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USE OF ENDOGENOUS BACTERIOCIN TO MANIPULATE RUMEN MICROBIAL ECOLOGY

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CRC SUGAR Industry Innovation through Biotechnology

Research Project Final Report

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1.0 SUMMARY

Ionophore antibiotics, such as monensin, are used routinely in the meat and livestock industry to improve animal health and growth. They are used particularly in feedlots, which are a growing feature of the industry. There is significant consumer concern regarding the use of antibiotics and their role in the emergence of drug resistant pathogens.

Bacteriocins produced by the rumen bacterium *Streptococcus bovis* were pursued as a possible source of endogenous antibacterial agents which could supplant the use of antibiotics in the cattle industry. Bacteriocins have the potential to project the same benefits as antibiotics without the negative impact on public perception, human health, animal health, and environmental concerns.

The chosen bacteriocin-producing *S. bovis* isolate (Sb15) was found to have a spectrum of activity similar to that of monensin. Methods for improving the consistency and levels of bacteriocin production were developed. A protocol for quantifying the concentration of bacteriocin was developed and used to verify that the level of activity of the bacteriocin produced by the wild-type isolate is equivalent to that of the commercially produced bacteriocin nisin. The bacteriocin's thermostability, resistance to proteases and activity spectrum marks it as being suitable for use in manipulating the rumen environment. Sequencing of the purified protein indicated that it was essentially identical to the well-characterised HC5 bacteriocin; however several amino acid residues could not be resolved and so the level of identity cannot be fully determined.

The fact that the identified bacteriocin is near-identical to HC5 reduces the attractiveness of this project to large agrichemical companies, who otherwise would see broader applications for the bacteriocin. Since the use of HC5 is not patented, however, it does not prevent its local development for the feedlot industry.

An economic evaluation confirmed *a priori* assumptions that batch fermentation with a wild-type strain would not be competitive compared to monensin. However, bacteriocin production from *S. bovis* fermentation on sugar mill streams can be competitive, if

1. The specific productivity is increased 5-fold through strain engineering;
2. The final biomass density is increased 10-fold by implementing a fed batch process with lactic acid removal using electrodialysis; and
3. The production is greater than 100 tonnes per annum

Attempts to enhance bacteriocin production through random mutagenesis failed due to a lack of a technique for effectively evaluating a large number of clones and/or techniques for enriching for superior clones. Industrial-style robotics-based screening and/or rational strain engineering will be required to generate superior strains. The potential of using eletrodialysis on whole broth was confirmed, though a full scale REED system and fed batch optimisation is required to establish, if a 10-fold improvement is feasible.

With a lengthy research phase ahead, the project was deemed inappropriate for the CRC SIIB to continue pursuing. However, the use of bacteriocins should still be considered a viable strategy for MLA to realise the aim of phasing out antibiotics use from animal production. Alternative production options, such as using bacteriocin producing bacteria as silage inoculants, may overcome production cost issues.

2.0 BACKGROUND

Antimicrobial agents are used extensively in the Australian meat and livestock industry as growth promotants and – to a lesser extent – for therapeutic/prophylactic purposes. Antimicrobial growth promotants (AGP) of the ionophore type (e.g., monensin) used in feedlots represent the largest group of antimicrobial agents used. In short term applications, ionophores reduce hydrogen production by fermentative bacteria, thereby reducing methane production and increasing feed efficiency. Ionophores also inhibit ammonia and lactic acid producers, thereby improving protein utilisation and reducing the risk of acidosis. In longer term applications (beyond a couple of months), resistance builds in the rumen and the direct benefits are lost. However, ionophores may continue to provide prophylactic benefits.

There is significant consumer concern, particular in Europe, regarding the use of AGP and their role in the emergence of drug-resistant pathogens. While there is no scientific evidence suggesting that ionophore use is associated with increased resistance to antibiotics relevant to human health, the same is clearly not the case for antimicrobial agents used for therapeutic/prophylactic purposes.

In this project, we explored the potential of replacing or supplementing current antimicrobial agents with bacteriocins occurring naturally in the rumen. Bacteriocins are a heterogeneous group of antibacterial peptides and proteins characterised by their ability to inhibit closely related, and sometimes more distantly related, strains of bacteria. The best-characterised bacteriocin is the food additive nisin, which is effective against not only food borne pathogens (e.g., clostridia) but also staphylococci and enterococci (Mantovani et al., 2001a). Although the mechanism is unclear, nisin has been shown to reduce rumen methanogenesis by 36% (Callaway et al., 1997).

Bacteriocin-like inhibitory substances (BLIS) have been detected in several genera of rumen bacteria, including *Butyrivibrio ssp*. and *Stretococcus bovis* (Iverson and Millis, 1976). Approximately 50 % of *S. bovis* strains isolated from the rumen produce bacteriocins; these are variable in specificity and potency (Mantovani et al., 2001a, Whitford et al., 2001, Xiao et al., 2004). The best-characterised, Bovicin HC5, has broad spectrum activity and high potency over a broad range of pH and culture conditions (Houlihan et al., 2004, Mantovani and Russell, 2003, Mantovani et al., 2001a, Mantovani et al., 2002). Bovicin HC5 has been shown to inhibit methane production of mixed ruminal bacteria (Lee et al., 2002) and ammonia production by the amino acid-fermenting ruminal bacterium *Clostridium aminophilum* (Mantovani and Russell, 2002). Bovicin HC5 was found to inhibit a variety of freshly isolated *S. bovis* strains, and, unlike nisin, resistance to HC5 did not develop (Mantovani et al., 2001b).

These results support the idea that endogenous bacteriocins have the potential to be used as ruminal additives. By providing an effective alternative, bacteriocins could be used instead of, or alongside, ionophore products as a strategy to prevent the build-up of resistance. Like ionophore antibiotics, bacteriocins can prevent or cure diseases, improve feed efficiency, and change the fat composition of livestock products (Teather and Kemp, 1999). There are also advantages to using bacteriocins over other antimicrobial agents. They are more precise in which bacteria are targeted; they are non-toxic and leave no residue in meat or milk (Teather and Kemp, 1999).

3.0 OBJECTIVES

The objectives that were stated in the original proposal were:

1. Identify *S. bovis* isolates producing bacteriocins and their antimicrobial spectrum
2. Characterise the bacteriocin(s) produced
3. Improve bacteriocin production through strain engineering (non-GMO)
4. Develop optimal fermentation and formulation strategy together with economic evaluation

The extent to which these objectives have been met:-

- Objective 1 has been completed. From the twenty-two *S. bovis* isolates examined, eight strains produced reasonably strong bacteriocin-like inhibitory substances (BLIS). One of these (Sb15) was chosen for further study. The spectrum of activity was assessed against a variety of pathogenic and non-pathogenic rumen bacteria.
- Objective 2 has been completed. This has included examining the thermostability and protease sensitivity, development of a method to purify and quantify the bacteriocin, and determining the sequence of the bacteriocin peptide.
- Objective 3 has been partially completed. Random mutagenesis was used in an attempt to improve bacteriocin production, however this was unsuccessful. Serial culturing and induction can successfully be used as methods for improved bacteriocin production levels.
- Objective 4 has been partially completed. A detailed economic analysis has been performed identifying a requirement for both enhanced specific productivity and increased titre as essential for a commercially viable process. Removal of lactic acid (essential to increase titre) by whole-broth electrodialysis during fermentation was demonstrated. At present, only spray drying for easy distribution has been considered as formulation.

4.0 METHODOLOGY

4.1 Organisms and Culturing

Twenty-two strains of *S. bovis* (Sb01-Sb12, Sb14-Sb20, AR03, AR25, YE01) were obtained from the Queensland Department of Primary Industries and Fisheries (DPI&F). The isolation, characterisation, conditions of growth and storage of these strains have been reported previously (Klieve et al., 1989; Klieve et al., 1999).

L. lactis subsp. cremoris MG1363 (Wegmann et al., 2007) was used as the target organism for initial screening for bacteriocin activity from the *S. bovis* isolates. This strain was chosen for two reasons: it is closely related to *S. bovis* and therefore likely to exhibit sensitivity to *S. bovis* bacteriocins; and it is microaerophilic rather than strictly anaerobic, which facilitates laboratory screening. A wide range of common ruminal bacteria and pathogenic bacteria were also provided by DPI&F to establish the sensitivity of indigenous ruminal bacteria to bacteriocins.

S. bovis and *L. lactis* cultures were grown on M17 agar (Oxoid Australia Pty Ltd, Thebarton, SA) supplemented with glucose (5 g L^{-1}) before being seeded into M17 broth (Oxoid Australia Pty Ltd, Thebarton, SA) supplemented with glucose (5 g L^{-1}) (M17G). Consecutive seed cultures were inoculated into broths as a 5 % inoculum. *S. bovis* and *L. lactis* were incubated overnight at 39°C and 30°C respectively.

Strains used for activity spectrum analysis (see below) were cultured in the following media: LB broth for *E. coli* (Sambrook et al., 1989); Preston broth (Oxoid Australia Pty Ltd, Thebarton, SA) for *Campylobacter*; Nutrient broth for *C. perfringens* (Oxoid Australia Pty Ltd, Thebarton, SA); and ruminal-fluid-based (RF) medium (Klieve et al., 1989) for all other strains. RF medium was supplemented with either lactate (*Megasphaera elsdenii*), maltose (Ruminococcus spp.), or cellobiose and glucose (remaining strains). Media were prepared anaerobically and dispensed under O_2-free CO_2 into Hungate tubes sealed with butyl rubber stoppers, then autoclaved for 45 minutes at 105°C. Broths were inoculated anaerobically and cultures were incubated until stationary phase at 39°C before being used as target organisms for growth antagonism assays (see below).

4.2 Bacteriocin Screening

For initial BLIS screening, each *S. bovis* strain was spot-innoculated onto an agar plate and incubated overnight at 39 °C. An overlay of M17 medium + 0.7 % agar (5 mL) infused with a 1/40 dilution of an overnight *L. lactis* culture (approximately 10^5 cells mL^{-1}) was poured over the agar plate and allowed to solidify. Plates were incubated at 30 °C and the diameter of clearing zones around the primary inoculation sites were measured after 24 and 48 hours. All strains were assayed in triplicate and zones of inhibition were measured using callipers.

4.3 Bacteriocin Sample Preparation

Several different methods were used to prepare bovicin samples. Crude filtrates were obtained by passing overnight cultures of *S. bovis* through a 0.22 μm sterile Millex-GP filter (Millipore, Cat. No. SLTGP033). To partially purify and concentrate extracts, an optimised protocol was developed from several previously-used methods (Yang et al., 1992; Whitford et al., 2001; Mantovani et al., 2002; Lee et al., 2002b). An overnight culture (600 mL) was centrifuged for 20 min. at 10,000 × *g* and 4 °C. The pellet was washed twice in sodium phosphate buffer (5 mM, pH 6.7; 160 mL for the first wash

and 40 mL for the second wash), re-centrifuging after each wash as described above. The pellet was then washed with 20 mL acidic NaCl (100 mM, pH 2), re-centrifuged and resuspended in 15 mL acidic NaCl then stirred for 2 h at room temperature to liberate bacteriocin from cell membranes. The cells were centrifuged as described above and the supernatant retained; the pellet was re-extracted 40 mL acidic NaCl as described above. The pellet was extracted a third time in 95 mL acidic NaCl. To concentrate the preparations, ammonium sulphate was slowly to the supernatants to a final concentration of 50 % w/v and the mixture stirred for 1 h at room temperature before being cooled to 4 ºC and centrifuged (15,000 x *g*, 20 min., 4 °C). The resulting pellet was dissolved in 4 mL acetic acid-NaOH buffer (1 M NaCl, 50 mM acetic acid, pH 4.5) and the resulting solution was re-centrifuged at 13,000 x *g*, 20 min., 4 °C. The final semi-purified, concentrated extract was prepared by ultrafiltration of the supernatant through a Vivaspin 3 kDa molecular-weight cut-off column (Vivascience). Each column was used to concentrate and purify 500 µl sample. Centrifugation at 11,000 rpm for 2-3 min. resulted in a 200 µl retentate. To this retentate, 300 µl acetic acid-NaOH buffer was added and the column re-centrifuged. This step was repeated twice and the final retentate was retained for analysis. For full protocol details, see Appendix 1.

4.4 Thermostability Assays

To test the thermostability of the BLIS in culture filtrates, samples were autoclaved at 121 °C for 20 min or subjected to one freeze-thaw cycle. Frozen samples were thawed for before use in well diffusion assays (see below).

4.5 Well Diffusion Assays

After inoculating a soft M17 agar with *L. lactis* (approximately 10^5 cells·mL^{-1}), 20 mL aliquots were pipetted into 90 mm Petri dishes. After drying, 5 mm diameter wells were cut into the agar using a custom-made hole punch. Samples of BLIS extract (60 µL) were pipetted into the wells and the agar plates were incubated overnight at 30°C. Diameters of zones of clearing were measured using callipers; the diameter of the well (5 mm) was subtracted to obtain the diameter of the zone of clearing. Untreated control extracts were tested in parallel. All samples were assayed in triplicate.

4.6 Proteolytic Enzyme Assays

Samples of crude extract were digested with proteolytic enzymes (12.1 U·mg^{-1} proteinase K, 4 U·mg^{-1} pronase E, or 0.062 mg·mL^{-1} trypsin). Activity was assessed by the disc diffusion method as described below (see Spectrum Analysis), using *L. lactis* as the target organism.

4.7 Disc Diffussion Assay

Disc diffusion assays were used for examining the inhibition spectrum of Sb15 BLIS. Target organisms were diluted 1:5 in 0.7 % agar (Difco, Cat. No. 214050). Five mL of this dilution was overlaid onto plates of the appropriate medium and allowed to solidify. Sterile antimicrobial discs (Oxoid, Cat. No. CT0998B) were placed on the surface of the medium and 20 µL of Sb15 bovicin sample (filtrate, autoclaved filtrate or semi-purified, concentrated extract) were added to each disc. Controls consisting of filtered medium were used to standardise for any inhibitory effects observed from the different media. In addition, crude extracts from a non-producing strain were used as a negative control to standardise the effects of other components in the media and the crude cell. Plates were incubated under anaerobic conditions and zones

of inhibition were measured after 24 hr using callipers. Where necessary, background inhibition was subtracted from inhibition zone measurements. All samples were assayed in triplicate.

4.8 Purification and N-terminal Amino Acid Sequencing

Concentrated, semi-purified samples prepared by ammonium persulfate precipitation (see above) were separated on acrylamide gels and BLIS activity was assayed essentially as described previously (Ansubel et al., 1997; Mantovani et al., 2002). Sample (15 µl) was electrophoresed (150 V, 90 min) through Tris-tricine SDS-PAGE gels containing 16.5 % acrylamide. Duplicate gels were prepared; one was stained using Bio-Safe™ Coomassie (Bio-Rad, Cat. No. 161-0786), and the second was overlaid with soft agar seeded with *L. lactis* and incubated overnight at 30 °C. Protein bands identified in Coomassie-stained gels were correlated to zones of clearing in the *L. lactis* lawns. A gel sample containing BLIS activity was excised from the stained Tris-tricine gel and submitted to the Australian Proteome Analysis Facility (Sydney, Australia) for N-terminal sequencing. The protein in the gel matix was passively eluted by incubating in SDS elution buffer overnight at 37 ºC. The sample was then loaded onto a Prosorb filter cartridge (Applied Biosystems) and washed with 3 x 100uL 0.1 % TFA to remove the SDS and reduce the background contamination. The sample was then subjected to 20 cycles of Edman N-terminal sequencing. Automated Edman degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing System and a Pulsed Liquid sequencing method.

4.9 Serial Culturing

A 10 mL culture was inoculated with 0.5 mL *S. bovis* Sb15 and grown at 39 ºC. Serial culturing was performed by transferring 0.5 mL into a fresh 10 mL broth at 24 hr intervals. Crude filtrate was used in disc diffusion assays as described above, using *L. lactis* as a target organism.

For each activity assay, a set of controls using a range of nisin concentrations was generated. A nisin standard curve was prepared. BLIS activity was standardised to a nisin activity to control for variation between experimental conditions. Similarly to the method described by Delgardo et al. (Delgado et al., 2005), the natural log of the concentration of the inhibitor (nisin) was plotted against the area of the zone of clearing. The slope of the resulting linear regression was used to transform the area of the BLIS clearing zone observed from Sb15 assays to obtain BLIS activity as a unit nisin activity.

4.10 Mutagenesis

An overnight *S. bovis* culture was centrifuged (4000 x *g*, 15 min, 4 ºC) and the resulting pellet washed in M17 supplemented with 5 % glucose (M17G), recentrifuged, and resuspended in M17G This cell suspension was used to inoculate a fresh M17G broth to an OD_{600} of 0.1. This culture was incubated at 39 ºC until mid-log phase (OD_{600} = 0.3 - 0.6), when the mutagen MNNG (N-methyl-N'-Nitro-N-Nitrosoguanidine) was added to a final concentration of 0, 50, 100 or µg/ml. Cultures were incubated overnight. A 1/100 dilution series was prepared and 50 µl of the 1/100 and 1/10 000 dilutions were spread over M17G agar plates. After overnight incubation, large colonies were picked and spotted on M17G agar for delayed antagonism assays (see above) using *L. lactis* as a target organism.

Enrichment methods using serial passaging to increase bacteriocin production and select for bacteriocin overproducers were attempted. This approach seeks to exploit the fact that tolerance genes are normally expressed from the same operon as the

bacteriocin; thus, a bacteriocin overproducer should also be more tolerant to bacteriocin, which in serial passaging should lead to enrichment of high producers.

Two eirichment protocols were used. Firstly, Sb15 cultures were incubated overnight at 39°C in the presence of MNNG (100 $\mu g \cdot mL^{-1}$). These were sub-cultured up to 13 times into M17G broth containing sterile bacteriocin positive supernatant (5%) and incubated overnight at 39°C. Disc diffusion assays (see above) were used to assess bacteriocin activity. Secondly, Sb15 cultures with 50, 100 and 150 $\mu g \cdot mL^{-1}$ of MNNG were incubated overnight at 39°C. An aliquot of these cultures (0.1 mL) was spread onto M17/5 $g \cdot L^{-1}$ glucose agar plates and incubated overnight at 39°C. Plaques from these plates were removed using 1 mL of M17G broth, pooled; 0.1 mL spread onto four M17G agar plates, which were incubated for 24 hours at 39°C. The cultures were repeatedly re-plated as described. Bacteriocin activity was tested at the 12th and 22nd repeat against the target organism L. lactis. Untreated controls were included.

4.11 Fermentation

Fermentations were performed in a 2 L Applikon bioreactor unit (Applikon, The Netherlands) controlled by an ADI 1025 bioconsole. The working volume was 1 L. An agitation rate of 300 rpm was used. The temperature was controlled at 39°C, and pH was controlled at 5.5 by the addition of 5 M NaOH and 5 M H_2SO_4.

S. bovis Sb15 was initially grown on M17 broth containing approximately 20 $g \cdot L^{-1}$ glucose. During the fermentation more glucose was fed along with a concentrated M17 broth mix. The concentrated M17 broth was fed to ensure that the vitamins and minerals were not being depleted from the fermentation. A feed rate of approximately 5 $g \cdot (L \cdot h)^{-1}$ glucose was estimated from sugar uptake rates achieved in previous experiments.

Lactose was removed using a custom-built REED electrodialysis device from Jurag Separations (Denmark). The REED system was based on a standard electrodialysis unit (PC Cell, Germany) with two cell pairs of 8cmx8cm anion and cation exchange membranes (Ameridia). In all experiments two cell pairs were used. The current was kept constant with the voltage varying to a maximum of 12 volts. Oscillation of the polarity was achieved by a custom-built oscillator with a variable timer. The unit operated at 9% efficiency, which is similar to what was previously achievable with this unit for pure solutions (Blinco, 2005).

Cell concentration was estimated by measuring the optical density at 600 nm (OD_{600}) by a BioChrom Libra S12 UV/Vis spectophotometer (BioChrom, UK). The optical density measurements were converted into biomass concentrations using a conversion factor of 1.0 A_{600} = 0.31 $g \cdot L^{-1}$, as determined experimentally separately for the bacteria.

Concentrations of glucose, LA, formate, acetate, and ethanol in the supernatant were determined by ion exchange HPLC (Waters, USA) equipped with a 7.8×300 mm HPX-87H fast acid column (Bio-Rad, USA). The mobile phase consisted of 0.008 N, helium degassed H_2SO_4 pumped at a flowrate of 0.6 $mL \cdot min^{-1}$. The column was maintained at 65°C. The peak elution profile was monitored with a Waters 484 differential refractive index detector (Milford, MA, USA) at 35°C. UV absorbing compounds were detected at 210 nm using a Waters 484 tuneable absorbance detector.

5.0 RESULTS

5.1 Identification of BLIS producing strains and their spectrum of activity

5.1.1 BLIS testing of isolates against *L. lactis*

An agar overlay method was used to identify strains that produced inhibitory substances. Eight of the twenty-two *S. bovis* strains tested inhibited growth of *L. lactis* MG1363, resulting in zones of clearing around inoculation sites (Figure 1). Replicate plates were treated with UV before applying the target organism; again, the same strains inhibited *L. lactis* MG1363 growth (data not shown).

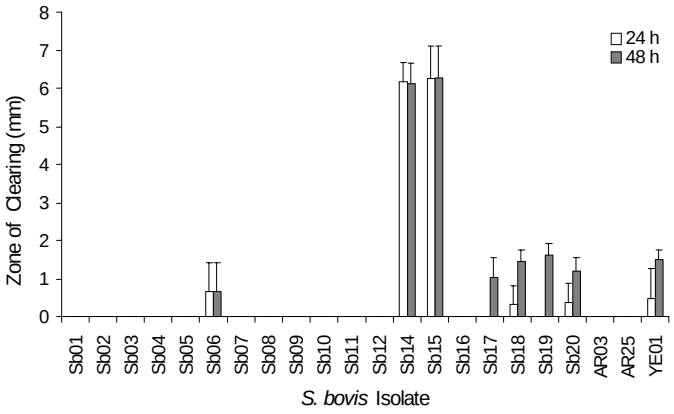

Figure 1: BLIS activity from *S. bovis* isolates as measured by zones of clearing in *L. lactis* lawns around the *S. bovis* colonies. Zones were measured at 24 and 48 hr after inoculation. Diameters of zones are given as average ± SE; n = 3.

5.1.2 Stability of Sb15 BLIS

Thermostability of BLIS in crude filtered extracts of SB15 culture filtrates was tested by (a) autoclaving and (b) freeze-thawing. Neither treatment had a significant effect on the size of the zone of inhibition (

Figure 2).

Resistance to cleavage by a variety of proteases was examined. Zones of clearing around SB15 supernatant extracts were measured after treating supernatant samples with Pronase E (a commercial mixture of proteases), trypsin and proteinase K. Clearing zones decreased in diameter after Pronase E and trypsin treatment, but were unaffected by proteinase K (

Figure 2).

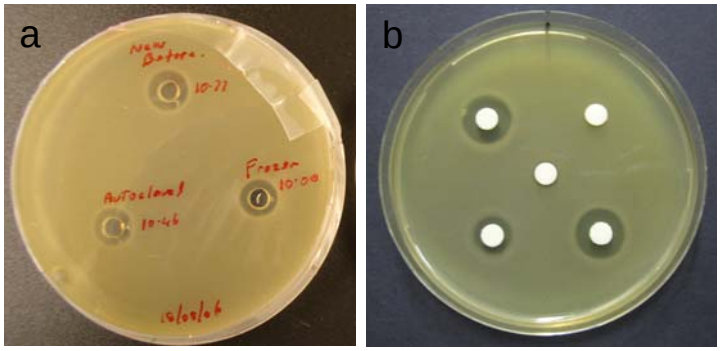

Figure 2: Stability of supernatant extracts from SB15 cultures. (a) Thermostability of – autoclaved or freeze-thawed samples were assayed using a well diffusion assay (b) Protease sensitivity determined using a disc diffusion assay

5.1.3 Sb15 Spectrum Analysis

The inhibitory activity of the Sb15 BLIS was tested against a range of bacterial including common rumen bacteria and pathogens (Figure 3). Several preparations were compared: culture filtrate (culture filtered through 0.22 µm membrane), autoclaved culture filtrate, and concentrated, semi-purified extract (ammonium precipitation). The BLIS inhibited a wide variety of bacteria, both Gram-positive and Gram-negative. Gram-positive species were generally more sensitive than Gram-negative strains. The concentrated extract was most effective, and in some cases concentration was required for efficacy.

Class	Organism	Gram stain	Comments	Zone of Clearing (mm)
Facultative anaerobes	*Escherichia coli* O157:H6	-	enteropathogenic	
	Escherichia coli	-	intestinal; non-pathogenic	
	Lactococcus lactis MG1363	+	fermentation; opportunistic pathogen	
Rumen/ intestinal symbionts	*Bacteroides ruminicola* ssp. *brevis*	-	rumen mutualist; fibrolytic	
	Bacteroides ruminicola ssp.	-	rumen mutualist; fibrolytic	
	Megasphaera elsdenii	-	rumen; lactate consumer	
	Bifidobacteria pseudolongum	+	intestinal mutualist	
	Bifidobacteria pseudolongum	+	intestinal mutualist	
	Butyrivibrio fibrisolvens	+	intestinal/ rumen mutualist	
	Lactobacillus sp. (YE08)	+	fermentation; gastro-intestinal symbiont	
	Lactobacillus sp. (YE06)	+	fermentation; gastro-intestinal symbiont	
	Ruminococcus albus	+	rumen mutualist; cellulolytic, H_2-producing	
	Ruminococcus bromii	+	rumen mutualist; cellulolytic	
	Ruminococcus flavefaciens	+	rumen mutualist; cellulolytic	
	Ruminococcus sp.	+	rumen mutualist; cellulolytic	
	Streptococcus bovis	+	rumen mutualist; acidosis	
	Streptococcus intermedius	+	commensalist; opportunistic pathogen	
	Clostridium butyricum	+	intestinal mutualist; meat spoilage	
Pathogens	*Campylobacter jejuni* (C70)	-	gastroenteritis	
	Campylobacter coli	-	gastroenteritis	
	Campylobacter jejuni (C838)	-	gastroenteritis	
	Clostridium perfringens (BR162)	+	enteritis/gangrene	
	Clostridium perfringens (BR163)	+	enteritis/gangrene	
	Clostridium perfringens (BR158)	+	enteritis/gangrene	
	Enterococcus avium	+	bacteremia	

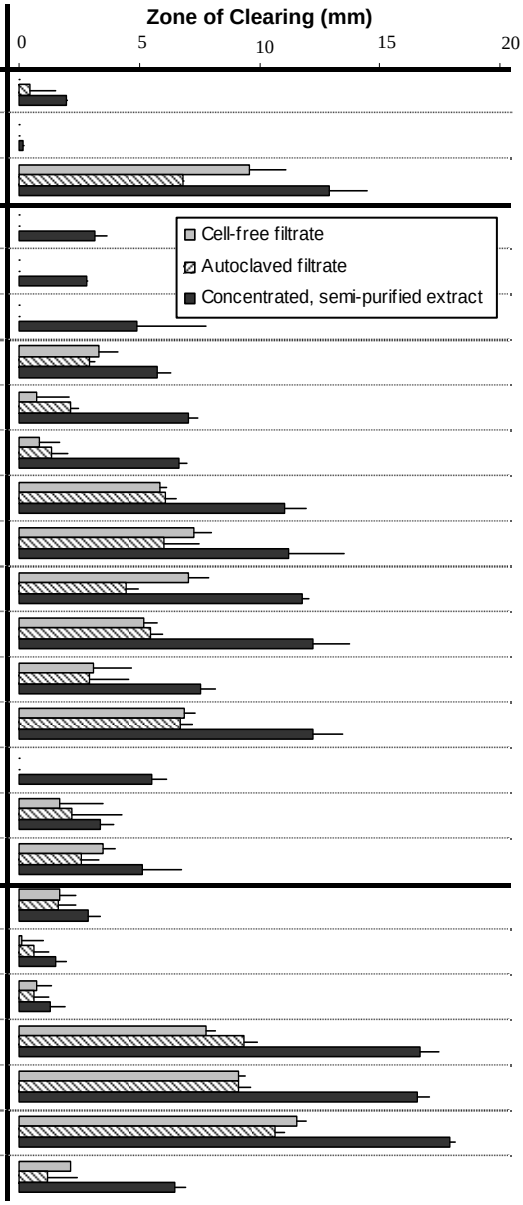

Figure 3: Activity of Sb15 BLIS against other bacteria. Zones of clearing around discs containing crude extract were measured after overnight incubation. Light grey bars, cell free filtrates; cross-hatched bars, autoclaved filtrate; dark grey bars, concentrated semi-purified extract.

5.1.4 Identification of SB15 bacteriocin

Preliminary studies showed that purification of bacteriocin from fermentation broth was difficult, presumably due to large amounts of peptides from yeast extract and peptone in the broth. Attempts to enrich the bacteriocin by ion exchange chromatography failed.

Instead bacteriocin was extracted from the cell membrane using acidic NaCl. Cells were recovered from broth by centrifugation and the pellet washed, prior to

extraction. Bacteriocin in the acidic NaCl cell wash solution was precipitated with ammonium sulphate (50%). The precipitate was redissolved in buffer and run through a 10 kDa spin-filter.

The BLIS activity was purified by separation of protein samples through polyacrylamide gels and identified by an agar overlay antagonism assay as described in the Materials and Methods. A zone of clearing in the *L. lactis* lawn corresponded to a specific protein band identified by Coomassie stain, thus identifying the bacteriocin protein (Figure 4). N-terminal sequencing was performed on this protein band; the resulting sequence is shown in Figure 5. A number of blank cycles were returned (designated by an 'X' in the sequence); re-sequencing did not resolve these amino acids. Lantibiotics like nisin are posttranslationally modified; common modifications are dehydration of Ser and Thr residues followed by lanthionine bridging with Cys. The corresponding residues are not resolved in conventional N-terminal sequencing.

The sequence of SB15 bacteriocin is almost identical to the published sequence of *S. bovis* HC5 bacteriocin. Indeed, it is possible that the Ser signal in position 7 and 12 in HC5 is a result of contamination with peptide that has undergone partial post-translational modification only. HC5 is similar to but distinct from a lantibiotics sequenced from *S. pyogenes*. HC5 has been studied extensively by James Russell from Cornell University. It is one of the few bacteriocins that have been tested as an anti-microbial growth promotant and it was found to be very potent in that application. The potency and spectrum data published are very similar to what we have observed for SB15.

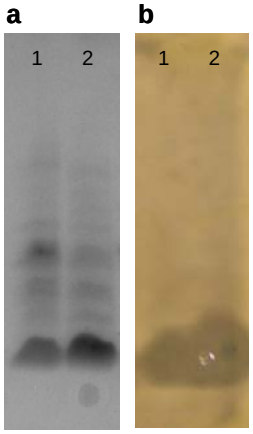

Figure 4: Identification of BLIS in polyacrylamide gels. (a) Coomessie stain of gel (b) *In situ* activity assay on duplicate gel. BLIS activity was identified by zones of clearing in *L. lactis* lawns. Lane 1 and 2 contain preparations from two different *S. bovis* cultures; lane 3 = Polypeptide SDS_PAGE molecular weight standards (BioRad, Gladesville, NSW).

```
S. pyogenes lantibiotic      VGSRYLCTPGSCWKLVCF--
S. bovis HC5 bovicin         VGXRYASXPGXSWKYVXF--
S. bovis SB15 bovicin        VGXRYAXXPGXXWKYVXFXX
```

Figure 5: Comparison of bacteriocin sequences from *S. bovis* SB15, *S. bovis* HC5 (Mantovani et al., 2002) and an *S. pyogenes* lantibiotic (Ferretti et al., 2001). Blank cycles are represented by 'X'.

5.1.5 Improvement of Consistency of Production

Large culture-to-culture variation was observed in Sb15 bacteriocin productivity. This is commonly the case for bacteriocin production. Serial culturing was used with the aim of stabilising bacteriocin production.

The *S. bovis* SB15 strain was subjected to serial culturing and BLIS activity was analysed at each sub-culture. Activity was standardised to a nisin control in order to account for experimental variation between culturing (see Materials and Methods). Bacteriocin activity was undetectable in the first and second cultures; however the yield increased rapidly during the third and fourth cultures and peaked by the seventh culture (Figure 6). The yield decreased slightly during subsequent cultures but remained high compared to cultures 1-3. The increase in yield was likely due to induction of bacteriocin gene expression, as has been observed during serial culture of nisin-producing cells (de Rutyer, 1996).

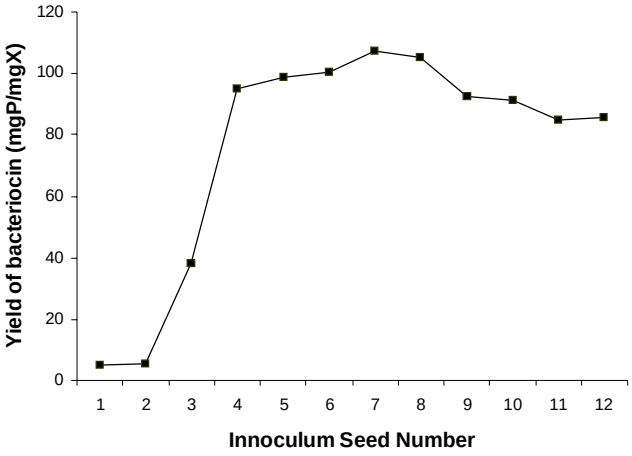

Figure 6: Bacteriocin yield during serial culturing. Yield was calculated as mg bacteriocin produced per mg biomass (mg P/mg X).

5.2 Approaches to improve bacteriocin production

Sb15 produce bacteriocin activity at a level similar to nisin producing *L. lactis*. This is inadequate for economic viable production (see later). We explored the potential of using random mutagenesis to enhance specific production and fed batch to increase total biomass.

5.2.1 Random mutagenesis

Bacteriocin production is measured by clearing assays, i.e., the ability to kill target cells on agar plates. Production is under self-induction control (Figure 6) and – in our hands – the most reliable estimates are found using disc assays after serial subculturing.

We first evaluated, if stab cultures could be used instead of disc assays. This is essential if we are to screen large number of mutated strains with no enrichment. Without enrichment, we would expect to have to screen 1,000s of colonies.

An Sb15 culture was incubated overnight at 39°C in the presence of 100 $\mu g \cdot mL^{-1}$ of the mutagen n-methyl-N'-nitro-nitrosoguanidine (MNNG). This culture was serially diluted and plated onto

M17/5 g·L^{-1} glucose agar. Ten colonies were selected at random and stored in glycerol at -80°C. The colonies were then

(a) spot/stab inoculated onto M17/5 g·L^{-1} glucose agar plates, incubated overnight at 39°C then overlaid with soft agar seeded with *L. lactis* and incubated at 30°C.
(b) inoculated in M17/5 g·L^{-1} glucose broth; with the 3rd sub-culture assayed using the disc method.

The stab inoculum results indicated that almost all of the mutated colonies had improved bacteriocin production over the untreated Sb15 (Table 1). The disc assay however revealed that the improvement (if any) is minimal. Thus, the apparent improvement probably reflected differences in induction conditions.

Table 1. Zone of clearing for random MNNG treated Sb15 colonies

Colony #	Stab inoculum on plate		Disc assay	
	Zone of clearing (mm)	S.E (±)	Zone of clearing (mm)	S.E (±)
1	10.6	0.4	4.7	0.4
2	13.0	0.1	4.7	0.4
3	9.6	1.9	4.7	0.3
4	15.2	1.2	5.2	0.4
5	7.9	1.9	0	0
6	12.5	0.4	5.2	0.4
7	13.3	0.7	5.1	0.6
8	13.0	0.3	5.1	0.6
9	12.3	1.3	5.2	0.7
10	13.9	0.8	5.5	0.5
Control	7.5	0.5	5.0	0.2

In light of this result, we decided to explore two enrichment methods. Both seek to explore the fact that tolerance genes are normally expressed from the same operon as the bacteriocin. Thus, a bacteriocin overproducer should also be more tolerant to bacteriocin, which in serial passaging should lead to enrichment of high producers.

Enrichment method #1

Sb15 cultures were incubated overnight at 39°C in the presence of MNNG (100 µg·mL^{-1}). These were sub-cultured into M17/5 g·L^{-1} glucose broth containing sterile bacteriocin positive supernatant (5%) and incubated overnight at 39°C. After 13 subcultures the "mutated" culture was compared to the control culture in terms of bacteriocin production. There was no change in the zone of clearing observed for broth from the mutated culture (9.97±0.83 mm) compared to control (10.30±1.16 mm),

Enrichment method #2

Sb15 cultures with 50, 100 and 150 µg·mL^{-1} of MNNG were incubated overnight at 39°C. An aliquot of these cultures (0.1 mL) was spread onto M17/5 g·L^{-1} glucose agar plates and incubated overnight at 39°C. Plaques from these plates were removed using 1 mL of M17/5 g·L^{-1} glucose broth, pooled; 0.1 mL spread onto 4 M17/5 g·L^{-1} glucose agar plates (designated M1, M2, M3 & M4), and then incubated for 24 hours at 39°C. The cultures were repeatedly re-plated as described. Bacteriocin activity was tested at the 12th and 22nd repeat against the target organism *L. lactis*. A comparison is made against untreated controls (designated C1 & C2).

The results from this method (Table 4) suggest that there is little, if any, improvement in bacteriocin production by the Sb15 cultures that are subjected to a mutagen and then put under pressure to produce bacteriocin by being in close competition with each other. At the end of the 22nd culture an M17/5 g·L^{-1} glucose broth was inoculated while in the presence of the Sb15

bacteriocin (i.e. was induced). This again supported the data which indicates that no change has been made with respect to bacteriocin production.

Table 2. Zone of clearing for MNNG treated Sb15 cultured on agar plates. Numbers are zone of clearing in mm ± standard error.

Culture	12th subculture		22nd subculture		
	Stab inoculum	Disc assay	Stab inoculum	Disc assay	Induced broth
C1	23.1±1.4	4.6±0.4	23.2±0.5	4.4±0.3	4.9±0.4
C2	23.6±2.4	4.4±0.8	20.9±0.8	4.0±0.3	5.8±0.3
M1	24.2±1.6	4.0±0.3	21.4±1.9	4.5±0.2	4.5±0.3
M2	26.0±1.4	4.4±0.5	23.3±0.8	3.9±0.2	4.6±0.2
M3	24.5±1.6	4.4±0.4	21.4±1.1	4.4±0.3	4.7±0.4
M4	24.8±1.7	4.0±0.6	22.4±0.7	4.5±0.2	5.2±0.2

5.2.2 Fed-batch Fermentation

Bacteriocin production is directly correlated with biomass production. Conventional batch fermentation suffers from low biomass yield due to lactic acid (LA) inhibition. This is true for all lactic acid bacteria and hence a problem faced by the Starter Culture industry. Various strategies such as cross-flow filtration have been used to retain biomass in the bioreactor while removing LA together with spent broth. More recently, the so-called REED (reverse electro-enhanced dialysis) approach (Jurag Separation APS, 2002; Rype 2003) has been developed to specifically remove small acids.

Application of REED in early stationary phase did not enhance biomass production, which levelled out at 2.5 $g \cdot L^{-1}$ in both control and REED cultures (Figure 7). Biomass production ceased at around 10 $g \cdot L^{-1}$ LA (Figure 7), which is only 25% of the expected level of 40 $g \cdot L^{-1}$ LA required for total growth inhibition. The feed medium is very rich and further studies are required to understand, why the cultures failed to reach higher densities. A significant slow down in glucose consumption was observed with the REED system (Figure 8) suggesting that cells were under suboptimal conditions, e.g., hypothermia in the large recirculation volume, hyperthermia in the flow cell or leakage of electrode solutions.

REED did achieve the desired reduction in LA concentration (Figure 8). The rate of reduction is slower than hoped for and the unit operated at only 9% efficiency, which is similar to what was previously achievable with this unit for pure solutions (Blinco 2005 – unpublished results). We attribute this low efficiency to the modification we had to make to the flow cell; dedicated REED systems operate at around 40% efficiency.

It is not possible on the basis of this study to draw any conclusions in regards to the utility of REED technology for this application. A dedicated REED pilot plant is required to evaluate the technology and feeding protocols need further optimisation to achieve the expected biomass yields.

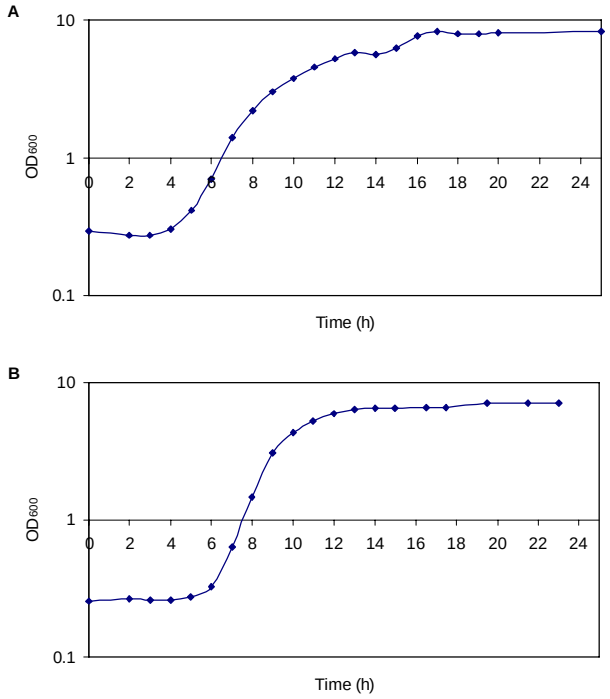

Figure 7: Comparison of the biomass production profiles of a (A) fed-batch and (B) fed-batch with ED

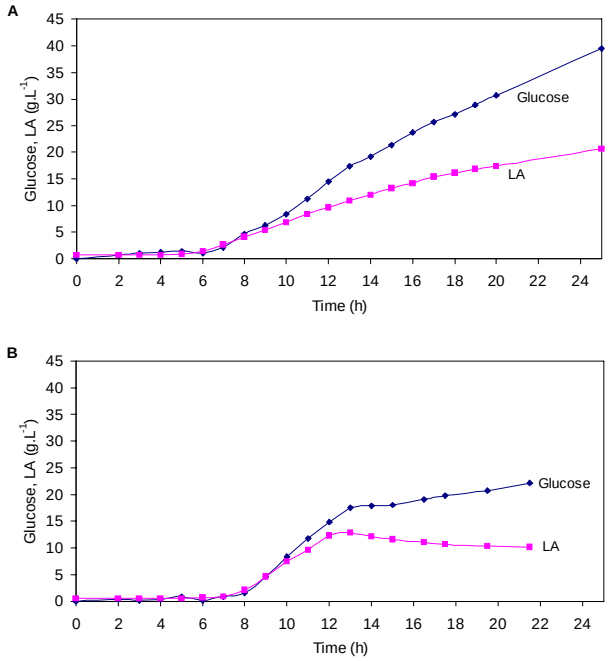

Figure 8: Comparison of the glucose consumption and lactate production profiles of a (A) fed-batch and (B) fed-batch with ED

5.3 Economic evaluation of bacteriocin production

Bacteriocin production has been subjected to a first pass economic analysis. As is the case with nisin, the final product is spray dried whole fermentation broth. The value of bacteriocin was set to US \$100 kg^{-1}, the price of monensin. We expect bacteriocin to be sold at a premium, but the current model does not consider sales and shipping.

The current level of bacteriocin is 2% of biomass, indicating a biomass value of US $2 kg^{-1}. It is impossible to produce biomass at that cost with an anaerobic organism. Thus, strain engineering is required to increase bacteriocin content. A rough calculation indicates that a biomass price of at least US $10 kg^{-1} is required for meaningful economics and we will assume that strain engineering can deliver this 5x improvement.

Four scenarios were explored:

1. Batch fermentation of 1:10 diluted molasses; 55 tpa bacteriocin
2. REED enhanced batch fermentation of 1:3 diluted molasses; 55 tpa bacteriocin
3. REED enhanced batch fermentation of concentrated juice (400 g/L); 55 tpa bacteriocin
4. As 3. but 110 tpa bacteriocin

The 1:10 dilution yields a sugar concentration of around 43 $g \cdot L^{-1}$; this can be fermented prior to complete growth inhibition by LA. REED has been used to achieve 10X higher LAB biomass levels in pilot studies at Chr. Hansen. Using molasses, however, we have to dilute to avoid inhibition by other compounds and 1:3 dilution (around 150 $g \cdot L^{-1}$ sugar) was considered a viable level. An alternative is to use concentrated juice, where we expect to be able to deliver the 10X improvement. The first three scenarios assumes 55 tpa bacteriocin production or approximately 50 % of the Australian market for monensin. The final scenario considers doubling the production.

For all processes, we assumed the following reaction stoichiometry (on a C-mole basis):

$$5.81\,\text{Sucrose} + 0.1\,\text{Urea} \rightarrow \text{Biomass} + 0.05\,CO_2 + 5.12\,\text{LA}$$

observed in previous batch studies. In reality, we expect the stoichiometry to be less favourable for high density batch and more favourable for REED batch, since high levels of LA encourage further LA production. A batch time of 13 hours was assumed for all processes. The specific growth rate in REED is higher (less LA inhibition), but a higher final biomass titre is required.

Process economics was evaluated using SuperPro designer supplemented with electrodialysis data from (Cushnie, 1994; Bailly, 2002) (Table).

The analysis highlights the problem of installed capital cost in low-density batch fermentation. Capital for the batch only process corresponds to around $4.6 per kg of sugar fermented. For comparison, NatureWorks' 150 mtpa PLA plant cost $2 per kg sugar fermented and included much more elaborate downstream processing and polymerisation as well as wastewater treatment. The proposed plant, however, is much smaller and the LA titre is limited to 40 $g \cdot L^{-1}$ compared to 160-180 $g \cdot L^{-1}$ in a LA production plant. With a capital:revenue ratio of 3.5, facility operating costs (depreciation, maintenance and insurance) accounts for an unhealthy 60% of operating costs.

REED technology can be used to increase biomass titre and thereby reduce capital costs. However, moving from a 250,000 L fermenter (batch) to a 25,000 L fermenter (batch, REED, juice) only reduces capital cost by a third, which is insufficient to make the process viable. Increased scale is required to make the option of REED-enhanced batch with juice viable and a doubling of production creates a viable process.

In summary, the key assumptions required to achieve a viable process are

1. Bacteriocin yield can be increased from 2% to 10%

2. REED can deliver a 10X biomass titre improvement when using concentrated juice
3. The full 110 tpa market for monensin in Australia can be secured or – alternatively – the much larger world market can be accessed.

Table 2: Economic evaluation of bacteriocin production

Assumptions	**BATCH**	**BATCH+REED**	**BR (Juice)**	**2X Juice**	
Biomass produced	554.5	554.5	554.5	1109	tpa
Yield molasses	16.5	16.5	7.095	7.095	kg/kg biomass
Labour	4383	4383	4383	4383	hpa
Utilities - fermenter+dryer					
Electricity	5785622	1727830	663143	1326287	kWh annual
Steam	174358464	46468464	12912564	25825127	kg annual
Utilities - REED					
Electricity		1900000	1900000	3800000	kWh annual
ED consumables		380000	380000	760000	$/annum
Facility OPEX					
Depreciation	5%	5%	5%	5%	Direct Fixed Capital
Maintenance	6%	6%	6%	6%	Direct Fixed Capital
Insurance	1%	1%	1%	1%	Direct Fixed Capital
Capital					
Fermenter + spraydrier	$17,895,000	$11,496,000	$8,844,000	$10,568,000	
REED		$2,800,000	$2,800,000	$5,600,000	
Start-up + working	$1,000,000	$1,000,000	$1,000,000	$2,000,000	
Total	$18,895,000	$15,296,000	$12,644,000	$18,168,000	
Operating					
Molasses @ $80/t	$731,940	$731,940	$983,544	$1,967,089	
Labour @ $65/h	$284,895	$284,895	$284,895	$284,895	
Utilities	$405,783	$191,582	$115,438	$230,877	
ED consumables		$380,000	$380,000	$760,000	
Facility dependent	$2,147,400	$1,715,520	$1,397,280	$1,940,160	
Total	$3,570,018	$3,303,937	$3,161,158	$5,183,020	
Cost per kg biomass	6.44	5.96	5.70	4.67	
Revenue @ $10/kg	$5,545,000	$5,545,000	$5,545,000	$11,090,000	
NPV (20%; 20 years)	($7,731,391)	($3,652,469)	($863,075)	$8,830,424	
IRR (20 years)	8%	13%	18%	32%	

6.0 OUTPUTS

- 8 BLIS$^+$ isolates were identified
- The Sb15 bacteriocin was found to have a spectrum of activity similar to that of monensin, an antibiotic currently used in the cattle industry
- The Sb15 bacteriocin is very thermo-tolerant and resistant to proteolytic enzymes. It is stable under conditions far in excess to what it would be subjected to in the rumen environment
- The activity of the Sb15 bacteriocin in crude supernatant extracts was found to be very high
- The Sb15 bacteriocin can be concentrated by precipitation; a new method was formulated for this protocol
- The amino acid sequence of the Sb15 bacteriocin was found to have strong similarity to an already published bacteriocin, indicating that it is not novel and cannot be protected by intellectual property
- The production of bacteriocin from Sb15 can be improved by serial culturing or induction, however levels required for economic viability were not achieved

- The first pass economic analysis indicates that bacteriocin production can be viable, if the titre is increased 5-fold.

7.0 INTELLECTUAL PROPERTY:

It appears that the Sb15 bovicin isolated during this study has the same amino acid sequence as the well-characterised HC5 bovicin. Therefore, the use of the Sb15 bacteriocin cannot be patented. There are no current patents for the use of *S. bovis* bacteriocins (including HC5), thus there is no limitation on using Sb15 bovicin.

The ownership or commercialisation of the IP from this project can be impacted by the isolates of *S. bovis* used in this study having been made available under an MTA agreement with the Animal Research Institute (ARI).

8.0 ENVIRONMENTAL AND SOCIAL IMPACTS:

There are no adverse environmental impacts from conducting the project. The potential environmental benefit from implementation of the findings from this project would be the replacement, or reduction, of antibiotic use in the cattle industry. The potential social impact would be the reduction of antibiotics in meat and milk, and hence the follow on down the food chain. This could lead to improved consumer confidence in these products and less incidence of antibiotic resistance.

9.0 EXPECTED OUTCOMES

For the sugar industry: The previous project on LA production by *S. bovis* established the ability of this organism to grow on low-nitrogen sources such as sugarcane processing by-products. As such, there is the potential to use sugarcane as a substrate to produce antimicrobial agents from *S. bovis*.

For the community: There is the potential for meat and milk products to be available without the use of antibiotics which can pass on through the food chain and have issues with antibiotic resistance.

10.0 FUTURE NEEDS AND RECOMMENDATIONS

Extensive studies of the closely related HC5 indicates that Sb15 bovicin would be a suitable candidate to replace monensin, thus realising MLA's objective of phasing out antibiotics use from animal production. However, achieving cost-effective production from sugar mill streams will require a lengthy research phase. Being non-core business, it is unlikely that anyone in the sugar industry will be interested in pursuing this research at this early stage.

Realising the 5X improvement in specific productivity through conventional random mutagenesis will require industrial-style robotics based-screening. Rational engineering using targeted modifications would be less expensive and feasible for academic research groups. Given the greatly reduced cost of genome sequencing, the first step should be 454 sequencing to quickly identify the full bovicin regulon necessary for targeted engineering.

Whole-broth electrodialysis using REED technology is a promising strategy for achieving high density fed batch cultures of lactic acid bacteria. The process, however, will need extensive optimisation at mini-pilot scale (10 L) with mill stream feedstocks to realise the 10X increase in biomass titre.

Use of Sb15 as a silage inoculant is an attractive alternative which has the potential of greatly reducing production costs. An initial estimate, however, indicates that in order to deliver the required level of bacteriocin, a total bacterial count in excess of the total rumen bacterial count is required. In order to avoid acidosis, it would be essential that the bacterial count in silage was reduced before feeding (e.g., through heating).

11.0 PUBLICATIONS ARISING FROM THE PROJECT

- Joachimsthal EL, Reeves R, Nielsen LK Ouwerkerk D, Klieve A, Vickers C (*In Preparation*) Screening and spectrum identification of bacteriocin production by various rumen isolates of *Streptococcus bovis*.
- Joachimsthal EL, Hung J, Reeves R, Nielsen LK (2007) Endogenous peptide production for antimicrobial application in the rumen. *In:* Proceedings from the Australian Microbiology Society Conference, 9–13 July, 2007, Adelaide, Australia, p. 112.

12.0 ACKNOWLEDGMENTS

- Dr. Athol Klieve and Ms. Diane Ouwerkerk of the Animal Research Institute (ARI) for providing access to their laboratory and library of rumen isolates.
- The MLA (Meat and Livestock Australia) for its financial support.

13.0 NOMENCLATURE

AGP	Antimicrobial growth promotants
BLIS	Bacteriocin-like inhibitory substance
ED	Electrodialysis
MNNG	n-methyl-N'-nitro-nitrosoguanidine

14.0 REFERENCES

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15.0 APPENDICIES

15.1 APPENDIX 1: Sample preparation protocol

1. Growth of cells O/N at 39°C in 600 mL of M17 broth
2. Centrifuge for 20 mins at 10,000×g at 4°C in 4×250 mL centrifuge tubes (supernatant kept for testing all supernatants are spun at 4000 rpm, 0.22 μm filtered, and stored at -20°C)
3. Add 40 mL of sodium phosphate buffer to each pellet, mix, split into 2×JA-21 tubes, and centrifuge for 20 mins at 10,000×g at 4°C, discard supernatant
4. Add 20 mL sodium phosphate buffer to each tube, centrifuge for 20 mins at 10,000×g at 4°C, discard supernatant
5. Add 10 mL acid salt wash (100 mM NaCl, pH 2) to each tube, mix and immediately centrifuge for 20 mins at 10, 000×g at 4°C, discard supernatant
6. Add 7.5 mL of acid salt wash (100 mM NaCl, pH 2) to each tube, adjust the pH to 2, mix and stir for 2 h at room temperature, centrifuge for 20 mins at 10,000×g at 4°C in JA-21 tubes, keep supernatant
7. Add 20 mL of acid salt, mix, stir for 2 h at room temperature, and centrifuge for 20 mins at 10,000×g at 4°C per JA-21 tube, keep supernatant
8. Repeat step 7 (keep supernatant) – final volume = 2×47.5 = 95 mL
9. Concentration of bacteriocin in the supernatant
   - 50% (w/v) of $(NH_4)_2SO_4$ is added slowly, stirred for 1 h at room temperature, then cooled to 4°C
   - Centrifuge at 15,000×g for 20 mins at 4°C
   - Remove supernatant by decanting slowly and carefully – collect pellet, and centrifuge at 4000 rpm in falcon tubes, add 1 mL of 1 M NaCl 50 mM acetic acid–NaOH buffer at pH 4.5 per 250 mL centrifuge tube, to dissolve the precipitate
   - Put into 1.5 mL eppendorf tube, centrifuge at 13,000×g and remove supernatant for further concentration (check if a precipitate has been formed, if a precipitate is visible then add more buffer to the pellet in an attempt to dissolve, and check if activity is present in this mixture)
   - Centrifuge in an ultrafiltration device – Vivaspin 3 kDa MWCO (500 μL, Vivascience, Cat. No. VSO192)

i. Rinse unit with ddH_2O
ii. Wash with pH 4.5 acetic acid–NaOH buffer to remove traces amounts of glycerine and sodium azide
iii. Add sample (500 μL)
iv. Centrifuge at 11,000 rpm at 4°C for 2-3 min (obtain a retentate of about 200 μL)
v. Add 300 μL pH 4.5 acetic acid–NaOH buffer, centrifuge again till approx. retentate of 200μl
vi. Repeat step (iv)×2
vii. Retain retentate for testing (denoted as "cell extract")