Effect of the addition of cellulolytic bacteria to ruminal bacteria on *in vitro* fermentation characteristics

Efecto de la adición de bacterias celulolíticas a bacterias ruminales sobre las características fermentativas in vitro

Efeito da adição de bactérias celulolíticas às bactérias ruminais nas características de fermentação in vitro

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Abstract

Background: Digestibility of fiber in the rumen is not due to enzymatic activity of individual bacteria, but rather to their interaction, which complements their enzymatic functioning. Thus, efficiency of fiber digestion depends on the diversity and density of cellulolytic bacteria. **Objective:** To estimate *in vitro* production of biogas, methane, and fermentative characteristics of cobra grass (*Brachiaria hibrido*) inoculated with ruminal bacteria (RB) in coculture with isolated cellulolytic bacteria (ICB) from bovine (ICB_{bov}) or water buffalo (ICB_{buf}). **Methods:** ICB_{bov} and ICB_{buf} were isolated from ruminal cellulolytic bacteria consortia using specific culture media for cellulolytic bacteria. Both were morphologically characterized and a Gram stain was performed. In the *in vitro* gas production test, the substrate was cobra grass and the inocula were ruminal bacteria (RB), ICB_{bov}, ICB_{buf}, $Coculture_{bov}$ (RB + ICB_{bov}) and $Coculture_{buf}$ (RB + ICB_{buf}). Biogas and methane (CH_4) production, as well as dry matter degradation (DMD) and neutral detergent fiber degradation (NDFD) were measured. A completely randomized design was used. **Results:** The ICB obtained were Gram positive cocci. Accumulated biogas production at 72 h from ICB_{bov} and ICB_{buf} was on average 42.11% of that produced by RB. The $Coculture_{bov}$ produced 14.24% more biogas than RB. The CH_4 production was lower in ICB_{bov} and ICB_{buf} than in RB, $Coculture_{bov}$ and $Coculture_{buf}$. The DMD and NDFD were not different among RB, $Coculture_{bov}$ and $Coculture_{buf}$. The ICB_{bov} degraded 37.10 and 96.34% more DMD and NDFD than ICB_{buf} ($p<0.05$). **Conclusion:** The use of ICB from bovine or water buffalo in coculture with RB does not improve *in vitro* production of biogas, DMD or NDFD with respect to RB alone.

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Keywords: *bacteria; biogas; bovine; buffalo; cellulolitic bacteria; coculture; fiber degradation; fiber; fermentation characteristics; gas production; in vitro fermentation; methane; rumen; ruminal bacteria.*

Resumen

Antecedentes: La digestibilidad ruminal de la fibra no se debe a la actividad enzimática individual de las bacterias sino a su interacción para complementar su funcionamiento enzimático. Así, la eficiencia de digestión de la fibra depende de la diversidad y la densidad de las bacterias celulolíticas. **Objetivo:** Estimar la producción de biogás, metano, y las características fermentativas *in vitro* del pasto cobra (*Brachiaria hibrido*) inoculado con bacterias ruminales (BR) en cocultivo con bacterias celulolíticas aisladas (BCA) de bovino (BCA_{bov}) o búfalo de agua (BCA_{buf}). **Métodos:** BCA_{bov} y BCA_{buf} se aislaron de consorcios bacterianos celulolíticos ruminales usando medios de cultivo específicos para bacterias celulolíticas. Ambas se caracterizaron morfológicamente y realizó tinción de Gram. En la prueba de producción de gas *in vitro*, el sustrato fue pasto cobra y los inóculos fueron bacterias ruminales (BR), BCA_{bov}, BCA_{buf}, $Cocultivo_{bov}$ (BR + BCA_{bov}) y $Cocultivo_{buf}$ (BR + BCA_{buf}). Se midió la producción de biogás y metano (CH_4), así como la degradación de la materia seca (DMS) y de la fibra detergente neutro (DFDN). El análisis estadístico se basó en un diseño completamente al azar. **Resultados:** Las BCA resultantes se identificaron como cocos Gram positivos. La producción de biogás acumulada a las 72 h por BCA_{bov} y BCA_{buf} fue en promedio 42,11% del producido por BR. El $cocultivo_{bov}$ produjo 14,24% más biogás que BR. La producción de CH_4 fue menor en BCA_{bov} y BCA_{buf} que en BR, $cocultivo_{bov}$ y $cocultivo_{buf}$. Las DMS y DFDN no mostraron diferencias entre BR, $cocultivo_{bov}$ y $cocultivo_{buf}$. La BCA_{bov} degradó 37,10 y 96,34% más DMS y DFDN que BCA_{buf} ($p<0,05$). **Conclusión:** El uso de BCA de bovino o búfalo de agua en cocultivo con BR no mejora la producción de biogás, DMS o DFDN *in vitro* respecto a BR.

Palabras clave: *bacteria; bacterias celulolíticas; bacterias ruminales; biogás; bovino; búfalo; características fermentativas; cocultivo; degradación de la fibra; fermentación in vitro; fibra; metano; producción de gas; rumen.*

Resumo

Antecedentes: A digestibilidade da fibra no rúmen não se deve à atividade enzimática individual das bactérias, mas sim à sua interação para complementar o seu funcionamento enzimático. Assim, a eficiência da digestão das fibras depende da diversidade e densidade das bactérias celulolíticas. **Objetivo:** Estimar a produção *in vitro* de biogás, metano e características fermentativas da gramínea de cobra (*Brachiaria hibrido*) inoculada com bactéria ruminal (BR) em cocultura com bactérias celulolíticas isoladas (BCI) de bovino (BCI_{bov}) ou búfalo de água (BCI_{buf}). **Métodos:** BCI_{bov} e BCI_{buf} foram isolados a partir de consórcios de bactérias celulolíticas ruminais utilizando meios de cultura específicos para bactérias celulolíticas. Ambos foram caracterizados morfologicamente, e foi realizada uma coloração de Gram. No teste de produção de gás *in vitro*, o substrato era erva de cobra e os inóculos eram bactérias ruminais (BR), BCI_{bov}, BCI_{buf}, $Cocultivo_{bov}$ (BR + BCI_{bov}) e $cocultivo_{buf}$ (BR + BCI_{buf}). Foram medidas a produção de biogás e metano (CH_4), bem como a degradação da matéria seca (DMS) e a degradação da fibra em detergente neutro (DFDN). Foi utilizado um desenho completamente aleatório. **Resultados:** BCIs eram cocos Gram positivos. A produção acumulada de biogás a 72 h de BCI_{bov} e BCI_{buf} foi em média 42,11% da produzida por BR. O $cocultivo_{bov}$ produziu 14,24% mais biogás do que o BR. A produção de CH_4 foi menor em BCI_{bov} e BCI_{buf} do que BR, $cocultivo_{bov}$ e $cocultivo_{buf}$. DMS e DFDN não eram diferentes entre BR, $cocultivo_{bov}$ e $cocultivo_{buf}$. O BCI_{bov} degradou 37,10 e 96,34% mais DMS e DFDN do que o BCI_{buf} ($p<0,05$). **Conclusão:** A utilização de BCI de bovino ou búfalo de água em cocultura com BR não melhora a produção *in vitro* de biogás, DMS ou DFDN no que diz respeito a BR.

Palavras-chave: *bactéria; bactérias celulolíticas; bactérias ruminais; biogás; bovino; búfalo; características fermentativas; cocultivo; degradação da fibra; fermentação in vitro; fibra; metano; produção de biogás; rúmen.*

Introduction

The distribution of molecules and their union within the cell wall of tropical forages affect the metabolic action of microorganisms. Tropical forages have high contents of hemicellulose, cellulose, pectin and lignin in the cell walls, accounting for 35 to 80% of its lignocellulosic biomass, which provides structural integrity to the forage (Trejo-López *et al*, 2018). This reduces its digestibility by ruminants and limits animal productivity in the tropics. The enzymatic complex of β-1-4 cellulases hydrolyzes cell walls and determines digestibility of tropical forages by ruminants. It should be noted that 10 to 35% of the energy consumed is absorbed as net energy since 20 to 70% of the cellulose is not digested. Few studies aimed at increasing the efficiency of fiber utilization in tropical forages have been reported (Barahona and Sánchez, 2005).

Ruminal anaerobic environment and its microorganisms are responsible for the digestion of structural carbohydrates (Cai *et al*, 2010; Sattar *et al*, 2018; Azizi *et al*, 2020) by degrading fiber through enzymatic digestion (Berny *et al*, 2019; Gudeta and Krishna, 2019; Liu *et al*, 2019). The potential cellulolytic bacteria in the rumen are *Bacteroides succinogenes*, *Clostridium*, *Trichonympha*, *Actinomycetes*, *Butyrivibrio fibrisolvens*, *Ruminococcus albus* and *Methanobrevibacter ruminantium* (Gudeta and Krishna, 2019). However, their cellulolytic potential varies with the species present and what the host eats (Qian *et al*, 2019; Gudeta and Krishna, 2019).

Several chemical, physical, and biological methods have been used to improve fiber digestibility in ruminant diets (Azizi *et al*, 2020). A biological method tested uses bacteria capable of degrading plant cell wall components (Harsini *et al*, 2019). Fiber digestibility in the rumen is not due to the enzymatic activity of individual bacteria, but rather to their interaction with other microorganisms (Sattar *et al*, 2018). Its efficiency depends on the diversity and density of microorganisms, including bacteria, protozoa, fungi, and archaea (Qian *et al*, 2019). Cellulolytic microorganisms can be used as probiotics in ruminant diets to improve digestion of fibrous components (Gudeta and Krishna, 2019). Therefore, the objective of this study was to estimate the *in vitro* production of biogas, methane (CH_4) and fermentative characteristics of cobra grass inoculated with ruminal bacteria (RB) in coculture with cellulolytic bacteria isolated (ICB) either from bovine or water buffalo.

Materials and Methods

Ethical considerations

All the procedures involving animals were in accordance with the ethical standards of Universidad Autónoma de Guerrero (Mexico) and were performed according to the protocols of the Federal Animal Health Law and NOM-062-ZOO-1999.

Isolated cellulolytic bacteria

This study is a sequel of previously published work on cellulolytic bacterial consortia (CBC) obtained from water buffalo and Swiss-bu cow (Herrera-Pérez *et al*, 2018; Torres-Salado *et al*, 2019), from which cellulolytic bacteria evaluated in the present study were isolated. The culture medium based on ruminal fluid (MRF) was described by Hungate (1950) and modified by Torres-Salado *et al* (2020). To isolate cellulolytic bacteria, a sterile solid culture medium [MRF + 0.2% carboxymethylcellulose (Sigma-Aldrich®, St Louis, MO, USA) + 2% agar (Sigma-Aldrich®, St Louis, MO, USA)] was prepared in sterile Petri dishes. The CBC was inoculated by the streak plate seeding method, and plates were placed in an anaerobic jar with GasPakTM (BD Bioxon®, Oaxaca, Oaxaca, Mexico). The anaerobic jar was placed in an incubator (Ecoshel 9082, Ciudad de Mexico, Mexico) for 72 h at 39 °C for development of colonies.

In sterile test tubes (18X150 mm), 9 mL of sterile MRF with cellobiose (0.2%; Sigma-Aldrich®, St Louis, MO, USA) (MFRC) were added under anaerobic conditions with CO_2. Colonies with good definition and isolation were transferred to a tube containing medium and incubated for 24 h at 39 °C. After incubation, a

sample was observed under a microscope (BX31, Olympus®, Allentown, Pennsylvania, USA) to identify bacterial morphologies. The process was repeated until a single morphology of ICB of water buffalo (ICB_{buf}) and Swiss-bu cow (ICB_{bov}) was obtained. The ICB was morphologically characterized according to Ramírez (2015), and Gram staining was conducted.

Substrate

Cobra grass (*Brachiaria hibrido*) was harvested at 56 d of regrowth and dehydrated at 60 °C until constant weight in an oven (Felisa® FE-293A, San Juan de Ocotán Zapopan, Jalisco, Mexico). The grass was then ground to pass 1 mm sieve in a Thomas-Wiley Mill (Thomas Scientific®, Swedesboro, NJ, USA). Bromatological composition of cobra grass was 7.5% crude protein, 69.05% neutral detergent fiber (NDF), 47.96% acid detergent fiber and 87.85% organic matter.

Inocula

1) RB = 5 mL ruminal bacteria from Swiss-bu cow ruminal fluid, centrifuged for 3 min at 1,157 *g* (Dehority *et al*, 1960). 2) ICB_{bov} = 5 mL ICB_{bov} incubated in MRF with cellobiose (0.2%) for 48 h. 3) ICB_{buf} = 5 mL ICB_{buf} incubated in MRF with cellobiose (0.2%) for 48 h. 4) $Coculture_{bov}$ = 5 mL RB and 5 mL ICB_{bov}; 5) $Coculture_{buf}$ = 5 mL RB and 5 mL ICB_{buf}.

Test of in vitro gas production

In serological vials (120 mL), 0.5 g cobra grass and 45 mL MFR medium were added. All the vials were maintained under anaerobic conditions with CO_2. They were hermetically sealed with a neoprene stopper (20 mm diameter) and an aluminum ring, then sterilized 15 min in an autoclave (All American® 1941X, Madison, Wisconsin, USA) at 121 °C and 15 psi. The vials were then inoculated and incubated for 72 h at 39 °C. Biogas production and CH_4 was measured following Menke and Steigass (1988) and modifications by Torres-Salado *et al* (2019).

Fermentative characteristics

Variables measured after incubation were pH (Herrera-Pérez *et al*, 2018), total bacterial count (Harrigan and McCance, 1979; Sánchez-Santillán *et al*, 2016), ammoniacal nitrogen (NH_3-N; McCullough (1967); DMD (Getachew *et al*, 2004; Hernández-Morales *et al*, 2018), NDFD (Sánchez-Santillán *et al*, 2015), and volatile fatty acids (VFA) as described by Cobos *et al* (2007).

Experimental design

A completely randomized design with five replications per inoculum was used.

Statistical analysis

The data were analyzed using the GLM procedure of SAS®, version 9.3 (2011). Average values were compared with the Tukey test ($p<0.05$).

Results

Morphology and Gram staining of ICB from water buffalo and Swiss-bu cow rumen CBC (Herrera-Pérez *et al*, 2018; Torres-Salado *et al*, 2019) indicated that they were Gram positive cocci. These cocci showed formation of diplococci and, occasionally, chains of three or more cocci.

Biogas production accumulated at 72 h by ICB_{bov} and ICB_{buf} represented on average 42.11% of that produced by RB ($p<0.05$). $Coculture_{bov}$ produced 14.24% more biogas accumulated at 72 h than RB ($p<0.05$). The ICB_{bov} and ICB_{buf} were not different ($p>0.05$) from RB in partial biogas production at 24 h, while $Coculture_{bov}$ produced 41.38% more biogas than RB ($p<0.05$). The ICB_{bov} and ICB_{buf} produced 14.07 and 26.30%, respectively, of the partial biogas produced by RB at 48 and 72 h ($p<0.05$). Moreover, neither of the cocultures was different from RB ($p>0.05$). The accumulated and partial production (48 and 72 h) of CH_4 showed that ICB_{bov} and ICB_{buf} produced less CH_4 than RB, $Coculture_{bov}$ or $Coculture_{buf}$ ($p<0.05$). However, partial production of CH_4 at 24 h by RB, ICB_{bov} and ICB_{buf} was not different ($p>0.05$; Table 1).

The DMD and NDFD were not different in RB, Coculture$_{bov}$ and Coculture$_{buf}$ ($p>0.05$). However, ICB$_{bov}$ degraded 37.10 and 96.34% more DMD and NDFD than ICB$_{buf}$ ($p<0.05$). The total bacterial count was not different in RB, Coculture$_{bov}$ and Coculture$_{buf}$; and ICB$_{bov}$ was not different from RB ($p>0.05$). The NH_3-N content of the culture medium was not different among inocula ($p>0.05$). The pH of the culture medium was different among inocula, which had pH within the range for RB (Table 2).

The concentration of VFA, acetate, propionate and butyrate were similar in RB, Coculture$_{bov}$ and Coculture$_{buf}$ ($p>0.05$). The average VFA of these inocula was 82.62% more than the VFA produced by ICB$_{bov}$ and ICB$_{buf}$ ($p<0.05$).

Table 1. Effect of adding isolated cellulolytic bacteria to ruminal bacteria on biogas and methane production (mL g-^{1} DM) using cobra grass as substrate.

Variable	ICB$_{bov}$	ICB$_{buf}$	RB	Coculture$_{bov}$	Coculture$_{buf}$	SEM
Biogas$_{24}$[1]	80.44^{c}	80.11^{c}	99.42^{bc}	140.56^{a}	119.21^{ab}	5.35
Biogas$_{48}$[2]	16.74^{b}	15.53^{b}	114.57^{a}	110.01^{a}	92.53^{a}	9.40
Biogas$_{72}$[3]	8.17^{b}	8.18^{b}	31.1^{a}	29.42^{a}	25.31^{a}	2.26
Biogas[4]	102.58^{c}	103.82^{c}	245.08^{b}	279.99^{a}	237.06^{b}	15.83
Methane$_{24}$[1]	11.03^{b}	12.67^{b}	15.1^{ab}	18.38^{a}	17.15^{a}	0.69
Methane$_{48}$[2]	7.96^{b}	6.65^{b}	25.71^{a}	26.56^{a}	27.76^{a}	2.00
Methane$_{72}$[3]	5.11^{b}	5.11^{b}	10.21^{a}	11.44^{a}	11.84^{a}	0.66
Methane[4]	24.10^{b}	23.52^{b}	51.54^{a}	57.19^{a}	56.74^{a}	3.47

Means with different superscript letters ($^{a, b, c}$) within rows indicate significant difference ($p<0.05$).
ICB$_{bov}$ = isolated bovine cellulolytic bacteria ($6.90x10^8$ cell mL^{-1}); ICB$_{buf}$ = isolated buffalo cellulolytic bacteria ($8.40x10^8$ cell mL^{-1}); RB = ruminal bacteria ($1.39x10^9$ cell mL^{-1}); Coculture$_{bov}$ = ICBbov and RB; Coculture$_{buf}$ = ICB$_{buf}$ and RB; SEM = Standard error of the mean; [1]partial production with 24 h incubation; [2]partial production with 24 to 48 h of incubation; [3]partial production with 48 to 72 h of incubation; [4]cumulative production.

Table 2. Effect of the addition of isolated cellulolytic bacteria to ruminal bacteria on *in vitro* fermentative characteristics of cobra grass substrate.

Variable	ICB$_{bov}$	ICB$_{buf}$	RB	Coculture$_{bov}$	Coculture$_{buf}$	SEM
DMD (%)	30.45^{b}	22.21^{c}	68.20^{a}	68.99^{a}	70.90^{a}	4.41
NDFD (%)	15.21^{b}	1.91^{c}	70.46^{a}	69.58^{a}	72.06^{a}	6.37
Bacteria (109 cell mL$^{-1)}$	0.80^{bc}	0.50^{c}	0.97^{ab}	1.04^{ab}	1.14^{a}	0.06
pH	6.85^{b}	6.88^{a}	6.61^{d}	6.63^{cd}	6.65^{c}	0.02
NH_3-N (mg dL$^{-1)}$	26.22	24.73	23.55	23.03	22.13	0.51
VFA (mM L$^{-1)}$	37.27^{b}	41.67^{b}	71.62^{a}	72.77^{a}	71.85^{a}	4.35
Acetate (mM L$^{-1)}$	18.20^{b}	21.18^{b}	35.43^{a}	33.00^{a}	31.57^{a}	1.21
Propionate (mM L$^{-1)}$	9.22^{b}	12.55^{b}	24.71^{a}	29.50^{a}	27.99^{a}	2.98
Butyrate (mM L$^{-1)}$	7.94^{d}	9.85^{c}	11.49^{ab}	10.27^{bc}	12.29^{a}	0.42

Means with different superscript letters ($^{a, b, c, d}$) within rows indicate significant difference ($p<0.05$). ICB$_{bov}$ = isolated bovine cellulolytic bacteria ($6.90x10^8$ cell mL^{-1}); ICB$_{buf}$ = isolated buffalo cellulolytic bacteria ($8.40x10^8$ cell mL^{-1}); RB = ruminal bacteria ($1.39x10^9$ cell mL-1); Coculture$_{bov}$ = ICB$_{bov}$ and RB; Coculture$_{buf}$ = ICB$_{buf}$ and RB; SEM = Standard error of the mean; DMD = dry matter degradation; NDFD= neutral detergent fiber degradation; NH_3-N = ammoniacal nitrogen; VFA = volatile fatty acids.

Acetate and propionate production showed no difference between ICB_{bov} and ICB_{buf} ($p>0.05$). The mean values of acetate and propionate of ICB_{bov} and ICB_{buf} were 59.08 and 39.71%, respectively, of the mean production of RB, $Coculture_{bov}$ and $Coculture_{buf}$ ($p<0.05$). The ICB_{buf} produced 24.06% more butyrate than ICB_{bov} ($p<0.05$; Table 2).

Discussion

Studies involving isolation of cellulolytic bacteria are based on genomic identification or metabolic tests (Qian *et al*, 2019; Xie *et al*, 2018; Hyung *et al*, 2018), but there is little research (Azizi *et al*, 2020; Gang *et al*, 2020) on the coculture of ICB with RB. In the present study, the ability of both ICB in coculture to increase fermentation characteristics and *in vitro* gas production was evaluated to determine whether they can be used as probiotics for ruminants. Although we are aware of the limitations of the technique used, it can be a useful method for determining its functionality because it describes the kinetics of microbial activity in response to the substrate and measures the effect of the inocula used (Williams, 2000; Harsini *et al*, 2019).

The ICB_{bov} and ICB_{buf} are strict anaerobic cocci that require fermentable carbohydrates for their growth (carboxymethylcellulose, cellobiose, fiber from cobra grass) producing acetate as a product of fermentation (Table 2). Based on the characteristics described and Bergey's Manual® of Systematic Bacteriology (Ezaki, 2015), ICB_{bov} and ICB_{buf} are classified within the genus *Ruminococcus*.

Biogas production of CH_4 (Table 1), DMD and NDFD (Table 2) did not show that ICBA potentiates DMD or NDFD of RB (coculture). Its use as a probiotic did not improve these variables; that is, fermentation and degradation of cobra grass did not improve. Azizi *et al* (2020) published similar results in *in vitro* tests with wheat straw inoculated with a coculture of RB and ICB from termite intestine; they found it was not different from RB alone.

Partial production of biogas makes it possible to infer the type of carbohydrates fermented during the incubation period. The production of biogas and CH_4 produced at 24 h was not different between RB and ICB because the cell content, that is, non-structural carbohydrates (Texta *et al*, 2019) and a certain protein fraction (Rodríguez *et al*, 2010) of cobra grass was fermented. After 48 h, differences in biogas production among inocula occurred because structural carbohydrates fermented, suggesting the capacity of cellulolytic bacteria to use these carbohydrates (González *et al*, 2011; Texta *et al*, 2019) and to interact with other cellulolytic bacteria. Increased biogas production is assumed to be the result of increased population of cellulolytic bacteria and fermentation of structural carbohydrates such as cellulose. Cellulose in grass produces acetate, 2 molecules of CO_2 and 8 of H^+ as fermentation products (Hungate, 1966), and it is the only carbon source, reflected in a higher production of biogas. Anaerobic fermentation of cobra grass requires a complex interaction of microorganisms (Deng *et al*, 2017; Torres-Salado *et al*, 2019) and we intended to manipulate it by adding ICBA to RB. *In vitro* biogas production values lower than those found in our study were reported in wheat straw inoculated with RB in coculture with ICB from termite intestine (Azzi *et al*, 2020) or ICB from Arabian horses (Harsini *et al*, 2019). In contrast, *in vitro* fermentation of corn silage inoculated with RB in coculture with *Lactobacillus plantarum*, *Enterococcus mundtii*, or *Enterococcus faecalis* (Gang *et al*, 2020) produced more biogas than the cocultures used in the present study.

Bacterial consortia are diverse communities that interact with each other and their environment to carry out interdependent physiological processes (Davey and O'Toole, 2000; Bader *et al*, 2010; Zuroff *et al*, 2013; Torres-Salado *et al*, 2019). When comparing the ICB of our study with CBC of bovine or buffalo origin (Torres-Salado *et al*, 2019; Herrera-Pérez *et al*, 2018) in the production of biogas from cobra grass, the results were similar. Thus, we infer that ICB require interaction with other cellulolytic

bacteria for heterofermentative activity due to food interdependence and cross-feeding (Sánchez-Santillán and Cobos-Peralta, 2016) because it includes hydrolysis, acidogenesis, syntrophic acetogenesis of volatile fatty acids and methanogenesis (Deng *et al*, 2017; Torres-Salado *et al*, 2019).

Ruminal CH_4 production involves energy losses (Liu *et al*, 2019; Gang *et al*, 2020) between 2 and 12% (Liu *et al*, 2019). The factors that determine CH_4 production are the bromatological characteristics of the substrate and the fermentation products of cellulolytic bacteria (Venegas *et al*, 2017; Torres-Salado *et al*, 2019). The different values in partial and accumulated production of CH_4 among inocula (Table 1) are due to NDF content of cobra grass, bacterial conformation of the inocula and production pattern of VFA. Acetate, CO_2 and H_2 are fermentation products of cellulolytic bacteria (Gang *et al*, 2020) generating a syntrophic relationship with methanogenic archaea (Liu *et al*, 2019; Torres-Salado *et al*, 2019), which use CO_2 and H_2 as a metabolic strategy and produce CH_4 (Torres-Salado *et al*, 2019). This is a consequence of increasing the fermentation of structural carbohydrates since their fermentation by cellulolytic bacteria will always produce CO_2 and H_2 that the archaea will use. However, the present study focused on improving the fermentation of these carbohydrates by manipulating the ruminal population. Values similar to our results were reported by Herrera-Pérez *et al* (2018) and Torres-Salado *et al* (2019) during cobra grass fermentation inoculated with RB in coculture with CBC.

The use of ICB in coculture with RB did not improve cobra grass DMD or NDFD (Table 2). These results agree with Azizi *et al* (2020), who mention that inoculation of fibrolytic bacteria in the rumen did not improve fiber digestion. This contradicts the study by Gang *et al* (2020), who reported that an increase in cellulolytic bacteria increases NDFD. The above can be attributed to the origin of the inoculum (RB and ICB), type of inoculum and conformation of the microorganism population (Abad-Guaman *et al*, 2015; Torres-Salado *et al*, 2019). Azizi *et al* (2020) reported an average of 38.36% DMD and 33.4% NDFD in wheat straw inoculated with RB in coculture with 3 ICB from termite intestine, while Harsini *et al* (2019) reported 41.30% DMD and 40.16% NDFD in wheat straw inoculated with RB in coculture with 3 ICB isolated from horses. These values are lower than the results of the present study. Torres-Salado *et al* (2019) reported 61.80 and 65.73% DMD, as well as 55.41 and 59.42% NDFD with bovine and buffalo CBC, respectively; these values are higher than those obtained with the ICB in our study. This supports the idea that ICB needs to interact with other bacteria to improve fiber degradation (Sánchez-Santillán and Cobos-Peralta, 2016).

Coculture$_{bov}$, coculture$_{buf}$ and RB showed lower pH levels than ICBs (Table 2) due to higher production of organic acids by hydrolysis of acetyl groups (Du *et al*, 2019). However, these pH values did not affect the enzymatic activity of cellulolytic bacteria, since values lower than 6.0 are required for their inhibition (Nagaraja, 2016). In total bacterial counts (Table 2), the lower values of IBSs compared to Cocultures are assumed to be attributed to a catabolic repression of IBSs due to the presence of glucose or other compounds in the medium that inhibited their enzymatic activity (Texta *et al*, 2019). In contrast, in RB and Cocultures, cross-feeding was present (Texta *et al*, 2019), reflected in the bacterial population for each type of inoculum. In contrast, other inocula interact by cross feeding (Texta *et al*, 2019). The inocula did not show differences in NH_3-N, which is the result of degradation of nitrogen compounds (Du *et al*, 2019), and in our study the population of cellulolytic bacteria was modified. Azizi *et al* (2020) reported 8.97 log10 total bacteria g^{-1}, pH 6.43 and 13.87 mg dL^{-1} of NH_3-N in culture medium with wheat straw substrate inoculated with RB in coculture with ICBs from termite intestine. These values are higher for total bacteria and lower in pH and NH_3-N than those of the present study.

The VFA are positively correlated with DMD and NDFD (Sánchez-Santillán and Cobos-Peralta, 2016). The VFA of cocultures and RB were higher than those of the ICBs because the DMD and NDFD were higher in cocultures and in RB (Table 2). The average production rate of acetate was 80.88% higher than that of propionate in the ICBs, while for the other inoculum the acetate production rate was 21.64% higher than that of propionate, confirming that cellulolytic bacteria mainly produce acetate during their metabolic path (Sánchez-Santillán *et al*, 2016). Gang *et al* (2020) reported 73.06, 24.07, 11.17, and 115 mM L^{-1} of acetate, propionate, butyrate and VFA in corn silage inoculated with RB in coculture with ICB from horses, and higher values in acetate and VFA, as well as values in propionate and butyrate similar to those in $Coculture_{buf}$ in our study.

We conclude that the use of ICB from bovine or water buffalo in coculture with RB does not improve production of biogas, DMD or NDFD with respect to RB. The ICBs do not produce a synergistic effect under the conditions of the present study. The ICBs do not have potential for use as a probiotic to enhance cobra grass degradation.

Declarations

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Conflict of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

Author contributions

Torres-Salado and Sánchez-Santillán designed the experiment and wrote the manuscript. Ayala-Monter carried out the experiment. Almaraz-Buendía performed the statistical analysis. All authors provided critical feedback of the writing and editing.

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