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A bacteriocin-mediated antagonism by ruminal lactobacilli against *Streptococcus bovis*

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Abstract

Cattle that were fed an all forage diet had higher numbers of *S. bovis* than lactobacilli (3×10^7 versus 4×10^3). Gradual adaptation of the cattle to 80% cereal grain caused only a modest decline in ruminal pH (always≥5.6), but there was a dramatic decrease in *S. bovis* and an increase in lactobacilli. The lactobacilli were more resistant to low pH than the *S. bovis* isolates, but pH alone could not explain the antagonism between ruminal *S. bovis* and lactobacilli. The ruminal lactobacilli were identified as *Lactobacillus fermentum*, a bacterium that produces a bacteriocin. Agar overlays, zones of clearing and batch culture growth experiments supported the hypothesis that *L. fermentum* was producing a bacteriocin that inhibited the growth of *S. bovis* in the rumen.

Keywords: Rumen; pH; Lactobacilli; *Streptococcus bovis*; Bacteriocin

1. Introduction

The interaction between ruminant animals and ruminal microorganisms is clearly symbiotic. The animal provides the microorganisms with food and a habitat for growth, and the microorganisms provide the animal with volatile fatty acids and microbial protein [1]. The rumen is well buffered by salivary secretions, but the ruminal pH can decline if the rate of fermentation is rapid. Ruminal acidosis is an ecological calamity that alters the types of microorganisms in the rumen, decreases the food intake, and in extreme cases even causes the death of the animal [2].

In the early 1950s, Hungate et al. [3] concluded that acute ruminal acidosis was caused by *Streptococcus bovis*. *S. bovis* normally accounts for less than 1% of the ruminal bacteria (approximately 10^7 cells/ml ruminal fluid), but it can dominate the population when soluble carbohydrates (e.g., starch or sugars) are plentiful. Homolactic fermentation produces very little ATP per hexose, but *S. bovis* has a very fast rate of fermentation and can generate more ATP per hour than other ruminal bacteria [1].

Animals can be gradually adapted to starch containing diets, and cross ruminal inoculation protected non-adapted animals from acute acidosis [4]. Mackie and Gilchrist [5] reported that adaptation increased the numbers of lactate-utilizing bacteria, but Allison [6] concluded that 'knowledge of... the adapted population is as yet incomplete and more data is needed.' Lactobacilli have often been impli-

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cated in ruminal acidosis, but they were generally assigned to a very acidic niche [7].

Many lactobacilli produce bacteriocins that inhibit other gram-positive bacteria [8], but the potential impact of bacteriocins on ruminal ecology has only recently received attention [9,10]. Preliminary experiments indicated that ruminal lactobacilli might be inhibiting the growth of *S. bovis*. Animals that were gradually adapted to starch never had a low ruminal pH, and there was a dramatic decrease in *S. bovis* and an increase in lactobacilli. The following experiments sought to define more precisely the nature of this antagonism.

2. Materials and methods

2.1. Cattle and diets

Four fistulated cows were assigned to two different diets. The forage diet was chopped timothy hay diet (9% crude protein, 32% neutral detergent fiber) and the cereal grain-forage diet was 80% cereal grain (16% crude protein, corn-soybean supplement, Agway Inc., Ithaca, NY, USA) and 20% chopped timothy hay. Each animal was fed 5 kg of diet twice a day, and the cattle were gradually transferred from the forage diet to the cereal grain-forage diet over a period of 10 days (10% change per day). Ruminal fluid was obtained approximately 2 h after feeding, and ruminal pH was measured immediately The ruminal fluid was squeezed through 8 layers of cheese cloth, transported to the lab, and incubated at 39°C for 30 min. Once the gas production had buoyed the feed particles, particle-free ruminal fluid was obtained from the middle of the flask.

2.2. Media

The minimal medium contained (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 240 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4 g of Na_2CO_3 and 0.3 g of cysteine hydrochloride (pH 6.7). MRS, a semi-selective medium for lactobacilli was adjusted to pH 5.4 [11]. The media were dispensed anaerobically into 18×150 mm tubes that were sealed with butyl rubber stoppers. Glucose (prepared anaerobically as a separate solution) was then added to achieve a final concentration of 10 mg/ml.

2.3. Enumeration and isolation

Ruminal fluid was diluted (10 fold increments) in triplicate on two different days ($n=6$) and spread onto the surface of minimal or MRS agar plates. The plates were anaerobically incubated at 39°C and colony-forming units were counted at 18 h. Sixty colonies (30 from each dietary treatment) were obtained from the minimal and MRS plates.

2.4. Bacteriocin production

Lactobacilli cultures from MRS plates (see above) were dotted on the surface of MRS agar plates and incubated anaerobically at 39°C in the glove box. Once colonies of approximately 3 mm diameter had formed, the plates were withdrawn from the glove box and overlayed with molten minimal medium agar (0.8%) that had been inoculated with approximately 10^6 *S. bovis* cells per ml. The plates were incubated aerobically for 16 h at 22°C, a temperature that does not allow *S. bovis* to grow. Once the bacteriocins had time to penetrate the agar, the plates

Table 1
Effect of diet and ruminal pH on the most probable number (MPN) of rapidly growing ruminal bacteria that could grow in minimal or MRS medium

Diet	Ruminal pH	MPN (per ml ruminal fluid)	
		Minimal	MRS
100% forage	6.8–6.7	3×10^7	4×10^3
80% cereal grain and 20% forage	6.0–5.6	2×10^3	5×10^7

The MPN were based on the dulicate samples from two animals ($n=4$).

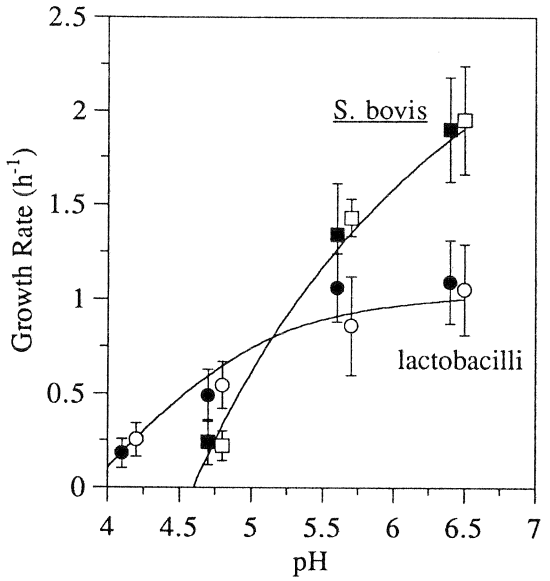

Fig. 1. The effect of pH on the growth of *S. bovis* (□, ■) and lactobacilli strains (○, ●) in vitro. The lactobacilli were grown in MRS and the *S. bovis* strains were grown in minimal medium that was supplemented with Trypticase (2 mg/ml). The open symbols show strains that were isolated from the cows fed only forage, and the closed symbols show strains that were isolated from cows fed cereal grain and forage. The bars show the standard deviations ($n = 15$).

returned to the glove box and incubated at 39°C for 6 h. Bacteriocin production was estimated from zones of clearing around the lactobacilli colonies.

Bacteriocin production was also estimated in anaerobic batch culture. *Lactobacillus fermentum* strain L8 was grown anaerobically in MRS broth (6 mg/ml glucose, 39°C, 24 h). The L8 cells were harvested by centrifugation (4000 × g, 22°C, 30 min), and the cell-free supernatant was discarded. L8 cells (approximately 400 μg protein/ml) and *S. bovis* inocula (approximately 15 μg protein/ml) were resuspended and incubated (39°C, 24 h) in minimal media lacking carbohydrate. Cellobiose (6 mg/ml) was then added, and the increase in optical density was determined (600 nm, 1 cm light path). Bacteriocin production was estimated from the difference in optical density (L8 plus *S. bovis* versus *S. bovis* alone).

2.5. *Streptococcus bovis strains*

Streptococcus bovis JB1 was isolated from a cow in Davis, CA, USA [12]. *S. bovis* 26 and K27FF4 were obtained from Colin Stewart, Rowett Research Institute, Aberdeen, Scotland and Albrecht Kistner, CSIR, Pretoria, South Africa, respectively [13].

2.6. *Other analyses*

Maximum specific growth rates were estimated from increases in optical density (600 nm, 18 mm tubes, 39°C). Fermentation products and glucose were assayed by high pressure liquid chromatography (Bio-Rad HPX-87H column, 0.017 N H_2SO_4 at a flow rate of 0.5 ml per min at 50°C, refractive index detector. D- and L-lactate were differentiated by enzymatic methods employing L- and D-lactate dehydrogenases [14].

2.7. *Statistics*

The effect of *L. fermentum* L8 bacteriocin *S. bovis* strains (gradually adapted to cereal grain versus only forage) were analyzed by a Students' *t*-test [15].

3. Results

3.1. *Most probable numbers of rapidly growing ruminal bacteria*

Cattle that were fed the forage diet had a constant and near neutral ruminal pH through the day (Table

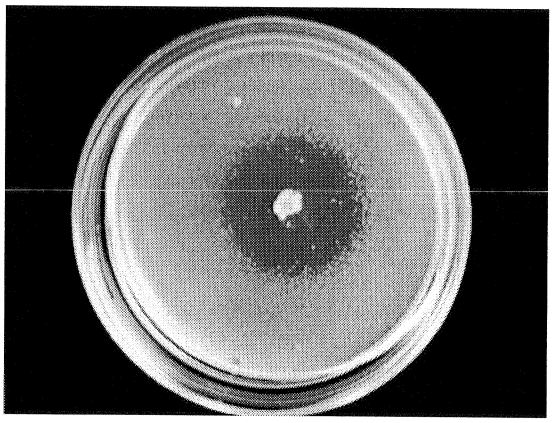

Fig. 2. The effect of *L. fermentum* L8 on the subsequent growth of *S. bovis* JB1 in a minimal medium agar overlay.

1). The ruminal fluid had 3×10^7 bacteria/ml that could grow in minimal medium after 18 h of incubation at 39°C. The number of ruminal bacteria that grew in MRS after 18 h of incubation at 39°C was only 4×10^3 per ml. Cattle that had been gradually adapted to the cereal-grain/forage diet had ruminal pH values that varied from 6.0 (prefeeding) to 5.6 (post-feeding) (Table 1). In this case, post-feeding plate counts for minimal medium and MRS were 2×10^3 and 5×10^7 per ml, respectively.

3.2. Taxonomy

All bacteria that grew rapidly on MRS plates were rod-shaped cells that produced a mixture of D- and L-lactate, acetate and ethanol. Pigment was never observed and final pH from glucose broth was approximately 3.7. Growth was observed at 39°C or 45°C. All of these bacteria could ferment glucose, sucrose, maltose, galactose, lactose, melibiose, xylose, arabinose, gluconate, and starch but not mannose, mannitol or cellobiose. Based on these taxonomic traits, all the rod-shaped bacteria appeared to be *Lactobacillus fermentum*.

All of the bacteria that grew rapidly on minimal plates were ovoid-shaped cells that produced a brilliant orange pigment and L- but not D-lactate. The final pH from glucose was approximately 4.2. Growth was observed at 39°C, 45°C but not 50°C and in the presence of 2% but not 6.5% sodium chloride. All of these bacteria could ferment glucose, cellobiose, lactose and starch but not xylose or arabinose. Based on these taxonomic traits, all of the ovoid-shaped bacteria appeared to be *S. bovis*.

3.3. pH resistance

Isolates presumptively identified as *S. bovis* grew even faster when the minimal medium was supplemented with Trypticase (2 mg/ml), but decreases in pH caused a decline in the maximum growth rate (Fig. 1). Isolates presumptively identified as *L. fermentum* never grew in minimal medium, but these bacteria were more resistant to decreases in pH than *S. bovis*. Bacteria from the cereal grain-forage fed cows were not more resistant to low pH than those from the forage-fed cows.

3.4. Bacteriocin production

L. fermentum isolates that had been grown anaerobically on glucose MRS plates, produced a diffusible substance that inhibited the growth of *S. bovis* (Fig. 2). All of the rod-shaped isolates produced zones of clearing, but the degree of growth inhibition varied. *S. bovis* JB1 was less susceptible than either *S. bovis* K27 or 26, and *L. fermentum* L8 seemed to cause the greatest and most consistent inhibition (Table 2).

Because *S. bovis* can utilize cellobiose as an energy source for growth and *L. fermentum* could not, it was possible to assay bacteriocin activity in anaerobic broth. When *L. fermentum* L8 was co-incubated with *S. bovis* strains for 24 h at 39°C, the subsequent

Table 2
The effect of ruminal lactobacilli on the growth of selected *Streptococcus bovis* strains

Lactobacilli strain	*Streptococcus bovis* strain		
	JB1	K27FF4	26
L1	+	+++	+++
L2	++	++	++
L3	+++	++	+++
L4	+	+	++
L5	+++	+	+++
L6	+++	+	+++
L7	−	+++	+++
L8	+++	+++	+++
L9	+++	+++	++
L10	−	+++	+
L11	+	+++	+
L12	+	+++	+
L13	+	+++	++
L14	−	++	++
L17	+	++	++
L19	+	++	++
L20	+	++	++
L21	+++	++	+++
L22	++	+++	+
L23	++	+++	+
L24	+	+++	−
L25	+++	++	+++
L26	−	++	+++
L27	−	+++	+++
L28	+	+	+++
L29	+	++	+++

The lactobacilli were grown on agar plates of MRS and these plates were overlayed with minimal medium containing approximately 10^6 *S. bovis* cells per ml. The zones of clearing were scored +++ (large zone) to − (no detectable zone).

growth of *S. bovis* on cellobiose was inhibited. Batch cultures of *S. bovis* JB1, K27FFA and 26 were inhibited 29, 43 and 14%, respectively. *S. bovis* strains from cows gradually adapted to cereal grain ($n=33$) and strains from cows receiving only forage ($n=24$) were both inhibited by L8, but there was considerable strain variation in each case ($41.5\pm25.9\%$ versus $51.6\pm23.5\%$, respectively)

4. Discussion

Animals receiving only forage had a near neutral ruminal pH, and the *S. bovis* count was 3×10^7, a value similar to that reported by Hungate [7]. Hungate noted a decline in *S. bovis* and an increase in lactobacilli when animals were severely acidotic, but this effect was explained by the 'ability of lactobacilli to grow in media too acid for streptococci' [7]. When our cattle were gradually adapted to cereal grain, *S. bovis* numbers declined 10 000-fold, but the ruminal pH was never less than 5.6. Since all of the *S. bovis* isolates grew rapidly at pH 5.6, pH alone could not explain the decline in *S. bovis* numbers.

Bryant et al. [16] found lactobacilli in the rumens of young calves, but mature ruminants had very few lactobacilli. When our animals were fed only forage, the number of rapidly growing lactobacilli was less than 10^4 per ml ruminal fluid. Gradual adaption to cereal grain caused a dramatic increase in lactobacilli. The ruminal lactobacilli were more resistant to low pH than the *S. bovis* isolates, but they only grew faster than *S. bovis* at pH values less than 5.15. This latter comparison supported the idea that some factor other than pH was affecting the relative success of these lactate-producing bacteria.

The substitution of cereal grain for hay would have decreased the supply of ruminally degraded amino acids [17]. The lactobacilli had an obligate requirement for peptides and amino acids, but the *S. bovis* isolates could utilize ammonia as a sole nitrogen source. Because the ruminal fluid never had less than 5 mM ammonia (data not shown), the inhibition of *S. bovis* could not be explained by nitrogen limitation.

Agar overlays indicated that the lactobacilli produced a diffusible substance that inhibited the growth of previously isolated *S. bovis* strains. Because these *S. bovis* strains had been isolated from different parts of the world, it appeared that this inhibitory factor was not highly strain specific. The lactobacilli differed in their ability to inhibit *S. bovis* and create a zone of clearing, and strain L8 seemed to be the most potent.

The idea that ruminal lactobacilli were producing a bacteriocin was corroborated by anaerobic batch culture growth experiments. When *L. fermentum* L8 cells were co-incubated with *S. bovis* JB1, K27FFA, and 26, the growth of *S. bovis* on cellobiose was inhibited. L8 seemed to inhibit *S. bovis* strains from cows receiving only forage (pH 6.7) to a greater extent than cows gradually adapted to cereal grain (pH 6.0–5.6), but strain variation precluded a statistically significant difference ($P>0.05$).

The inability of the L8 bacteriocin to completely inhibit the subsequent growth of *S. bovis* batch cultures is consistent with the bacteriocin mode of action. Bacteriocins translocate ions (presumably protons) across the cell membrane and dissipate protonmotive force [8]. When *S. bovis* was exposed to increasing amounts of monensin, an ionophore that is sodium proton antiporter, non-growth energy dissipation increased, and the cells grew less efficiently [18]. Optical density always decreased, but only very high concentrations of monensin completely inhibited growth.

Previously isolated ruminal lactobacilli were identified as *Lactobacillus vitulinus* or *Lactobacillus ruminis* [19], but these species produce only L-lactate and are unable to ferment xylose. A variety of lactobacilli can produce D- as well as L-lactate, but most lactobacilli do not produce large amounts of acetate and ethanol when glucose is the fermentation substrate [20]. The ability of the ruminal lactobacilli to ferment xylose narrowed the taxonomic classification to *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus collinoides*, *Lactobacillus hilgardii*, *Lactobacillus vaccinostercus*, and *L. fermentum*, but only *L. fermentum* is able to grow at 45°C. Some strains of *Lactobacillus confusus* can grow at 45°C, but none of these strains can utilize lactose.

L. fermentum has been isolated from yeast, milk products, sour dough, fermenting plant material, manure, sewage and the mouth and feces of man [20]. Krogh [21] isolated lactobacilli from the rumen that resembled *L. fermentum*, but these strains did

not ferment arabinose. Based on this criterion, Krogh [21] indicated that these strains were 'intermediates between *L. brevis* and *L. fermentum*.' Krogh's isolates did not ferment starch, but all of our strains grew rapidly on this carbohydrate.

DeKlerk and Smit [22] characterized a bacteriocin from *L. fermentum* that could inhibit the growth of other lactobacilli, but this bacteriocin was a complex structure that had both lipocarbohydrate and proteinaceous components. The bacteriocin was inactivated by either trypsin or pepsin but was stable at 96°C for 30 min. The bacteriocin was purified by dialysis and column chromatography, but separation of the lipocarbohydrate and protein components caused an almost complete loss of activity. Culture supernatants that were diluted more than 4-fold did not show bacteriocidal activity, but the ratio of free and cell-bound bacteriocin was not determined.

Ionophores have been used as ruminal additives since the 1970s, and these compounds inhibit gram-positive ruminal bacteria [23]. The ability of ruminal ionophores to increase feed efficiency and animal performance has remained relatively stable, but ionophores can be toxic to animals [24]. Bacteriocins also show maximal activity against gram-positive bacteria, and nisin, the best characterized bacteriocin, has been approved as a human food additive [8]. Further work is needed to see if the *L. fermentum* bacteriocin has activity against other gram-positive ruminal bacteria.

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