Dairy One, Ithaca, NY). [2] Estimated using BCNR Model 2016; CP = Crude protein. OM = Organic Matter. NDF= Neutral Detergent Fiber. ADF= Acid Detergent Fiber. TDN = Total Digestible Nutrients. NEm = Net Energy for Maintenance. NEg = Net Energy for Gain.



Procedure & Techniques (Batch culture incubation , IVOMD, GC, TBEM)
Measurements (VFA, NH_3, CH_4, Protozoa Count & Viability)
Similar to Exp 1

Methodology

Experiment 1 - Culture conditions, effect of yeast

Data was analyzed through PROC MIXED (SAS 9.4, SAS Inst. Inc., Cary, NC) using a factorial split plot design with repeated measures arrangement.

Main plot = Steer ➔ Main Treatment ➔ Yeast addition
Sub plot = Bottle ➔ Sub treatment ==> Culture condition

Experiment 2 – Effect of feed additives on methane and protozoa viability.

CRD using MIXED procedure of SAS, with additive as fixed effect (treatment) and the random effect of steer.
EU = Average of 6 bottles (and tubes) ➔ 3 bottles / Trt, 2 individual incubations in triplicate)

Gas, pH, total protozoa, and protozoa viability ➔ repeated measures with additive, hour, and their interaction as fixed factors, and steer (day) as random factor. When significant, value at hour 0 was included as a covariable.

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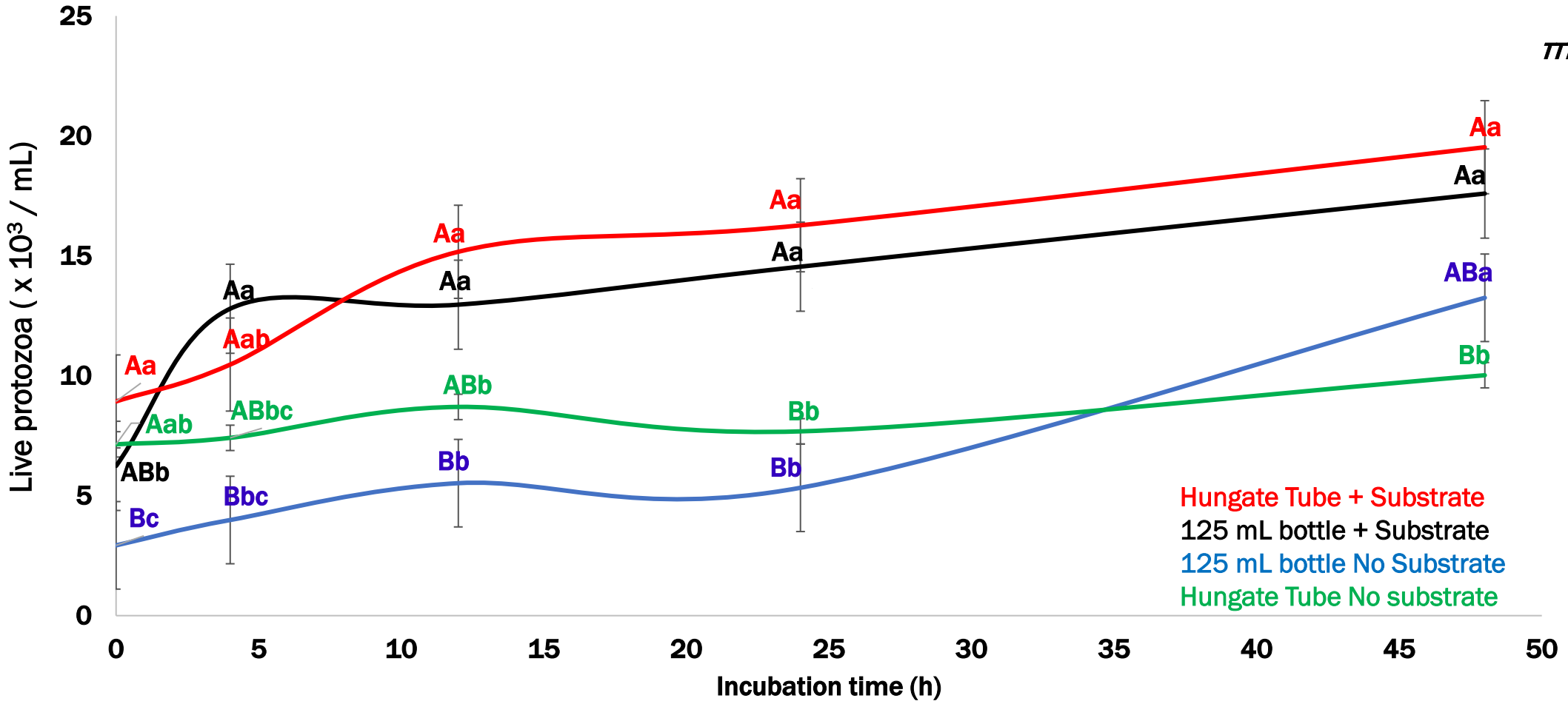
Results - Experiment 1

Effect of incubation conditions on ruminal protozoa count and viability

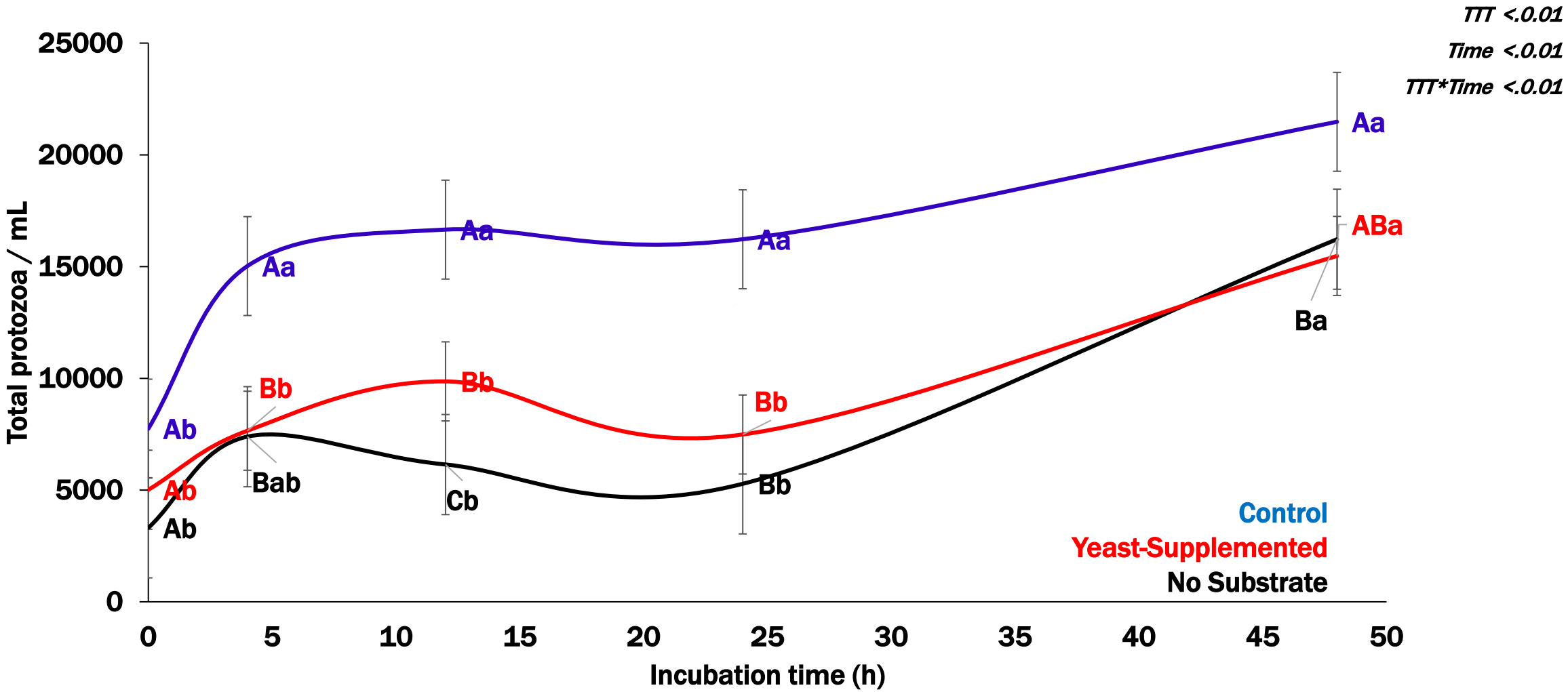
TTT <.0.01
Time <.0.01
*TTT*Time <.0.01*



Different capital letters indicate a significative difference between treatments at each time point
Different Lower letters indicate a significative difference between time points within each treatment

Results - Experiment 1

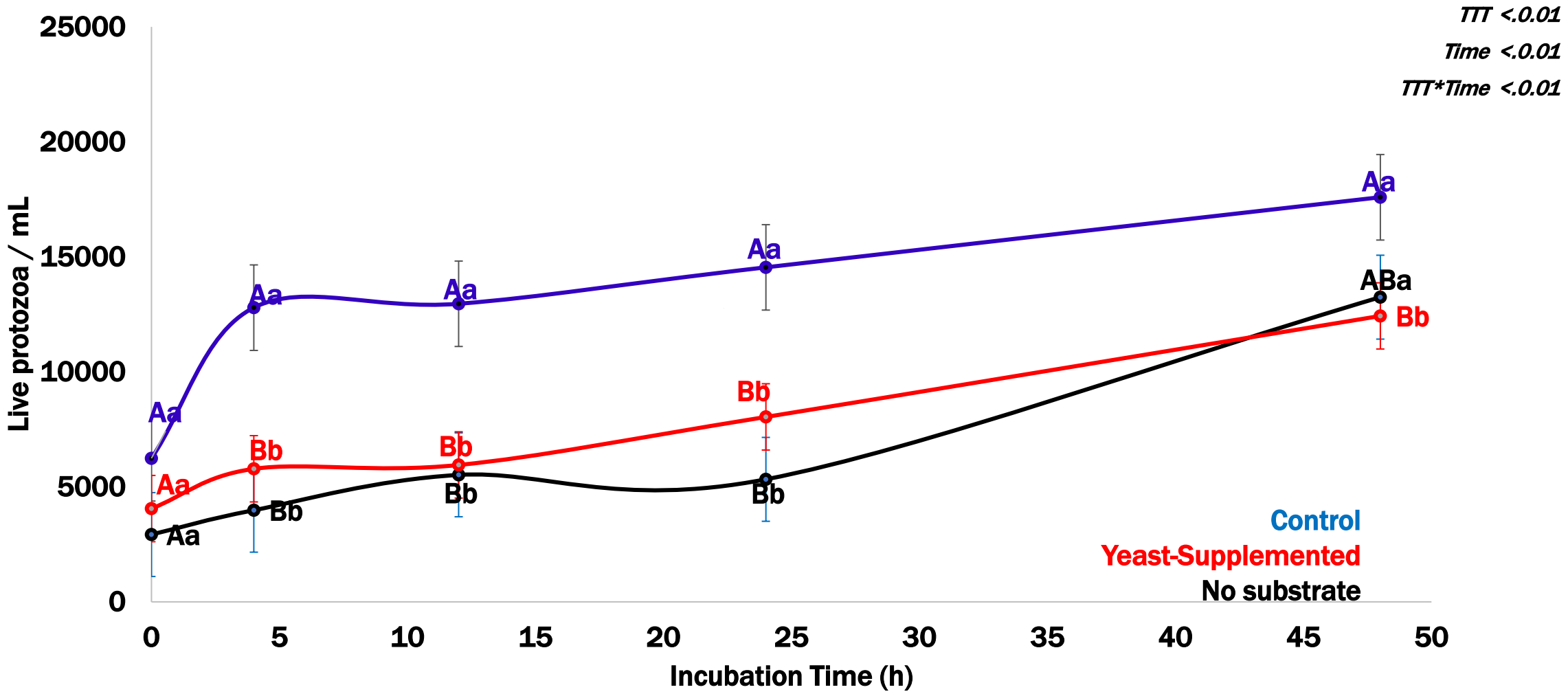
Effect of yeast fermentation product on Total ruminal protozoa count



Different capital letters indicate a significative difference between treatments at each time point
Different Lower letters indicate a significative difference between time points within each treatment

Results - Experiment 1

Effect of yeast fermentation product on ruminal protozoa viability



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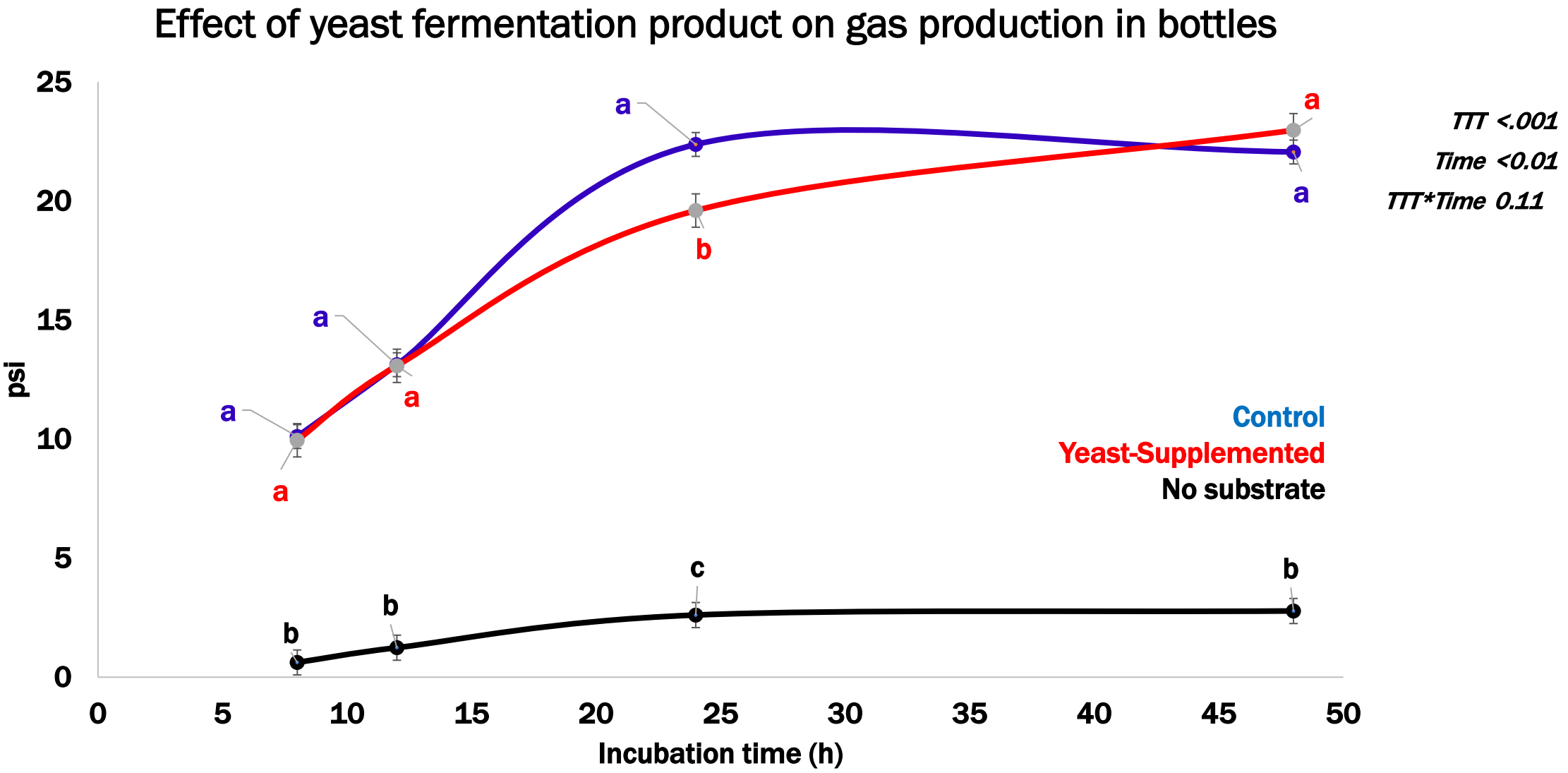
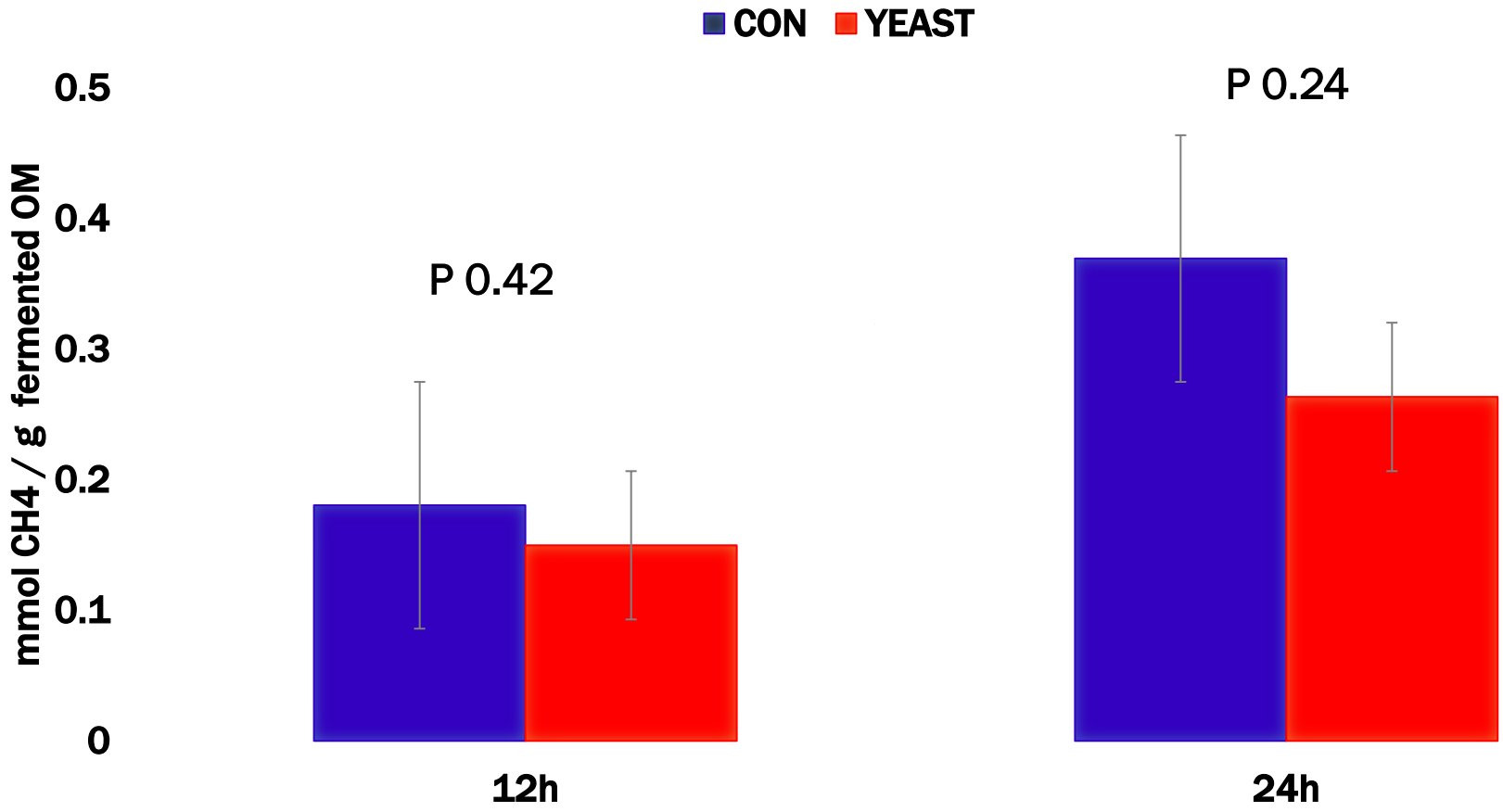


Results - Experiment 1

Effect of yeast fermentation on Methane production product in bottles

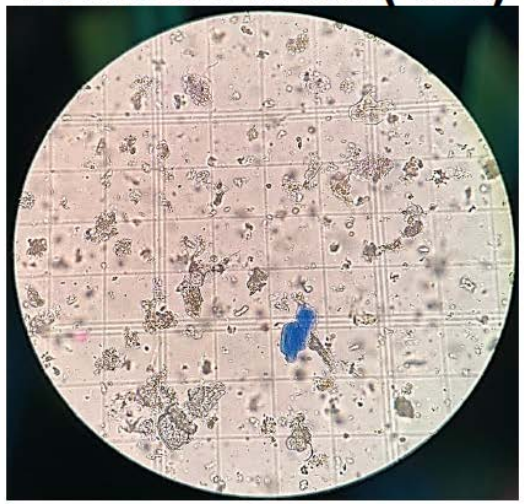


Results - Experiment 1

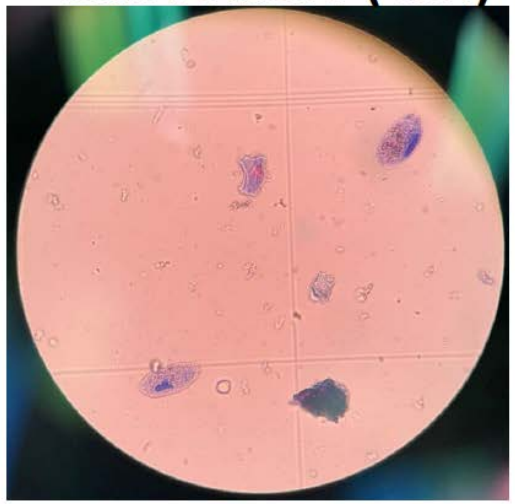
Control Steers (12h)

Yeast Steers (12h)

Control Steers (48h)



Yeast Steers (48h)



Results - Experiment 2

Effect of additive inclusion on ruminal fermentation parameters

	Treatments (Mean*)				Pr > F	
Variable	**CON**	**CNSE**	**ST**	**TAN**	**Trt**	**SEM**
VFA, mM						
Acetate	***41.75^{b}***	64.41^{a}	63.11^{a}	67.21^{a}	< 0.01	5.84
Propionate	***12.15^{b}***	20.83^{a}	20.69^{a}	21.15^{a}	< 0.01	1.31
Butyrate	***6.17^{b}***	9.94^{a}	9.25^{a}	10.51^{a}	< 0.01	1.07
A:P	***3.62^{a}***	3.07^{b}	3.11^{b}	3.12^{b}	0.01	0.19
BCVFA	2.49	3.14	2.94	3.53	0.11	0.55
TOTAL VFA	***62.59^{b}***	98.56^{a}	98.51^{a}	102.67^{a}	< 0.01	4.70

CON = Control Treatment. CNSE = Cashew nutshell extract ST = Saponins + Tannins. TAN =Tannins

*2 independent incubations (n=6). Different letters in a row indicates differences between means (Tukey-Kramer P <0.05);.

Results - Experiment 2

Effect of additive inclusion on ruminal fermentation parameters

	Treatments (Mean*)				Pr > F	
Variable	**CON**	**CNSE**	**ST**	**TAN**	**Trt**	**SEM**
VFA, mol/100 mol						
Acetate	66.63	65.22	66.18	65.26	0.57	1.08
Propionate	18.81^{b}	20.11^{ab}	***22.9^{a}***	20.07^{ab}	0.01	0.87
Butyrate	9.87	9.92	9.48	10.02	0.51	0.39
BCVFA	4.05^{a}	3.02^{b}	***2.51^{c}***	3.25^{b}	0.01	0.43
A:P	3.61^{a}	3.07^{b}	***2.97^{c}***	3.12^{b}	0.02	0.89

CON = Control Treatment. CNSE = Cashew nutshell extract ST = Saponins + Tannins. TAN =Tannins

*2 independent incubations (n=6). Different letters in a row indicates differences between means (Tukey-Kramer P <0.05);.

Results - Experiment 2

Effect of additive inclusion on ruminal gas production and methane

	Treatments (Mean*)				Pr > F	
Variable	**CON**	**CNSE**	**ST**	**TAN**	**Trt**	**SEM**
IVOMD	86.21	84.38	84.27	84.11	0.29	0.01
NH_3-N, mM	11.25	12.38	10.35	10.14	0.96	3.71
Methane						
CH_4mg mL^{-1}	1.68^{a}	1.76^{a}	$\mathit{1.13^{b}}$	$\mathit{0.78^{c}}$	0.05	0.01
CH_4mmol $gOMF^{-1}$	0.186^{c}	0.189^{c}	0.127^{b}	$\mathit{0.087^{a}}$	0.01	< 0.01

CON = Control Treatment. CNSE = Cashew nutshell extract ST = Saponins + Tannins. TAN =Tannins

IVOMD = In vitro organic matter digestibility; CH_4mg mL^{-1}= Methane concentration. **CH_4mmol $gOMF^{-1}$** = millimoles of methane per gr of organic matter fermented; *2 independent incubations (n=6). Different letters in a row indicates differences between means (Tukey-Kramer P <0.05);.

Results - Experiment 2

Effect of additive inclusion on ruminal gas production and pH

	Treatments*					Effects			
Variable	CON	CNSE	ST	TAN	SEM	COV¥	Trt	hour	Trt*hour
Gas (psi)									
24 h	7.7^{Xa}	7.69^{Xa}	7.32^{Xa}	7.66^{Xa}	0.58	-	<0.01	<0.01	<0.01
48 h	15.76^{Yb}	15.88^{Yb}	*7.63^{Xa}*	*2.28^{Zb}*					
Ruminal pH									
0 h	6.69^{a}	6.72^{a}	6.78^{a}	6.7^{a}	0.03	0.84	0.33	<0.01	0.75
24 h	6.8^{a}	6.79^{a}	6.82^{a}	6.82^{a}					
48 h	6.39^{b}	6.37^{b}	6.42^{b}	6.47^{b}					

CON = Control Treatment. CNSE = Cashew nutshell extract ST = Saponins + Tannins. TAN =Tannins

*2 independent incubations (n=6). Different letter (a b c) in a column indicates differences between times within the same treatment. Different capital letter (X Y Z) in a row indicates differences between treatments within the same time - Adjustment for Multiple Comparisons: Tukey-Kramer (P <0.05)

¥ When significant, value at hour 0 was included as a covariable.

Results - Experiment 2

Effect of additive inclusion on ruminal protozoa viability

Variable	Treatments					Effects			
	CON	CNSE	ST	TAN	SEM	COV¥	Trt	hour	Trt*hour
Total protozoa (x 10^4 ml^{-1})									
0 h	2.94	2.78	2.54	2.43	0.4	0.68	0.04	0.81	0.1
24 h	3.54	2.22	2.29	1.61					
48 h	2.92	3.04	1.49	1.43					
Protozoa Viability									
0 h	0.89	0.82	0.85	0.85	0.04	0.78	0.03	0.24	0.13
24 h	0.66^{Y}	0.60^{XY}	0.56^{Y}	0.65^{Y}					
48 h	0.66^{X}	0.69^{X}	0.45^{Y}	0.48^{Y}					

CON = Control Treatment. CNSE = Cashew nutshell extract ST = Saponins + Tannins. TAN =Tannins

*2 independent incubations (n=6). Different letters in a row indicates differences between means (Tukey-Kramer P <0.05);.

¥ When significant, value at hour 0 was included as a covariable.

Discussion

- Reduction of PROT and VIAB with yeast (cite 2 papers)
- Reduction of PROT and VIAB with Tannins (cite 2 papers)
- Reduction of PROT and VIAB with Saponins (cite 2 papers)
- Changes in CH4 with yeast, tannins and saponins
- Changes in IVOMD with yeast, tannins and saponins
- Changes in gas with yeast, tannins and saponins
- Changes in VFA, NH3 with yeast, tannins and saponins

Discussion

- Count with TBEM Vs Methyl-green-formalin-saline solution (MFS),
- Advantages of the Trypan Blue Exclusion Method (TBEM) for assessing ruminal protozoa viability

Outline

- Introduction
- Materials and Methods
- Results & Discussion
- Conclusions
- Acknowledgments

Conclusions & Summary

- Incubation during 24 hours in 125 mL bottle with substrate (diet) result in higher protozoa viability than Hungate tubes.

- Even with diet addition (substrate), viable protozoa were detected and counted with TBEM

Conclusions & Summary

- Dietary YEAST, and addition of TAN and CIT promoted a significative reduction of total protozoa, and protozoa viability, as well as gas production, and CH4 (mol/g of fOM) in ruminal fluid.

- Reduction in protozoa viability coincided with a reduction of CH4 gas, and fiber digestibility.

- TBEM is a viable, simple, rapid, and cost-efficient methodology to evaluate RPC and RPV after a nutritional challenge in RF, showing membrane disruptions associated with apoptosis.

Conclusions & Summary

- Alive and dead protozoa were successfully detected, identified and counted using TBEM under the evaluated experimental conditions and feed additives
- Total protozoa number detected here was similar to previous reports in cows ($1.5x10^4$) and steers ($3.7x10^3$), Therefore TBEM is suitable to quantify RPC and RPV when culturing filtered RF in 125mL bottles following our standard batch culture incubation.

- TBEM is a viable, simple, rapid, and cost-efficient methodology to evaluate RPC and RPV after a nutritional challenge in RF, showing membrane disruptions associated with apoptosis.

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