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Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population *in vitro*

Anuraga Jayanegara*, Gunjan Goel[1], Harinder P.S. Makkar[2], Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany

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ABSTRACT

This study aimed to investigate the effects of purified hydrolysable (chestnut and sumach) and condensed tannins (mimosa and quebracho) on methane production, rumen fermentation and microbial population structure. The tannins were extracted and purified from their original plant sources, and were characterized for their protein precipitation capacity. The purified tannins were added into 380 mg hay:concentrate substrate (70:30 w/w) at three different concentrations (0.5, 0.75 and 1.0 mg/ml). *In vitro* incubation was carried out for 24 h at 39 °C in 100 ml calibrated glass syringe containing 30 ml of medium (10 ml rumen liquor and 20 ml double strength buffer). Parameters measured after the incubation were gas production, methane concentration, short chain fatty acids (SCFA: C_2, C_3, C_4, *iso*C_4, C_5, *iso*C_5, total SCFA and ratio of C_2/C_3), *in vitro* organic matter digestibility (IVOMD) and microbial population structure. The experiment was performed in three runs, represented by two incubation units per run. Results revealed that the protein precipitation capacity of chestnut and sumach tannins was greater than that of mimosa and quebracho tannins. An interaction between different tannins and doses existed with regard to methane concentration ($P \leq 0.05$). All the tannins decreased methane concentration either linearly or quadratically, but their magnitudes were different; the magnitude of decrease was greater for the hydrolysable tannins than the condensed ones, and correlated with their protein precipitation capacity. Increasing levels of all tannins decreased IVOMD by following a quadratic pattern ($P \leq 0.05$) and there was a tendency that the condensed tannins decreased IVOMD more than the hydrolysable tannins ($P \leq 0.1$). All the purified hydrolysable and condensed tannins decreased total methanogen population ($P \leq 0.05$) than that of control when added at 1.0 mg/ml; the decrease ranged from 22.3 to 36.7% from control. Additions of purified tannins at all levels generally decreased *Fibrobacter succinogenes* population ($P \leq 0.05$). Sumach tannins at all addition levels decreased the population of *Ruminococcus flavefaciens* ($P \leq 0.05$), and the magnitude of decrease was much greater than those of other tannins. It is concluded that hydrolysable tannins had a greater effect in reducing methane emission with less adverse effect on digestibility than those of condensed tannins.

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; GHG, greenhouse gas; NDF, neutral detergent fibre; IVOMD, *in vitro* organic matter digestibility; PF, partitioning factor; qPCR, quantitative real-time polymerase chain reaction; SCFA, short-chain fatty acid.

* Corresponding author. Present address: Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, Bogor Agricultural University, 16680 Bogor, Indonesia.

E-mail address: anu_jayanegara@yahoo.com (A. Jayanegara).

[1] Present address: Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan 173234, India.

[2] Present address: Animal Production and Health Division, Food and Agriculture Organization of the United Nations, Rome, Italy.

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1. Introduction

Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are important greenhouse gases (GHG) in the atmosphere, and their global atmospheric concentrations have considerably increased especially during the last century (Monteny et al., 2006). Accumulation of these gases raises the earth's temperature and contributes to global warming (Rosenzweig et al., 2008). Agricultural sector is among the major sources of GHG in which about 0.20–0.35 of the global GHG emission is originated from the sector. Approximately 0.70 of methane emission arises from anthropogenic sources in which agriculture accounts for about two-third from the figure (EPA, 2010). Enteric fermentation from ruminants, globally, is estimated to account for between one-quarter and one fifth of anthropogenic methane emissions (Thorpe, 2009).

On the other hand, apart from their contribution to global warming, ruminants are capable of converting fibrous materials such as grasses and agricultural by-products into high quality foods such as milk and meat. Other non-food products from the animals could also be utilized for human benefit such as skin, wool, faeces, *etc.* With regard to the context of ruminant production, methane emission from enteric fermentation is not only associated with environmental problems, but it also represents considerable amount of energy losses from the animals. It has been estimated that around 0.06–0.10 of the gross energy of the ruminant diet is lost through methane (Immig, 1996). Therefore, developing feeding strategies to minimize methane emissions is desirable both for conserving the environment as well as for increasing the efficiency of energy utilization.

A number of nutritional attempts have been made to lower enteric methane emission from ruminants as reviewed by some authors (Takahashi et al., 2005; Beauchemin et al., 2008; Hristov et al., 2013). With a rapidly growing concern on food safety, natural compounds such as those originated from plants for mitigating the methane emissions are preferable over the synthetic ones (Makkar et al., 2007). Accordingly, essential oils, saponins and polyphenols or tannins are the prospective plant natural compounds for mitigating methane emissions (Benchaar and Greathead, 2011; Bodas et al., 2012; Jayanegara et al., 2014). With regard to tannins, previous studies have reported that feeding of tannin-containing forages to ruminants reduced methane emissions (*e.g.*, Puchala et al., 2005; Animut et al., 2008). However, in most of those studies, the reduction in methane was obscured by changes in forage composition and quality that may affect the emission as well. Thus, there is a considerable uncertainty about the effectiveness of tannin-containing forages to reduce enteric methane emissions from ruminants.

In the present study, other confounding components were omitted by extracting and purifying tannins from some plant sources, *i.e.* chestnut (*Castanea* sp.), sumach (*Rhus typhina*), mimosa (*Mimosa tenuiflora*) and quebracho (*Schinopsis balansae*). The first two plants were rich in hydrolysable tannins and the others were rich in condensed tannins; both tannin types may elicit different responses on rumen methanogenesis due to their distinct chemical structures in which such kind of study is limitedly investigated to date. Although tannins particularly hydrolysable tannins may cause toxicity responses to ruminants when consumed at excessive amounts, they provide beneficial effects when used at low to moderate concentrations (Reed, 1995). Condensed tannins are not usually toxic to ruminants since they are not absorbed (Reed, 1995), but they may bind parts of the nutrients irreversibly, making them unavailable. Also they can bind to gastrointestinal tract, causing adverse effects (Makkar et al., 2007); this is a drawback of condensed tannins as opposed to that of hydrolysable tannins. The working hypotheses were that the purified tannins would reduce methane production and that different forms of the tannins would elicit different response on methane reduction *in vitro*.

2. Materials and methods

2.1. Extraction and purification of tannins

Chestnut and sumach plant materials were collected from the botanical garden of University of Hohenheim, Stuttgart, Germany. Mimosa and quebracho materials were collected from Mongolia in conjunction with other plants used in our previous study (Jayanegara et al., 2009). Extraction of tannins from the plant materials (1 g each in 50 ml of aqueous methanol, 1:1 v/v) was done in an ultrasonic water bath at 135 W (Branson 3210, Connecticut, USA) for two successive periods of 25 min each, pooling both the supernatants, and centrifugation at 10,000 × *g* for 10 min at 4 °C (Makkar, 2003). The purification procedure of tannins was carried out by using a Sephadex LH-20 column, according to the modified method from Makkar and Becker (1994). Supernatant obtained from the extraction procedure was added with 1 mg/g of ascorbic acid to prevent oxidation of tannins. This supernatant was passed through a swollen slurry of Sephadex LH-20 prepared in aqueous methanol (1:1, v/v). The Sephadex LH-20 was washed slowly on a sintered glass funnel with approximately 1.5 l of aqueous methanol (1:1, v/v) under gravity to remove non-tannins from the Sephadex. The tannins remained on the Sephadex LH-20 and gave it a brown colour. Tannins were eluted using aqueous acetone (7:3, v/v). Acetone was removed under vacuum at about 30 °C and then the aqueous solution containing tannins was lyophilized. The tannins were characterized and used in the subsequent *in vitro* rumen fermentation experiment.

2.2. Characterization of purified tannins

Characterization of purified tannins from chestnut, sumach, mimosa and quebracho were done by butanol–HCl-method (Makkar, 2003) and by protein precipitation method using blue dye bound bovine serum albumin (BSA) (Asquith and Butler,

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