Tannin-tolerant bacteria from crossbred Holstein x Zebu cows

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Abstract – The objective of this work was to isolate and characterize tannin-tolerant ruminal bacteria from crossbred Holstein x Zebu cows fed a chopped mixture of elephant grass (*Pennisetum purpureum*), young stems of "angico-vermelho" (*Parapiptadenia rigida*), and banana tree (*Musa* sp.) leaves. A total of 117 bacteria strains were isolated from enrichment cultures of rumen microflora in medium containing tannin extracts. Of these, 11 isolates were able to tolerate up to 3 g L^{-1} of tannins. Classical characterization procedures indicated that different morphological and physiological groups were represented. Restriction fragments profiles using *Alu*1 and *Taq*1 of 1,450 bp PCR products from the 16S rRNA gene grouped the 11 isolates into types I to VI. Sequencing of 16S rRNA PCR products was used for identification. From the 11 strains studied, seven were not identifiable by the methods used in this work, two were strains of *Butyrivibrio fibrisolvens*, and two of *Streptococcus bovis*.

Index terms: *Butyrivibrio fibrisolvens*, *Streptococcus bovis*, anti-nutritional polyphenolics, ruminant nutrition.

Bactérias tolerantes a taninos obtidas de vacas mestiças Holandês x Zebu

Resumo – O objetivo deste trabalho foi isolar e caracterizar bactérias ruminais tolerantes a taninos obtidas de vacas mestiças Holandês x Zebu alimentadas com dieta composta por capim-elefante (*Pennisetum purpureum*) picado com ramos novos de angico-vermelho (*Parapiptadenia rigida*) e folhas de bananeira (*Musa* sp.). Um total de 117 cepas bacterianas foram isoladas a partir de cultivos de enriquecimento da microbiota ruminal em meio contendo extrato de taninos. Destas, 11 foram capazes de tolerar até 3 g L^{-1} de taninos. Procedimentos clássicos de caracterização indicaram que diferentes grupos, morfológicos e fisiológicos, estavam representados. Perfis dos fragmentos de restrição com *Alu*1 e *Taq*1 dos produtos de PCR de 1.450 bp do gene 16S rRNA agruparam os 11 isolados nos tipos I a VI. O sequenciamento dos produtos PCR 16S rRNA foi utilizado para identificação. Das 11 estirpes estudadas, sete não foram identificáveis pelos métodos utilizados neste trabalho, duas eram estirpes de *Butyrivibrio fibrisolvens* e duas de *Streptococcus bovis.*

Termos para indexação: *Butyrivibrio fibrisolvens*, *Streptococcus bovis*, polifenóis antinutricionais, nutrição de ruminantes.

Introduction

The low nutritive value of tropical forages is a severe constraint to enhance ruminant productivity, and has been gaining importance due to the growing demand for higher productivity and animal protein. Environmental challenges prevent the use of large pasture areas, which may result in emission of greenhouse gases from ruminant livestock. According to literature (Jones et al., 1994; Molan et al., 2001), vegetal fiber degradation could be augmented by manipulating ruminal microbiota or via protein supplementation to diet. Leguminous trees, shrubs, and herbs have significant potential to increase the nutritive value of ruminant diet, and, simultaneously, the feedstuff conversion rate into milk and meat, when content in plant secondary metabolites is neutralized by the gut microbiota (Ammar et al., 2009; Waghorn, 2008). This potential is mostly due to the higher crude protein content from legumes, in comparison to grasses. Condensed tannins in forage legumes improve the nutrition of sheep by reducing ruminal degradation of plant protein and increasing crude protein flow to the intestine (Molan et al., 2001; Vasta et al., 2010). However, the effects of condensed tannins in forage legumes on rumen bacterial populations in vivo are still poorly understood (Molan et al., 2001; Arcuri et al., 2003; Vasta et al., 2010). Several agronomically promising legume species, such as *Acacia*, *Mimosa*, and *Sesbania* spp., have limited use as feedstuff because of the presence of anti-nutritional

factors (ANFs), including nonprotein amino acids (Reed et al., 2001), saponins, oxalic acid, fluoroacetate, and a range of different tannin and related phenolic structures (Kumar & Vaithiyanathan, 1990; Waghorn, 2008; Patra & Saxena, 2010).

Tannins are toxic, high molecular weight, hydrolysable or condensed polyphenols that exist in a wide variety of plant species. In grazing ruminants, high concentrations of tannins adversely affect nutrition. Tannins produce an astringent taste, which alters the palatability of diets, thus reducing overall feed intake (Patra & Saxena, 2010). Tannins also produce an insoluble protein-tannin complex that is poorly digested in the rumen and lower digestive tract, which inhibits microbial enzymes involved in fiber degradation (Smith & Mackie, 2004). Joanisse et al. (2007) found a negative relationship between the percentage of a temperate legume shrub cover in forest soils and fiber degrading beta-glucosidase activity. Similarly, Khiaosa-Ard et al. (2009) observed that treatments of feed with condensed tannins reduced ruminal fiber and crude protein degradation. However, effectively using feedstuff is still possible, due to the complex microbiota found in the gastro-intestinal tract, whose composition is mainly determined by the available diet (O'Donovan & Brooker, 2001; Ammar et al., 2009; Khiaosa-Ard, 2009).

Low levels of tannins (< 5% dry weight) can benefit ruminants by protecting protein from bacterial deamination (Waghorn, 2008; Patra & Saxena, 2010), and preventing bloat (Min et al., 2005). Ruminal microbial populations (bacteria, protozoa, and fungi) are not yet fully understood, especially in regard to the mechanisms involved in cell wall degradation of tropical feedstuffs rich in secondary compounds. Therefore, decreasing the effect of tannins would allow several fodder trees to be incorporated into different ruminant farming systems, which could improve the nutritional status, productivity, and, subsequently, profitability of animals, besides decreasing environmental footprint and allowing a more environmentally friendly ruminant production.

The objective of this work was to isolate and characterize tannin-tolerant ruminal bacteria from crossbred Holstein x Zebu cows fed a chopped mixture of elephant grass, young stems of "angico-vermelho", and banana tree leaves.

Materials and Methods

Three crossbred Holstein x Zebu dry cows, fistulated in the rumen, were fed a chopped mixture of elephant grass (*Pennisetum purpureum* Schumach.), young leaves and stems of "angico-vermelho" [*Parapiptadenia rigida* (Benth.) Brenan], and banana tree (*Musa* sp.) leaves. The experimental procedure, with some modifications, followed that of Belenguer et al. (2010). "Angico-vermelho" or *P. rigida* is a native leguminous tree, commonly found in Brazil. It was included as part of the animals' feedstuff due to its abundance in pastures and recognized tannin levels. The feeding mixture was prepared on a fresh matter base, with the addition of 50% of elephant grass and approximately 25% of *P. rigida* and *Musa* materials, until 3 g kg^{-1} of condensed tannins were introduced to the diet.

Animals were kept in individual, partially shaded stalls of about 100 m^2, located at the husbandry area of the Coronel Pacheco Experimental Station of Embrapa Gado de Leite, and were observed daily to assure that they were in perfect health conditions. Animals were allowed to adapt to the tannin-containing diet for 21 days, before sampling started (Smith & Mackie, 2004). All animals were provided with the assigned ad libitum feed every morning at 8:00h. The experimental diet was thoroughly mixed and fed to the animals. During the adaptation period, increased proportions of *P. rigida* or *Musa* materials were progressively added to the diet, until the planned 25%. Mineral mixture and water were available ad libitum.

Condensed tannin extracts were prepared and added to the culture media in the laboratory (Arcuri et al., 2003). Stems from *Mimosa artemisiana*, as well as banana leaves, were collected, immediately stored in the dark at 4°C at the Coronel Pacheco Experimental Station, and taken to the rumen microbiology laboratory. The samples were frozen in liquid nitrogen, and ground in a pre-chilled ceramic mortar and pestle. Tannins were extracted from plant samples (Hagerman, 1988). Variable amounts of plant material (10 to 25 g) were briefly suspended in 10 mL of 80% (v v^{-1}) aqueous ethanol per 1 g sample of dry matter, stirred for 30 min and stored overnight at 4°C. The suspension was filtered through Whatman filter paper nº 40 using slight vacuum. Condensed tannins were separated from low molecular weight tannins, other phenolics and pigments using 100 g Sephadex LH-20 dextran resin

(Pharmacia Biotech AB, Uppsala, Sweden) in 80% aqueous ethanol. After elution with 50% acetone, two extractions with ethyl acetate using Rotavapor (Büchi Company, Flawil, Switzerland), and lyophilization (Freeze Dry System, Labconco Corporation, Kansas City, Missouri, USA), the purified condensed tannin powders were stored at 4ºC in the dark.

Rumen contents were collected via cannulae, passed through sterile cheesecloth into sterile pre-warmed (40°C) thermos flasks, and taken in styrofoam boxes to the laboratory for bacterial isolation (Prado et al., 2010).

Standard anaerobic procedures were initially used to enrich tannin-tolerant populations (Odenyo et al., 2001; Arcuri et al., 2003). In order to do so, 20 mL aliquots of the rumen fluid were transferred into a sterile homogenizer under continuous flux of CO_2 to which 180 mL of the anaerobic dilution solution (ADS) (Arcuri et al., 2003) were added. The suspension was homogenized for 3 min under CO_2. From the homogenized suspension, 1 mL aliquots were immediately transferred into tubes (18 mm x 150 mm) (Bellco Glass, Inc., Vineland, New Jersey, USA) containing 9 mL ADS, and serially diluted down to 10^{-6}. From this dilution, 1 mL aliquots were transferred into 9 mL of GSM medium containing final concentrations of 1, 2 and 3 g L^{-1} of tannins (Odenyo et al., 2001) in 10 mL total volume. Tubes were incubated at 39°C for 48 hours. The tannin plus GSM medium procedure was replicated at least twice. Then, 0.1 mL aliquots from cultures grown in the presence of 2 or 3 g L^{-1} of condensed tannins were transferred to roll tubes. Roll-tubes used for isolation were prepared according to Odenyo et al. (2001). The medium contained (per 100 mL volume) 2.0 g of agar, 0.4 g of cellobiose, 0.2 g of tryptose, 0.1 g yeast extract, 0.05 g of glucose, 0.05 g of soluble starch, 30 mL of clarified rumen fluid, 5 mL of mineral 1, 5 mL of mineral 2, 0.1 mL resazurin (1%), 2 mL cysteine-sulfide (1.25%), 5 mL of $NaCO_3$ (8%), and 52.9 mL of distilled water. To increase the number of tannin-tolerant strains, 2 or 3 g L^{-1} of tannin extract were added. The tubes were incubated at 39ºC for three days (Nelson et al., 1997). Isolated colonies were picked under CO_2 and transferred into tubes containing glucose medium, and incubated at 39ºC overnight. Isolates were transferred back to GSM medium containing the same amounts of tannin extract to confirm their ability to grow in the presence of tannin extracts. Tubes were incubated at 39ºC for three days. Cultures from the latter tubes were transferred back into GSM liquid medium, and grown overnight at 39°C. Finally, 1 mL aliquots were transferred into sterile, anaerobic 20% glycerol solution, and stored at -80°C.

Bacterial isolates were characterized according to Nelson et al. (1997) and Odenyo et al. (2001), with some modifications, by Gram staining and motility (fresh aliquots diluted into ADS solution) through examination on phase-contrast microscopy (Leitz Laborlux, Leica Microsystems, Wetzlar, Germany). The growth rate of each isolate was determined in glucose medium, and the growth measured as a direct proportion to the increase in light absorbance at 600 nm (OD_{600} nm) every hour for eight hours. The ability of isolates to use different carbohydrates as carbon sources was tested by using the GSM medium, and substituting glucose with each of the following sugars: arabinose, cellobiose, dextrin, fructose, glucose, lactose, mannitol, maltose, raffinose, rhamnose, sucrose, trehalose, and xylose at 0.3% (w v^{-1}). Tubes were inoculated with 0.3 mL (from cultures grown into glucose-containing GSM tubes, OD_{600} nm = 0.8) of the test isolate, and incubated at 39ºC for up to 24 hours.

Bacterial DNA was extracted by thermal lysis (Nunes et al., 1999) after growth into GSM broth for 18 hours at 39ºC. Aliquots of 1 mL were centrifuged at 5,000 x *g* for 4 min. Pellets were rinsed three times with 500 µL TE buffer, pH 7.5 (10 mmol L^{-1} Tris-HCl pH 7.5; 1 mmol L^{-1} EDTA), resuspended into 100 µL TE, boiled for 10 min, and centrifuged at 12,000 x *g* for 2 min. Supernatants were stored at -20ºC.

The polymerase chain reaction was performed using a termocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA) programmed for initial cycle at 94ºC for 5 min, followed by 30 cycles (94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min), and final extension at 72ºC for 5 min. The reactions were carried out in 50 µL volume consisting of 1X PCR buffer, 1.5 mmol L^{-1} of $MgCl_2$, 10 µmol L^{-1} of each dNTPs, 20 pmol of each primer (20 µmol L^{-1}), 3 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, California, USA), and 100 ng of bacterial DNA, estimated by spectrophotometric analysis Nanodrop ND-1000, (Thermo Scientific Inc., Wilmington, Delaware, USA). Amplicons from the 16S rRNA gene of about 1,450 bp were obtained using universal primers FD

(5'-GAGTTTGATCCTGGCTCAG-3') and RD (5'-AAGGAGGTGATCCAGCC-3') (Odenyo et al., 2001). The PCR products were separated using 1.5% agarose gel (w v^{-1}), and dyed with ethidium bromide (0.005%, w v^{-1}). DNA bands were visualized under ultraviolet light, and photographed using the Eagle Eye II Photo-documentation System (Stratagene, La Jolla, California, USA). After this procedure, 10 µL of each amplification reaction were digested with *Alu*1 and *Taq*1 (Promega Co., Madison, Wisconsin, USA). Digestions were performed in a termocycler at 37ºC for four hours, followed by inactivation at 65ºC for 15 min, for *Alu*1, and at 80ºC for 20 min for *Taq*1

Restriction fragments were separated in 2.0% (w v^{-1}) agarose gel into TBE 1X (0.089 M Tris; 0.002 M EDTA, pH 8.0; 0.089 mol L^{-1} boric acid), under a 125 V field. Bands were dyed with ethidium bromide (0.005%, w v-1), and registered using Eagle Eye II Photo-documentation.

Sequencing of the 16S rRNA gene was performed based on Odenyo et al. (2001). The PCR products generated, as described above, were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, United Kingdom), and were submitted to sequencing reaction using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, Buckinghamshire, United Kingdom), and thermocycler GeneAmp PCR System 9700. Reaction products were purified by precipitation into isopropanol, and injected into the automatic sequencer MegaBACE 1000 DNA Analysis System (GE Healthcare, Buckinghamshire, United Kingdom). Near full-length 16S rDNA sequences (1,450 bp) were analyzed using the LaserGene package (DNASTAR, Madison, Wisconsin, USA). BLAST searches were performed from the GenBank database (http://www.ncbi.nlm.nih.gov) to identify the samples.

Results and Discussion

Feeding cows with a condensed tannins-containing diet allowed the isolation of tannin-tolerant bacterial populations, as described by Smith & Mackie (2004). The authors found that after three weeks of tannin diets the proportion of tannin-resistant bacteria increased significantly. A total of 117 strains were isolated from liquid media containing condensed tannin extracts. The present study characterized 10% of this collection, that is, 11 bacterial isolates, which were initially selected for their Gram reaction and ability to tolerate either 2 or 3 g L^{-1} of condensed tannins.

PCR products of about 1,450 bp of the 16S rRNA gene obtained from the 11 isolates were digested with the restriction enzymes *Alu*1 and *Taq*1. The restriction profiles of these 11 isolates, of both endonucleases, agreed well. These profiles grouped the 11 bacterial isolates into 6 different PCR-RFLP types, as shown in Table 1. Type II, which contained isolates 2, 3, 18, and

Table 1. Identification from sequences comparison (GenBank), morphology, gram staining, motility, carbohydrate fermentation ability and length fragment after digestion with *Taq*1 of the 11 bacteria isolates[(1)].

Isolate	Type	Genus	Morphology	Gram stain	Motility	Tannin	A	Cb	C	D	F	G	Gl	L	Mn	M	R	Rh	S	T	X	Length fragment
						(g L^{-1})																(bp)
1	I	*Butyrivibrio fibrisolvens*	Long rods in chains	Variable	No	3	±	+	-	+	+	+	+	±	-	+	+	-	+	+	+	750–600–200
2	II	Not identified	Long rods	Positive	No	3	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	800–350–230
3	II	Not identified	Diplococci	Positive	No	3	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	800–350–230
18	II	Not identified	Long rods	Positive	No	2	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	800–350–230
21	II	Not identified	Long rods	Positive	No	2	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	800–350–230
7	III	*Streptococcus bovis*	Diplococci	Positive	No	2	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	800–600–200
37	III	*Streptococcus bovis*	Diplococci	Positive	No	2	-	+	-	+	+	+	+	+	-	+	+	-	+	+	-	800–600–200
20	IV	Not identified	Short rods	Variable	High	2	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	800–650–550–350–200
86	V	*Butyrivibrio fibrisolvens*	Curved rods	Negative	High	2	+	+	-	+	+	+	+	+	nd	+	+	+	+	±	+	700–350–200–170
90	VI	Not identified	Curved rods	Negative	High	2	±	+	-	+	+	-	+	+	+	+	+	+	+	±	+	900–470
92	VI	Not identified	Curved rods	Negative	High	2	+	+	-	+	+	-	+	+	+	+	+	+	+	±	+	900–470

[(1)]A, Arabinose; Cb, Cellobiose; C, Cellulose; D, Dextrose; F, Fructose; G, Galactose; Gl, Glucose; L. Lactose; Mn, Mannitol; M, Mannose; R, Raffinose; Rh, Rhamnose; S, Sucrose; T, Trehalose; X, Xylose. -, no growth; ±, very low growth; +, fast growth; nd, not determined.

21, was predominat. Type III corresponded to isolates 7 and 37, whereas type VI grouped isolates 90 and 91. Isolates 1, 20 and 86 were distinct types I, IV and V, respectively.

Sequencing of the 16S rDNA PCR products allowed identification of the isolates belonging to types I and V as *Butyrivibrio fibrisolvens*. *B. fibrisolvens* is a Gram-positive bacterium that can stain Gram-negative (Yang et al., 2010). This nomenclature has been used to identify a genetically and functionally diverse group of metabolically ubiquitous bacteria, usually mobile when isolated from the rumen of cattle and sheep (Stewart et al., 1997; Boeckaert et al., 2008). This can explain the identification of strains type I (isolate 1) and type V (isolate 86) as the same species, in spite of their differences. Isolate 1 was described as long rods in chains, Gram variable (meaning that the Gram reaction could provide results according to the age of the culture), and nonmotile, whereas isolate 86 was described as curved rods, Gram-negative, and highly motile (that is, with fast movements), and resulted different in relation to their digestion profiles (Table 1). These isolates were able to tolerate condensed tannins in the medium – isolate 1 tolerated up to 3 g L^{-1}. This strain showed the slowest growth in glucose, with a longer lag phase, and did not grow to the same level as the other isolates (Figure 1). It also poorly fermented arabinose and lactose, and could not ferment cellulose, mannitol, and rhamnose. This indicates that isolate 1 may require some growth factor, which was not identified in this study.

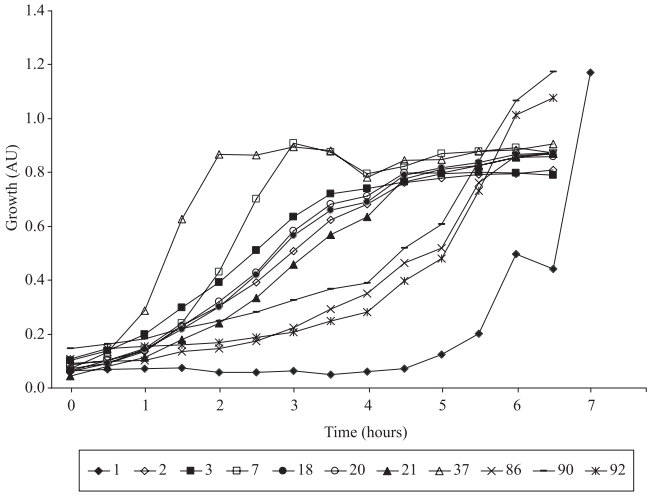

Figure 1. Growth of tannin-tolerant bacteria isolates on glucose medium measured as light absorbance at 600 nm. Numbers indicate original identification of each isolated strain.

Isolate 86, also identified as *B. fibrisolvens*, can grow better than isolate 1 in glucose. It poorly fermented trehalose, and, like the other isolates, was also not able to degrade cellulose. *Butyrivibrio* sp. is known as a genus that participates in a number of the most important biochemical processes in rumen and other digestive cavities, including cellulolysis, in variable extents. Other butyrate-producing species, which also have been described as pertaining to the *Butyrivibrio* group, do not have these biochemical abilities (Kopecny et al., 2003; Fukuda et al., 2005). *B. fibrisolvens* is described as one of the microorganisms that isomerize linoleic acid into the conjugated linoleic acid (CLA), a potent anti-carcinogenic and fat-modulator natural product, credited to be the fastest one (Fukuda et al., 2005). Vasta et al. (2010), using a quantitative experimental approach, also found that the *B. fibrisolvens* populations were higher in the rumen of lambs fed a tannin-supplemented diet than in the control diet. Due to tolerance to tannins and other polyphenolics, this group has been considered a potential candidate to be used as probiotics, because of its functional and nutraceutical properties, such as the increment of cis-9, trans-11 conjugated linoleic acid (Fukuda et al., 2006). Furthermore, Khiaosa-Ard et al. (2009) concluded, from their study on feeding condensed tannins to ruminants, that only the treatment containing condensed tannins suppressed methane formation and shifted microbial populations toward bacteria at the cost of protozoa.

Isolates 2, 18, and 21, described as long rods, showed the same fragment length profiles; however, they were grouped with isolate 3, described as diplococci. All stained Gram-positive, and were nonmotile. Isolates 2 and 3 tolerated 3 g L^{-1} of tannins. All type II isolates fermented the same carbohydrates, and grew in glucose in very similar ways (Figure 1). However, none of these isolates could be identified, which demonstrates that bovines kept in Brazil's southeastern region have ruminal microbial populations that are yet unknown. Therefore, the findings of this study reassure the importance of descriptive works to explore natural biodiversity, which could lead to innovative approaches to animal production in the tropics.

Isolates grouped into type III were identified as *Streptococcus bovis*. Isolates 7 and 37 were described as diplococci, Gram-positive and nonmotile, and tolerated only up to 2 g L^{-1} of condensed tannins. From

the 15 carbohydrates tested, isolate 7 did not ferment cellulose, whereas isolate 37 did not ferment arabinose, cellulose, mannitol, rhamnose, and xylose. Isolates grew differently in the presence of glucose, which was measured as a direct proportion to the increase in light absorbance at 600 nm (Figure 1). Isolate 37 showed the fastest growth in glucose in comparison to the other isolates studied, in which a sharp exponential growth was achieved in only two hours, followed by a relatively long stationary phase. This kind of growth is typical of *S. bovis*, and is considered one of its competitive advantages, used to overcome other species when soluble sugars are available in the rumen, causing acute acidosis (Russel & Strobel, 1989). The second fastest growth in glucose was achieved by isolate 7, which agrees with this characteristic of *Streptococcus* sp.. Regarding fermentation patterns, three divisions for *S. bovis* are described in literature (Ruoff et al., 1989); among these, the group biotype I is able to ferment mannitol, as isolate 7 from the present study. Tannin tolerance has been described for the genus *Streptococcus* (O'Donovan & Brooker, 2001). Becker et al. (2008) described the species *Streptococcus gallolyticus*, previously identified as *S. bovis* and presenting tannases, which was isolated from fecal samples of marsupials, such as koalas, known to feed on vegetables rich in polyphenolics, i.e., to tolerate and degrade gallic acid, a precursor to polyphenolics, including condensed tannins. *S. gallolyticus* (*S. caprinus*) was resistant in vitro to at least 7% (w v^{-1}) tannic acid and 4% (w v^{-1}) acacia condensed tannin, levels 10-fold greater than those tolerated by *S. bovis* (O'Donovan & Brooker, 2001). Similarly, Rusniok et al. (2010) described the ability of this species to adapt to the presence of tannins in the rumen, due to its capacity to use a broad range of carbohydrates, as found in the present study (Table 1), especially those present in the plant cell wall, and to express several hydrolytic enzymes, including tannases and other phenolic compounds decarboxylases that should contribute to the detoxification of the digestive tract. Nelson et al. (1997) also observed that *Streptococcus* was able to tolerate tannin. Therefore, the different fermentation patterns found among isolates 7 and 37, in the present study, may indicate a new species not previously described in literature. By using *Lotus corniculatus*, a condensed tannin-rich temperate forage as part of the diet, Molan et al. (2001) found that the average populations (after 8 to 120 hours) of *Clostridium proteoclasticum*, *B. fibrisolvens*, *Eubacterium* sp., and *S. bovis* decreased, in comparison to the control treatment without forage. This indicates that there may be a species-specific effect of condensed tannins on bacteria in the rumen, and that the type of condensed tannin may also specifically interfere with microorganisms.

Type IV included isolate 20, a short rod, Gram variable, highly motile strain. Regarding motility, this group was comparable to isolates 86, 90, and 92, and fermented all the tested carbohydrates, except cellulose. However, it did not tolerate more than 2 g L^{-1} of tannins. This isolate could not be identified by its 16S rDNA sequencing.

Type VI grouped two isolated strains described as Gram-negative, highly motile, curved rods. In the rat gastrointestinal environment, Smith & Mackie (2004) found that tannins selected for Enterobacteriaceae and Bacteroides species. The authors used dot-blot quantification to confirm that these Gram-negative bacterial groups predominated in the presence of dietary tannins, and that there was a corresponding decrease in the Gram-positive *Clostridium leptum* group and in other groups. Mechanisms for Gram-negative higher tolerance to tannins were suggested by Zoetendal et al. (2008), which are beyond the scope of this study. In the present study, the Gram-negative isolates could not be identified, but both tolerated up to 2 g L^{-1} of tannins, and could not ferment cellulose or galactose. Isolate 90 grew poorly in arabinose and trehalose, whereas isolate 92 grew poorly only in the latter. Figure 1 shows that, although isolate 90 grew better (i.e., had higher optical density) than isolate 92, throughout the entire growth assay using glucose, their inocula size was different and favored isolate 90, which may have caused the difference in their growth curves. These isolates could not be identified either. Therefore, the present study reinforces the need to increase research efforts to provide the basis for manipulating the complex gastro-intestinal microbiota.

Conclusions

1. Bovines fed tannin-containing diets harbor different bacterial populations, which are able to tolerate or degrade tannins.

2. Some isolates are not yet described by literature; from 11 sequenced isolates, two are identified as *Butyrivibrio fibrisolvens*, and two as *Streptococcus bovis*.

3. Isolate 1, identified as *B. fibrisolvens*, is able to tolerate up to 3 g L^{-1} of condensed tannins, an ability not previously described in the literature for this species.

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