ISOLATION AND CHARACTERISATION OF TANNIN-RESISTANT BACTERIA FROM THE RUMEN OF FERAL GOATS AND CAMELS

A thesis

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by

Anita Sardiana Tjakradidjaja

Ir. – IPB – Bogor – Indonesia

Dip.Ag.Sc. – UNE – Armidale – Australia

M.Rur.Sc. – UNE – Armidale – Australia

SCHOOL OF AGRICULTURE, FOOD AND WINE

THE UNIVERSITY OF ADELAIDE

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Anita Sardiana Tjakradidjaja
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Summary

Low availability and poor nutrient quality of tropical grasses result in low levels of animal production. Browse/shrub legumes such as mulga (*Acacia* sp.) and calliandra (*Calliandra calothyrsus*) can be used as supplements to improve animal production. However, their utilisation is limited by the presence of antinutritional compounds, such as tannins. Tannins are polyphenolic compounds that are capable of binding other nutrients to form stable complexes. Tannins comprise hydrolysable (HT) and condensed tannins (CT) with CTs being the major form found in the legumes. Their concentration in feeds determines their effect on animal production. Low levels of tannin (< 40 g.kg DM^{-1}) protect feed protein from degradation by rumen microbes, thereby increasing the amount of protein passing through the rumen, and reducing the potential for bloat. However, high concentrations of tannin retard animal production through the inhibition of enzyme activities of some rumen bacteria, decreasing the availability of protein and fibre, and reducing feed intake. Tannins in high concentrations also bind nutrients such as protein and carbohydrates; tannin-protein or tannin-carbohydrate complexes are difficult to digest by rumen microbes or by enzymes secreted by ruminants in the gastrointestinal tract. This decreases the availability of protein and carbohydrate for the animals, reducing feed intake and decrease animal production.

Several methods have been developed to overcome tannin problems in livestock feeds with a focus on the use of biological treatments. Such treatments emphasise the involvement of rumen microorganisms residing in the rumen of feral animals or of animals adapted to feeds with high tannin contents. This approach resulted in the isolation of the tannin-tolerant bacteria, *Streptococcus gallollyticus* and *Selenomonas ruminantium* K2, from the rumen of feral goats. However, these bacteria, alone or in combination, were unable to mimic the effect of whole rumen fluid on tannin detoxification.

These bacteria may not, therefore, be the only ruminal species that are resistant to, or degrade tannins. Other tannin-resistant (TR) bacteria may also exist in the rumen. Although each of these TR-bacteria may have specific characteristics, they may interact synergistically to promote digestion of tannin-containing browse legumes. Therefore, the present project was aimed at isolating TR-bacteria from the rumen contents of feral goats and camels, and studying their ecology.

To achieve these objectives, the research project is divided into three sections as follows :

1. Optimising the method for extracting tannin from legume leaves and selecting the appropriate method to measure tannin content.
2. Isolating TR-bacteria in rumen fluid samples from feral goats and camels, and characterising them phenotypically and by molecular characterisation using restriction analysis of amplified 16S rDNA and 16S rDNA sequence analysis.
3. *In vitro* studies of dry matter (DM) disappearance of mulga and calliandra with populations of TR-bacteria grown in a monoculture or in a co-culture system.

Extracts of condensed tannins (CTs) were used as substrates for isolating TR-bacteria from feral goat and camel rumen fluids. CTs were extracted from legumes using 70% acetone, and used as a substrate in bacterial enrichment studies.

The amount of soluble tannin extracted from the samples varied among plant species with mulga containing less free tannin than calliandra. Factors such as processing methods (fresh, freeze-drying and oven-drying), or physical and chemical treatments (autoclaving and phenol extraction) are tested for their effect on yield of extractable tannin. Freeze-drying was selected for processing mulga and calliandra leaves before extraction of CT by the method of Terrill *et al*. (1992).

Measurement of tannins using different methods yielded different values of extractable tannin from the same legume. Since vanillin-HCl, butanol-HCl and H_2SO_4

methods are based on different principles, the standard used, the solvents, and other factors were tested for each method. The H_2SO_4 method was selected to measure the amount of free tannin extracted from the legumes. This selection was made on the basis that the results were comparable to those produced by the butanol-HCl assay procedure, but the H_2SO_4 method would have less interference from water in the microbiology studies. The amount of extractable tannin was expressed as equivalents to quebracho tannins when 70% acetone was used to dissolve the tannin extracts and the standard.

Twenty TR-bacteria were successfully isolated from the rumen fluid of feral goats and camels using extractable CT of freeze-dried mulga and calliandra, as well as hydrolysable tannin (tannic acid). These isolates were divided morphologically into several groups : Gram-positive cocci (Group 1), Gram-positive cocci/rods (Group 2 and 4), Gram-negative cocci (Group 3), Gram-negative curved rods (Group 5) and Gram-negative slender rods (Group 6).

These bacteria were identified by physiological and biochemical characterisations, and API testing as *Streptococcus* sp. (Group 1), *Lactobacillus* sp. (Group 2 and 4), *Selenomonas ruminantium* (Group 5) and *Butyrivibrio* sp. (Group 6). A further identification using restriction analysis of amplified 16S rDNA with four restriction endonucleases (*Alu*I, *Hae*III, *Msp*I and *Taq*I) characterised bacteria that belong to the *Streptococcus* sp. (Group 1) and *Selenomonas ruminantium* (Group 5). Confirmation of genera of other TR-bacteria was made after integrating the RFLP analysis with amplified 16S rDNA sequence analysis. This identified the bacteria as : *Lactobacillus* sp. (Group 2 and 4), and *Butyrivibrio* sp. (Group 6). The genus of Gram-negative coccus (Group 3) was identified as *Escherichia* (*E. coli*) *coli*; however, a further clarification is necessary for the identification Group 3 isolate. These results provide an example of phenotypic identification and molecular characterisations using restriction analysis of amplified 16S rDNA and 16S rDNA sequence analysis for identifying TR-isolates from feral goat and camel rumen fluids.

The TR-bacteria had different capabilities of degrading mulga and calliandra as indicated by results of studies on *in vitro* DM degradability using monocultures of the TR-bacteria. The highest DM degradabilities of both legumes were obtained in cultures inoculated with *Butyrivibrio* spp. (G23A and G53C), and *S. gallolyticus*. These TR-bacteria are the main TR-bacteria degrading mulga and calliandra. The other TR-bacteria, *Lactobacillus* spp. (G43C and G33A), *E. coli* (C43C) and *Sel. ruminantium* K2, used metabolic products from these main TR-bacteria. Co-culture between the main TR-bacteria (*Butyrivibrio* spp. (G23A and G53C) *or S. gallolyticus*) with the bacteria that utilize the metabolic products of tannin degradation (*Lactobacillus* spp. (G43C and G33A), *E. coli* (C43C) or *Sel. ruminantium* K2) could improve mulga and calliandra degradation.

Calliandra leaves were more degraded than mulga leaves because calliandra leaves have smoother physical characteristics, and lower concentrations of fibrous components and protein/fibre bound tannins than mulga leaves. Grinding the sample leaves and incubating cultures for 48 h improved degradation of legume leaves; incubation for 48 h was also suitable for TR-bacteria to achieve optimum growth and enzyme secretions.

TR-bacterial characteristics and populations, and the nature of legume leaves are important factors affecting degradation of legumes containing tannins. Factors associated with TR-bacterial characteristics are the presence and activity of enzymes for degrading nutrients and antinutrients from the legumes, the presence of substances protecting TR-bacteria from the antinutrients present in the legume, and the bacterial growth characteristics during the period of legume degradation. All these factors, consequently, affect TR-bacterial populations in cultures that contain legumes with tannins. Factors related to the nature of legume leaves are the physical characteristics, nutrient composition and concentration, especially the fibrous compounds and the types of tannins or other antinutrients.

It is concluded that *Streptococcus gallollyticus* and *Selenomonas ruminantium* are not the only tannin-resistant bacteria. Other bacteria from the rumen of feral goats and camels also tolerate tannins extracted from freeze-dried mulga and calliandra leaves, including

Butyrivibrio sp., *Lactobacillus* sp. and *E. coli* (Gram-negative coccus). These bacteria differ in their abilities to digest the legumes. However, improvement in the legume digestibility can result from the interactions between these tannin resistant bacteria.

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List of Abbreviations

Abs	absorbance
ADF	acid detergent fibre
ADL	acid detergent lignin
bp	base pair
BHI	brain heart infusion
CF	crude fibre
CP	crude protein
CT(s)	condensed tannin(s)
DM	dry matter
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra acetic acid
ed. (s)	editor(s)
Edit.	edition
EE	ether extract
eg.	*exempli gratia* (for example)
et al.	*et alia*
etc.	*et cetera*
FD	forage - Dehority medium
g	gram
h	hour
HT(s)	hydrolysable tannin(s)
ie.	that is
Kb	kilobase pair
kg	kilogram
l	litre
m	metre
µg	microgram
µl	microlitre
mg	milligram

mM	millimolar
min	minute
M	molar
MW	molecular weight
N	normal
NDF	neutral detergent fibre
NFE	nitrogen free extract
ng	nanogram
nm	nanometer
OD	optical density
OM	organic matter
PCR	polymerase chain reaction
pmol	picomole
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rotations per minute
sd	standard deviation
sec	second
sem/SEM	mean standard error
sp./spp.	species
TAE	Tris acetate EDTA buffer
Tr-bacteria	tannin-resistant bacteria
VFA	volatile fatty acid
$v.v^{-1}$	volume per volume
$w.v^{-1}$	weight per volume
$w.w^{-1}$	weight per weight