Research in Veterinary Science 90 (2011) 26–30

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Pediococcus acidilactici isolated from the rumen of lambs with rumen acidosis, 16S rRNA identification and sensibility to monensin and lasalocid

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ARTICLE INFO

Article history:
Received 6 September 2008
Accepted 5 May 2010

Keywords:
Rumen acidosis
Pediococcus acidilactici
Rumen bacteria
Streptococcus bovis

ABSTRACT

A lactic-acid producing bacterium was isolated from the rumen of lambs with rumen acidosis. The cells were Gram-positive, nonmotile, nonsporing, catalase negative spherical, 1.5–2.0 μm in diameter, and occur in pairs and tetrads. Analysis of 16S ribosomal RNA indicated that the rumen bacterium was a strain of *Pediococcus acidilactici* with 99% of nucleotide homology. This bacterium was sensible to monensin and lasalocid at the unique dose tested of 300 ppm. The concentration of lactic acid and DM degradation decreased ($P < 0.05$) when monensin or lasalocid were added to the culture media after 24, 48 and 72 h of incubation. In contrast, total VFA concentration and pH were higher ($P < 0.05$) in the culture media added with the ionophores. Up to now *S. bovis* is considered the main ruminal bacterium related with rumen acidosis, but the importance of *P. acidilactici* should be also reconsidered in experimental studies focused on the control rumen acidosis.

1. Introduction

When ruminants are fed high-cereal grain diets, the rate of lactic acid and VFA production can be so fast that ruminal pH decreases. Ruminal acidosis depresses food intake and animal efficiency, and in extreme cases even causes death of the animal (Asanuma and Hino, 2002). Rumen acidosis is initiated by the proliferation of lactate-producing bacteria (Tung and Kung, 1993). The rumen bacterium *S. bovis* is of particular interest because of its role in the development of lactic acidosis in cattle and sheep fed an excess of starch (Whitford et al., 2001). However, it has been suggested that other rumen bacteria may be more important sources of lactate (Owens et al., 1998). In recent years, some rumen bacteria have been re-classified based on their 16S rRNA sequences. For example, *Bacteroides amylophilus* is now *Ruminobacter amylophilus* and *Bacteroides succinogenes* is now *Fibrobacter succinogenes* (Krause and Russell, 1996). The sensitivity of 16S rRNA methodology has been enhanced by the polymerase chain reaction, and new kits and equipments that make genetic methodologies for bacteria classification easier to use than phenotypic methodologies (i.e. gram stain, cell shape, motility, nutritional requirements and fermentation products). In the present study, we isolated a rumen bacterium from lambs with rumen acidosis; this bacterium shares phenotypical characteristics of the species *Streptococcus bovis*, but its 16S rRNA revealed a different classification.

2. Materials and methods

2.1. Source of lactate-producing rumen bacteria

Ten male lambs were fed a diet high in carbohydrates of easy fermentation (see Table 1). These animals had an average rumen pH of 6.4 before fed them with this diet. Rumen acidosis was considered as a rumen pH iqual or below 5.8 after 3 h post feeding. Most of the lambs (nine) started to present rumen acidosis 5 d after the exposure to the experimental diet. After 30 d, five of these lambs were selected as the source of rumen fluid for isolation of lactate-producing rumen bacteria.

The rumen fluid (300 mL) was obtained with an esophagus probe 3 h after morning feeding. The ruminal fluid was strained through three layers of cheesecloth and immediately used for inoculation of selective media for lactate-producing bacteria.

Streptocel broth and solid media (Dioxon) were used as enrichment media for isolation. These media contain (g/L): peptone from casein, 15.0 g; peptone from soy meal, 5.0; sodium chloride, 4.0; sodium citrate, 1.0; L-cystine,0.2; sodium sulfite, 0.2; D(+) glucose, 5.0; sodium azide, 0.2; crystal violet, 0.0002; the solid medium has also agar–agar (13.0 g). The media were prepared according with the company instructions; 30 g/L of distilled water were autoclaved 15 min at 121 °C and it was dispensed (4.5 mL) in 13 × 100 mm culture tubes while CO_2 was bubbled into the culture tubes.

Culture tubes containing 9 mL of Streptocel broth were inoculated by triplicate with 1.0 mL of rumen fluid of each lamb with ru-

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doi:10.1016/j.rvsc.2010.05.006

Table 1
Composition of the experimental diet.

Ingredient	g per kg (DM basis)
Sorghum	448
Corn	275
Soy bean meal	139
Corn silage	57
Corn stover	61
Mineral mix[a]	20
EM (Mcal kg^{-1})[b]	2.9
PC (%)[b]	12.0

[a] Contains (per 1000 g) calcium, 130 g; phosphorus, 50 g; sodium, 109 g, chlorine, 200 g; iron, 4.3 g; magnesium, 3.3 g; manganese, 0.2 g; copper, 80 mg; cobalt, 67 mg; iodine, 4 mg; zinc, 8 mg.
[b] According to NRC, Nutrient Requirements of Sheep, Sixth Revised Edition, 1985.

men acidosis. The tubes were incubated at 39 °C for 3 d, and then 1.0 mL were transferred to fresh Streptocel broth media and incubated in the same conditions. This procedure was repeated one more time in order to enhance the enrichment procedure. All inoculations were done inside a Class II biosafety cabinet (vertical flow, Labconco) under a CO_2 phase. Petri dishes with Streptocel solid medium (15 mL) were inoculated (by the pour-plate method) in triplicate with 0.1 mL of the third enrichment culture obtained. The Petri dishes were placed in an anaerobiosis jar with GasPack (hydrogen plus carbon dioxide generator) and incubated at 39 °C for 48 h.

2.2. Isolation of lactate-producing bacterium

By using disposable inoculating loops, colonies were isolated from Petri dishes with well-spaced colonies and placed in culture tubes containing 4.5 mL of Streptocel broth medium, and incubated at 39 °C for 48 h. Smears of isolates were stained using the gram method and observed microscopically in an Olympus contrast microscope at magnification 100X.

2.3. Lyophilization procedure

The pure bacteria obtained (on the basis of cell morphology and size, and Gram reaction) were pour-plated on solid medium and incubated at 39 °C for 48 h, the biggest colonies were transferred, with an inoculating loop into serum bottles containing 100 mL of Streptocel broth medium. The inoculated serum bottles were incubated at 39 °C for 72 h; then they were frozen at −10 °C for 48 h before freeze-drying. The samples were lyophilized in a freeze dry system (Labconco, Freezone, 6 L model) for 72 h at −40 °C under a pressure vacuum of 133×10^{-3} mBar.

From here, when samples of the isolated bacterium were required (for chemical or genetic analyses), they were obtained from the samples preserved by lyophilization.

In order to find out the required rehydration time of the lyiophilizedresp freeze-dried bacterium before start metabolic or genetic study; the lyophilized bacterium (0.1 g) was rehydrated during 4, 12 and 24 h at 39 °C in 9.9 mL of GCS–RF anaerobic broth medium, following the procedure described by Cobos et al. (2007) and Hungate (1969). After each period the viable bacteria concentration was estimated by the most probable number method (Harrigan and McCance, 1979) with three repetitions and dilution from 10^{-1} to 10^{-13}.

Molecular characterization of the rumen bacterium isolated. A pure culture of the rumen bacteria isolated and conserved by lyophilization, was hydrated (0.1 g in 9.9 mL of Streptocel broth medium, 24 h at 39 °C). This culture (0.1 mL) was pour plated in Petri dish containing Streptocel solid medium and incubated 48 h at 39 °C. The colonies obtained were used for DNA extraction.

DNA extraction was performed following the instructions of the "Wizard Genomic DNA Purification Kit" (Promega A 1120). Amplification of the 16S rRNA was carried out using the polymerase chain reaction method. The sequences of the primers were:

8F : AGAGTTTGATCMTGGCTCAG, and
1492r : TACGGYTACCTTGTTACGACTT

The amplification reaction was carried out using Taq DNA Polymerase in Storage Buffer B (Promega M1661). Reaction parameters were: 5 min cycle of pre-denaturation at 94 °C; denaturation 30 s at 94 °C; annealing 20 s at 52 °C; extension 90 s at 72 °C. After 34 cycles a final cycle of post-extension for 7 min at 72 °C. The amplified fragments were stained with ethidium bromide and observed by an agarose gel electrophoresis at 1% (Sambrook et al., 1989).

2.4. Phylogenetic tree

The purified PCR products were sequenced at the Service of Sequence of the Universidad Autonoma Metropolitana-Iztapalapa. The 800 bases sequence obtained was compared with sequences of the genus *Pediococcus, Lactobacillus, Aerococcus* y *Tetragenococcus, Enterococcus y Streptococcus* from the data base of the Gen Bank using the Blast program (Altschul et al., 1997).

Finally, the identified bacterium was phylogenetically analyzed using the Neighbor-Joining y Kimura 2 method (Saitou and Imanishi, 1989) and the Phylo_win program (Galtier et al., 1996), the grouping stability was estimated using a 1000 bootstrap replicates (Efron and Gong, 1983; Felsenstein, 1985).

2.5. Metabolic characteristics with or without ionophores

This study was performed in culture tubes (18 × 150 mm) containing 9 mL of glucose-rumen fluid broth medium (G-RF). The G-FR broth was prepared under anaerobic conditions following the procedures described by Cobos et al. (2007).The medium G-RF contains (each 100 mL): distilled water, 52.1 mL; clarified rumen fluid, 30 mL, mineral solution I, 5 mL; mineral solution II, 5 mL; sodium carbonate solution (0.8 g/L), 5 mL; resazurin solution (0.01 mL/L), 0.1 mL; tripticase-peptone, 0.2 g; yeast extract, 0.1 g; cystein–sulfide solution (0.25 g/L), 2 mL; and glucose, 0.5 g.

The treatments evaluated were: T1 = (control), inoculation of 0.5 mL of the isolated lactate-producing bacteria by triplicate in culture tubes (18×150 mm) containing 9.0 mL of G-FR broth medium and 0.25 g (dry matter basis) of the diet used to fed the lambs with ruminal acidosis; T2 = T1 + monensin, 30 ppm, and T3 = T1 + lasalocid, 40 ppm. The variables measured after 0, 24, 48 and 72 h of incubation were: dry matter degraded, pH, and volatile fatty acids (VFA), lactic acid, ammonia and bacteria concentration.

At the end of each incubation period; non degraded DM was recovered by filtration on Whatman paper No. 41, dried 24 h at 60 °C, and weighted to estimate percent of DM degraded. For VFA analysis, 5 mL of the incubated culture was centrifugated at 10,000 rpm for 10 min to precipitate small feed particles and bacteria, 2 mL of the supernatant was transferred to disposable microcentrifuge tubes (2.5 mL capacity) containing 0.5 mL 25% methaphosporic acid (4:1proportion), the sample was mixed with a vortex, after 30 min, the samples were centrifugated at 14,000 rpm for 10 min. Two mL of the supernatant was transferred to chromatographic vials and analyzed without further preparation. The VFA (acetate, propionate and butyrate) analysis was done in a Clarus 500 (Perkin–Elmer) chromatograph with flame ionization detector (FID) and auto sampler, with the following conditions: injection volume, 1 μL; column, 15m × 0.32 mm Elite capillary FFAP; oven, injector and column temperature, 80, 250