

Long-term encapsulated nitrate supplementation modulates rumen microbial diversity and rumen fermentation to reduce methane emission in grazing steers

Yury Tatiana Granja-Salcedo^{1*}, Rodolfo Maciel Fernandes^{1,2}, Rafael Canonenco de Araujo³, Luciano Takeshi Kishi⁴, Telma Teresinha Berchielli^{1,5}, Flávio Dutra de Resende^{1,2}, Alexandre Berndt⁶, Gustavo Rezende Siqueira^{1,2}

¹ Department of Animal Science, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Univ Estadual Paulista–UNESP, Jaboticabal, SP, Brazil.

² Department of Animal Science, Agência Paulista de Tecnologia dos Agronegócios (APTA), Colina, SP, Brazil.

³ GRASP Ind. & Com. LTDA, Curitiba, PR, Brazil

⁴ Department of Technology, Faculdade de Ciências Agrárias e Veterinárias (FCAV), Univ Estadual Paulista–UNESP, Jaboticabal, SP, Brazil.

⁵ INCT/CA–UFV-Department of Animal Science, Viçosa, MG, Brazil.

⁶ Embrapa Southeast Livestock, São Carlos, SP, Brazil.

***Correspondence:** Dr. Yury T. Granja-Salcedo. yurygranjasalcedo@gmail.com

Supplemental material

Table S1. Ingredients and chemical composition of supplements offered and forage nutritional value during each period.

<i>Ingredient, g/kg DM</i>	Dry season (135 d)		Rainy season (168 d)		Finished (104 d)	
	UR	EN	UR	EN	UR	EN
Soybean meal	198.9	198.2	0.0	0.0	69.8	69.3
Ground corn	654.9	652.4	814.0	809.3	876.4	870.0
Encapsulated Nitrate	0.0	99.2	0.0	139.0	0.0	48.4
Urea	34.2	0.0	47.9	0.0	14.8	0.0
Calcareous	61.7	0.0	86.1	0.0	26.6	0.0
Mineral supplement	50.3	50.2	52.0	51.7	12.4	12.3
<i>Supplement chemical composition, %</i>						
Crude protein	26.0	26.0	21.0	21.0	16.0	16.0
NDT	71.0	71.0	69.0	69.0	79.0	79.0
<i>Forage nutritional value, g/kg DM</i>						
Crude protein		45.6		111.3		97.2
Ether extract		12.6		14.0		18.2
Neutral detergent fibre		722.8		658.6		683.5
Acid detergent fibre		436.0		351.1		349.0

Table S2. Median and interquartile range of the number of observed operational taxonomic units (OTUs), richness (Chao1 and ACE) and diversity estimators (Shannon Wiener and Simpson) by rumen bacteria and archaea population in grazing Nellore Steers after thirteen months of supplementation with Urea (URS) or encapsulated nitrate (ENS).

	URS	ENS	p-value
Bacteria			
Total OTUs	2030±80	2060±317	0.9048
Chao-1	2175±119	2199±273	0.8923
ACE	2190±125	2191±227	0.6342
Shannon	5.893±0.20	5.906±0.48	0.9137
Simpson	0.966±0.02	0.968±0.01	0.8906
Archaea			
Total OTUs	457.18±51	439.32±27	0.7381
Chao-1	498.47±47	471.64±34	0.7925
ACE	489.46±27	490.32±27	0.9183
Shannon	1.587±0.17	1.479±0.41	0.3874
Simpson	0.959±0.01	0.951±0.01	0.5962

Table S3. Median and interquartile range of the bacterial abundance at phylum level (a) and Euryarchaeota:Bacteria ratio (b) in grazing Nellore Steers after thirteen months of supplementation with Urea (URS) or encapsulated nitrate (ENS).

Bacterial phylum	Supplement		p-value
	URS	ENS	
Actinobacteria	0.145±0.02	1.494±0.24	0.0447
Bacteroidetes	41.331±11.61	39.339±6.65	0.7302
Cyanobacteria	0.238±0.40	0.342±0.22	0.5556
Fibrobacteres	2.181±1.06	3.283±2.22	0.0412
Firmicutes	23.005±6.13	30.498±8.31	0.0017
Lentisphaerae	0.436±0.49	0.503±0.51	0.1197
Proteobacteria	2.581±1.26	7.985±1.90	0.0392
Spirochaetes	2.335±1.25	1.149±1.04	0.1256
SR1	0.753±0.42	0.836±0.35	0.6124
Synergistetes	0.079±0.05	0.098±0.06	0.1933
Tenericutes	1.163±0.21	0.639±0.17	0.6382
TM7	0.853±0.31	0.793±0.24	0.6904
Verrucomicrobia	6.985±0.46	1.126±0.82	0.0031
WPS.2	0.409±0.14	0.283±0.11	0.7038
Unassigned	16.303±3.55	11.581±3.48	0.0101

Differences were considered significant at $p < 0.05$ using the Wilcoxon test.

Table S4. Spearman correlations (r) and P-values (p) among microbial groups and methane emissions in grazing Nellore Steers after thirteen months of supplementation with Urea (URS) or encapsulated nitrate (ENS).

Amplicon	Microbial taxa	DMI, kg/d				CH4, gr/d				CH4, gr/kg tDMI				CH4, gr/kg sDMI				CH4, gr/kg fDMI				Rumen pH			
		URS		ENS		URS		ENS		URS		ENS		URS		ENS		URS		ENS		URS		ENS	
		r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Archaea	Euryarchaeota																							-0.77	0.04
	<i>Methanobacterium</i>																								
	<i>Methanobrevibacter</i>																								
	<i>Methanomassiliicoccus</i>																								
	Actinobacteria	0.99	0.01																						
	Bacteroidetes			0.87	0.02																				
	Cyanobacteria					0.75	0.04																		
	Fibrobacteres	0.99	0.01																						
	Firmicutes																								
	Proteobacteria																								
	Spirochaetes	0.90	0.01																						
	Tenericutes																								
	Verrucomicrobia																								
	BF ratio																								
	<i>BactBlautia</i>																								
	<i>Bacteroides</i>																								
	<i>Barnesiella</i>	-0.77	0.04	0.71	0.05																				
	<i>Clostridium_III</i>																								
	<i>Clostridium_IV</i>																								
	<i>Clostridium_XIVa</i>																								
	<i>Duganella</i>																								
	<i>Eubacterium</i>																								
	<i>Fibrobacter</i>																								
	<i>Kandleria</i>	0.83	0.03																						
	<i>Lactobacillus</i>																								
	<i>Mitsuokella</i>																								
	<i>Mogibacterium</i>																								
	<i>Olsenella</i>																								
	<i>Paraprevotella</i>																								
	<i>Prevotella</i>																								
	<i>Pseudobutyrivibrio</i>	-0.71	0.05																						
	<i>Roseburia</i>																								
	<i>Ruminococcus</i>																								
	<i>Selenomonas</i>																								
	<i>Sphaerochaeta</i>																								
	<i>Succinimonas</i>	0.71	0.05																						
	<i>Succinivibrio</i>																								
	<i>Treponema</i>																								
	<i>Veillonella</i>																								
Bacteria																									

CH4 = methane; sDMI = supplement dry matter intake; fDMI = forage dry matter intake; tDMI = total dry matter intake. Only significant P < 0.10 correlations are shown.

Table S4. Spearman correlations (r) and P-values (p) among microbial groups and rumen fermentation parameters in grazing Nellore Steers after thirteen months of supplementation with Urea (URS) or encapsulated nitrate (ENS).

Table S4. Spearman correlations (r) and P-values (p) among microbial groups and methane emissions in grazing Nellore Steers after thirteen months of supplementation with Urea (URS) or encapsulated nitrate (ENS).

Amplicon	Microbial taxa	Valerate, %				Total VFA, mmol/L			
		URS		ENS		URS		ENS	
		r	p			r	p	r	p
Archaea	Euryarchaeota					0.89	0.02		
	<i>Methanobacterium</i>	-0.77	0.04						
	<i>Methanobrevibacter</i>					0.77	0.04		
	<i>Methanomassiliicoccus</i>								
	Actinobacteria							0.93	0.01
	Bacteroidetes			-0.70	0.05			0.64	0.07
	Cyanobacteria								
	Fibrobacteres			-0.81	0.03				
	Firmicutes					-0.64	0.07	-0.67	0.07
	Proteobacteria			0.78	0.04				
	Spirochaetes								
	Tenericutes			-0.67	0.07				
	Verrucomicrobia	-0.67	0.07						
	BF ratio					0.83	0.03		
	<i>BactBlautia</i>					0.66	0.07		
	<i>Bacteroides</i>	0.71	0.05						
	<i>Barnesiella</i>	0.66	0.07	-0.94	0.01			0.71	0.05
	<i>Clostridium_III</i>								
	<i>Clostridium_IV</i>								
	<i>Clostridium_XIVa</i>			0.89	0.02				
	<i>Duganella</i>								
	<i>Eubacterium</i>								
	<i>Fibrobacter</i>					0.77	0.04		
	<i>Kandleria</i>	-0.77	0.04	0.66	0.07				
	<i>Lactobacillus</i>								
	<i>Mitsuokella</i>	-0.94	0.01			0.89	0.02		
	<i>Mogibacterium</i>					0.83	0.03		
	<i>Olsenella</i>					0.77	0.04		
	<i>Paraprevotella</i>								
	<i>Prevotella</i>								
	<i>Pseudobutyryrivibrio</i>					-0.94	0.01		
	<i>Roseburia</i>	-0.71	0.05	0.77	0.04				
	<i>Ruminococcus</i>								
	<i>Selenomonas</i>	-0.83	0.03	0.89	0.02				
	<i>Sphaerochaeta</i>					-0.94	0.01		
	<i>Succinimonas</i>	0.66	0.07					-0.94	0.01
	<i>Succinivibrio</i>								
	<i>Treponema</i>								
	<i>Veillonella</i>	-0.83	0.03	0.77	0.04				

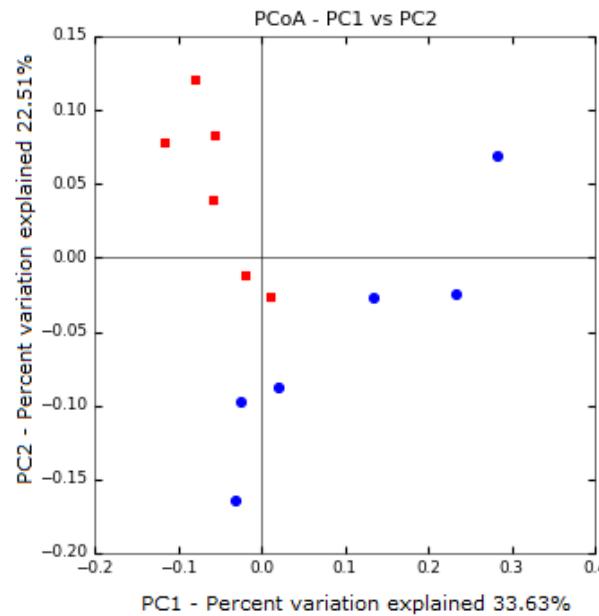


Figure S.1. Weighted Unifrac diversity principal coordinate analysis (PCoA) to explore dissimilarities in microbial composition among grazing Nellore Steers after a long time (thirteen months) of supplementation with encapsulated nitrate (blue circle) or urea (red square). Unifrac test = 0.061.