# Final report

## The Probio-TICK Initiative


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# Abstract

The Probio-TICK Initiative was a Cooperative Research Centre Project (CRC-P) co-funded by MLA with the aim of controlling cattle tick and buffalo fly, the two major economic diseases affecting the northern beef industry. The project has produced and tested a natural topical treatment for cattle tick. This was achieved by mining an established microbial library owned MST comprising bacteria recovered from soil in northern Australia and applying research in microbiology, fermentation, chemistry and parasitology. The treatment, termed Probio-TICK, contains up to 9 microbes shown to have relevant *in vitro* activity and characterised by gene sequencing and chemical analysis of their metabolites. Field efficacy studies, with the microbes applied to the coat of the cattle in liquid (water and oil) suspensions, provided evidence that the product can significantly reduce tick burdens. The results indicate that a probiotic approach to ectoparasite control on cattle may offer many advantages over traditional methods, saving on labour costs and avoiding chemical residues and resistance. Because the microbes are endemic there are no environmental issues and manufacture could also stimulate a local industry. The technology behind Probio-TICK is the subject of a provisional patent.

# Executive summary


## Background

Ectoparasites (ticks and buffalo fly) of cattle are estimated to cause major economic loss exceeding $250M p.a. to profitable beef production in northern Australia. Management of these pests relies on the dual approach of host genetics and chemicals. The former is challenging in view of market demand for better eating quality, and the latter faces the loss of efficacy due to acaricide resistance and the ever-present risk of residues in edible tissues. One approach to circumvent both these shortcomings is to exploit endemic, biologically active microbes which can supplement the host animal's ectomicrobiome and work to protect the animal against ectoparasite infestation.

Probio-TICK is a novel concept that applies the well-accepted health benefits of human "inner-health" probiotics to the "outer-health" of cattle hides by boosting the animal's innate resistance to pest invasion. Probio-TICK contains a community of beneficial microbes with known biological activity against ectoparasites which is applied to the hide of cattle. In particular, MST has identified a collection of organisms, endemic to northern Australia, that demonstrate acaricidal and chitinase activity *in vitro*. Chitin is a critical structural protein present in most life cycle stages of ectoparasites.

The Probio-TICK Initiative was funded through AusIndustry's CRC-P scheme, with Microbial Screening Technologies as the project lead and MLA and Macquarie University as contributing partners. The results of the research and early field studies will be used in further clinical development of Probio-TICK for use in the Australian beef industry and has potential to be applied internationally.

## Objectives

a. Isolate and identify microbes known to produce metabolites that are active against arthropods (ticks and insects) which parasitise cattle.
b. Apply such microbes to cattle in order to change the composition of the hide microbiome.
c. Demonstrate that microbes applied in this way cause a reduction in ectoparasite numbers.

The project has achieved all three of these objectives.

## Methodology

Candidate microbes from Northern Australian soil samples were tested in the laboratory for insecticidal and chitinase activity and further tested in in vitro acaricidal and buffalo fly models. By combining several microbes with positive attributes, two formulations of Probio-TICK were tested in a safety/feasibility field trial (GEN I), followed by two controlled efficacy and safety trials using tick-infested cattle (GEN II and GEN IIIa).

## Results/key findings

The project achieved proof of concept of a completely novel means of parasite control in just 3 years.

Microbes were selected and conditions for their larger scale growth were optimised. Laboratory testing against ticks and buffalo fly confirmed that the majority of the microbe extracts were active. The selected microbes were combined and prepared for application on cattle. The trial of an initial formulation showed that the product was safe and the proved the feasibility of topical application. Microbial samples collected from the hides in this trial were used to show that the microbes were incorporated into the ectomicrobiome of cattle

Following further refinement, two formulations containing between 5 and 9 microbial isolates, one oil-based (GEN II) and one aqueous (GEN IIIa), were used in field trials and shelf-life studies. The cattle in the trials were exposed to field tick challenge. For the oil-based backliner (trial code MST 002) tick burdens declined from Day 20 compared with untreated or placebo controls, showing a statistically significant difference in favour of Probio-TICK on Day 41. Cattle sprayed with the aqueous preparation (MST 004) had significantly lower tick counts on Days 13 and 27 (on average across Days 13, 20 and 27 tick counts were reduced by half, with a 73% reduction on Day 27 alone). On Day 27, 4 of the 20 cattle in the control (untreated) groups were rescue treated with a chemical acaricide because of excessive tick burdens (>150 ticks on one side).

The results show that natural control of ticks on cattle using carefully selected and curated endemic soil microbes can aid in the control of cattle tick during field challenge. The advantage of this product is that it has no chemical residues, and is safe to cattle, people and the environment. It has potential as an organic control agent for ticks and possibly other external parasites.

## Benefits to industry

The Probio-TICK Initiative has provided proof-of-concept that a sustainable microbial probiotic can be developed that will provide protection against cattle tick. Probio-TICK has potential to provide a lower cost, sustainable, eco-friendly, long-term solution to cattle pests. It is estimated that a commercial product will return $22M to producers assuming an adoption rate of 15% measured against the tick vaccine benchmark of 90% protection for 1 year. A provisional patent was submitted in April 2020 to protect the IP of the invention.

## Future research and recommendations

The future is to enter the second phase of work to build the scientific and business case for Probio-TICK. The scientific case will be underpinned by additional supportive studies and further product refinement. The supporting studies are underway or in planning. They include an 'establishment' trial (MST 006) to determine the appropriate treatment interval and to help elucidate the mode of action and a third field trial of a dust formulation to test if self-administration of Probio-TICK is feasible in remote areas (MST 007). Additional work will be to develop a 'Good Manufacturing Practice' formulation of Probio-TICK for wider field testing and shelf life work. Publication of the project results in peer-reviewed journals will assist the scientific credentials of the work. The business case is designed to position Probio-TICK as commercially attractive. It will include establishing a defined product registration pathway with APVMA. An adoption pathway supported by beef producer surveys will also inform the market appetite which, together with the GMP scale up will provide a commercial basis for investment by a third party.

Funding avenues are being explored to assist in continuing development work.

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# 1. Background

Ectoparasites (ticks and buffalo fly) of cattle are estimated to cause major economic loss exceeding $250M p.a. to profitable beef production in northern Australia. Management of these pests relies on the dual approach of host genetics and chemicals. The former is challenging in view of market demand for better eating quality, whereas the latter faces the loss of efficacy due to resistance and the ever-present risk of residues in edible tissues. Vaccines have been suggested as alternative control agents, however a commercial vaccine failed in the market place and, despite decades of research, other vaccine candidates are yet to reach the market. One approach to circumvent both these shortcomings is to exploit endemic, biologically active microbes which can supplement the host animal's ectomicrobiome and work to protect the animal against ectoparasite infestation. An advantage of this approach is that animals do not have to be injected as required by a vaccine and self-administration may be an option that would suit many management systems in northern Australia.

Probio-TICK is a novel concept that applies the well-accepted health benefits of human "inner-health" probiotics to the "outer-health" of cattle hides by boosting the animal's innate resistance to pest invasion. Probio-TICK contains a community of beneficial microbes with known biological activity against ectoparasites which is applied to the hide of cattle. In particular, MST has identified a collection of organisms, endemic to northern Australia, that demonstrate acaricidal, insecticidal and chitinase activity *in vitro*.

The Probio-TICK Initiative was funded through AusIndustry's CRC-P scheme, with Microbial Screening Technologies as the project lead and MLA and Macquarie University as contributing partners. The results of the research and early field studies will be used to further clinical development of Probio-TICK for future commercial use.

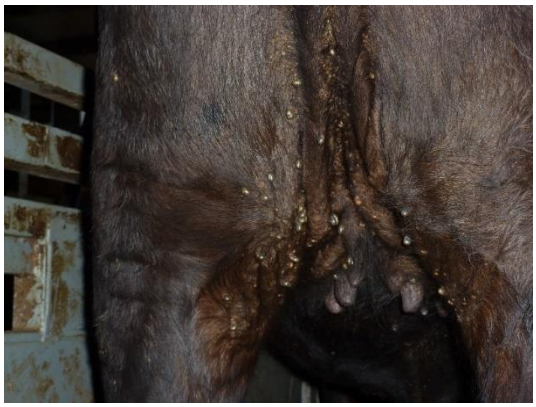

Fig. 1
A yearling from Trial MST 002 infected with cattle ticks from natural exposure in the field in North Queensland.

# 2. Objectives

The project had 3 objectives:

*a.* Isolate and identify microbes known to produce metabolites that are active against arthropods (ticks and insects) which parasitise cattle
b. Apply such microbes topically to cattle in order to change the composition of the hide microbiome
c. Demonstrate that microbes applied in this way cause a reduction in ectoparasite numbers.

The project has achieved all three of these objectives.

Work activities were reported across 4 milestones and for this Final Report are organised based on the project objectives (a, b, c, as above) plus a report on progress in planning for commercial development (d). This report is organised under the following topics:

a. Isolate and identify microbes known to produce metabolites that are active against arthropods (ticks and insects) which parasitise cattle:
   - Assess MST microbe library including activity and potential mode of action of microbes
   - Conduct bioassays to determine activity of microbes against cattle tick and buffalo fly *in vitro*
   - Assemble GEN I product with 5 microbes profiled by gene sequencing and growth characteristics

b. Apply such microbes topically to cattle in order to modify the composition of the hide microbiome:
   - Collect microbes from cattle hides and describe the endogenous microbiome
   - Evaluate GEN I on cattle for safety, feasibility of topical application and assimilation into microbiome

c. Demonstrate that microbes applied in this way cause a reduction in ectoparasite numbers:
   - Test GEN II on cattle exposed to tick infection for safety and efficacy
   - Test GEN IIIa microbes on cattle exposed to tick infection for safety and efficacy

d. Plans for commercial development:
   - Prepare and submit provisional patent
   - Develop plans for commercial development, including APVMA and meetings with companies.

# 3. Methodology

## 3.1 Project Methodologies

The project used a wide range of methodologies in the laboratory, field and in IP protection. The majority of the laboratory work was performed at MST with staff and equipment deployed to achieve the outputs. Third parties were engaged to perform other specialist tasks including additional laboratory work and field work.

Each set of methods was successfully applied to achieve the project objectives.

### 3.1.1 Microbes

Microbes in the MST collection were reviewed by searching for relevant characteristics in the database. All of the microbes were derived from soil recovered from northern Australia. All isolates were Actinomycetes in pure culture and characterised biologically and chemically. The desirable characteristics of isolates for ectoparasite control were as follows: recovered from soil in Northern Australia; *in vitro* activity against arthropod parasites; lack of *in vitro* toxic effects on mammalian cells; good growth characteristics; a secondary metabolite profile that includes relevant activity; chitinase activity. Using these filters, the collection of about 30,000 candidates was reduced down to 30 that were investigated further.

Chemical analysis and structural elucidation of secondary metabolites was undertaken at Macquarie University to define the chemotaxonomy of selected cultures and strengthen the IP.

Extracts of the 9 most promising microbes were tested for activity against cattle parasites *in vitro* (removed from their host and tested in the laboratory). Activity against cattle tick was performed by using a larval packet test[1]. In this test, freshly hatched larvae of *Rhipicephalus australis* were exposed to extracts of each of 9 selected microbial cultures and mortality rate assessed. Mortality of buffalo fly *Haematobia exigua* was measured *in vitro* following topical application of each of 6 microbial extracts onto adult flies. In both tests known actives were successfully used as positive controls to confirm the validity of the assays.

The collection of 30 microbes was provided to University of Western Australia for total genome sequencing.

Generation I (GEN I) comprised 5 of the most promising microbes from the MST soil collection. Production of the microbes in the laboratory was optimised and scaled up. The general system employed a grain-based growth medium and incubation at 28°C for a period of 5 - 7 days. Spores and actively growing Actinomycetes were collected, concentrated, adjusted to a standard concentration and combined into a suitable formulation for application onto animals. This methodology was also used for the production of later generations of Probio-TICK.

### 3.1.2 Cattle microbiome

The Bos-TICK project was initiated in parallel to the field trials to provide information on the composition of the cattle ectomicrobiome, about which little is known. The data will add to the scientific basis for the Probio-TICK concept and will form a baseline for future studies on establishment of the Probio-TICK microbes. Resident skin microbes were also considered as an additional potential source of novel microbes with anti-tick activity that could contribute to Probio-TICK. Collection of microbes in the Bos-TICK project was achieved by vacuuming the coats of 200 cattle in 30 locations, mainly in Queensland, to provide 1,000 samples. The microorganisms were packaged and sent to the MST laboratory. 200 samples were cultured and some further characterised by 16S sequencing. This collection represents the largest collection of cattle skin samples known.

For the trial on cattle in MST 001, GEN I organisms were applied to the coats of 5 cattle and microbiome assessed 7, 21 and 42 days after treatment. The protocol was approved by NSW Department of Primary Industry Animal Care and Ethics on 31 August 2018.

Samples were analysed at Macquarie University using 16S RNA gene amplification to identify the taxonomic composition of the ecto-microbiome.

### 3.1.3 Efficacy of Probio-TICK

GEN II (with 5 organisms) and GEN IIIa (with 9 organisms) were developed for use in field trials to test efficacy against natural challenge with ticks. The effect of Probio-TICK on eggs laid by treated female ticks was also assessed in the laboratory. Soils from which the microbes were recovered originated from northern Queensland, Western Australia and the Northern Terrritory.

[1] Anon (1971) Pl Prot Bull FAO 19: 15, cited by Roulston *et al* (1981) Aus vet J 57/8: 362

The two safety/efficacy field trials used a common methodology to measure efficacy of Probio-TICK on cattle exposed to natural cattle tick infestation. The trials also acted as product safety studies. The protocols were consistent with the internationally accepted protocols and were conducted in Northern Queensland by a third party contract research provider, Eurofins.

The trial protocols had the following details:

Cattle with predominantly *Bos taurus* genetics were maintained on farm for the trial. 20 cattle received Probio-TICK, 10 received vehicle control and 10 were untreated. Probio-TICK ($10^8$ of each microbe per animal per dose) was given in 5 applications of 100 mL at weekly intervals (Day 0, 6, 13, 20, 26). Tick counts and egg analysis were conducted on days 0, 6, 13, 20, 26 and 41 (and Day 56 for one trial). Health and skin condition were observed and reported during the 5-week trial. Skin swabs were taken on Day 1 and Day 41 to assess the microbiome. Trial MST 002 tested an oil backline formulation (GEN II). Trial MST 004 tested an aqueous body spray (GEN IIIa).

For the egg analysis, engorged adult female ticks were collected from cattle in this trial and sent to the Brisbane laboratory of QDAF. All ticks were viable on arrival and were identified as *Rhipicephalis australis*. They were incubated at approximately 27°C and 85% relative humidity for a period of 7 days and parameters including number and weight of ticks, egg hatch weight and viability of hatched eggs were assessed and reported.

Statistical analysis of the trial data was performed by a separate third party provider.

Trials were performed under appropriate ethics and permit requirements, namely the Queensland Government Department of Agriculture and Fisheries Animal Ethics Committee AEC Application Reference Number SA 2019/10/715 and CA 2020/02/1357 for trials MST 002 and MST 004, respectively.

### 3.1.4 Commercial development

Microbes are owned by MST and further chemical characterisation (metabolite profile and genome) undertaken to strengthen the IP position. A Patent Attorney was employed to prepare the patent. Background work included patent searches and literature searches.

A consultant was engaged to plan steps in registration of a future product. This will be used to establish a registration pathway with the Australian Pesticides and Veterinary Medicines Authority (APVMA). Commercial companies were interviewed and a group of northern beef producers interviewed about the market needs for Probio-TICK. This work is ongoing.

# 4. Results

## 4.1 Results

### 4.1.1 Microbes

The MST microbe library provided a rich source of microbes and the database provided a ready method for searching and sorting. Members of this collection were analysed for fermentation performance, metabolite production, bioactivity and spore characteristics. The microbe and chemistry aspects of the work has seen successful sorting and selection of a small group of candidate microbes from a collection of over 30,000 potential candidates. From the 30 microbes chosen, 1,000 distinct chemicals were isolated and many characterised. The 30 selected microbes were genetically fingerprinted with total genome sequencing.

The GEN I panel of 5 microbes was grown up and used in the first animal field trial in Goulburn, NSW which focussed on safety and ease of application (MST 001). On further analysis, the collection fell below desirable levels for spore count, longevity and chitinase activity and so alternative organisms from the original 30 were chosen for Gen II (see 4.1.3).

MST 001 trial was conducted to observe the health of cattle subjects after treatment vs. untreated controls, and assess the longevity of the GEN I microbes on cattle. The microbiome study is reported below. The cattle had no adverse health effects based on body condition score, body mass and skin appearance.

Fig 2. A collection of 9 Probio-TICK microbe cultures following growth on grain in plastic bags and prior to processing to enrich for microbes.

*In vitro* trials

The packet test on tick larvae was validated with known positive controls. All of the 9 microbial extracts tested showed efficacy >36% and 7 of the 9 achieved efficacies between 71 and 100%.

Tests against buffalo fly (% mortality with topical application) validated the effect of positive control chemicals. The efficacy of all six microbial extracts exceeded 60% and four of the extracts were 100% effective.

### 4.1.2 Cattle Microbiome

The work was conducted in two steps:

*Bos-TICK Survey*

The metagenomics of samples collected from Northern Queensland clearly demonstrated that, despite geographic distances between the properties, there was a large overlap in microbial taxonomic classes and genera indicating that although the diversity of microbes in the ectomicrobiome is large, it does not show significant geographic effects. While the population was not identical to Day 42 of the analysis of ProbioTICK Gen I, both populations showed a complex and diverse ecto-microbiomes.

The major findings were that Actinomycetes are an identifiable sub-population of the ecto-microbiome of the cattle hide and haircoat (~1% of total bacterial population) and can therefore be replaced by Actinomycetes belonging to *Streptomyces,* such as those in Probio-TICK, without unduly altering the ecto-microbiome. Based on their genetic fingerprint, bacterial populations of a cohort of ten animals in a herd were tightly clustered with individuals showing very similar microbial classes and genera confirming comparable ecto-microbiome diversity (Figure 3). Further, the diversity of classes and genera from the metagenomics analysis is consistent with the bacterial strains isolated by conventional microbiology.

*Animal study with GEN I (MST 001)*

Microbiome analysis was performed on 189 samples, including baseline (pre GEN I treatment) and post treatment (9 regions of animals' coats were sampled in both cases) as well as 26 hide samples and 2 farm dust samples. The baseline and dust samples had a similar population pattern, while the vacuumed animals and post treatment samples were distinct.

The conclusions from microbiome work are that:

- We have vastly expanded the knowledge of the cattle ecto-microbiome
- The ecto-microbiome of cattle comprises a similar population of organisms between farms.
- The populations differ between pre- and post-treatment.
- Together these data suggest that the microbes included in Probio-TICK will be able to establish ectodermal populations on treated cattle and that altering the microbiome is a feasible approach.
- Trial MST 001 included cattle that were provided a good plane of nutrition and the changes in microbiome may have also reflected their improved health. This suggests that the microbiome reflects hide health.
- The 3 generations of Probio-TICK have now been used on 45 cattle with no adverse effects on health or skin at the site of application.

### 4.1.3 Efficacy of Probio-TICK

In the trial of the oil-based backliner (GEN II, MST 002) tick burdens declined from Day 20 compared with untreated or placebo controls and tick counts were significantly lower on Day 41 (64%).

Cattle sprayed with the aqueous preparation (GEN IIIa, MST 004) had significantly lower tick counts on Days 13 and 27 (on average across Days 13, 20 and 27 tick counts were reduced by half; with a 73% reduction on Day 27 alone). On Day 27, 4 of the 20 cattle in the control groups were rescue treated with a chemical acaricide because of excessive tick burdens (>150 ticks). No Probio-TICK – treated animals required additional treatment.

In both studies the effect on ticks lasted 2 to 3 weeks after the last treatment.

Eggs were successfully harvested and collected from ticks but neither preparation affected egg hatch rates.

Probio-TICK use in these trials shows that there was a delay in onset of effect (14 -20 days) suggesting that it takes time for microbes to spread and establish at tick predilection sites. Tick numbers remained low in treated animals showing that efficacy persists for 2-3 weeks after treatment. These time periods coincide with the time between infestation with tick larvae and their development through the nymphal stage to become adults. The data suggest that the Probio-TICK microbes affect the establishment and/or development of larvae and/or nymphs so that the effect on adult tick numbers is seen 2-3 weeks afterwards. Consistent with this hypothesis is that chitin is a structural protein that is essential for tick integrity, growth and development and the chitinase produced by the microbes may disrupt tick development.

From these studies, we conclude that by using a protocol of weekly treatments, Probio-TICK reduced tick numbers to the extent that such a product would assist with parasite control in field situations and that the product can protect cattle against tick infestation.

### 4.1.4 Commercial Development

A Provisional Patent was submitted to the Australian Patent Office on 15 April 2020 (application number 2020901107).

A plan was developed to inform the registration pathway, although the meeting with APVMA is on hold. Seven Chairs of RBRCs affiliated with NABRC were interviewed on the desirability of a natural tick control product. All were supportive and some keen to support a preparation suitable for remote use.

# 5. Key findings

## 5.1 Major findings

The project results lead to the following findings:

- Access to a large, characterised microbial collection proved to be a major strength of the project. This approach saved years of development time and cost and it provided the opportunity to re-sample the collection during the project to refine each generation of Probio-TICK.
- The Probio-TICK microbes have the potential to be incorporated in the cattle microbiome.
- Two studies of cattle challenged with natural tick infection showed efficacies in the 65-70% range. The two studies used two different formulations and different microbe collections (with some overlap).

- Probio-TICK use shows that there was delay in onset of effect (14 -20 days) suggesting that it takes time for microbes to migrate and establish at tick predilection sites. Further, efficacy persists for 2-3 weeks after treatment. It is hypothesized that the ProbioTICK microbe community interrupts the establishment of larval ticks and, possibly, nymphs on cattle, meaning that the effect is only seen 2 – 3 weeks after treatment, when exposed larvae and nymphs would be expected to have reached engorged adult-hood.
- Probio-TICK is safe to use on cattle.

# 6. Conclusion and recommendations

## 6.1 Conclusions

- The Probio-TICK project has shown that endemic microbes from Northern Australia when applied to the hide of cattle can aid the control of cattle tick. This represents a completely novel approach to control. Probio-TICK has potential to control cattle tick in enterprises using organic production with *Bos taurus* or other cattle.
- The Probio-TCK invention and project IP have been protected by a Provisional Patent.
- The product is ideal for Australian local manufacture.

## 6.2 Recommendations

The scientific case should be underpinned by further supporting studies and product refinement. Some supporting studies are underway or in planning. They include an 'establishment' trial to determine the longevity of the microbes on the cattle hide after application of Probio-TICK to inform on treatment intervals (MST 006). A dust formulation using GEN IIIb microbes will test if self-administration of Probio-TICK is feasible in remote areas (MST 007). Additional work will be to develop a GMP formulation of Probio-TICK for wider field testing and shelf life work.

The business case is designed to position Probio-TICK as an attractive development investment for a third party. In addition to the proven efficacy portfolio the case will include establishing a defined registration pathway with APVMA. In addition, an adoption pathway supported by beef producer surveys will also inform on the market appetite which, together with the GMP scale up and product costings, will provide a commercial basis for investment.

The aim now is to seek funding to take Probio-TICK to the next stage. This includes approaches to CRCNA, CRC-P, AWI and MDC.