Faisury Ossa[1],
Martha Arcos[1],
Tito E. Díaz[2] and
Wolfgang Pittroff[3]

Cell wall degradation of Bouteloua repens in vitro by pure cultures of R. flavefaciens and F. succinogenes isolate from cattle grazing tropical lowland pastures in Colombia

ABSTRACT

The capacity to degrade the cellular wall of *Bouteloua repens* of 12 isolates of *Ruminococcus flavefaciens* and 17 isolates of *Fibrobacter succinogenes* was determined. A procedure was developed to standardize both, the inoculum and the substrate concentration for *in vitro* incubations of pure culture isolates. The ruminal bacteria were isolated from either *Bos indicus* cattle from the Alto Magdalena region of Colombia, grazing *Bouteloua repens*, and Sanmartinero cows (a Colombian criollo breed) from the eastern lowlands (Llanos Orientales) of Colombia, grazing *Brachiara spp.* The isolates identity was confirmed using molecular methods. All isolates of *F. succinogenes* showed a high capacity to degrade cell wall preparations of *Bouteloua repens*, than those of *R. flavefaciens* (P<0.0001). The effect of individual isolated on cell wall digestibility was also highly significant (P<0.0001). Native isolates surpassed considerably the reference strains of *R. flavefaciens* ATCC 19208 (12.83% vs. 6.83%) and *F. succinogenes* ATCC 19169 (13.77% vs. 7.94%) on their capacity to digest *Bouteloua repens* cellular wall. This difference was highly significant (p<0.05) for all isolates. Findings suggest that in tropical conditions native ruminal bacteria has developed a high capacity to degrade lignocellulose. Further research about the potential usefulness of specific inocula or enzyme extracts developed from such isolates in improving the degradability of low quality forages by cattle or what industrial applications can be improved by the use of these products.

Key Words: Enzyme complex, interspecific studies, inoculums, lignocellulose, native microbial isolates.

1. Programa Nacional de Fisiología y Nutrición Animal, CORPOICA, A. A. 240142 Las Palmas, Bogotá, Colombia, Sur América.
e-mail: fossa@corpoica.org.co,
martha_ab@email.com 2. Subdirección de Investigación Estratégica. CORPOICA, A.A. 240142 Las Palmas, Bogotá, Colombia, Sur América.
3. Ruminant System Laboratory, University of California, Davis.

RESUMEN

Título: **Degradación in vitro de *Bouteloua repens* por cultivos de *R. flavefaciens* y *F. succinogenes* aislados de ganado alimentado con pastos tropicales en Colombia**
Se determinó la capacidad de degradación de la pared celular de *Bouteloua repens* de 12 aislados de *Ruminococcus flavefaciens* y 17 de *Fibrobacter succinogenes*. Se desarrolló un procedimiento para estandarizar la concentración, tanto del inóculo como de los sustratos, de las incubaciones *in vitro* de cultivos puros de los aislados. Las bacterias fueron aisladas del ganado *Bos indicus*, procedente de la región del Alto Magdalena, que pastoreaban Teatino (*Bouteloua repens*) y del ganado Sanmartinero (una raza nativa) procedente de los Llanos Orientales de Colombia, que pastoreaban *Brachiaria spp.* La identificación del aislado fue confirmada usando métodos moleculares. Todos los aislados de *F. succinogenes* mostraron mayor capacidad de degradación de las preparaciones de pared celular de *Bouteloua repens* que los de *R. flavefaciens* (P<0.0001). La capacidad de los aislados individuales para degradar la pared celular también difirió significativamente (P<0.0001). Los aislados nativos sobrepasaron considerablemente la capacidad para digerir la pared celular de *Bouteloua repens* de las cepas de referencia de *R. flavefaciens* ATCC19208 (12.83% vs. 6.83%) y *F. succinogenes* ATCC19169 (13.77% vs. 7.94%), diferencia que fue altamente significativa (p<0.05) para todos los aislados. Los resultados sugieren que, en condiciones tropicales, las bacterias ruminales nativas tienen una alta capacidad para degradar la lignocelulosa. Se requiere mayor investigación sobre la utilidad potencial del inóculo o de extractos enzimáticos desarrollados a partir de tales aislados, a fin de aumentar la degradabilidad por el ganado de forrajes de baja calidad o para definir las aplicaciones industriales pueden ser mejoradas por el uso de estos productos.

Palabras claves: complejo enzimático, estudios interespecíficos, inóculos, lignocelulosa, aislados nativos.

INTRODUCCIÓN

THE ADAPTATION of cattle to nutritional environments characterized by poor-quality feed resources is the result of genetic selection in the host animal and of symbiosis with microbial species, especially those adapted to feeds characterized by high cell wall content and low digestibility. The total digestion of forages results from the capacity of microbial species to colonizate the particule and its celulollityc activity.

This evolutionary interaction is still poorly understood, and efforts to manipulate the composition of microbial species reviewed by Jouany *et al.* (2000), or the application of biotechnology (enzyme characterization, construction of transgenics review by Forsberg *et al.*, (1999), have had variable success. The ruminal microorganims can work eficiently in consorcios, as mixed systems. (Arcos y Díaz, 2000). For this reason, the problems of intraespecific competitivity and sintropism it should look to try strategies of manipulation or genetic manipulation (Díaz, 2002). The ability to increase the digestion of low quality feedstuffs by sustainable manipulation of the rumen ecosystem is a goal for tropical cattle production systems, currently with low levels of productivity. The studies reported in this work were designed to isolate and identify rumen bacteria, with high capacity to digest native tropical forages in Colombia, where cattle are mainly fed on forage. The Llanos Orientales, a tropical savannah ecosystem located at the East of the Andes, is one of the most important cattle production regions of the country. Most of its area is

covered by native pastures (predominantly *Paspalum spp.*), but extensive areas of improved pastures (mainly *Brachiaria spp.*) have been brought into production. The dry section of the Alto Magdalena is another important cattle production region, where the most common grasses are *Bouteloua repens* and *Botriochloa pertusa*, characterized by their high content of cell wall.

Cell wall degradation in the rumen, as well as the digestion and fermentation of the associated structural carbohydrates, depends on the presence of ruminal microorganisms and their enzymatic activity (Ørskov and Ryle, 1990). On the other hand, the ruminal microbial population, and its capacity to degrade natural substrates, depends upon factors associated with chemical and structural characteristics of the plant (Hoover, 1985; Díaz, 1993). Forage cell wall content determines, in a variable degree, the digestibility and nutritional value of these resources (Van Soest *et al.*, 1991). It has been shown that in ruminant diets with high fiber content, the cellulolytic bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* predominate (Dehority, 1965; Coen and Dehority, 1970). It may be hypothesized that in tropical grassland ecosystems, cattle well adapted to low quality feed in their diet through most of the year, have developed specific symbiotic relationships with cellulolytic bacteria, which have a high capacity for digestion of structural carbohydrates. This hypothesis was tested by evaluating the ability of native isolates of *R. flavefaciens* and *F. succinogenes* to degrade *in vitro* structural carbohydrates of low quality forages. These isolates were obtained from the ruminal fluid of Sanmartinero (a criollo breed) cattle grazing *Brachiaria spp.* in the Llanos Orientales, and Brahman cattle grazing *Bouteloua repens* in the Alto Magdalena region. The main objectives were: (a) to ascertain the capacity of pure cultures of bacteria isolated from cattle commonly grazing on tropical pastures in Colombia, in order to degrade preparations of structural carbohydrates; (b) to standardize an *in vitro* protocol to determine the cell wall degradation of forages by pure cultures of bacteria; (c) to establish a basis for the development of inocula and/or enzymatic extracts with increased capacity for the digestion of structural carbohydrates.

Materials and methods
Bacterial cultures

Ten isolates of both, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, obtained from the rumens of Brahman cattle from the region of Alto Magdalena, were included in this study. Additionally, two isolates of *R. flavefaciens and seven* of *F. succinogenes* obtained of purebred criollo cattle from the Llanos Orientales were also included (Table 1). Bacteria were cultivated under conditions described in previous studies (Hungate, 1969; Bryan, 1972). Native isolates of *R. flavefaciens* and *F. succinogenes* were identified by macroscopic and microscopic characteristics (Moore and Moore, 1977; Hobson, 1988), and their identity were confirmed using standard protocols of the polymerase chain reaction (PCR) (Ossa, 1999). *Ruminococcus flavefaciens* was identified using the primers RF-1L (5'CATTTATGCCC-TATTTACCG3') and RF-1R (5' TGT-CATTATGTTGTCTGCTG 3'), while oligonucleotides FS-1L (5' ATGGA-CACGTACCTGCCGAC 3') and FS-1R (5'TAATGCCACCAACGACAAGC 3') were used for identification of *F. succinogenes* (Ossa, 1999).

Standardization of the technique for in vitro *digestion of forage cell wall by pure cultures of rumen bacteria*

Inoculum. The technique was based on the procedure reported by Dehority (1965) and Dehority and Scott (1967), for the digestibility of forage dry matter using *Bouteloua repens* cell wall (CW). Dry forage was treated with neutral detergent fiber solution eliminating cellular content, following the method of Van Soest *et al.*, (1991).

A culture of *Ruminococcus flavefaciens* was used (OD$_{640}$ = 0.2 equivalent to 4x10^8 cfu ml^{-1}), after 16 to 18 hours of incubation in cellobiose medium (Arcos, 1998; Bryant and Burkey, 1953). Different inoculum volumes of this pre-

paration were added to 10 ml of medium with 40% rumen fluid, to obtain a final inoculum concentration of 1, 2, 3, 4, and 5% (V/V). The liquid medium was prepared as described by Hungate (1969), using 0.5% (W/V) of *Bouteloua repens* cell wall (particulate size equal to 1 mm) as substrate. Since several studies of digestibility of dry matter by pure cultures have demonstrated that the maximal rate of digestion occurs at 0.5% forage concentration, this value was selected as substrate concentration for the inoculum standardization (Dehority and Scott, 1967; Osborne and Dehority, 1989; Ørskov and Ryle, 1990). Six replications were included for each concentration of inoculum, three of which were incubated at 39°C for 48 hours and the other three for 72 hours. At the end of the incubation probes were placed at – 20°C for 45 minutes to inactivate the fermentation process (Van Soest *et al.*, 1991). Tube contents were then treated with 100 mlL of neutral detergent fiber solution and boiled for 60 minutes. The obtained solution was filtered in crucibles of 40 mm of porosity. The obtained residues were washed with 400 ml of distilled water at 90°C, 2 ml of alcohol and dried at 60°C for 16 hours. The percentage digestibility of each sample was calculated from the initial and residual cell wall mass.

Standardization of the concentration of used substrate. Experiments were performed using concentrations of *Bouteloua repens* cell wall of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0% (W/V), maintaining a concentration of inoculum from *R. flavefaciens and F. succinogenes* of 4% (V/V) and an OD$_{640}$ of 0.2. To determine the effect of incubation time on digestibility, six replicates of each concentration were subjected to different periods of incubation. Three replicates were incubated for 48 hours, whereas, the other three replicates were incubated for 72 hours (Ifkovits *et al.*, 1965; Dehority and Scott, 1967).

Table 1. *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* isolates used in this study

Microbial species	Isolate or breed of origin	Number of isolates	Ecosystem
Ruminococcus flavefaciens	ATCC 19208	1	Temperate region (U.S.)
	Brahman	10	Alto Magdalena, Colombia
	Sanmartinero	2	Llanos Orientales, Colombia
Fibrobacter succinogenes	ATCC 19169	1	Temperate region (U.S.)
	Brahman	10	Alto Magdalena, Colombia
	Sanmartinero	7	Llanos Orientales, Colombia

Digestibility of Bouteloua repens *by different cultures of* Ruminococcus flavefaciens *and* Fibrobacter succinogenes

R. flavefaciens ATCC19208, *F. succinogenes* ATCC19169, and each of the native isolates of *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* (Table 1), were diluted to an OD_{640} of 0.2 ($4x10^8$ cfu ml^{-1}) from cultures of 16 to18 hours of growth. A final concentration of 4.0% (V/V) of inoculum was added to 10 mlL of medium with 4.0% (W/V) of *Bouteloua repens* cell wall. Samples were incubated for a period of 48 hours at 39°C and further procedures were performed as mentioned above.

Statistical analysis

Data were analyzed using linear models. The fixed factors considered were: geographical location, bacterial species, isolates, and the interaction between location and species. Isolates occurred within location in the associated cattle breed; consequently, their effect was modeled as nested within location. Since isolates are specific to species, isolation was not included in the model for the analysis of the species by the location interaction effect. The following models were fitted.

For the analysis of the isolates effect the model used was:

$$y_{ijk} = \mu + \lambda_i + \sigma_j(\lambda_i) + \varepsilon_{ijk}$$

Where *y* denotes degradability of a cell wall preparation of *Bouteloua repens* after 48 hours of incubation, μ is the population mean, λ_i denotes the effect of the i-th location, σ_j denotes the effect of the j-th isolates within the i-th location, and ε_{ijk} denotes residual error. Both factors were considered fixed; thus, MSE was the error term in the corresponding F tests for both factors. Multiple comparisons were conducted using simultaneous confidence intervals for mean differences in order to control for experiment-wise error rate.

For the analysis of the location and species effects the model used was:

$$y_{ijk} = \mu + \lambda_i + \phi_j + (\lambda\phi)_{ij} + \varepsilon_{ijk}$$

Where *y* denotes degradability as above, μ is the population mean, λ_i denotes the effect of the i-th location, ϕ_j denotes the effect of the j-th species, $(\lambda\phi)_{ij}$ represents the effect of the interaction between location and microbial species, and ε_{ijk} is the residual error.

In order to estimate the difference in degradability of *Bouteloua repens* by isolates in this project relative to a reference strain of the same microbial species, one-tailed Dunnett t-tests were performed with the reference strains as standard. The specific hypothesis evaluated was, that within species, the bacteria isolated in Colombia exhibited significantly higher degradation capacity of purified cell wall preparations of *Bouteloua repens* when compared to the reference strain. The model with isolates as a fixed factor was fitted separately for each microbial species.

Data of the substrate and inoculum standardization experiments, obtained from replicates of the same preparation at two different incubation lengths, were first analyzed with a mixed model for repeated measures with replicate as a random factor. However, a likelihood ratio test (p=0.0991 or higher) did not suggest an improvement over a fixed model by taking into account the covariance among observations. Accordingly, data were analyzed with a fixed factor model, as follows:

$$y_{ijk} = \mu + \chi_i + \tau_j + (\chi\tau)_{ij} + \varepsilon_{ijk}$$

Where μ is the population mean, χ_i is the effect of i-th inoculum or substrate concentration, τ_j is the effect of the j-th incubation time (48 or 72 hours), $(\chi\tau)_{ij}$ ισ the main effect interaction, and ε_{ijk} is the random error. For both sets of standardization experiments, incubation time and the main effect interaction were not significant and thus, eliminated from the model. Consequently, all further experiments were conducted with a 48 hour incubation time.

Quadratic regression models were fitted to quantify the relation of cell wall degradability to substrate and inoculum concentration. Inspection of Type 1 sums of squares revealed the quadratic term to be significant in both cases. All procedures were carried out with version 8.1 of SAS software (SAS®,1999).

Results
Standardization of in vitro *digestion by pure cultures*

Inoculum. Inoculum concentration was significantly related to (P < 0.0001) in vitro cell wall digestibility of *Bouteloua repens* (Figure 1). Variations in inoculum concentrations (1, 2, 3, 4, and 5% [V/V]) produced digestibility values between 8.35 to 13.51% (P < 0.05). The lowest degradation (8.35%) of *Bouteloua repens* cell wall was obtained using the 1% inoculum concentration. Digestibility tends to increase with 2, 3 and 4% inoculum concentrations. Maximum digestibility (13.51%) was achieved when the concentration of inoculum was 4% (V/V). When 5% (V/V) inoculum was used, digestibility decreased and was not significantly different from the value ob-
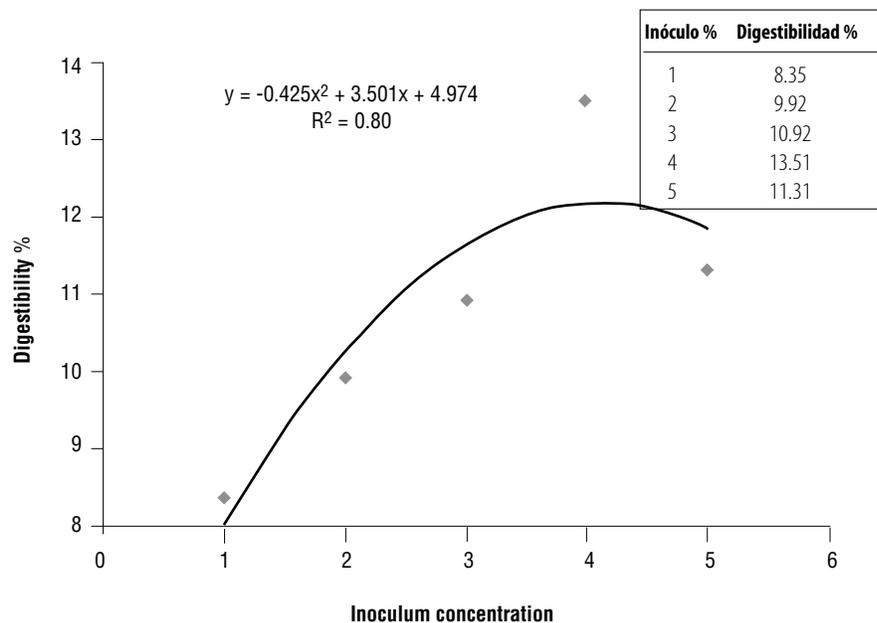

Inóculo %	Digestibilidad %
1	8.35
2	9.92
3	10.92
4	13.51
5	11.31

$y = -0.425x^2 + 3.501x + 4.974$
$R^2 = 0.80$

Figure 1. Effect of inoculum concentration on the *in vitro* digestibility of *Bouteloua repens* cell wall by a pure culture of *Ruminococcus flavefaciens*.

tained with the 3% inoculum. The association between the digestibility of *Bouteloua repens* cell wall and varying concentrations of inoculum was described by a quadratic regression model:

The ecuation, $y = -0.42.5X^2 + 3.501X + 4.974$ (X denoting inoculum concentration), with $R^2 = 0.80$.

Substrate. *Bouteloua repens* cell wall composition was 5.9% lignin, 26.9% cellulose and 24.5% hemicellulose. Digestibility of this cell wall by *Ruminococcus flavefaciens* cultures increased at substrate concentrations of 0.5, 1, 2, 3, and 4% (W/V) and decreased at the 5% (W/V) substrate level ($P < 0.05$) (Figure 2). The relationship between the digestibility of *Bouteloua repens* cell wall and the concentration of substrate could be described by a quadratic model of the form:

$Y = 0.0184x^2 + 0.9404X + 7.781$, with $R^2 = 0.91$.

Incubation time. There were no differences ($P > 0.05$) in the amount of digested cell wall between the 48 and 72 hour incubations times. The digestibility obtained with *R. flavefaciens* cultures was 10.39% at 48 hours and 10.37% at 72 hours, whereas the *F. succinogenes* culture achieved a digestibility level of 11.43% and 11.39% at 48 and 72 hours, respectively.

In vitro *degradation of* Bouteloua repens *cell wall by* Ruminococcus flavefaciens *and* Fibrobacter succinogenes

All degradation values obtained fell into a relatively narrow range (11.09 to 12.83% for *R. flavefaciens*, and 12.00 to 13.77% for *F. succinogenes*, respectively (Tables 2 and 3). Nevertheless, an effect of strain was presented. In average, the digestibility obtained by *Fibrobacter* isolated was higher than that obtained by *Ruminococcus* (Table 4) which was highly significant ($P<0.0001$). In general, the digestibility observed at incubations with isolates from the Alto Magdalena region was higher than those observed with bacteria isolated in the Llanos Orientales (Table 4). This location effect was also highly significant ($P<0.0001$). The interaction between location and species was significant at $p=0.011$; however, the ranking of microbial species did not change across regions; therefore, no sliced effect tests were performed.

Native isolates were compared against their corresponding reference strains (*R. flavefaciens* ATCC 19208; *F. succinogenes* ATCC 19169). All native isolates produced a significantly higher degradability
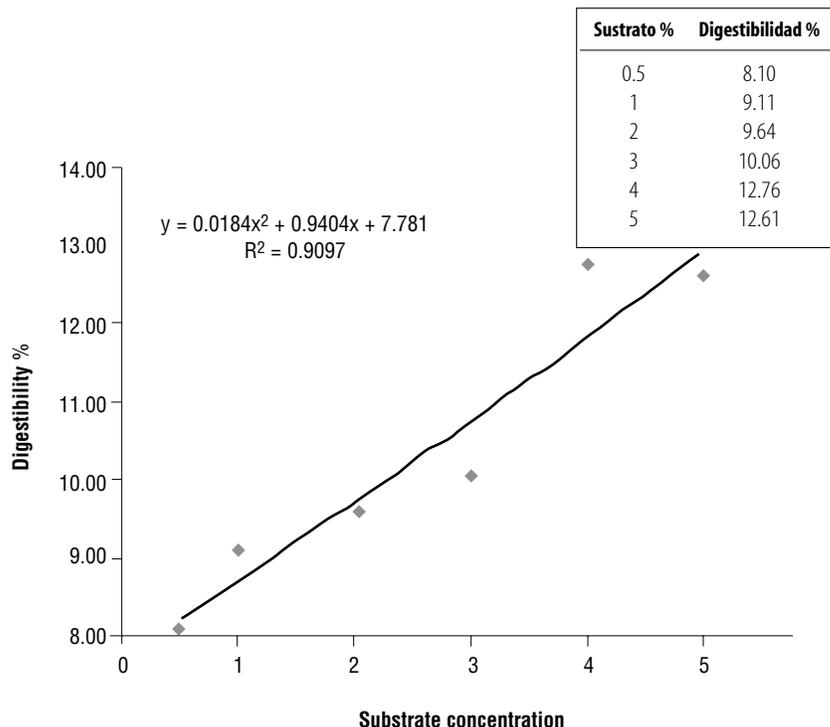

Sustrato %	Digestibilidad %
0.5	8.10
1	9.11
2	9.64
3	10.06
4	12.76
5	12.61

$y = 0.0184x^2 + 0.9404x + 7.781$
$R^2 = 0.9097$

Figure 2. Effect of substrate concentration on the *in vitro* digestibility of *Bouteloua repens* cell wall by a pure culture of *Ruminococcus flavefaciens*.

Table 2. Comparison of native isolates of *Ruminococcus flavefaciens* with a reference strain in terms of degradability of *Bouteloua repens* cell wall preparations after 48 h of incubation

Location	Isolate	Difference with reference[a]	Mean degradability[a]	Contrast[b]
Llanos Orientales				
	LR5	4.97	11.8±0.06	***
	LR6	4.26	11.09±0.02	***
Alto Magdalena				
	MR1	5.63	12.46±0.03	***
	MR2	4.99	12.81±0.03	***
	MR3	5.49	11.82±0.05	***
	MR4	5.92	12.32±0.03	***
	MR5	5.82	12.75±0.03	***
	MR6	6.00	12.65±0.04	***
	MR7	5.70	12.83±0.04	***
	MR8	5.82	12.53±0.04	***
	MR9	5.10	12.65±0.04	***
	MR10	5.98	11.93±0.03	***
Reference	ATCC 19208		6.83±0.03	

[a] All values in percent.
[b] $P < 0.05$ in simultaneous one-tailed Dunnett t tests compared against the reference strain.

of *Bouteloua repens* cell wall preparations, with the differences being greater than 50% in all cases (Tables 2 and 3).

Discussion
Standardization of in vitro *digestion by pure cultures*

Reductions in the inoculum concentration affects substrate degradation negatively. When inoculum concentrations were low (1, 2 and 3% [V/V]), the bacteria capability to degrade *Bouteloua repens* cell wall decreased. The maximum digestion at 4% (V/V) inoculum agrees with Coen and Dehority (1970), who observed maximum degradation and utilization of hemicellulose from intact bromegrass by pure cultures at an inocu-

Table 3. Comparison of native isolates of *Fibrobacter succinogenes* with a reference strain in terms of degradability of *Bouteloua repens* cell wall preparations after 48 h of incubation

Location	Isolated	Difference with reference[a]	Mean degradability[a]	Contrast[b]
Llanos Orientales				
	LF1	4.99	12.94±0.03	***
	LF2	4.88	12.82±0.05	***
	LF3	4.80	12.73±0.03	***
	LF4	4.90	12.85±0.04	***
	LF5	4.10	12.01±0.02	***
	LF6	4.93	12.87±0.02	***
	LF7	5.01	12.95±0.04	***
Alto Magdalena				
	MF1	5.31	13.25±0.04	***
	MF2	5.70	13.51±0.02	***
	MF3	5.17	13.11±0.03	***
	MF4	5.72	13.66±0.04	***
	MF5	5.39	13.33±0.04	***
	MF6	4.98	12.92±0.03	***
	MF7	5.84	13.78±0.04	***
	MF8	5.12	13.06±0.05	***
	MF9	5.30	13.24±0.04	***
	MF10	5.56	13.5±0.03	***
Reference	ATCC19169		7.94±0.03	

[a] All values in percent.

[b] $P < 0.05$ in simultaneous one-tailed Dunnett t tests compared against the reference strain.

Table 4. Effect of microbial species and geographic location of origin on degradability of cell wall preparations of *Bouteloua repens* by pure cultures (least square means)

Effect	Degradability in 48 hours[a]	Contrast[b]
Species		
Ruminococcus flavefaciens	11.96±0.07	B
Fibrobacter succinogenes	13.04±0.01	A
Location		
Alto Magdalena	12.09±0.07	B
Llanos Orientales	12.91±0.04	A

lum concentration of 3.9% (V/V), $OD_{640} = 0.2$. However, Dehority and Scott (1967) found maximum degradation of hemicellulose of intact forage substrates at $OD_{640} = 0.2$, 2% (V/V), while Dehority (1965) found highest isolated hemicellulose degradation and utilization by pure inocula at a concentration of 1.4% (V/V) $OD_{640} = 0.1$; on the other hand, Dehority (1968) determined that the maximum capacity of *R. flavefaciens* isolates to degrade and use isolated hemicellulose from fresh grass at $OD_{600} = 0.1$, 1% (V/V) inoculum concentration. Thus, these results confirm that the concentration of bacterial inoculum is an important factor influencing *in vitro* digestibility forage. Fiber digestion is in large measure dependent on population size of cellulolytic bacteria (Shi *et al*., 1997), showing the importance of establishing the optimum inoculum concentration to evaluate the capacity of pure microbial cultures to degrade forages.

The observation that the ability of *Ruminococcus flavefaciens* to degrade *Bouteloua repens* cell wall varied quadratically with the concentration of the substrate, with a peak digestibility at 4% (W/V) inoculum concentration, suggests that in batch culture this parameter follows first order kinetics respect to substrate concentration, when degradation is limited by the amount of substrate available (Shi *et al*., 1997). However, the activity of *R. flavefaciens* diminished when the concentration of *Bouteloua repens* CW was raised to 5% (W/V). It

could arise from the likely competition brought about by an excess of bacterial numbers which would need to establish dominance with regard to degradation sites. The finding that incubation beyond 48 hours did not increase digestibility, agrees with previous work, suggesting that *in vitro* bacteria realize most if not all, of their growth during the first 48 hours of incubation (Ifkovits *et al*., 1965).

In vitro *degradation of* Bouteloua repens *cell wall by different cultures of* Ruminococcus flavefaciens *and* Fibrobacter succinogenes

Isolates of *R. flavefaciens* varied in their ability to degrade cell wall of *Bouteloua repens* (Table 2). This observation is in agreement with results from other studies of *in vitro* digestion of structural carbohydrates by pure cultures, which demonstrated differences between isolated of *R. flavefaciens* in the capacity to digest cellulose and hemicellulose (Dehority, 1965; Dehority and Scott, 1967; Coen and Dehority, 1970). These differences are associated to the enzymatic potential of each bacterial isolate, a feature that probably is genetically determined. Differences in digestive ability allow the classification of *R. flavefaciens* into low, medium and high degraders of structural carbohydrates (Dehority and Scott, 1967). Similar results were obtained for *in vitro* digestibility by isolated *F. succinogenes* strains (Table 3).

The higher capacity for degradation of *Bouteloua repens* cell wall by isolates from the Alto Magdalena as compared to those from the Llanos Orientales (Table 4) is probably the result of adaptive modifications of their enzyme complexes that permits a higher degradation of this forage. *Bouteloua repens* is the most prevalent source of forage in the Alto Magdalena region and it is not commonly found in the Llanos Orientales. The most important substrate for isolates from Llanos Orientales is *Brachiaria spp*. Reference strains used in this study had been originated from temperate ecosystems (U.S.) where forages are higher in soluble sugars and lower in cell wall. This suggests that variations in activity of the enzymes to degrade *Bouteloua repens* cell wall involve environmental conditions inherent to the ecosystem from which the isolate is extracted, and in a large part, may be related to the major forage source found in the ecosystem. This suggestion is sup-

ported by previous studies by Akin *et al*. (1989), who reported significant differences in the capacity of inocula isolated from cattle in the temperate zone (US) and the tropical ecosystem of Australia to degrade Bermudagrass (*Cynodon dactylon*) dry matter (64 vs. 75% degradability, respectively.)

Besides regional differences, this study also established differences between genera of cellulolytic bacteria. Native isolates of *Fibrobacter* had higher digestibility of *Bouteloua repens* cell wall than *Ruminococcus isolates* (Table 4). Differences in the capacity of cellulolytic bacteria to degrade a substrate depend on the activity of constitutive or inducible enzymes and the production of these enzymes per unit of cell weight (Pardee 1962; Pollock, 1962; Dehority, 1968). Further, species rate and capacity of adherence to fiber particle is important in subsequent substrate degradation (Valérie *et al*., 1990). Adherence has been the focus of numerous studies that have shown marked variation under different experimental conditions (Bhat *et al*., 1990; Miron *et al*., 1994; Morris, 1998), suggesting structural mechanisms dependent on genera of bacteria. The facility to hydrolyze structural carbohydrates by enzyme complexes is inherent to the species of interest as reported by Wood *et al*. (1982) and Van Gylswyk *et al*. (1984). In these studies, it was shown that the enzyme complex of *F. succinogenes* has a greater capacity to hydrolyze crystalline cellulose than that of *R. flavefaciens*. It is explained by the predominance of *F. succinogenes* in the rumen, when concentration of crystalline cellulose in the diet of the animal is high. Miron *et al*. (1994) demonstrated that the nature of the substrate alters the degradative ability of different bacterial species. Shi *et al*. (1997) showed that substrate degradation by *F. succinogenes* and *R. flavefaciens* generates different concentrations of soluble sugars, acetate and succinate and this affects the ability of different species to digest forage cell wall.

In our experiments, native isolates of *F. succinogenes*, as those of *R. flavefaciens*, exhibited a high capacity to degrade *Bouteloua repens* cell wall. In studies conducted by our research group, the *in situ* dry matter digestibility of *Bouteloua repens* ranged between 32.60 and 34.07% (Chamorro, personal communication). Further, it was observed that the *in vitro* digestibility of *Bouteloua repens* cell wall by mixed cultures varied between 31.50 and 33.90%. These results may be attributed to the synergistic enzyme activity exhibited by populations of bacteria, fungi and protozoa in the rumen (Dehority, 1991). Degradation obtained in mixed cultures illustrates the degradation of structural carbohydrates, due to the synergistic action of different species of cellulolytic bacteria (Fondevila and Dehority, 1996), while *in vitro* digestibility by pure cultures demonstrates the intrinsic ability of a microorganism species to degrade specific substrates. Although, total degradation in this study is the result of the exclusive action of pure cultures of bacteria, the values for digestibility of *Bouteloua repens* in vitro by isolates of *F. succinogenes* and *R. flavefaciens* are similar to results seen in situ, if it is considered that the utilized material is cell wall, and not total dry matter, which also contains soluble macromolecules (soluble carbohydrates and protein). The high concentration of lignin in the cell wall of this forage (5.9%) has a major effect on *in vitro* digestibility, by impeding the accessibility of bacteria to digestible cellulose and hemicellulose (Van Soest, 1993; Wilson and Mertens, 1995). Access to digestible substrate is easy *in situ* as a result of the physical action of ruminal fungi on forage cell wall, and possibly, by the presence of an esterase of a particular genus of fungi that breaks hemicellulose-lignin bonds (Gordon and Phillips, 1989). Additionally, studies with mixed cultures indicate that the activity of cellulolytic enzymes produced by certain species of protozoa contribute significantly to the rapid degradation of forage (Akin, 1988). Evidence exists that the capacity to degrade forage is greater in mixed cultures than in pure cultures. Some bacteria have the capacity to use free components produced by other microorganisms in the medium, reducing the accumulation of fermentation products. This, in turn, results in a forage degradationn increment (Miron *et al*., 1994).

Conclusions

The standardized protocol presented here offers advantages over the technique of *in vitro* digestibility of dry matter by mixed cultures, developed previously by our group (Programa de Fisiología y Nutrición Animal, C.I. Tibaitatá, Corpoica, Colombia; unpublished data). Substrate size, microbial inoculum and culture medium volume utilized in the *in vitro* digestion by pure cultures were lower than those of previous methods, resulting in reduced consumption of these resources. Thus, our standardized process consumed substantially less resources and produced data with higher reproducibility. It is reflected in the small standard errors in our measurements.

The results obtained show that the forage species *Bouteloua repens*, a typical representative of tropical forages, has low digestibility, presumably because of its high content of lignin. Native isolates of *F. succinogenes* and *R. flavefaciens* have an extraordinarily high capacity to degrade *Bouteloua repens* cell wall compared to reference strains, supporting the hypothesis that native cellulolytic microorganisms, in their adaptation to a tropical ecosystem, have developed a special capacity to degrade forage with high fiber contents. Comparisons between species and locations of origin of isolated are critical in the evaluation of digestion capacity of ruminal microbes. The techniques implemented establish a basis for future *in vitro* studies digestibility by pure cultures of other ruminal microorganisms. Further work is required to understand the synergistic activity of specifically adapted ruminal microbes in the digestion of tropical forages and to establish appropriate techniques to profit by these isolates in the improvement of nutritional management of cattle fed on tropical pastures. It is conceivable that the implications of this work are also relevant for pasture based cattle production systems of the temperate zone.

B I B L I O G R A P H Y

Akin, D. E. 1988. Biological structure of lignocellulose and its degradation in the rumen. Anim. Feed Sci. Technol. 21:295-310.

Akin, D. E.; Gordon, G. L. R.; Rigsby, L.L. 1989. Comparative fiber degradation by mixed rumen fungi from Australian and U.S.A. cattle. Anim. Feed Sci. Technol. 23:305-321.

Arcos, B. M. and Díaz, M. T. 2000. Cell wall digestibility of *Bouteloua repens* by pure and mixes culture of rumen fungi and bacteria isolates from grazing cattle in the tropics. Rev: Reproduction Nutrition Development 2:190.

Arcos B. M. 1998. Aislamiento, conservación y evaluación de la cinética de crecimiento y actividad celulolítica de cepas de *Fibrobacter succinogenes* de bovinos en pastoreo de gramíneas tropicales. Tesis de Maestría, Pontificia Universidad Javeriana, Bogotá, Colombia.

Bhat, S.; Wallace, R. J.; Orskov, E. R. 1990. Adhesion of cellulolytic ruminal bacteria to barley straw. Appl. Environ. Microbiol. 56:2698-2703.

Bryant, M. P.; Burkey L. A. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36: 205-217.

Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. 25:1324-1328.

Coen, J. A.; Dehority, B. A. 1970. Degradation and utilization of hemicellulose from intact forages by cultures of rumen bacteria. Appl. Microbiol. 20:362-368.

Dehority, B. A. 1965. Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. J. Bacteriol. 89:1515-1520.

Dehority,B. A.; Scott, H. W. 1967. Extent of cellulose and hemicellulose digestion in various forages by cultures of rumen bacteria. J. Dairy Sci. 50:1136-1141.

Dehority, B. A. 1968. Mechanism of isolated hemicellulose and xylan degradation by cellulolytic rumen bacteria. Appl. Microbiol. 16:781-786.

Dehority, B. A. 1991. Effects of microbial synergism on fiber digestion in the rumen bacteria. Fed. Proc. 32:1819-1825.

Díaz, T. E. 1993. Evaluation of cellulolytic activity during *in situ* digestion of corn stover. Journal Paper of the Iowa State University. 26:524.

Díaz, T. E. 2002. Fuentes de recursos utilizados en alimentación animal. *En*: *Medicina Veterinaria y Zootecnia en Colombia*. Capítulo 18, Fundación Edivez. p. 555-562.

Fondevila, M.; Dehority, A. 1996. Interactions between *Fibrobacter succinogenes*, *Prevotella ruminicola*, and *Ruminococcus flavefaciens* in the digestion of cellulose from forages. J. Anim. Sci. 74:678-684.

Forsberg, C. W.; Egbosimba, E. E. and S. MacLellan. 1999. Recent Advances in biotechnology of rumen bacteria-review. Asian-Australasian Journal of Animal Science 12(1):93-103.

Gordo, L. R.; Phillips, M. W. 1989. Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. Appl. Environ. Microbiol. 7: 1703.

Hobson, P. N. 1988. *The rumen microbial ecosystem*. Elsevier, London and New York, pp. 527.

Hoover, W. H. 1985. Chemical factors involved in ruminal fiber digestion J. Dairy Sci. 69:2755 -2766.

Hungate R. E. 1969. A roll tube method for cultivation of strict anaerobes. Methods in Microbiology. 3B:117-132.

Ifkovits, R. W.; Ragheb, H. S.; Barnes, R. F.; Packett, L. V. 1965. A pure-culture inoculum method for evaluation of forage cellulose digestibility. J. Anim. Sci. 13:1092-1099.

Jouany, J.P.; Michalet-Doreau, B. and M. Doreau. 2000. Manipulation of the rumen ecosystem to support high-performance beef cattle - Review. Asian-Australasian Journal of Animal Science. 13(1):96-114.

Miron, J.; Duncan, S. H.; Stewart, C. S. 1994. Interactions between rumen bacterial strains during the degradation and utilization of the monosacharides of barley straw cellwalls. J. Appl. Bacteriol. 76:282-287.

Moore, V. H. and Moore, E.C. 1977. The Virginia Polytechnic Institute and State University Anaerobe. Laboratory Blacksburg, Virginia.

Morris E. J. 1998. Characteristics of the adhesion of *Ruminococcus albus* to cellulose. FEMS Microbiol. Lett. 51:113-118.

Osborne, J. M.; Dehority, B.A. 1989. Synergism in degradation and utilization of intact forage cellulose, hemicellulose, and pectin by three pure cultures of ruminal bacteria. Appl. Environ. Microbiol. 55:2247-2250.

Ørskov, E. R. and M. Ryle. 1990. Energy Nutrition in Ruminants. Elsevier Applied Science Publishers, London, New York.

Ossa, F. 1999. Identificación molecular de bacterias celulolíticas ruminales y degradación de la pared celular de *Bouteloua repens* por cepas nativas de *Ruminococcus flavefaciens*. Tesis de Maestría, Pontificia Universidad Javeriana, Bogotá, Colombia.

Pardee, A. B. 1962. The synthesis of enzymes. *In*: I.C. Gunsalus and R. Y. Stanier (ed.) *The Bacteria*. Vol. 3. Academic Press Inc., New York, p. 577-630.

Pollock M. R., 1962. Exoenzymes. *In*: I.C. Gunsalus and R. Y. Stanier (ed.) *The Bacteria*. Vol. 4. Academic Press Inc., New York, p. 121-178.

Russell, J. B.; Dombwroski, D.B. 1980. Effects of pH on the efficiency of growth by pure cultures of rumen bacteria in continuos culture. Appl. Environ. Microbiol. 39:604-610.

Russell, J. B.; Wilson, D. B. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? J. Dairy Sci. 79:1503-1509.

SAS-STAT User's Guide. 1999. SAS Institute Inc., Cary (NC), USA.

Shi, Y.; Odt, L. C.; Weimer J. P. 1997. Competition for cellulose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate-limited conditions. Appl. Environ. Microbiol. 63:734-742.

Valérie, R.; Fonty, G.; Komisarczuk-Bony, S.; Gouet, P. 1990. Effects of physicochemical factor on adhesion to cellulose avicel of the ruminal bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* subsp. succinogenes. Appl. Environ. Microbiol. 56:3081-3087.

Van Gylswyk, N.O.; Schwartz, H. M. 1984. Microbial Ecology of Cellulose and Hemicellulose Metabolism in Gastrointestinal Ecosystems. National Chemical Research Laboratory. Pretoria, South Africa. p. 588-599.

Van Soest, P. J.; Robertson, J.; Lewis, B. 1991. Symposium: Carbohydrate methodology, metabolism and nutritional implications in dairy cattle. J. Dairy Sci. 74:3583-3597.

Van Soest, P. J. 1993. Cell wall matrix interactions and degradation session synopsis. *In*: H.G. Jung et al (ed.) *Forage Cell Wall Structure and Digestibility*. ASA-CSSA and SSSA, Madison (WI), USA, p. 377-395.

Wilson, J. R.; Mertens, D. R. 1995. Crop quality and utilization. Crop Science. 35:251-259.

Wood, T. M., Wilson, C. A.; Stewart, C. S. 1982. Preparation of the cellulase from the cellulolytic anaerobic rumen bacteria *Ruminococcus albus* and its release from the bacterial cell wall. Biochem. J. 205:129-37.