RESEARCH LETTER

Bovicin HC5 inhibits wasteful amino acid degradation by mixed ruminal bacteria *in vitro*

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Abstract

Streptococcus bovis HC5 produces a broad spectrum lantibiotic (bovicin HC5) that inhibits pure cultures of hyper ammonia-producing bacteria (HAB). Experiments were preformed to see if: (1) *S. bovis* HC5 cells could inhibit the deamination of amino acids by mixed ruminal bacteria taken directly from a cow, (2) semi-purified bovicin was as effective as *S. bovis* HC5 cells, and 3) semi-purified and the feed additive monensin were affecting the same types of ammonia-producing ruminal bacteria. Because purified and semi-purified bovicin HC5 was as effective as *S. bovis* HC5 cells, it appeared that bovicin HC5 was penetrating the cell membranes of HAB before it could be degraded by peptidases and proteinases. Mixed ruminal bacteria that were successively transferred and enriched nine times with trypticase did not become significantly more resistant to either bovicin HC5 ($50\,AU\,mL^{-1}$) or monensin ($5\,\mu M$), and amplified rDNA restriction analysis indicated that bovicin HC5 and monensin appeared to be selecting against the same types of bacteria.

Introduction

In ruminants, feedstuffs are subjected to fermentation in the rumen before intestinal degradation (Hungate, 1966). This fermentation gives them the ability to digest cellulose, but some products (ammonia and methane) are deleterious. Ruminal amino acid deamination robs amino acids from the animal because the rate of ammonia production is often greater than the amount that can be used to synthesize microbial protein (Nocek & Russell, 1988). Nutritionists have combated this degradation using proteins that are naturally insoluble or using heat treatments that decrease solubility. However, these approaches increase the cost of the ration significantly (Ferguson, 1975). The ionophore, monensin, can also decrease amino acid degradation (Russell & Strobel, 1989; Yang & Russell, 1993), but the European Union banned the use of this antibiotic as a feed additive in 2006 (Russell & Houlihan, 2002).

Early studies suggested that carbohydrate-fermenting bacteria accounted for most of the ammonia produced in the rumen, but even the most active pure cultures had rates of amino acid deamination that were tenfold lower than mixed ruminal bacteria (Bladen *et al*., 1961). In the 1980s, some obligate amino acid-fermenting bacteria were isolated from the bovine rumen that had very high rates of ammonia production (Paster *et al*., 1993). More recently, Attwood and colleagues showed that wild and domesticated ruminants in New Zealand had additional hyper ammonia-producing bacteria (HAB), but their properties were similar to the first three isolates (Attwood *et al*., 1998).

All but one HAB (*Fusobacterium necrophorum*) are Gram-positive bacteria (Paster *et al*., 1993; Attwood *et al*., 1998), and *in vitro* experiments indicated that they could be inhibited by nisin, a lantibiotic produced by *Lactococcus lactis* (Callaway *et al*., 1997). However, the addition of nisin to the diet of a cow did not decrease ruminal ammonia or protect amino acids, and it became apparent that ruminal bacteria could rapidly become nisin resistant (Russell & Mantovani, 2002). *Streptococcus bovis* HC5 produces a lantibiotic (bovicin HC5) that can inhibit bacteria that are nisin-resistant, and pure culture experiments indicated that bovicin HC5 could inhibit HAB *in vitro* (Mantovani & Russell, 2002).

The following experiments were designed to see if: (1) *S. bovis* HC5 cells could inhibit the deamination of mixed ruminal bacteria taken directly from a cow, (2) semi-

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purified bovicin was as effective as *S. bovis* HC5 cells, and (3) semi-purified bovicin and the feed additive monensin were affecting the same types of ammonia-producing ruminal bacteria.

Materials and methods

Microorganisms and growth

Streptococcus bovis JB1 and *S. bovis* HC5 were grown anaerobically in a culture broth (39 °C) as previously described (Mantovani *et al*., 2002). The mixed ruminal bacteria were grown anaerobically in a basal medium containing salts, vitamins, and minerals (Cotta & Russell, 1982). When indicated, the cultures were supplemented with trypticase (15 mg mL^{-1}). The basal medium was prepared under O_2-free CO_2 and was dispensed into anaerobic bottles (30 mL) that were sealed with butyl rubber stoppers. The media pH was adjusted to 6.7 with NaOH.

Mixed ruminal bacteria

Inocula were obtained from fistulated dairy cows (*c*. 480 kg body weight) fed *Brachiaria decunbens* hay (6.5% crude protein, 40.7% acid detergent fiber, and 72.7% neutral detergent fiber) *ad libitum* and 1.0 kg of corn silage once daily. Mixed ruminal contents (2 h after feeding) were strained through four layers of cheesecloth and incubated at 39 °C for 20 min. Once the feed particles had been buoyed to the top of the flask by fermentation gases, mixed ruminal bacteria were obtained from the center of the flask. The mixed rumen bacteria were harvested anaerobically by centrifugation (1710 ***g***, 10 min, 25 °C) and washed in basal media. The pellet was resuspended and the OD was adjusted to 1.6. The pH was adjusted to 6.7 with NaOH (1 M).

Bacteriocin preparation

The procedure of Yang *et al*. (1992) was used to dislodge bovicin HC5 from the surface of stationary phase *S. bovis* HC5 cells, and this procedure is now commonly used to extract large amounts of bacteriocins from lactic acid bacteria. Experiments using preparative scale HPLC methods (C-18 column, 6.0 × 150 mm, 5 μm Shimadzu, 0.1% trifluoroacetic acid, 22 °C), similar to those previously described (Mantovani *et al*., 2002) indicated that the procedure of Yang *et al*. (1992) yielded only one peak with antimicrobial activity. Briefly, stationary phase *S. bovis* HC5 cultures (1 L, *c*. 400 μg mL^{-1}) were harvested by centrifugation (1710 ***g***, 10 min, 4 °C). The cell pellets were washed and resuspended in acidic sodium chloride (100 mM, pH 2.0, 39 °C, 2 h). The cell suspensions were then recentrifuged (1710 ***g***, 10 min, 4 °C), and Tween 80 (0.1% v/v) was added in the cell-free supernatant. Previous work suggested that *S. bovis* HC5 cultures grown with Tween 80 had more cell-free bovicin HC5 than untreated controls, but subsequent experiments showed that this nonionic detergent only augmented activity of the cell-free bacteriocin, presumably by enhancing its dispersion (Houlihan & Russell, 2006). Preliminary experiments indicated that the concentration of Tween 80 remaining in the semi-purified preparation of bovicin HC5 (< 0.03% final concentration) had no effect on the growth or ammonia production of any of the HAB. The semi-purified preparation of bovicin HC5 was assayed for antibacterial activity by serially diluting the extract in distilled water (twofold increments), and placing each dilution (50 μL) in agar wells. The agar was inoculated with *L. lactis* American Type Culture Collection 19435 (*c*. 10^6 CFU mL^{-1}). Activity units (AU mL^{-1}) were calculated from the reciprocal of the highest dilution showing a visible zone of clearing.

Ammonia production and specific activity

Mixed ruminal bacteria were resuspended (20 mL, $OD_{600\,nm}$ *c*. 1.6) in anaerobic basal medium to determine deamination activity. The culture broth was supplemented with 15 mg mL^{-1} trypticase and bottles were incubated (39 °C) for 6 h to determine the initial rate of ammonia production, or 24 h to determine total ammonia accumulation. The specific activity of ammonia production was based on the change in ammonia and cell protein after 6 h of incubation. The ammonia production was determined in cell-free supernatants (8000 ***g***, 5 min, 5 °C) using a colorimetric assay (Chaney & Marbach, 1962). Six times as much reagent was used to overcome cysteine interference, and ammonium chloride was used as the standard. Cells harvested by centrifugation (8000 ***g***, 5 min, 5 °C) were digested with dilute NaOH (0.2 N, 100 °C, 10 min) and assayed by the method of Lowry *et al*. (1951).

Enrichment and selection of amino acid-fermenting bacteria

Mixed ruminal bacteria that had been harvest by centrifugation (1710 ***g***, 15 min) were serially diluted (tenfold increments) into anaerobic basal media (10 mL) and incubated at 39 °C for 24 h. Final $OD_{600\,nm}$ was measured and the ammonia concentration was determined as described above. Ammonia-producing bacteria were enriched by subsequent transfers (10% inoculum, v/v) in a medium containing trypticase (15 mg mL^{-1}). To verify the effect of inhibitors on ammonia accumulation, some enrichments contained either monensin (5 μM) or semi-purified bovicin HC5 (50 AU mL^{-1}).

Amplified rDNA restriction analysis (ARDRA)

Mixed ruminal bacteria were harvested by centrifugation (1710 ***g***, 15 min). Total DNA was isolated using basic

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protocols for extraction of bacterial DNA (Ausubel *et al.*, 1997). 16S rRNA genes were amplified using the primers 16SF (5′-AGAGTTTGATCMTGG-3′) and 16SR (5′-TAC CTTGTTACGACTT-3′). The PCR protocol had an initial denaturation step (1 min, 94 °C) followed by 36 cycles of denaturation (1 min, 94 °C), annealing (1 min, 55 °C), and extension (1 min, 72 °C) in a Sprint Thermocycler (PTC 100 Programmable Thermal Controller, MJ Research Inc.) using GoTaq DNA polymerase (Promega®, Madison, WI). After a terminal extension step (7 min, 72 °C), samples were kept at 15 °C for 10 min. The PCR products were digested with HaeIII (Promega) for 5 h (37 °C). Restriction fragments were separated on Sigma® High Resolution agarose A-8455 (2.5%, w/v) at 80 V and stained with ethidium bromide.

Statistical analysis

All incubations and determinations were performed two or more times, in triplicate. When error bars are given, they refer to the SD.

Results

Effect of *S. bovis* cells

When mixed, ruminal bacteria were harvested by centrifugation and resuspended in basal media (850 μg cell protein mL^{-1}); the initial rate of ammonia production (0–6 h) from trypticase (15 mg mL^{-1}) was *c.* 25 nmol NH_3 min^{-1} mg^{-1} protein (Fig. 1a). The addition of *S. bovis* JB1 cells, a non-bacteriocin-producing strain, had little, if any, impact on the ammonia production rate, but even relatively small amounts of *S. bovis* HC5 cells (16 μg cell protein mL^{-1}) caused as much as a 40% decrease in specific activity. Total ammonia accumulation after 24 h of incubation was also affected by *S. bovis* HC5 but not *S. bovis* JB1 cells (Fig. 1b). Cultures containing *S. bovis* JB1 cells (16 μg cell protein mL^{-1}) that were added with 256 AU mL^{-1} of purified bovicin HC5 showed a decrease in specific activity and total ammonia accumulation similar to the ones added with *S. bovis* HC5 cells (Fig. 1). Ammonia production from *S. bovis* JB1 or HC5 alone was < 5 mM (data not shown).

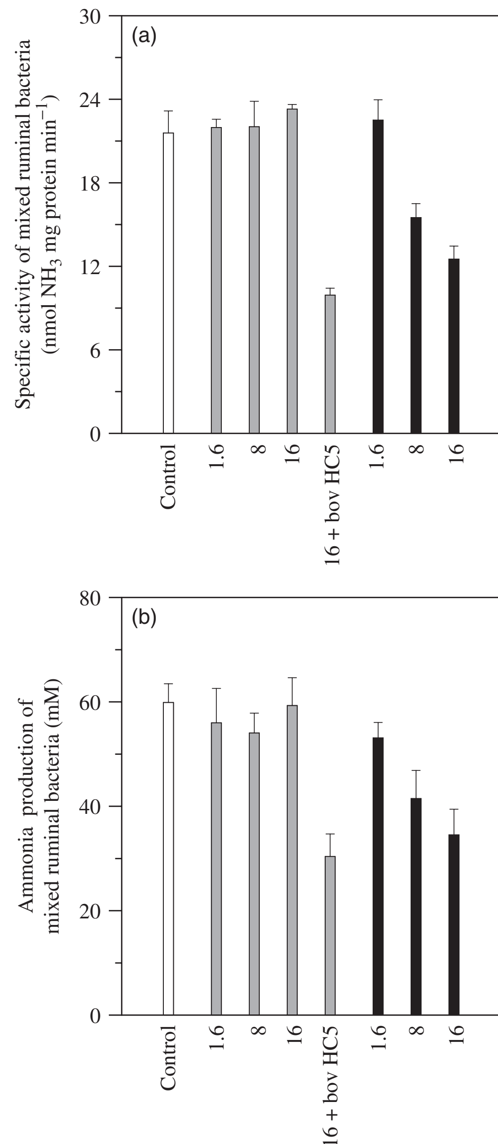

Fig. 1. The effect of *Streptococcus bovis* JB1 (gray bars) and *S. bovis* HC5 (black bars) on the specific activity of ammonia production (a) and ammonia accumulation (b) of mixed ruminal bacteria. In each case, trypticase was added as an amino acid source (15 mg mL^{-1}). Control without *S. bovis* is shown as open bar. Numbers at the bottom indicate the amount of *S. bovis* cell protein (μL mL^{-1}) added. Cultures containing *S. bovis* JB1 cells added with 256 AU mL^{-1} of purified bovicin HC5 are also indicated (16+bov HC5).

Effect of semi-purified bovicin HC5 and monensin

When bacteriocin extracts were applied to a reverse phase HPLC C-18 column, a main absorbance peak showing antimicrobial activity was observed, which confirmed the homogeneity of the bacteriocin preparation (Fig. 2). Semi-purified bovicin HC5 was also able to inhibit the initial rate of ammonia and total ammonia accumulation after 24 h of incubation, and the greatest effects were observed when the amount of bovicin was < 50 AU mL^{-1} (Fig. 3a). Low concentrations of monensin (< 3 μM) had a greater impact on initial rate of ammonia production than total ammonia accumulation, and at least 4 μM was needed to get the same decrease as the one observed with 50 AU mL^{-1} of semi-purified bovicin HC5 (Fig. 3b).

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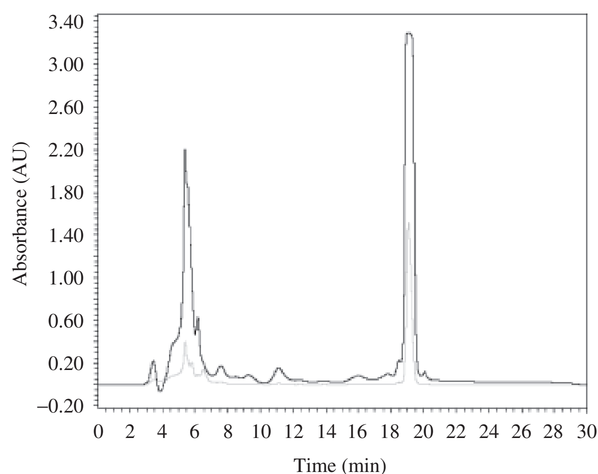

Fig. 2. Chromatogram of a reverse-phase HPLC analysis of bovicin HC5 extracts. Bovicin HC5 extracts were applied to a C-18 column and the elution was carried out with 35–50% linear gradient of acetonitrile and water containing 0.1% trifluoroacetic acid. Flow rate was 1 mL min^{-1}. Absorbance was measured at 214 nm (upper line) and 280 nm (bottom line).

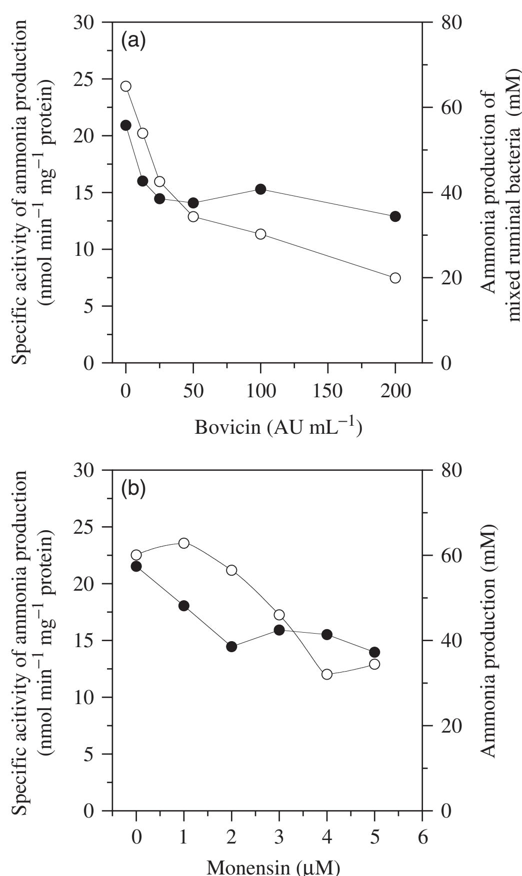

Fig. 3. The effect of semi-purified bovicin HC5 (a) and monensin (b) on the ammonia production of mixed ruminal bacteria showing specific activity of ammonia production (open circles) and ammonia accumulation after 24 h (closed circles). Trypticase was added as an amino acid source (15 mg mL^{-1}).

Enrichment of ammonia-producing bacteria

When mixed ruminal bacteria were transferred sequentially (10% v/v) on a daily basis in basal medium containing only trypticase (15 mg mL^{-1}) as an energy source for growth, the ammonia production gradually increased from 40 to > 60 mM (Fig. 4a), and the OD was 0.9 or greater (Fig. 4b). If the transfers contained either bovicin HC5 (50 AU mL^{-1}) or monensin (5 μM), the ammonia production was always < 30 mM (Fig. 4a), and the final OD (after nine transfers) was < 0.5 (Fig. 4b). ARDRA indicated that bovicin HC5 and monensin were selecting against the same types of bacteria (Fig. 5).

Discussion

The ionophore, monensin, has been used as a growth-promoting antibiotic for > 30 years in the beef cattle industry (Russell & Strobel, 1989), and the FDA has also approved its use in lactating dairy cattle (Fairfield *et al.*, 2007). The benefit of monensin was originally linked to its ability to inhibit hydrogen-producing bacteria and the subsequent decreases in the production of methane, but this effect on ruminal fermentation only explains approximately one-third of its benefit (Russell & Strobel, 1989). Early work indicated that monensin could also decrease ammonia accumulation *in vitro* and *in vivo* (Richardson *et al.*, 1976). The idea that monensin could spare amino acids from wasteful ruminal degradation was supported by the observation that improvements in the feed efficiency of beef-lot cattle were only observed if the animals were growing rapidly (large needs for amino acids), and the diets had been supplemented with soybean meal rather than urea (Lana *et al.*, 1997).

Monensin resistance initially appeared to be correlated with the presence of an outer membrane (Russell & Strobel, 1989). However, it should be noted that some Gram-negative bacteria need a period of adaptation before a high degree of monensin resistance is observed, and some Gram-positive bacteria are relatively monensin resistant (Callaway & Russell, 1999, 2000; Callaway *et al.*, 1999; Rychlik & Russell, 2002). Adaptation and selection experiments indicated that monensin resistance was associated with an accumulation of extracellular polysaccharide, and this hydrophilic material has the ability to exclude the hydrophobic monensin molecule (Callaway & Russell, 1999; Rychlik & Russell, 2002). *Clostridium aminophilum* has the ability to become at least moderately monensin resistant (Krause & Russell, 1996). Because *C. aminophilum* did not become resistant to the bacteriocin of *Butyrivibrio fibrisolvens* (Rychlik & Russell, 2002) or bovicin HC5 (Mantovani &

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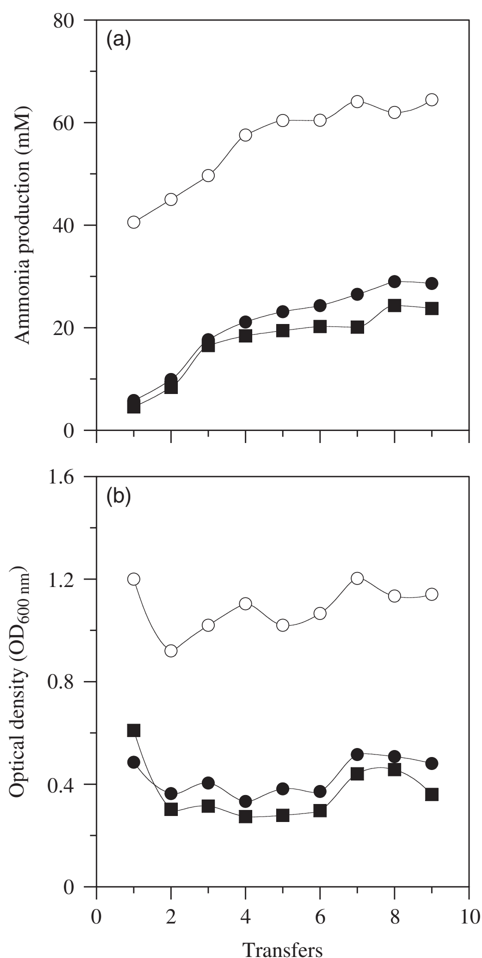

Fig. 4. The enrichment of amino acid-fermenting ruminal bacteria (open circles) and the effects of monensin (closed squares) and semi-purified bovicin HC5 (closed circles) on ammonia accumulation (a). Cultures (10% inoculum, v/v) were transferred at 24 h intervals. Bovicin HC5 and monensin were used at 50 AU mL^{-1} and 5 μM, respectively, and trypticase was added as an amino acid source (15 mg mL^{-1}).Optical densities are shown in (b).

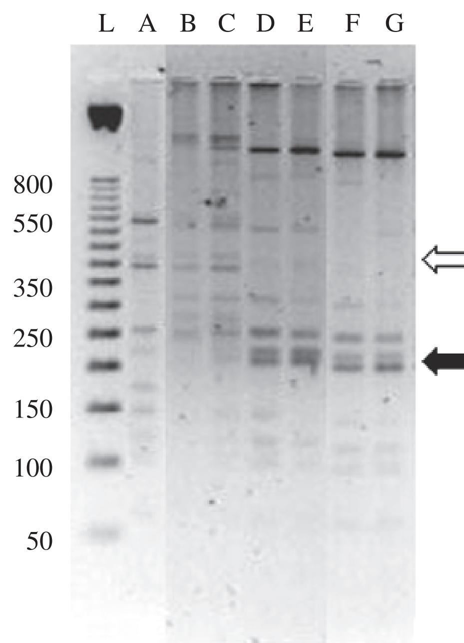

Fig. 5. Agarose gel electrophoresis showing the amplified 16S rRNA gene restriction analysis of mixed ruminal bacteria that were transferred with bovicin HC5 (50 AU mL^{-1}) and monensin (5 μM). Bovicin HC5 was 50 AU mL^{-1} and monensin was 5 μM. The open arrow shows bands that decreased after bovicin HC5 or monensin was added. The closed arrow shows bands that increased after bovicin HC5 or monensin was added. L, DNA ladder, 50 bp DNA (Promega); A, before enrichment; B, control after five transfers with trypticase; C, control after nine transfers with trypticase; D, bovicin HC5 after five transfers with trypticase; E, bovicin HC5 after nine transfers with trypticase; F, monensin after five transfers with trypticase; G, monensin after nine transfers with trypticase.

Russell, 2002), it appeared that bacteriocin and monensin resistance were not necessarily correlated.

Krause & Russell (1996) used rRNA probes to detect *Peptostreptococcus anaerobius*, *Clostridium Sticklandii*, and *C. aminophilum* in the ruminal fluid of a cow fed timothy hay, and each species accounted for *c.* 1% of the rRNA. Attwood *et al.* (1998) also detected *P. anaerobius*, but they did not find *C. sticklandii* or *C. aminophilum* and identified several other HAB in wild and domestic ruminants from New Zealand. These results indicate that HAB are diet dependent, and it is unlikely that all HAB have been cultured.

A key factor affecting HAB could be cereal grain and bacteriocin-producing bacteria. A mathematical model based on the kinetics of ammonia production indicated that HAB numbers in cattle fed timothy hay could be as high as 10%, but cattle fed 90% cereal grain had ammonia production rates so low that HAB were not needed (Rychlik & Russell, 2000). The potential involvement of bacteriocins was supported by the observation that mixed bacteria from cows fed 90% grain strongly inhibited a triculture of HAB *in vitro*, but no inhibition was seen with mixed bacteria from the cows fed timothy hay.

Because cell-free bacteriocins can be degraded by extracellular peptidase and proteinases (Jack *et al.*, 1995; Mantovani *et al.*, 2002), it is not surprising that cell-free bacteriocins cannot be readily detected in ruminal fluid. However, recent work with bovicin HC5 indicates that cell-associated activity is at least as potent as the cell-free activity, and it is much more resistant to degradation (Xavier *et al.*, 2008). Given these latter results, we first chose to examine the effect and of *S. bovis* HC5 cells on the amino acid deamination and ammonia accumulation of mixed ruminal bacteria (Fig. 1). Results indicated an inhibition by *S. bovis* HC5 cells as great as the one observed with the feed-additive monensin (5 μM), and similar results were attained if

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purified bovicin HC5 was added to cultures containing *S. bovis* JB1 cells, a non-bacteriocinogenic strain. Moreover, semi-purified bovicin HC5 alone (50 AU mL^{-1}) was able to inhibit the ammonia production of our mixed ruminal bacteria (Fig. 3), and this result indicates that bovicin HC5 penetrated the cell membranes of sensitive bacteria before it was degraded.

The ability of bacteria to be selected or become resistant to antibacterial agents is well known, but mixed ruminal bacteria that were successively transferred and enriched nine times with trypticase did not become significantly more resistant to either bovicin HC5 (50 AU mL^{-1}) or monensin (5 μM). The ammonia accumulations of the treated cultures were always much lower than untreated controls (Fig. 4), and ARDRA indicated that bovicin HC5 and monensin appeared to be selecting against the same types of bacteria. Bacteria that persisted in the bovicin HC5- and monensin-treated cultures had approximately the same ARDRA pattern. However, it should be noted that the positions of the bands in lanes D and E of Fig. 5 are slightly different from the bands in lanes F and G. Hence, one cannot say for sure that bovicin and monensin are selecting for the same type of bacteria. Further work will be needed to define these latter bacteria, but *Megasphaera elsdenii* is a likely candidate. *Megasphaera elsdenii* is not an obligate amino acid-fermenting bacteria or HAB *per se*, but it is highly monensin resistant and can grow on serine and threonine (Rychlik *et al.*, 2002).

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