CARISSA MICHELLE GOLTARA BICHARA

ESTUDO *IN VITRO* DE EXTRATOS VEGETAIS AMAZÔNICOS COMO MODULADORES DA BIOHIDROGENAÇÃO DE ÁCIDOS GRAXOS E INIBIDORES DA DEGRADAÇÃO PROTEICA E METANOGÊNESE EM FLUIDO RUMINAL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

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Neuza Maria Brunoro Costa

Cristina Mattos Veloso

Luciana Navajas Rennó

Hervé Louis G.Rogez
(Coorientador)

Josefina Bressan
(Orientadora)

Aos meus pais, José Walton e Elisete Bichara

e irmãos Débora Bichara e Walton Junior,

Dedico

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SUMÁRIO

LISTA DE ABREVIATURAS

ADF	*Acid detergente fiber* (fibra em detergente ácido-FDA)
BH	*Biohydrogenation*
C16:0	*Palmitic acid*
C18:0	*Stearic acid* (ácido esteárico)
*t*11-C18:1	*Vaccenic acid* (ácido vacênico)
C18:1 *n*-9	*c*9-18:1, *oleic acid* (ácido oleico)
C18:2 *n*-6	*c*9,*c*12-18:2, *linoleic acid* (ácido linoleico)
C18:3 *n*-3	*c*9,*c*12,*c*15-18:3, *α-linolenic acid* (ácido α-linolênico)
C20:5 *n*-3	*Eicosapentaenoic acid* – EPA (ácido eicosapentanóico)
*c*9,*t*11-CLA	*Rumenic acid* (ácido rumênico)
CH_4	*Methane* (metano)
CLA	*Conjugated linoleic acid* (ácido linoleico conjugado)
CT	*Condensed tannins* (taninos condensados)
DO	*Desmodium ovalifolium*
FA	*Fatty acid* (ácidos graxos)
GEE	Gases causadores do efeito estufa
HT	*Hydrolysable tannins* (taninos hidrolisáveis)
IE	*Inga edulis*
LP	*Lipolysis* (lipólise)
MUFA	*Monounsaturated fatty acids* (ácidos graxos monoinsaturados - AGMI)
NDF	*Neutral detergente fiber* (fibra em detergente neutro-FDN)
PSC	*Plant secondary compounds*
PT	Polifenóis Totais
PUFA	*Polyunsaturated fatty acid* (ácidos graxos poli-insaturados-AGPI)
SFA	*Saturated fatty acid* (ácidos graxos saturados-AGS)
TAG	*Triacylglycerols* (triacilgliceróis)
UFA	*Unsaturated fatty acid* (ácidos graxos unsaturados-AGI)
VFA	*Volatile fatty acids*

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RESUMO

BICHARA, Carissa Michelle Goltara, D.Sc. Universidade Federal de Viçosa, outubro de 2014. **Estudo *in vitro* de extratos vegetais amazônicos como moduladores da biohidrogenação de ácidos graxos e inibidores da degradação proteica e metanogênese em fluido ruminal**. Orientadora: Josefina Bressan. Coorientador: Hervé Louis Ghislain Rogez.

O objetivo desse trabalho foi avaliar os efeitos *in vitro* de extratos vegetais de duas leguminosas amazônicas na proteção dos ácidos graxos poli-insaturados (AGPI) contra a biohidrogenação ruminal, na fermentação microbiana, na inibição da degradação proteica e metanogênese, via produção de amônia e metano (CH_4), respectivamente. Extratos de folhas de *Inga edulis* (IE) e *Desmodium ovalifolium* (DO) foram incubados com silagem de capim como substrato, nas concentrações 0 (C_0; controle), 5 (C_5), 10 (C_{10}), 50 (C_{50}) e 100 (C_{100}) mg de extrato liofilizado por g de matéria seca (MS), a 39 ºC, com uma mistura de fluido ruminal:tampão (v/v) e óleo de linhaça. Após diferentes tempos de incubação (3, 6, 9 e 24 horas), o perfil lipídico, perfil de ácidos graxos voláteis (AGV's), produção de amônia e metano no fluido ruminal foram determinados. O processo de biohidrogenação do ácido α-linolênico (C18:3 *n*-3) foi retardado na presença de ambos os extratos. Após 3 horas de incubação, a diminuição do desaparecimento do ácido α-linolênico variou de 23 a 65% com o extrato de IE e 15 a 61% com extrato de DO. Similarmente, o desaparecimento do ácido linoleico (C18:2 *n*-6) após 3 h foi reduzido em 19 a 69% e 13 a 56% com uso de IE e DO, respectivamente. Ácido rumênico (*c*9,*t*11-C18:2), um importante ácido linoleico conjugado (CLA) e primeiro intermediário do processo de biohidrogenação do ácido linoleico, aumentou significativamente (P<0,05) com a suplementação de ambos os extratos no tratamento C_{10} nas primeiras 3 h e com C_{10} e C_{50} após 6 h de incubação. A concentração de AGV's foi afetada somente com elevadas concentrações de extratos (C_{50} e C_{100}), onde houve uma diminuição na concentração dos principais AGV's no fluido ruminal. Em relação à produção de amônia, os extratos reduziram a concentração de amônia ruminal, comparado ao controle, em 8 a 38% e 8,6 a 47% para IE e DO, respectivamente. Ambos os

extratos também apresentaram efeitos antimetanogênicos no fluido ruminal, atingindo reduções de 20 a 40% quando expresso em mmol CH_4/mmol de glicose fermentada. Esses resultados demonstram que o uso de extratos de *Inga edulis* e *Desmodium ovalifolium* retardam a biohidrogenação ruminal durante as primeiras horas de incubação e apresentam potencial como aditivos alimentares visando a redução da produção de metano e amônia em ruminantes, permitindo assim, aobtenção de alimentos provenientes de ruminantes mais saudáveis para o consumo humano, com menos impacto ao meio ambiente.

ABSTRACT

BICHARA, Carissa Michelle Goltara, D.Sc. Universidade Federal de Viçosa, october, 2014. ***In vitro* study of amazonian plant extracts as modulators of biohydrogenation of fatty acids and inhibitors of protein degradation and methanogenesis in ruminal fluid.** Adviser: Josefina Bressan. Co-adviser: Hervé Louis Ghislain Rogez.

The objective of the present study was to investigate the effect of phenolic-rich extracts from two Amazonian plant species on *in vitro* ruminal by-pass of polyunsaturated fatty acids (PUFA), rumen microbial fermentation, inhibition of ammonia and methane production. Leaf extracts of *Inga edulis* (IE) and *Desmodium ovalifolium* (DO) were incubated with grass silage as substrate, at 0 (control; C_0), 5 (C_5), 10 (C_{10}), 50 (C_{50}) and 100 (C_{100}) mg of lyophilized extract per g of feed dry matter, at 39 ºC in a rumen fluid:buffer mixture, together with linseed oil. After different incubation times: 0, 3, 6, 9 and 24h, the fatty acid profile present in the fermentation fluid, total volatile fatty acids (VFA), methane and ammonia production were analyzed. The α-linolenic acid (C18:3 *n*-3) biohydrogenation process was retarded in the presence of IE and DO extracts. After 3 hours, the α-linolenic acid disappearance was reduced by 23 to 65% with IE and 15 to 61% with DO. Accordingly, the linoleic acid (C18:2 *n*-6) disappearance after 3 hours was reduced by 19 to 69% and 13 to 56% for IE and DO, respectively. Rumenic acid (*c*9,*t*11-C18:2), an important conjugated linoleic acid (CLA) isomer and first intermediate of the biohydrogenation process of linoleic acid, significantly increased ($P<0.05$) with both extracts for treatment C_{10} in the first 3 hours of incubation, and for most of the treatments after 6 hours of incubation. Total VFA concentrations were affected by dietary treatment only at the highest concentrations (C_{50} and $C1_{00}$), which where there was a decrease in the concentration of the major VFA's ruminal. Relative to the control diet, the phenolic-rich extract reduced the rumen fluid ammonia level from 8 to 38% and 8.6 to 47% for IE and DO, respectively. Both legumes also presented antimethanogenic effects in the ruminal fluid, levels ranging from 10 to 40% were found when expressed mmol CH4/mmol fermented hexoses. These

results lead us to conclude that phenolic-rich extracts from *Inga edulis* and *Desmodium ovalifolium* slow down the ruminal fatty acid biohydrogenation during the first hours of incubation and has potential as feed additives to reduce methane and ammonia production in ruminants, allowing healthier meat and dairy products, with less impact on the environment.

1 **INTRODUÇÃO GERAL**

Durante as últimas décadas, recomendações para reduzir a ingestão de gorduras saturadas, como aquelas provenientes de alimentos derivados de ruminantes (carne, leite e manteiga) foram feitas por diversas organizações relacionadas à nutrição e saúde. Em 2003, a Organização Mundial da Saúde (OMS) concluiu que a ingestão de ácidos graxos saturados (AGS) está diretamente relacionada ao risco de doença cardiovascular.

A meta atual é restringir a ingestão de AGS para menos de 10% do consumo diário de energia e menos de 7% para os grupos de alto risco (OMS, 2003; Santos et al., 2013). No entanto, estas recomendações para redução da gordura saturada foram fortemente criticadas por focar demais somente na redução de AGS ao invés de enfatizar o aumento do consumo de gorduras mais saudáveis, ou seja, a gordura com maior proporção de ácidos graxos insaturados (AGI), tais como óleos de oliva, amendoim, milho, soja, entre outros. Diversos estudos têm demonstrado que a substituição de AGS da dieta por ácidos graxos mono (AGMI) e poli-insaturados (AGPI) é considerada uma estratégia para o melhor controle da hipercolesterolemia e consequente redução da chance de eventos clínicos (Santos et al., 2013, Mozaffarian, 2010), assim como para a melhora da sensibilidade à insulina (Summers et al., 2002) e diminuição do risco de Diabetes *mellitus* tipo 2 (Van Dam, 2002).

Embora os produtos derivados de ruminantes sejam conhecidos por seu alto teor de AGS e sejam fortemente associados ao aumento do risco de doença cardiovascular (DCV) e doença arterial coronariana (DAC), eles também contribuem significativamente para a oferta de ácidos graxos com potenciais efeitos de promoção à saúde, entre os quais dois são de particular interesse: o *cis*9, *trans*11-C18:2 (ácido rumênico), um ácido linoleico conjugado (*conjugated linoleic acid* - CLA) e o ácido vacênico (*trans*11-C18:1). De fato, os produtos de ruminantes são a principal fonte de CLA para consumo humano (Chin et al., 1992; Lawson et al., 2001).

O CLA é parcialmente sintetizado no rúmen por bactérias celulolíticas, principalmente por *Butyrivibrio* spp, durante o processo de biohidrogenação do ácido linoleico (*cis*9,*cis*12-C18:2). No entanto, a maior proporção de CLA presente em carne e leite é via formação endógena, produzido nos tecidos e glândulas mamárias pela ação da enzima Δ9-dessaturase, tendo como substrato o ácido vacênico, o qual é o segundo intermediário da biohidrogenação do ácido linoleico. Esse processo ocorre de forma natural no rúmen e tem como objetivo a transformação de AGPI provenientes da dieta dos animais em AGS, que serão posteriormente absorvidos, transferidos para o leite e também armazenados nos tecidos.

Portanto, o AGI que escapar do metabolismo microbiano no rúmen, assim como os intermediários da biohidrogenação, fluem através do retículo-rúmen e têm o potencial de melhorar o valor nutricional de produtos de ruminantes (Or-Rashid et al., 2009), sendo esta uma estratégia para aumentar a qualidade nutricional desses alimentos para o consumo humano.

Estudos têm demonstrado que o consumo de *cis*9, *trans*11-CLA pode reduzir o risco de câncer, diminuir a aterosclerose, melhorar a imunidade e modificar o metabolismo energético em modelos animais (Park et al., 1999; Parodi, 1999; Belury, 2002; Pariza, 2004; Palmquist et al., 2005). Embora os resultados com humanos sejam variáveis, há fortes evidências de que este CLA apresente importantes efeitos biológicos.

A manipulação do ecossistema microbiano ruminal é uma opção viável para atender as expectativas do ponto de vista de benefícios para a saúde, como para a preservação do meio ambiente. A eficiência da fermentação ruminal possibilita modular a biohidrogenação, bem como minimizar a produção de gás metano ruminal (Wallace, 2004).

A produção animal é uma fonte significativa de emissão de gases causadores do efeito estufa (GEE) em todo o mundo, gerando gás carbônico (CO_2), metano (CH_4) e óxido nítrico (N_2O). Estimativas apontam que a pecuária contribui com uma porcentagem entre 7 e 18% das emissões antrópicas globais de GEE (Hristov et al., 2013).

Dados do Ministério da Ciência, Tecnologia e Inovação (MCTI, 2013) mostram que o setor da Agropecuária foi o que mais contribui para as emissões de GEE no Brasil em 2010, representando 35%. O Brasil, por ser detentor do maior rebanho comercial de bovinos do mundo e por utilizar forrageiras tropicais como base da alimentação destes animais, tem sido indicado como importante produtor de CH_4. Esse dado torna-se preocupante pelo fato que, segundo informações do Painel Intergovernamental sobre Mudanças Climáticas (IPCC, 2007), o CH_4 apresenta um potencial 21 vezes maior de aquecimento global que o CO_2.

Nas últimas décadas, uma série de aditivos, tais como ionóforos e probióticos, foram introduzidos na nutrição de ruminantes, principalmente com a finalidade de melhorar a eficiência de conversão alimentar e aumentar a produtividade animal. No entanto, o uso da maioria destes compostos tornou-se um problema muito debatido por organizações de consumidores na Europa, em razão da qualidade do produto e segurança alimentar.

Quatro antibióticos de uso rotineiro na alimentação de gado (bacitracina de zinco, espiramicina, virginiamicina e fosfato de tilosina) foram proibidos na União Europeia desde o ano de 1999, porque esta prática tem sido relacionada à resistência de patógenos bacterianos a estas drogas (União Europeia, 1998).

Uma alternativa tem sido o uso de plantas ou extratos vegetais ricos em compostos bioativos como aditivos naturais e, portanto, uma alternativa à essa problemática do uso de antibióticos. Alguns extratos vegetais com uma alta concentração de compostos fenólicos (Broudiscou et al., 2002; Cabiddu et al., 2010; Jayanegara et al., 2011) e especificamente taninos (Woodward et al., 2001; Min et al., 2005) mostraram-se eficientes na redução da metanogênese (Beauchemin et al., 2007; Bodas et al., 2008; Garcia-Gonzales et al., 2010), aumento do conteúdo de CLA (Durmic et al., 2008; Wood et al., 2010; Toral et al., 2011), menor degradação proteica (Hess et al., 2006) e produção de amônia (Williams et al., 2011).

A Amazônia, com sua enorme biodiversidade, possui um grande potencial na exploração de novos compostos bioativos. Extratos de plantas

nativas desta região, particularmente espécies utilizadas na medicina popular, como *Byrsonima crassifolia*, *Inga edulis*, *Davilla kunthii* e *Cecropia palmata*, já foram avaliadas quanto ao teor de compostos fenólicos e atividade antioxidante (Silva et al., 2007). Contudo, estas plantas ainda não foram exploradas com a finalidade de modular o metabolismo ruminal.

Logo, o estudo de plantas amazônicas pode levar à identificação de novas fontes de fitoquímicos de interesse para serem empregados em sistemas de pastagem sustentável, visando melhorar a qualidade nutricional da carne e leite para consumo humano e com menos impacto ao meio ambiente.

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CAPÍTULO I

POLYPHENOLIC COMPOUNDS AS A MODULATOR OF FATTY ACID BIOHYDROGENATION IN THE RUMEN: A REVIEW

POLYPHENOLIC COMPOUNDS AS A MODULATOR OF FATTY ACID BIOHYDROGENATION IN THE RUMEN: A REVIEW

C.M.G., Bichara[a], J. Bressan[b], H. Rogez[c], Q.C. Dan Van[d], Y. Larondelle[d]

[a]Instituto da Saúde e Produção Animal, Universidade Federal Rural da Amazônia. Avenida Presidente Tancredo Neves, nº 2501 Bairro: Montese, 66.077-830, Belém, Pará, Brasil.

[b]Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, Avenida PH Rolfs s/n, Campus Universitário, 36571-900, Viçosa, Minas Gerais, Brasil.

[c]Faculdade de Engenharia de Alimentos, Universidade Federal do Pará, Avenida Perimetral s/n, 66.095-780, Belém, Pará, Brasil.

[d]Institut des Sciences de la Vie, Unité de Biochimie de la Nutrition, Université Catholique de Louvain, Croix du Sud, 2/8, B-1348, Louvain-la-Neuve, Belgium.

ABSTRACT

The fatty acid (FA) metabolism in the rumen has a major influence on the FA composition of milk and meat-producing ruminants because when dietary material enters in the rumen it undergoes a wide range of chemical changes performed by the microbial population and lipids are extensively altered in the rumen, resulting in marked differences in the FA profile of lipids in the diet. Ruminal microorganisms transform lipids entering the rumen via two major processes, lipolysis and biohydrogenation. Nutritional strategies for the enrichment of ruminant products stimulated many studies in the last decades and currently being achieved. Much of this research was targeted at identifying the intermediates formed during biohydrogenation process (such as conjugated linoleic acid - CLA), as well as to optimize the FA content of these products by decreasing their saturated FA (SFA) content and increasing their *n*-3 FA in particular. The inclusion of plant secondary compounds in the diet of ruminants, such as polyphenolic compounds, can influence the metabolism of ruminal microorganisms increasing the flow of polyunsaturated FA (PUFA) and CLA from the rumen, subsequently accumulated into tissue lipids and milk fat, and increasing the benefits to human health. Then, there is increasing interest in the crucial role of exogenous factors, such as the animal diet, as modulators on ruminal biohydrogenation.

Keywords: conjugated linoleic acid, lipid metabolism, plant extract, plant secondary metabolites, ruminal fermentation.

1 **INTRODUCTION**

Ruminants have a digestive anatomy and physiology markedly different to that of monogastric animals. They have three additional digestive organs at the anterior end of the tract, called the rumen, reticulum and omasum, which allow the microbial population to extract and the host to absorb energy from fibrous plant material not otherwise available to mammalian enzymes. Digestion of food particles in the rumen occurs by a combination of microbial fermentation and physical breakdown during rumination. The rumen environment is characterized by 10^{10} bacteria, 10^{6} protozoa, 10^{4} fungi and yeasts per ml of live liquor, a temperature of 38–39 ºC, a normal range of pH between 6.0 and 6.7, and a redox potential of −250–450 mV (Berchielli et al., 2006). Changes in these conditions, mainly on pH, may influence the microbial population and its fermentation products. As a result of the location of the rumen, anterior to the abomasum, feedstuffs consumed by ruminants are exposed to microbial attack prior to gastric and intestinal digestion. The rumen is essentially a fermentation chamber in which microbial attack helps digest the diet.

Diets consumed by lactating dairy cows are low in fat, generally containing about 3-5% of lipids (Jenkins, 1993; Lock; Bauman, 2004). The FA profile of the feed supplement is of great importance since higher levels may adversely affect rumen microbial fermentation, so the general recommendation is that total dietary fat should not exceed 6-7% of dietary dry matter (DM) (Bauman; Corl; Peterson, 2003; Berchielli et al., 2006). The predominant PUFA in ruminant diets are C18:3 *n*-3 (*c*9,*c*12,*c*15-18:3, α-linolenic acid) and C18:2 *n*-6 (*c*9,*c*12-18:2, linoleic acid). The first is present in galacto- and phospholipids derived mainly from chloroplast membranes of forage crops, and the latter is a major component present in triacylglycerols (TAG) of oilseeds and concentrates that are used to feed the dairy cows.

Nevertheless, these components are also present at low concentrations in milk and meat-producing ruminants.

The consumption of dairy or meat products from ruminants is often associated with an increased incidence of coronary heart disease in human (Menotti et al., 1999), due to high concentrations of saturated fatty acids (SFA) in these products. This fact is due to the fate of dietary lipids during rumen fermentation, where they follow two major processes: lipolysis (LP) and biohydrogenation (BH) (Harfoot, 1978; Palmquist; Jenkins, 1980; Jenkins, 1993), undergoing hydrolysis and saturation, respectively. Then microbial population converts the unsaturated FA (UFA) from diet to SFA. This microbial activity leads to synthesis of long chain FA as the pool for conjugated linoleic acid (CLA), conjugated linolenic (CLNA) isomers and other BH intermediates. The interest in modulating the BH is that not allowing its full pathways, compounds with potential health benefits, as PUFA (*e.g.*, *n*-3 and *n*-6 PUFA) and CLA, can be accumulated in tissue lipids and milk fat, improving the nutritional composition for human consumption.

This review aimed to present an overview of biohydrogenation pathways in the rumen, factors influencing bypass of nutraceutical FA, and a critical discussion of the effects of polyphenolic compounds in the biohydrogenation process.

2 **LIPOLYSIS OF DIETARY LIPIDS**

Lipids entering the rumen are first transformed by microbial lipases in a process called lipolysis, the initial step in lipid metabolism that occurs rapidly (Dawson et al., 1977) and is a necessary step for the biohydrogenation of UFA.

The microbial lipases are extra-cellular enzymes that hydrolyze the ester linkages found in TAG, galacto- and phospholipids, causing the release of free FA (Dawson et al., 1977). After lipolysis, UFA undergo BH by ruminal microorganisms. This process also produces glycerol and galactose, which are converted to volatile fatty acids (VFA), mainly propionate and butyrate. A likely hypothesis on the existence of lipolysis is that rumen bacterial species

that carry out these reactions have special ability to hydrolyze TAG in order to use glycerol (and galactose) as an energy source.

The number of microorganisms capable of hydrolyzing esters is low, and their activity is highly specific (Henderson, 1971; Fay et al., 1990). Various bacterial strains of *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* are capable of hydrolysing the ester bond, but *B. fibrisolvens* lipase hydrolyses phospholipids, *A. lipolytica* hydrolyses only tri- and di-glycerides, and their rates of hydrolysis differ.

Lipase activity also occurs in ciliatae protozoa (mainly *Epidinium* spp.), although their contribution is less than that of bacteria. Rumen fungi have not been shown to have a role in the lipolysis of dietary lipids in the rumen (Harfoot; Hazlewood, 1988).

3 **BIOHYDROGENATION OF UNSATURATED FATTY ACIDS**

BH is a process that converts UFA to SFA via isomerization to *trans* FA intermediates, followed by hydrogenation of the double bonds (Harfoot; Hazlewood, 1988). This process involves only a few rumen bacterial species that carry out these reactions as a protection mechanism against the toxic effect of UFA (Lock; Bauman, 2004). Indeed, the antimicrobial effect of dietary lipids is associated with the degree of unsaturation of the FA, PUFA being more toxic for biohydrogenating bacteria than monounsaturated FA (MUFA) (Maia et al., 2010). This activity is mainly associated with bacteria attached to feed particles, rather than with those in free liquid. Bacteria are largely responsible for BH in the rumen (Harfoot; Hazlewood, 1988; Jenkins et al., 2008).

The major pathways for the BH of C18:3 *n*-3, C18:2 *n*-6 and C18:1 *n*-9 (*c*9-18:1, oleic acid) proceed according to the scheme shown in Figures 1, 2 and 3. Pathways of C18:3 *n*-3 and C18:2 *n*-6 involve an initial isomerization of the *c*12 double bond, resulting in the formation of a conjugated *c*9,*t*11 FA, which then undergoes hydrogenation of its *cis* double bond(s) leaving *t*11-C18:1 (vaccenic acid) as the penultimate product. Finally, this is hydrogenated to C18:0 (stearic acid). Isomerization of the *c*12 double bond is

considered a prerequisite step in the BH of FA containing a *c*9,*c*12 double bond system. The enzyme, linoleate isomerase, responsible for the conjugation of the *c*9,*c*12 double bond structure of 18:2 *n*-6 and 18:3 *n*-3, has been studied in a limited number of bacterial species. This enzyme is bound to the bacterial cell membrane and shows an absolute substrate requirement for a *c*9,*c*12 diene system and a free carboxyl group. It thus produces *c*9,*t*11-C18:2 (or *c*9,*t*11-CLA, rumenic acid) and *c*9,*t*11,*c*15-C18:3 from C18:2 *n*-6 and C18:3 *n*-3, respectively. In contrast, *t*11-C18:1 is an intermediate from both C18:2 *n*-6 and C18:3 *n*-3. Some BH intermediates can escape from the rumen, and the two major ones are *t*11-C18:1 and *c*9,*t*11-CLA. BH is extensive, and for most diets, it varies between 70-95% and 85-100% for C18:2 *n*-6 and C18:3 *n*-3, respectively (Doreau; Ferlay, 1994), indicating that C18:0 is the major FA leaving the rumen. C18:1 *n*-9 can be biohydrogenated to C18:0 (Harfoot; Hazlewood, 1988). The extent of its BH is lower compared to C18:2 *n*-6 and C18:3 *n*-3 and varies between 58-87% (Doreau et al., 2009).

Actually, BH processes are considerably more complex than imagined because a remarkable range of *trans*-C18:1, C18:2 and C18:3 containing one or more trans double bonds have been identified in the rumen outflow, absorbed and incorporated into ruminant fat. Characterizing the BH process in more detail represents a major challenge, being difficult or impossible to elucidate the metabolic origins of a specific minor BH intermediate. Nevertheless, recent *in vitro* and *in vivo* studies have provided additional insight into possible biochemical pathways accounting for the formation of specific intermediates during the BH of C18:3 *n*-3 (Figure 1), C18:2 *n*-6 (Figure 2) and C18:1 *n*-9 (Figure 3) (Shingfield et al., 2010).

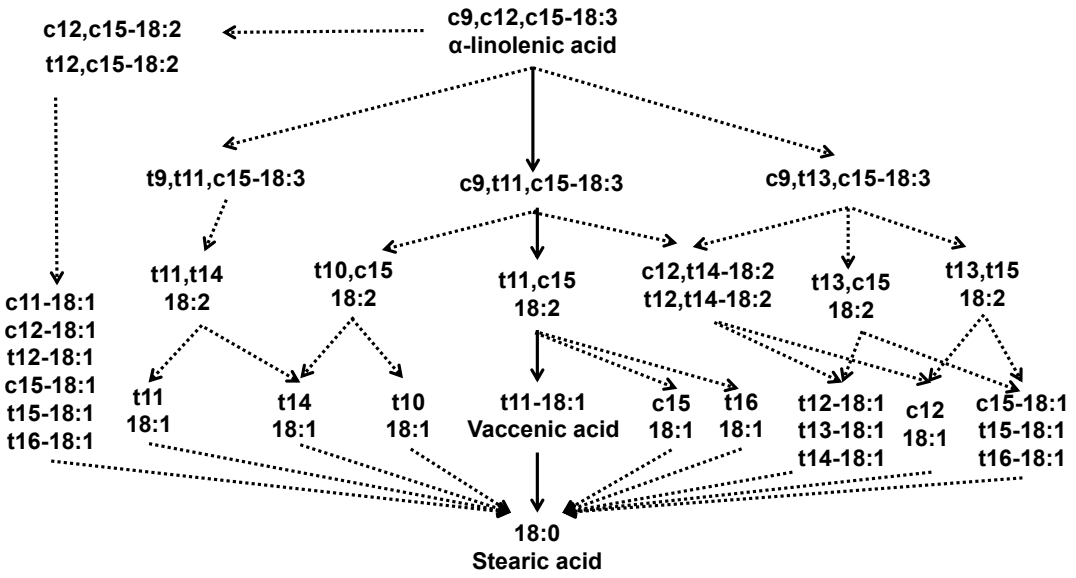

Figure 1 - Putative pathways describing C18:3 *n*-3 metabolism in the rumen. Arrows with solid lines highlight the major biohydrogenation pathway, and arrows with dashed lines describe the formation of minor FA metabolites (Shingfield et al., 2010).

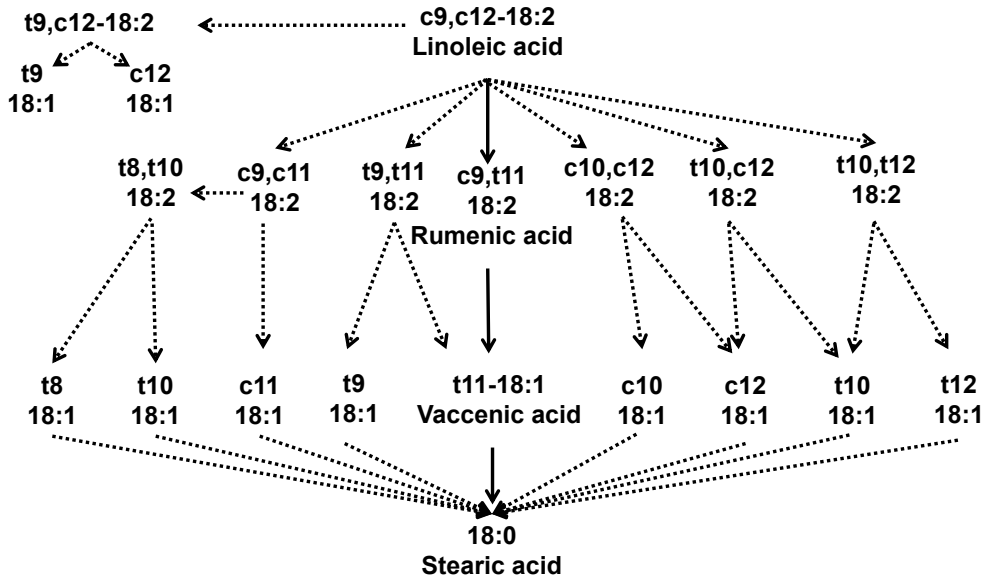

Figure 2 - Putative pathways describing C18:2 *n*-6 metabolism in the rumen. Arrows with solid lines highlight the major biohydrogenation pathway, and arrows with dashed lines describe the formation of minor FA metabolites (adapted from Shingfield et al., 2010).

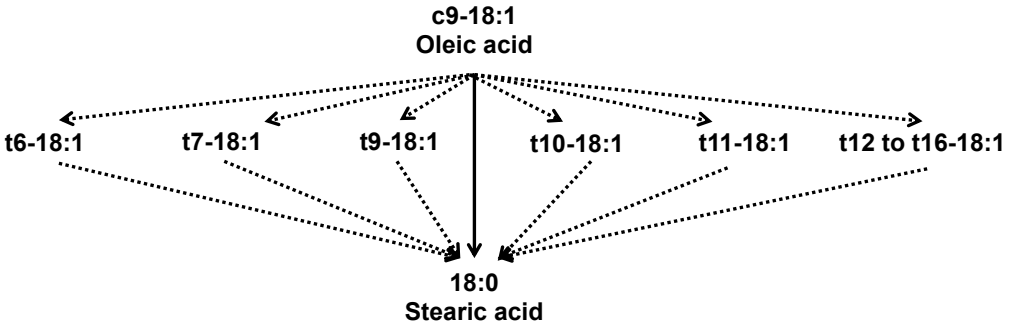

Figure 3 - Putative pathways describing C18:1 *n*-9 metabolism in the rumen. Arrows with solid lines highlight the major biohydrogenation pathway, and arrows with dashed lines describe the formation of minor FA metabolites (adapted from Shingfield et al., 2010).

3.1 MICROORGANISMS INVOLVED IN BH

The rumen ecosystem consists of a highly diverse collection of anaerobic microorganisms with the majority (70–80% of the microbial matter in the rumen) attached to feed particles in the digesta (McAllister et al., 1994). Rumen bacteria play the main role in lipid metabolism in the rumen (Harfoot; Hazlewood, 1988; Jenkins et al., 2008).

For many years, the only bacterial species known to be capable of biohydrogenation was *Butyrivibrio fibrisolvens*. Since then, a range of diverse bacteria has been isolated, showing different capacities to biohydrogenate UFA. However, recent evidence leads to the conclusion that *B. fibrisolvens* is of principal importance in ruminal BH, as already shown by Polan et al. (1964) who investigated more than 20 strains of ruminal bacteria. *B. fibrisolvens* was shown to form *c*9,*t*11-CLA and *t*11-C18:1, but not C18:0, from C18:2 *n*-6. The bacteria involved in the different steps of BH were first simply divided into two groups, A and B (Kemp; Lander, 1984). Group A bacteria which can isomerize and hydrogenate C18:2 *n*-6 to *t*11-C18:1 (i.e., *B. fibrisolvens* and other cellulolytic bacteria, as *Ruminococcus albus* and *Ruminococcus flavefaciens*), and group B bacteria which can saturate *t*11-C18:1 to C18:0 (i.e., *Fusocillus* spp., *Clostridium proteoclasticum*) (Kemp; Lander, 1984). *Clostridium proteoclasticum* was recently reclassified as

Butyrivibrio proteoclasticus (Moon et al., 2008), and it is the mainly bacteria from the rumen capable of converting MUFA to SFA.

From this, it appears that no species catalyzed the full sequence of reactions to convert C18:2 *n*-6 and C18:3 *n*-3 to C18:0. Environmental factors have a great influence on production of *trans* C18:1 and CLA, mainly the ruminal pH, that has to be maintained above 6.0 because cellulolytic bacteria are sensitive to acid condition.

The contribution of protozoa on the BH is not clearly defined. It was suggested that their participation was due to activity of ingested bacteria (Singh; Hawke, 1979). However, Devillard et al. (2004) observed that the CLA and *t*11-C18:1 content of rumen protozoal cells was 4–5 fold higher than that in bacteria, which suggests that protozoa may also be a major pool of these intermediates in the rumen. It has been known for many years that protozoal lipids contain proportionally more UFA than the bacterial fraction, although they appear not to synthesize these two FA from C18:2 *n*-6. It has been argued that the high UFA content of protozoa results from the ingestion of plant particles, especially chloroplasts (Wright, 1959; Stern et al., 1977). This cannot explain the high concentration of CLA and *t*11-C18:1 in protozoa because these FA are absent in plant material. Therefore, bacterial activity must be involved and the most likely explanation is that protozoa do not form CLA and *t*11-C18:1, but they are very efficient in incorporating intermediates formed by bacteria, contributing to a significant proportion of these components to the duodenum of ruminants.

3.2 CONJUGATED FATTY ACID ISOMERS

Conjugated linoleic acid (CLA) are a group of geometric and positional isomers of linoleic acid (C18:2 *n*-6). They have double bonds which are separated by a single carbon-carbon bond (conjugated unsaturation) instead of the usual methylene interrupted double bond system. CLA isomers include both *cis*-*cis*, *cis*-*trans* and *trans*-*trans* geometry with double bonds at 9 and 11, 10 and 12 or 11 and 13 positions. The predominant natural isomer in humans and animals is the *c*9,*t*11-CLA (Parodi, 1977; Britton et al., 1992;

Chin et al., 1992; Parodi, 1994). ‘‘Rumenic acid’’ was proposed by Kramer et al. (1998) as the common name for this acid. Rumenic acid is considered to be the most biologically active CLA isomer because it is the predominant isomer incorporated in membrane phospholipids (Ha et al., 1990). It is usually the predominant CLA isomer found in the rumen and in milk (comprises more than 90%), but many others are present, as *t*9,*t*11-C18:2 (Shingfield et al., 2003; Palmquist et al., 2005; Chilliard et al., 2007) and *t*10,*c*12 isomer (Bauman; Griinari, 2001; Shingfield; Griinari, 2007) for a total of 32 isomers identified (Bessa et al., 2000; Kramer et al., 2004; Shingfield et al., 2008).

Metabolic pathways for formation of these isomers are directly related to diet, for example, by high-starch feeding or fish or vegetable oil supplementation, *t*10,*c*12 can becomes a major intermediate (Bauman; Griinari, 2001).

3.2.1 **Endogenous synthesis of CLA**

As seen previously, *t*11-C18:1 and *c*9,*t*11-CLA are the major BH intermediates that partialy escape from the rumen to be found in the later portions of the gastro-intestinal tract, namely the small intestine. However, *c*9,*t*11-CLA is only a transient intermediate in rumen BH, whereas *t*11-18:1 tends to accumulate because its reduction appears to be rate-limiting in the complete BH sequence of PUFA. As a consequence, a relatively small portion of CLA formed in the rumen escapes further ruminal BH and is subsequently absorbed from the digestive tract. Thus, a major portion of the CLA in milk and tissue lipids must originate from another source. Griinari and Bauman (1999) proposed that endogenous synthesis could be an important source of c9,*t*11-CLA in milk fat, with synthesis involving the enzyme Δ9-desaturase and *t*11-18:1 as the substrate (Figure 4). In growing ruminants, adipose tissue has the greatest activity of Δ9-desaturase, but in lactating ruminants, the mammary gland appears to be the major site of endogenous synthesis of CLA.

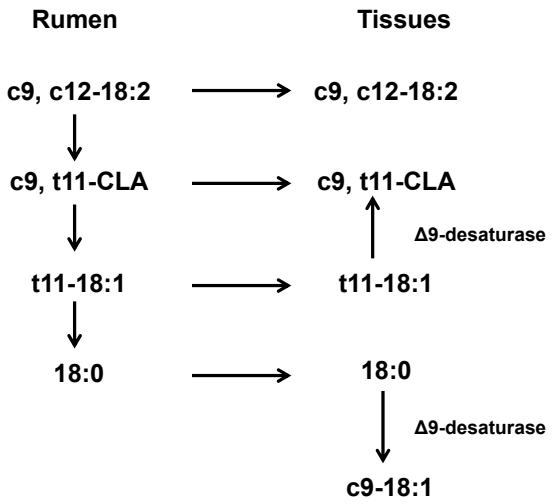

Figure 4 - Pathways of *c*9,*t*11-CLA biosynthesis (Griinari; Bauman 1999).

3.2.2 **Biological activities of CLA**

The role of CLA in animal metabolism is currently under strong investigation. The already known effects can be very diverse, and they are not fully integrated into a comprehensive framework of knowledge. The interest of the scientific community was primarily focused on its anticarcinogenic action (Pariza; Hargraves, 1985; Ha et al., 1987).

Since then, the inhibitory action of CLA in several chemical induced carcinogenesis models was demonstrated, including epidermal (Belury, 2002), mammary (Ip et al., 1991, 1994; Thompson et al., 1997) and gastrointestinal carcinomas (Ha et al., 1990; Liew et al., 1995). Physiological concentrations of CLA inhibit proliferation of several human cancer cell lines *in vitro* (Shultz et al., 1992a, 1992b; Schonberg; Krokan, 1995). A study suggested that CLA might act by antioxidant mechanisms (Ha et al., 1990), others suggested inhibition of nucleotide synthesis (Shultz et al., 1992a) or inhibiting carcinogen activation, as opposed to direct interaction with the pro-carcinogen, scavenging of electrophiles or selective phase I detoxification pathways (Liew et al., 1995). However, there might be more than one mechanism by which CLA influences carcinogenesis (Pariza et al., 2000).

CLA has been accounted for many human beneficial effects in reducing risk of atherosclerosis, different types of cancer, hypertension,

improvement of immune function (Whigham et al., 2000; Bhattacharya et al., 2006; Park, 2009; Koba; Yanagita, 2013) and also the ability to reduce body fat while enhancing lean body mass (Park; Pariza, 2007).

It should be kept in mind that the multiple biological effects seen in animal models have been produced by feeding mixtures of CLA isomers, in particular *c*9,*t*11 and *t*10,*c*12-CLA in approximately equal amounts, and evidence indicates that these isomers induce different effects.

4 POLYPHENOLIC COMPOUNDS IN PLANTS, THEIR MANIPULATION AND EFFECTS ON PUFA RUMINAL BIOHYDROGENATION

In search of natural alternatives to increase nutritional quality products from ruminants, there is interest on bioactive properties of plant secondary compounds (PSC) and metabolites. Bioactivity is defined as the capacity of a compound to elicit pharmacological or toxicological effects in humans and animals (Bernhoft, 2010). Bioactive plants and plant compounds, when included as feed or food components, have a broad range of effects in the animal, from health promoting and beneficial for animal production up to toxic or even lethal effects.

In studies with ruminants, the PSC have been tested mainly for their antimicrobial properties and potential to influence N metabolism, methane production, fibre utilization, biohydrogenation, etc. Cowan (1999) grouped antimicrobial compounds from plants into five classes: phenolics, terpenoids/essential oils, alkaloids, lectins and polypeptides and polyacetylenes. There is good evidence that PSC from at least some of these classes affect microorganisms found in the rumen and, the compounds that have received the most attention are the essential oils, saponins and tannins.

The amount of CLA and *t*11-C18:1 hydrogenated to C18:0 can be affected by conditions in the rumen, the type and the concentration of dietary PUFA, and by dietary polyphenolic substances such as tannins which irreversibly inhibit the process (Harfoot et al., 1973, Vasta et al., 2008). The effect of tanniniferous feeds on milk fat and protein composition varies markedly depending on the concentration of tannins present in the feeds.

Condensed tannins (CT) in high concentrations (>75 g/kg DM) generally have adverse effects on animal performance, as a depressed voluntary feed intake, digestive efficiency and animal productivity. However, moderate concentrations (20-45 g/kg DM) might have positive effects, as beneficial effects on protein metabolism in ruminants, decreasing rumen degradation of dietary protein and increasing absorption of amino acids in the small intestine (Aerts et al., 1999; Barry; McNabb, 1999).

Tannins affect ruminal BH probably due to its antimicrobial effects. The CT have been shown to inhibit the growth of many ruminal bacteria, and selective activity could be beneficial nutritionally by altering the ruminal BH process and hence enhancing the intermediate products of interest flowing from the rumen, improving the nutritional quality from ruminants-derived products. The antimicrobial activities of tannins are assigned to the interactions of tannins with the extracellular enzymes secreted and the cell wall of bacteria causing morphological and functional changes, reducing the activity of ruminal bacteria.

Several studies have demonstrated the effects of these compounds on the growth of cellulolytic and proteolytic ruminal bacteria. According to McAllister et al. (2005); Patra and Saxena (2011) this effect is correlated with molecular weight, which tannins with low molecular weight have higher inhibitory effects on rumen microorganisms.

Recently, polyphenolic compounds, and more particularly tannins, have been tested for their effect on ruminal BH, showing different sites of action, including a reduced PUFA biohydrogenation, an accumulation of *t*11-C18:1, and/or a decreased concentration of C18:0 (Figure 5). The following will be described the *in vitro* and *in vivo* studies that have been tested different sources of polyphenolic-rich plants and their effect on ruminal BH.

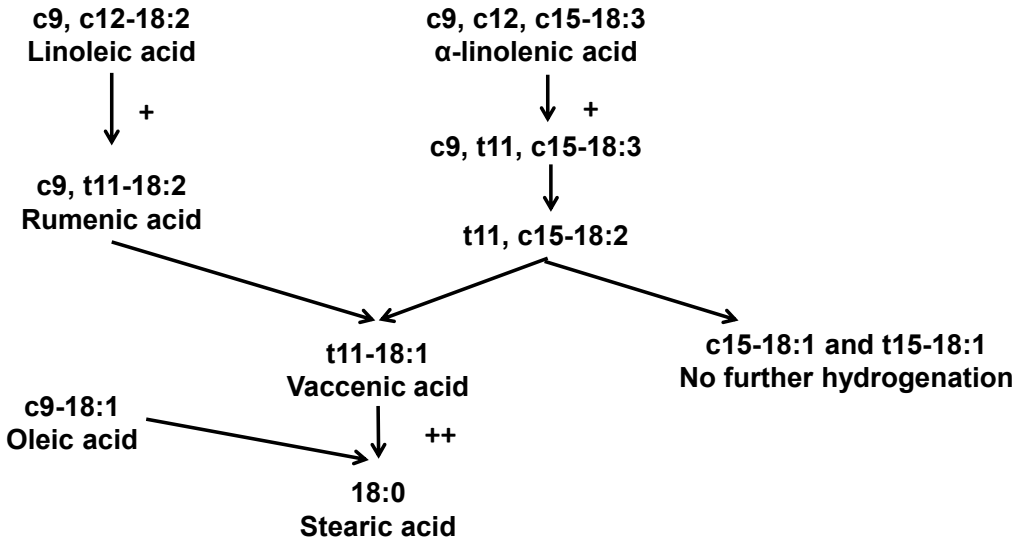

Figure 5 - Possible sites of action of polyphenolic compounds in ruminal biohydrogenation: (+) inhibition of first step of BH (Cabiddu et al., 2009 and 2010; Jayanegara et al., 2011); (++) inhibition of last step of BH (Khiaosa-Ard et al., 2009; Vasta et al., 2009a, 2009b and 2010).

4.1 *IN VITRO* STUDIES

In vitro studies (Table 1) have suggested that feeding tannins to ruminants can favorably alter ruminal BH of dietary C18:2 *n*-6, enhancing accumulation of *t*11-C18:1 in the rumen and thereby the content of some human health promoting FA in dairy or meat products.

Durmic et al. (2008) with a study involving 91 Australian plants investigated the effects of natural compounds on the bacteria and ruminal BH and the results suggest that some plants selectively inhibit targeted ruminal bacteria involved in BH (six plants had selective inhibition of *B. fibrisolvens*), and that some can inhibit saturation of C18:2 *n*-6, CLA and *t*11-C18:1 *in vitro*. This suggests the potential to increase output of CLA and *t*11-C18:1 from the rumen, and to improve the UFA content in animal derived food products.

Vasta et al. (2009a) suggested that tannins can reduce ruminal BH because they inhibit activity of rumen microorganisms. Indeed, they found that the presence of these polyphenolic compounds in rumen liquor (above 0.6 mg/ml rumen fluid) results in a 23% increase of *t*11-C18:1, 16% decrease of C18:0, but with no change in the proportion of CLA. The authors

suggested that several changes in BH process occur because tannins act selectively on bacterial strains.

Khiaosa-Ard et al. (2009) investigated the effects of condensed tannins (CT), either via extract or plant-bound, and saponin extract on ruminal BH of C18:3 *n*-3 and concluded that only the CT-extract diet led to a different profile in the effluent compared with the control diet with *t*11-C18:1 being considerably increased at cost of C18:0.

Cabiddu et al. (2010) aimed to compare the effects of two species with different plant polyphenol compositions, *Vicia sativa* and *Trifolium incarnatum*, at two different stages (vegetative and reproductive), on lipolysis and PUFA BH in the rumen. They concluded that bound phenols (BP) and tannic polyphenols (TP) had a negative effect on lipolysis and BH, but TP showed a greater negative correlation than BP for both forages.

Jayanegara et al. (2011) studying 27 tropical forages (mainly tree and shrub leaves), characterized by different phenolic profiles demonstrated to have the potential to modify ruminal FA BH towards lowering the disappearance of PUFA and the appearance of C18:0 and increasing the production of the major CLA. Leaves of avocado (*Persea americana*) were particularly interesting, because they changed the BH pattern at a moderate total extractable polyphenols (TEP) content of 73 g/kg DM. Then, in the tropical feedstuffs investigated, TEP have an impact on ruminal FA BH and are associated with an increased bypass of PUFA and the generation of CLA.

In a recent paper, Jayanegara et al. (2012) investigate the effect of 18 alpine forage plant species on ruminal FA BH and its relationship to the polyphenols, and concluded that *Castanea sativa* demonstrated to reduce the disappearance of C18:3 *n*-3 and C18:2 *n*-6, caused the highest appearance of *c*9,*t*11-CLA and *t*11-C18:1 and led also to the lowest appearance of C18:0.

4.2 *IN VIVO* STUDIES

In the experiment of Priolo et al. (2005), the intramuscular FA of lambs fed with sulla (*Hedysarum coronarium*) contained higher levels of CLA, eicosapentaenoic acid (C20:5 *n*-3, EPA) and MUFA, and lower levels of C16:0, C18:1 and total SFA than that of lambs fed a concentrate diet. Moreover, the *n*-6/*n*-3 ratio was more than three-times lower in the meat of the sulla-fed lambs than in the meat of the concentrate-fed lambs, resulting in a FA composition that was more beneficial to human health compared to the first group of animals (Table 2). In another study, Cabiddu et al. (2009) concluded that CT in sulla at flowering are conducive to lower *c*9,*t*11-CLA and *t*11-C18:1 but also lower total *trans* FA, *n*-6/*n*-3 ratio and higher C18:3 *n*-3 and C18:2 *n*-6 in milk fat.

Vasta et al. (2009b), evaluating the effect of herbage or concentrate feeding and dietary tannin supplementation on FA metabolism and composition in sheep ruminal fluid, plasma, and intramuscular fat, concluded that the supplementation of quebracho (*Schinopsis lorentzii*) tannins reduces ruminal BH, in particular, the last step of the BH was inhibited to a larger extent than the previous steps, leading to accumulation of *t*11-C18:1 in ruminal fluid. Also, the meat of the tannin-supplemented lambs had greater percentages of PUFA and decreased percentages of SFA. In another study, evaluating the effects of tannins on ruminal BH in lambs, Vasta et al. (2010) observed similar results: supplementation of quebracho increased *t*11-C18:1 in the rumen, without affecting the concentration of C18:0. The *B. proteoclasticus* population was lower, and *B. fibrisolvens* and protozoa populations were higher in the rumen of lambs fed the tannin supplemented diet than in controls. These results suggested that quebracho tannins altered BH by changing ruminal microbial populations.

Khiaosa-ard et al. (2011) aimed to determine if forages from biodiverse alpine swards, rich in various PSC affect ruminal FA BH when used as incubation substrate (24 h) or as donor cow's feed (2 to 10 weeks). They found a change in the FA BH pattern by enhancing *t*11-C18:1 in ruminal fluid lipids *in vivo* but not when incubated *in vitro*.

Toral et al. (2011), using tannins as feed additives in high producing dairy ewes, concluded that the hydrolysable tannin (HT) extracts added to a diet containing sunflower oil had no effect on ruminal fermentation and animal performance, or an important impact on milk FA profile in lactating ewes. They suggested that the low dose tested (10 g/kg DM), the type of tannins (1:1 quebracho CT and *Castanea sativa* HT extracts), or both have been responsible for the lack of change in milk *t*11-18:1 and CLA content.

Rana et al. (2012) investigated the effect of supplementation of *Terminalia chebula,* a polyphenol-rich plant extract at different concentrations and concluded that total MUFA and PUFA content in *Longissimus dorsi* muscle were enhanced by 25 and 35%, respectively, whereas SFA was reduced by 20% thereby improving the desaturation index. Δ9-desaturase activity also increased by 47% resulting in an enhancement of total CLA content (58.73%) in muscle.

5. CONCLUSIONS

It is clear that plant extracts have the potential to be exploited for strategic feeding of milk and meat-producing ruminants in order to improve the FA profile considered beneficial for human health. The observed effects are varied and often contradictory. This is most likely because of differences on the plant extracts used, concentrations and also the basal diet. Then, there is a need to identify bioactive plants and compounds, establish suitable doses, mode and frequency of application, as well as the mechanism of action in the animal and then, become the basis for achieving more sustainable, less expensive and healthier ruminant derived human food.

Table 1 – The effects of polyphenolic compounds on ruminal biohydrogenation: *in vitro* studies.

Tannin source	Diet/Dosage	Lipid source	Duration	Effects	Reference
CT extracts of *Ceratonia siliqua*; *Acacia cyaanophylla* and *Shinopsis lorentzii.*	Hay Hay + Concentrate (1:1) (2 Levels of CT: 86.5 and 145 g/kg DM)	-	12h	↑ *t*11-C18:1; ↓ C18:0	Vasta et al. (2009a)
CT extracts of *Onobrychis viciifolia* (OV) and *Acacia mearnsii* (AM)	OV: dried *Onobrychis viciifolia* AM: grass-clover hay + CT extract (1.12 g CT/d)	Linseed oil (0.39 g/d)	10 d (Rusitec)	↑ *t*11-C18:1; ↓ C18:0	Khiaosa et al. (2009)
Vicia sativa and *Trifolium incarnatum*	1 g DM	-	6h	Tannins from forage species: ↓ lipolysis and BH.	Cabiddu et al. (2010)
27 tropical forages (Darmaga, Indonesia)	200 mg of DM	Linseed oil (50 mg/g DM)	24h	*Persea americana:* changes the BH pattern at a moderate TEP content. TEP were negatively related with the disappearances of C18:3 and C18:2 e C18:1 CLA was positively correlated with TEP; TEP ↓ BH on the first step.	Jayanegara et al. (2011)
18 alpine forage (south-eastern Switzerland)	200 mg of DM	Linseed oil (50 mg/g DM)	24h	*Castanea sativa* ↓ disappearance of C18:3; C18:2 and C18:0 TEP, CT and HT: ↓ disappearance of C18:3 and C18:2 and appearance of C18:0; ↑ appearance of *t*11-C18:1; HT: ↑ appearance of CLA.	Jayanegara et al. (2012)

DM: dry matter; TEP: total extractable phenols; CT: condensed tannins; HT: hydrolysable tannins; ↓ reduction; ↑ increase.

Table 2 – The effects of polyphenolic compounds on ruminal biohydrogenation: *in vivo* studies.

Tannin source	Diet/Dosage	Experiment	Lipid source	Effects	Reference
Hedysarum coronarium L.	Group 1 (control): oats hay: concentrate (15:85) Group 2: fresh *H. coronarium* Group 3: fresh *H. coronarium* + PEG	Lambs	-	In meat: ↑CLA , MUFA, C22 :5 EPA; ↑ *n*-6/*n*-3 ratio; ↓ SFA	Priolo et al. (2005)
Hedysarum coronarium L.	1.5 kg DM with a foreseen CT concentration of 40 g/kg DM	Ewes	-	In milk: CT ↓ *c*9,*t*11-CLA, *t*11-C18:1, total trans FA, *n*6/*n*3 ratio; ↑ C18:3 and C18:2	Cabiddu et al. (2009)
Schinopsis lorentzii	Group 1: fresh herbage Group 2: concentrate-based diet. Within each treatment, 1/2 received supplementation providing 40 g/kg of CT in DM.	Ewes	-	In ruminal fluid: CT ↓ BH ➔ ↓ C18:0 and ↑ *t*11-C18:1; In muscle: ↑ PUFA and ↓ SFA	Vasta et al. (2009b)
Schinopsis lorentzii	Group 1 (control): concentrate Group 2: concentrate with supplemental tannins at 64 g/kg DM	Lambs	-	In ruminal fluid: ↑ *t*11-C18:1; ↓ *t*11-C18:1/18:0 ratio	Vasta et al. (2010)
Alpine forage (Switzerland)	Highland alpine site with free grazing	Cows	-	In ruminal fluid: ↑ *t*11-C18:1, ↓C18:0; Trend to ↑ C18:3	Khiaosa et al. (2011)
1:1 (w/w) mixture of *Schinopsis lorentzii* (CT) and *Castanea sativa* (HT)	Alfalfa hay: concentrate (40:60); 10 g tannins/kg DM	Ewes	Sunflower oil (20 g/kg DM)	In milk: tannin supplementation did not impact the concentration of MUFA, SFA and PUFA.	Toral et al. (2011)
Aqueous extract of *Terminalia chebula*	Isocaloric and isonitrogenous diet	Male goat kids	-	In muscle: ↑ PUFA; ↓SFA and ↑ total CLA	Rana et al. (2012)

DM: dry matter; CT: condensed tannins; HT: hydrolysable tannins; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: Saturated fatty acids; PEG: polyethylene glycol; ↓ reduction; ↑ increase.

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CAPÍTULO II

CARACTERIZAÇÃO QUÍMICA, PERFIL DE ÁCIDOS GRAXOS, TEORES DE POLIFENÓIS TOTAIS E TANINOS CONDENSADOS DE LEGUMINOSAS DA AMAZÔNIA BRASILEIRA COM POTENCIAL PARA USO NA ALIMENTAÇÃO ANIMAL

CARACTERIZAÇÃO QUÍMICA, PERFIL DE ÁCIDOS GRAXOS, TEORES DE POLIFENÓIS TOTAIS E TANINOS CONDENSADOS DE LEGUMINOSAS DA AMAZÔNIA BRASILEIRA COM POTENCIAL PARA USO NA ALIMENTAÇÃO ANIMAL

Chemical composition, polyphenolic and tannin contents of legumes species from the Amazonian region for use in animal nutrition

Bichara, C.M.G.[1]; Catherine[2], R.; Rogez, H.[3]; Bressan, J.[4]; Larondelle, Y[2].

[1]Instituto da Saúde e Produção Animal, Universidade Federal Rural da Amazônia, Av. Presidente Tancredo Neves, nº 2501, 66.077-90 Belém-PA, Brasil;

[2]Faculdade de Engenharia de Alimentos, Universidade Federal do Pará, Av. Perimetral s/n, 66.095-780 Belém-PA, Brasil;

[3]Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, Av. Peter Henry Rolfs, s/n 36570-900 Viçosa-MG, Brasil;

[4]Unité de Biochimie de la Nutrition, Université Catholique de Louvain, Croix du Sud, 2/8, B-1348 Louvain-la-Neuve, Belgium.

RESUMO

O objetivo deste trabalho foi determinar a composição química, o perfil de ácidos graxos e o teor de polifenóis totais e taninos condensados de dez espécies de leguminosas encontradas na Amazônia brasileira, com potencial para uso na nutrição animal. As folhas liofilizadas apresentaram teores de matéria seca e cinzas totais variando de 909,50 a 962,84 g/kg e 39,76 a 96,18 g/kg de matéria seca (MS), respectivamente. O teor de proteína bruta variou de 125,01 a 245,85 g/kg MS, sendo que *Centrosema pubecens* e *Senna alata* apresentaram os maiores teores (>200 g/kg MS). Em relação ao extrato etéreo, a espécie *S. alata* apresentou o maior teor (91,73 g/kg), enquanto a espécie *Inga thibaldiana* o menor (23,51 g/kg MS). Os teores de fibra variaram de 177,03 a 593,76 g/kg de fibra em detergente neutro e 96,41 a 449,72 g/kg de fibra em detergente ácido. Os teores de lignina variaram de 20,05 a 104,60 g/kg MS, exceto para as espécies de *Inga*, que apresentaram teores >177,86 g/kg MS. Os teores de ácidos graxos totais variaram de 12,51 (*Desmodium ovalifolium*) a 21,52 g/kg (*S. alata*), sendo que a espécie *C. pubecens* apresentou as maiores proporções de C18:3 *n*-3 e *D. ovalifolium* de C18:2 *n*-6. As leguminosas apresentaram concentrações em

polifenóis totais variando de 24,83 a 147,22 g eq. ácido gálico/kg MS, sendo as leguminosas do gênero *Inga* (*I. heterofila*, *I. thibaldiana* e *I. edulis*) as que apresentaram os maiores teores (>133,96 g EAG/kg MS). Os teores de taninos condensados totais variaram de 0,07 a 48,81 g eq. cianidina/kg MS. As leguminosas estudadas apresentam composição química com potencial para estudos na área de nutrição animal.
Palavras chave: compostos fenólicos, leguminosas, plantas Amazônicas, proteínas.

ABSTRACT

The nutrient composition, polyphenolic and tannin contents have been determined in ten Amazonian leaves plant species, with potential for use in animal nutrition. Lyophilized leaves of plants under investigation showed dry matter and total ash ranging from 909.50 to 962.84 g/kg and 39.76 to 96.18 g/kg Dry matter (DM), respectively. Crude protein ranging from 125.01 to 245.85, where *Centrosema pubecens* and *Senna alata* showed the highest values (>200 g/kg MS). Regarding the ether extract, *S. alata* showed the highest concentration (91.73 g/kg DM) and *Inga thibaldiana* the lowest (23.51 g/kg DM). The fiber content ranged from 177.03 to 593.76 g/kg DM for neutral detergent fiber and 96.41 to 449.72 g/kg DM for acid detergent fiber. Lignin contents ranged from 20.05 to 104.6 g/kg DM, except for the *Inga* species, which showed levels >177.86 g/kg DM. The total fatty acid content ranged from 12.51 (*Desmodium ovalifolium*) to 21.52 g/kg DM (*S. alata*), and the species *C. pubecens* showed the highest proportions of C18:3 *n*-3 and *D. ovalifolium* of C18:2 *n*-6. Legumes presented higher levels of total polyphenols from 24.83 to 147.22 g Eq. gallic acid/kg DM; legumes of the genus *Inga* (*I. heterofila*, *I. thibaldiana* and *I. edulis*) showed the highest values (>133.96 g GAE/kg DM). Total-condensed tannins content ranged from 0.07 to 48.81 g Eq. cyanidin/kg DM. The Amazonian legumes showed interesting chemical composition with potential for studies in animal nutrition.
Key words: Amazonian plants, crude protein, legumes, polyphenolic compounds.

1 **INTRODUÇÃO**

Os ruminantes desempenham um papel importante em muitos sistemas agropecuários, principalmente em áreas tropicais. Porém, os sistemas de alimentação são frequentemente baseados em volumosos, apresentando forrageiras tropicais que não fornecem quantidades suficientes de nutrientes para a produção máxima dos animais. Sem suplementação adequada, tais dietas resultam em insuficiente consumo de forragem, baixa eficiência de conversão alimentar e baixo desempenho dos animais.

Uma alternativa é a introdução de leguminosas nas pastagens, promovendo incremento da produção animal, pelo aumento da quantidade e qualidade da forragem, principalmente por seu maior valor nutricional, relacionado à fixação biológica de nitrogênio e, muitas vezes, por seus teores de taninos (Williams et al., 2011). Assim, leguminosas forrageiras, árvores e arbustos estão sendo, cada vez mais, usadas como suplementos para suprir essas deficiências nutritivas para o rebanho.

Taninos condensados (TC) ou proantocianidinas (PA) são compostos fenólicos que formam complexos particularmente com proteínas e assim, podem agir anti-nutricionalmente em elevadas doses (>50 g/kg MS) e reduzir a ingestão voluntária de alimentos (Barahona et al., 1997). Em contraste, dietas contendo <50 g/kg MS podem ser benéficas em termos metabólicos de utilização proteica, devido à redução da degradação de proteínas no rúmen e aumento do fluxo de proteínas não degradadas para o intestino delgado (Waghorn et al., 1987; Wang et al., 1994; Min et al., 2003). TC são os tipos de taninos mais facilmente encontrados em leguminosas, sendo o outro grupo (taninos hidrolisáveis-HT) raramente encontrado em forragens. Estruturalmente, TC são polímeros complexos de unidades de flavanóis (flavan-3-ols, flavan-3,4-diols e biflavans) unidas por ligações carbono-carbono, ocorrendo normalmente nos vacúolos das células vegetais (Hagerman; Butler, 1991).

A Amazônia apresenta uma grande biodiversidade em vegetais com potencial para fornecer novos fitoquímicos, apresentando capacidade antioxidante, antimicrobiana, anticancerígena, dentre outras, cuja

valorização pode apresentar vantagens para o desenvolvimento sustentável da região. Entretanto, pouco se sabe sobre as características químicas e teores de compostos fenólicos de leguminosas da Amazônia brasileira. Por isso, esse trabalho teve como principal objetivo a caracterização química, identificação do perfil de ácidos graxos, assim como determinação dos teores de polifenóis totais e taninos condensados de leguminosas encontradas no Norte do Brasil.

2 **MATERIAL E MÉTODOS**

2.1 COLETA DAS PLANTAS

Folhas de 10 espécies de leguminosas encontradas na Amazônia brasileira (Quadro 1) foram coletadas manualmente, por especialistas da Embrapa Amazônica Oriental, (EMPRAPA/Belém-PA), os quais foram os responsáveis pela identificação das espécies, no período de janeiro de 2012, em três diferentes locais: Belém (capital do Estado do Pará, Brasil), Igarapé-Açu (cerca de 121 Km da capital) e Paragominas (distante aproximadamente 215 Km da capital Paraense). O material foi transportado ao laboratório, no máximo 12 horas após as coletas. As folhas foram cortadas em pedaços de, aproximadamente, 1,5 cm^2 para posteriores etapas de congelamento e liofilização. As folhas liofilizadas foram trituradas em moinho de facas com tamanho de partículas aproximado de 1 mm e estocadas a -20 ºC. A caracterização química das amostras foi realizada no Laboratório de Bioquímica da Nutrição, na Universidade Católica de Louvain-La-Neuve, Bélgica.

Nome científico	Nome comum	Porte	Pontos de coletas
Arachis pintoi	Amendoim forrageiro	Herbácea perene	3
Calopogonium mucunoides	Calopogônio	Forrageira perene	3
Centrosema pubecens	Centrosema comum	Herbácea perene	2
Desmodium ovalifolium	Desmódio	Subarbusto	2
Inga heterophylla	Ingá-miúdo	Árvore	2
Inga thibaudiana	Ingá-barata	Árvore	2
Inga edulis	Inga-cipó	Árvore	2
Pueraria phaseloides	Kudzu tropical	Herbácea perene	3
Senna alata	Fedegoso gigante	Árvore pequeno porte	2
Senna obtusifolia	Fedegoso	Herbácea	2

Quadro 1 – Identificação das leguminosas amazônicas coletadas.

2.2 CARACTERIZAÇÃO QUÍMICA E PERFIL DE ÁCIDOS GRAXOS

Realizou-se a determinação da matéria seca (MS) mediante a secagem em estufa à 105ºC durante 16 horas (adaptação dos métodos 967.03 e 930.15; AOAC, 1995); cinzas por meio da incineração em mufla à 550ºC durante 16 horas (adaptação dos métodos 923.03, 967.04 e 942.05; AOAC, 1995); proteína bruta pelo método Kjeldahl (N*6.25) (adaptação dos métodos 981.10 e 991.20; AOAC, 1995); extrato etéreo (Directive 98/64/EC; Commission of the European Communities, 1998); fibra em detergente neutro (FDN) (Van Soest et al., 1991); fibra em detergente ácido (FDA) (adaptação do método 973.18; AOAC, 1995). FDN e FDA foram determinadas sequencialmente e expressos sem cinza residual. FDN foi determinado com uso de α-amilase, porém sem adição de sulfato de sódio. Lignina foi determinada por extração ácida com H_2SO_4 72%. O teor de hemicelulose foi calculado como a diferença entre FDN e FDA e celulose sendo a diferença entre FDA e lignina. Para estimativa dos carboidratos totais, foi usada a equação proposta por Sniffen et al. (1992) e para estimativa dos carboidratos não-fibrosos, a equação preconizada por Hall et al. (1999).

Para a determinação do perfil de ácidos graxos (AG), os lipídeos foram extraídos seguindo o método de Folch, modificado por Christie (1982) e C13:0 usado como padrão interno. AG foram metilados em solução KOH em metanol (0,1 mol/L) a 70 ºC durante 60 minutos e solução HCl em metanol (1,2 mol/L) à 70 ºC por 20 minutos e finalmente extraídos com

hexano. Os ésteres metílicos de AG (EMAG) foram separados e quantificados em cromatografia gasosa (CG Trace ThermoQuest, Thermo Finnigan, Milão, Itália), equipado com detector de ionização de chama e coluna capilar de sílica fundida (100 m x 0.25 mm diâmetro interno). O sistema usou H_2 como gás carreador a uma pressão constante de 200 KPa. Os picos foram identificados e quantificados por comparação do tempo de retenção com padrões de EMAG puros (Alltech Associates Inc., Deerfield, IL).

2.3 POLIFENÓIS TOTAIS E TANINOS CONDENSADOS

Os compostos fenólicos foram extraídos das folhas liofilizadas (0,5 g), em duplicata, com 10 mL de uma solução extratora composta por acetona:água:ácido acético (50:49:1, v:v:v), em tubos de 15 mL. Os tubos foram transferidos para banho-maria a 40 ºC, durante 1 hora e após, centrifugados a 5000x*g* durante 21 minutos a 4 ºC. A concentração de polifenóis totais foi determinada pelo método colorimétrico Folin-Ciocalteau (Singleton; Rossi, 1965), a 755 nm e o resultado expresso em gramas de equivalentes em ácido gálico por quilograma de matéria seca (g EAG/kg MS).

Os taninos condensados foram determinados pelo método butanol-HCl, como descrito por Mupangwa et al. (2000). Primeiramente, 0,5 g de folhas liofilizadas foi extraída duas vezes com 5 mL de solução acetona: água (70:30 v:v) em tubos à temperatura ambiente durante 30 minutos e então centrifugados à 3000×*g* durante 15 min à 5ºC. Para a quantificação dos taninos condensados extraíveis, uma alíquota de 150 µL do sobrenadante foi adicionada a 3 mL de solução butanol-HCl (95:5 v:v), seguida por agitação e aquecimento a 90 ºC por 60 minutos. A leitura foi realizada a 550 nm. Para quantificação dos taninos condensados ligados, os resíduos resultantes da extração foram liofilizados e, em seguida, 50 mg de cada amostra foram pesados e adicionados de 10 mL de solução reagente butanol:HCl, seguindo de aquecimento a 90 ºC por 60 minutos, resfriamento e centrifugação à 3000x*g* por 10 min e o sobrenadante lido à 550 nm. Os

taninos condensados totais foram a soma de ambas as frações (extraível e ligada), expressa em gramas de equivalentes em cianidina por quilograma de matéria seca (g EC/kg MS).

3 **RESULTADOS**

3.1 CARACTERIZAÇÃO QUÍMICA E PERFIL DE ÁCIDOS GRAXOS DE LEGUMINOSAS BRASILEIRAS

As espécies estudadas no presente trabalho variaram substancialmente na composição química, conforme visualizado na Tabela 1.

Tabela 1 – Teores de matéria seca (MS), cinzas totais, proteína bruta, extrato etéreo, carboidrato total e carboidrato não fibroso em folhas liofilizadas de leguminosas amazônicas.

Espécie	**Composição química (g/kg MS)**					
	Matéria seca	**Cinzas**	**Proteína bruta**	**Extrato etéreo**	**Carboidrato total**	**Carboidrato não fibroso**
Arachis pintoi	934,40	89,90	179,76	45,69	684,74	272,18
Calopogonium mucunoides	921,02	59,62	179,92	68,72	691,74	350,80
Centrosema pubecens	940,91	62,80	204,27	48,38	684,55	210,42
Desmodium Ovalifolium	924,45	50,93	125,01	32,08	792,52	250,20
Inga heterofila	962,84	39,76	162,91	42,41	754,91	162,82
Inga thibaldiana	940,79	44,68	149,18	23,51	782,63	236,68
Inga edulis	934,71	47,29	179,30	27,49	745,92	267,45
Pueraria phaseloides	929,08	69,55	171,07	43,83	715,55	320,53
Senna alata	936,11	96,18	245,85	91,73	566,24	305,35
Senna obtusifolia	909,50	94,10	160,40	54,77	690,74	513,71
Erro padrão da média	3,10	4,64	7,10	4,43	14,18	20,92

Os resultados de matéria seca variaram de 909,50 a 962,84 g/kg MS, o que representa, em porcentagem, uma umidade de 3,8 a 9,1% nas folhas liofilizadas. Os teores de cinzas variaram de 39,76 a 96,18 g/kg MS. Dentre as plantas estudadas, *C. pubecens* e *S. alata* apresentam os maiores teores de proteínas (>200 g/kg MS). Em relação ao extrato etéreo, *S. alata* apresentou o maior teor (91,73 g/kg), enquanto a espécie *I. thibaldiana* apresentou o menor (23,51 g/kg MS).

Em relação aos teores de fibra (Tabela 2), *D. ovalifolim*, *I. heterofila* e *I. thibaldiana* apresentaram os maiores teores de FDN (>500 g/kg MS) e FDA (>400 g/kg MS). O conteúdo de lignina foi baixo, variando de 20,05 a 104,60, exceto para as espécies de *Inga*, os quais apresentaram teores de 177,86 (*I. edulis*), 238,12 (*I. heterofila*) e 248,12 g/kg MS (*I. thibaldiana*). Os teores de hemicelulose e celulose variaram de 77,63 a 161,28 e 76,36 a 213,01 g/kg MS, respectivamente.

Tabela 2 – Teores de fibra, lignina, hemicelulose e celulose em folhas liofilizadas de leguminosas amazônicas.

Espécie	Teores de Fibra (g/kg MS)		Lignina	Hemicelulose	Celulose
	FDN	FDA			
Arachis pintoi	412,56	251,28	69,51	161,28	181,77
Calopogonium mucunoides	340,94	230,24	55,00	110,70	175,24
Centrosema pubecens	474,13	318,61	100,94	155,51	217,68
Desmodium Ovalifolium	542,32	400,92	104,60	141,39	296,32
Inga heterofila	593,76	449,72	238,12	143,64	211,00
Inga thibaldiana	545,95	437,23	248,11	108,73	189,12
Inga edulis	478,48	348,95	177,86	129,53	171,09
Pueraria phaseloides	395,01	267,09	54,07	127,93	213,01
Senna alata	260,89	183,26	41,31	77,63	141,94
Senna obtusifolia	177,03	96,41	20,05	80,62	76,36
Erro padrão da média	28,87	24,85	17,87	6,38	12,34

FDN: fibra em detergente neutro; FDA: fibra em detergente ácido; MS: matéria seca.

O perfil lipídico das leguminosas mostrou-se bastante interessante em nível nutricional, apresentando teores totais de ácidos graxos variando de 12,51 (*D. ovalifolium*) a 21,52 g/kg (*S. alata*) (Tabela 3). Somente a espécie *S. alata* apresentou teores superiores a 20 g de AG totais/kg MS. *C. pubecens*, *A. pintoi* e *S*. *obtusifolia* apresentaram as maiores proporções de C18:3 *n*-3, assim como os maiores teores de AG insaturados (>63%). Baixas concentrações de C18:3 *n*-3 foram encontradas em *D. ovalifolium* e nas espécies *thibaldiana* e *edulis* do gênero *Inga*. Essas duas espécies também apresentaram os menores teores de AG insaturados (aproximadamente 49%). *D. ovalifolium* apresentou o maior teor de C18:2 *n*-6 (16,31 g/kg MS), seguido pelas espécies *C. pubecens* e *A. pintoi.* Para o C18:1 *n*-9, *D. ovalifolium*, juntamente com *I. heterofila* e *I. thibaldiana* apresentaram os maiores valores. A razão AGI:AGS variou de 0,98 a 1,75 entre as espécies estudadas.

Tabela 3 – Perfil lipídico das folhas liofilizadas de leguminosas amazônicas.

Espécie	Total AG g/kg MS	% AG Total								
		C6 – C15:0	C16:0	C18:0	C18:1 t9	C18:1 *n*-9	C18:2 *n*-6	C18:3 *n*-3	∑AGI	AGI: AGS
Arachis pintoi	18,66	6,75	17,87	4,35	0,015	2,32	12,69	46,71	63,42	1,72
Calopogonium mucunoides	16,66	8,84	16,55	5,85	0,07	2,51	11,11	44,22	59,84	1,48
Centrosema pubecens	18,24	6,30	13,48	8,08	0,04	2,04	12,02	46,79	63,32	1,71
Desmodium ovalifolium	12,51	9,88	17,70	6,94	0,07	4,63	16,31	33,41	56,76	1,29
Inga heterofila	14,09	10,08	12,48	13,66	0,20	4,87	6,71	39,37	54,70	1,19
Inga thibaldiana	12,14	13,60	14,34	12,54	0,29	4,62	8,29	33,95	49,90	0,98
Inga edulis	13,18	13,14	19,40	8,04	0,07	2,81	10,29	33,91	49,87	0,99
Pueraria phaseloides	16,70	9,82	16,88	7,86	0,22	2,40	11,88	38,10	55,97	1,27
Senna alata	21,52	7,64	19,65	3,71	0,13	1,00	10,11	45,49	59,14	1,44
Senna obtusifolia	19,85	7,53	18,34	4,61	0,12	2,06	13,75	46,20	63,68	1,75
Erro padrão da média	0,71	0,55	0,54	0,72	0,02	0,29	0,59	1,25	1,14	0,06

AG: ácidos graxos; ∑AGI: somatória dos AG insaturados.

3.2 TEORES DE POLIFENÓIS TOTAIS (PT) E TANINOS CONDENSADOS (TC)

Os teores de PT variaram de 24,83 a 147,22 (Tabela 4), sendo que as leguminosas do gênero *Inga* apresentaram os maiores teores (>130 g EAG/kg MS). *A. pintoi*, *C. pubecens* e *P. phaseloides* apresentaram os menores teores de PT (<30 EAG/kg MS). Grandes diferenças também foram encontradas em relação aos TC, sendo que os teores totais variaram de 0,07 a 48,81 g EC/kg. *C. mucunoides* e *P. phaseloides* foram as duas únicas espécies que não apresentam teores de TC na fração extraível. *I. edulis* foi a espécie que apresentou maiores teores em ambas as frações e, consequentemente, a maior concentração de TC totais, seguida das outras duas espécies (*I. heterofila* e *I. thibaldiana*) e do *D. ovalifolium.*

Tabela 4 - Teores de polifenóis totais (PT) e taninos condensados (TC) das folhas liofilizadas de leguminosas amazônicas.

Espécie	**Polifenóis totais – PT (g EAG/kg MS)**	**Taninos condensados - TC (g EC/kg MS)**		
		Extraível	***Ligado***	***Total***
Arachis pintoi	29,60	4,73	1,34	6,07
Calopogonium mucunoides	54,16	nd	0,07	0,07
Centrosema pubecens	24,83	0,08	0,12	0,20
Desmodium ovalifolium	96,22	21,95	9,15	31,10
Inga heterofila	133,96	21,67	6,41	28,08
Inga thibaldiana	147,22	34,03	7,47	41,49
Inga edulis	136,30	39,06	9,75	48,81
Pueraria phaseloides	25,82	nd	0,25	0,25
Senna alata	50,33	1,26	0,17	1,37
Senna obtusifolia	47,89	0,42	0,30	0,72
Erro padrão da média	9,76	2,72	0,73	3,42

MS: Matéria seca; EAG: equivalente em ácido gálico; EC: equivalente em cianidina; nd: não detectado; MS: matéria seca.

4 **DISCUSSÃO**

Os teores de proteína bruta são de grande importância na alimentação de ruminantes, uma vez que as proteínas, assim como compostos nitrogenados não proteicos, são degradados pelos microrganismos presentes no rúmen, servindo aos mesmos como fatores de crescimento para formar a proteína microbiana e, posteriormente, servirão como fonte de proteína para o ruminante (Kozloski, 2002). Leguminosas são vegetais que apresentam, naturalmente, elevados teores de nitrogênio, uma vez que são transformadoras do nitrogênio atmosférico em nitrogênio orgânico que, por sua vez, se transformará no solo em formas de nitrogênio absorvíveis pelas plantas. Por esse motivo, leguminosas forrageiras são utilizadas em regiões tropicais e subtropicais como suplementos de proteína para ruminantes pastando com volumosos de baixa qualidade, especialmente durante a estação seca.

Hess et al. (2008), estudando quatro diferentes espécies de leguminosas tropicais, cultivadas em solos colombianos, encontraram teores de proteínas de 138 a 198 g/kg, enquanto Sturm et al. (2007), também trabalhando com leguminosas de solos colombianos, encontraram concentrações inferiores, variando de 89 a 115 g/kg. Vitti et al. (2005), estudando três leguminosas brasileiras (*Leucaena leucocephala*, *Sesbania sesban* e *Cajanus cajan*), encontraram teores de 153, 153 e 170 g/kg, respectivamente. Assim, as leguminosas caracterizadas neste trabalho apresentaram teores proteicos elevados, uma vez que oito espécies apresentaram concentrações superiores a 160 g/kg MS (somente *D. ovalifolium* e *I. thibaldiana* apresentaram teores de 125,01 e 149,18 g/kg MS, respectivamente).

A fibra também apresenta um papel muito importante na alimentação dos ruminantes, uma vez que é fonte de energia e potencializadora dos processos fermentativos. Vitti et al. (2005) encontraram teores de FDN variando de 699 a 721; Hess et al. (2008) de 437 a 597 e Sturm et al. (2007) de 205 a 431 g/kg para diferentes leguminosas. Um nível mínimo de fibra na dieta é necessário para ótima produção e saúde de vacas leiteiras.

Insuficiente quantidade de fibra ou a falta de uma determinada quantidade de fibra efetiva na dieta, pode resultar em baixo pH ruminal, decréscimo da eficiência microbiana, diminuição da porcentagem de gordura do leite, e pode ameaçar a saúde das vacas (Mooney; Allen, 1997).

Em relação à lignina, Sturm et al. (2007) encontraram valores de 22 a 80 e Hess et al. (2008) de 39 a 56 g/kg MS. Os valores encontrados para a maioria das espécies brasileiras estão próximos a esses níveis, exceto para as espécies de *Inga*, onde *I. edulis, I. heterofila* e *I. thibaldiana* que apresentaram concentrações de 177,86, 238,12 e 248,12 g/kg MS, respectivamente. Nesse sentido, Duarte (2007) também encontrou concentrações elevadas para o gênero *Inga*, porém, para outra espécie, a *I. subnuda* (273 g/kg MS).

Os lipídeos compõem, aproximadamente, 43% do extrato etéreo, sendo o restante constituído por matéria não saponificável, ceras e clorofilas, sem valor nutricional para os ruminantes (Van Soest, 1994). Concentrações elevadas de lipídeos podem reduzir o consumo e a digestibilidade dos alimentos, motivo pelo qual as concentrações de extrato etéreo na matéria seca da dieta de ruminantes não deve ser superior a 70 g/kg (Palmquist; Jenkins, 1980). Vitti et al. (2005) encontraram teores variando de 29 a 36 g/kg para as leguminosas tropicais brasileiras, valores esses próximos das leguminosas aqui estudadas, exceto para a espécie *S. alata*, a qual apresentou uma concentração bastante superior (91,73 g/kg MS).

O tipo de lipídeo em vegetais varia de acordo com sua localização na planta. Nas folhas, os lipídeos mais comuns são galactolipídeos, constituídos por glicerol, galactose e AG poli-insaturados e os fosfolipídeos, associados às membranas biológicas. Os AG como o C18:3 *n*-3 e o C18:2 *n*-6 compõem a maior parte dos AG presentes nas forrageiras (Van Soest, 1994; Bauman et al. 1999). A dieta natural dos herbívoros, constituída por gramíneas e leguminosas, possui baixo teor de lipídeos e situa-se entre 10 e 40 g/kg MS. O perfil lipídico dos alimentos de ruminantes influencia diretamente a ação das bactérias ruminais e, consequentemente, o perfil lipídico dos produtos derivados (carne e leite). Os lipídeos da dieta são extensivamente

hidrolisados no rúmen, sofrendo o processo de lipólise e biohidrogenação. Os AG insaturados, devido à sua ação tóxica às bactérias ruminais, influenciam diretamente a biohidrogenação ruminal. Assim, quanto maior a ingestão de AG poli-insaturados na dieta, maior a probabilidade de ácidos graxos intermediários desse processo que são benéficos à saúde humana serem encontrados nos alimentos derivados de ruminantes. Não há na literatura dados do perfil lipídico de leguminosas cultivadas na Amazônia brasileira.

Jayanegara et al. (2012), avaliando diferentes grupos de plantas do sudeste da Suíça, determinaram teores de 10,8 a 14,2 g de AG totais/kg MS em três espécies de leguminosas (*Anthyllis vulneraria*, *Hedysarum hedysaroides* e *Trifolium badium*), concentrações relativamente inferiores ao encontradas nas leguminosas brasileiras, que variaram de 12,51 a 21,52 g/kg MS. *C. pubecens, A. pintoi e S. obtusifolia* foram as leguminosas que apresentaram os maiores teores de C18:3 *n*-3 (>46% dos AG totais) e *D. ovalifolium* apresentou o maior teor de C18:2 *n*-6 (16,3%).

Silva, Rogez e Larondelle (2007) estudando o processo de extração dos compostos fenólicos das folhas de *I. edulis* encontraram teores de 125,3 g EAG/kg, bem próximo ao valor encontrado neste trabalho. Silva et al. (2007) avaliando as concentrações de compostos fenólicos em diferentes partes de 15 plantas amazônicas (folhas, cascas, caules, frutos e sementes) encontraram teores de PT nas folhas variando de 9,8 a 45,5 g EAG/kg de folha fresca. Souza et al. (2008) encontraram teores variando de 44,2 a 63 g EAG/kg MS nas folhas de quatro espécies amazônicas (*Byrsonima crassifolia*, *Davilla kunthii*, *Davilla rugosa* e *Inga edulis*). Silva et al. (2007) confirmaram que os polifenóis são encontrados em elevadas concentrações nas folhas das plantas. Isso se deve à aceleração da biossíntese pela exposição à luz, servindo como um mecanismo de filtração contra a radiação UV-B, processo anteriormente descrito por Harborne e Williams (2000).

Compostos fenólicos de plantas são bastante conhecidos por impactar no metabolismo microbiano ruminal, interferindo na metanogênese (Broudiscou et al, 2002; Busquet et al., 2006; Patra et al., 2006; Garcia-

González et al., 2010). Mais recentemente foi comprovada a participação de taninos como moduladores na biohidrogenação ruminal (Durmic et al., 2008; Khiaosa-Ard et al., 2009; Vasta et al., 2009a; Vasta et al., 2009b).

Leguminosas são conhecidas por apresentar teores de taninos relativamente elevados (Barahona et al., 2003). Mupangwa et al. (2000) avaliando os teores de taninos condensados totais, extraíveis e ligados em três leguminosas tropicais do Zimbábue (*Cassia rotundifolia*, *Lablab purpureus* e *Macroptilium atropurpureum*) encontraram concentrações de TC totais de 29,5, 16,9, 12,4 g eq. tanino mimosa/kg MS, respectivamente. Vitti et al. (2005) encontraram para as leguminosas brasileiras concentrações de 0,5; 12,7 e 23,5 g eq. catequina/kg MS. Sturm et al. (2007), com leguminosas da Colômbia, encontraram valores variando de 0 (não detectado) à 263 g/kg MS. Jackson e Barry (1996), avaliando diferentes leguminosas tropicais da Colômbia, reportaram concentrações de TC totais variando de 22,2 a 264,8 g eq. *locus pedunculatus*/kg MS.

Jakson e Barry (1996) estudando os taninos em folhas de leguminosas forrageiras tropicais encontraram que, na maioria das plantas, 70 a 95% do TC totais encontravam-se sob a forma extraível. Resultados similares foram encontrados por Terril et al. (1992) e por Douglas et al. (1993), em forrageiras temperadas. No presente estudo, a maioria das plantas apresentou a mesma característica, com a maior parte sendo de taninos extraíveis, exceto para as espécies *C. pubecens, C. mucunoides* e *P. phaseloides*, onde grande parte dos TC apresentou-se de forma ligada. Esses resultados estão de acordo com os estudos de Jones et al. (1973), os quais afirmaram que, devido aos taninos condensados serem normalmente encontrados nos vacúolos das células vegetais, isso justifica a elevada capacidade de extração dos TC em plantas forrageiras.

5 **CONCLUSÕES**

Os dados obtidos nesse trabalho são de grande importância para a caracterização das leguminosas amazônicas, sendo o perfil lipídico desses vegetais descritos pela primeira vez. As leguminosas apresentam uma

composição química interessante, podendo ser consideradas, por exemplo, fontes de compostos fenólicos, como as espécies do gênero *Inga*. Esses resultados demonstram um grande potencial do uso dessas leguminosas na nutrição de ruminantes, visando maior eficiência ruminal e, como consequência, aumentando o valor nutritivo e nutracêutico de alimentos provenientes de ruminantes.

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CAPÍTULO III

EFFECT OF DIFFERENT CONCENTRATION OF LEAF EXTRACTS FROM TWO AMAZONIAN TROPICAL LEGUMES (*INGA EDULIS* AND *DESMODIUM OVALIFOLIUM*) ON THE RUMINAL BIOHYDROGENATION *IN VITRO*

EFFECT OF DIFFERENT CONCENTRATION OF LEAF EXTRACTS FROM TWO AMAZONIAN TROPICAL LEGUMES (INGA EDULIS AND DESMODIUM OVALIFOLIUM) ON THE RUMINAL BIOHYDROGENATION *IN VITRO*

Bichara, C.M.G.[1]; Bressan, J.[2]; Rogez, H.[3]; Dang Van, Q.C.[4] ; Focan, M.[4]; Larondelle, Y[4].

[1]Instituto da Saúde e Produção Animal, Universidade Federal Rural da Amazônia. Avenida Presidente Tancredo Neves, nº 2501 Bairro: Montese, 66.077-830, Belém, Pará, Brasil.

[2]Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, Avenida PH Rolfs s/n, Campus Universitário, 36571-900, Viçosa, Minas Gerais, Brasil.

[3]Faculdade de Engenharia de Alimentos, Universidade Federal do Pará, Avenida Perimetral s/n, 66.095-780, Belém, Pará, Brasil.

[4]Institut des Sciences de la Vie, Unité de Biochimie de la Nutrition, Université Catholique de Louvain, Croix du Sud, 2/8, B-1348, Louvain-la-Neuve, Belgium.

ABSTRACT

The objective of the present study was to investigate the effect of phenolic-rich extracts from two Amazonian legumes on *in vitro* ruminal bypass of polyunsaturated fatty acids (PUFA) and appearance of biohydrogenation products. Leaf extracts of *Inga edulis* (IE) and *Desmodium ovalifolium* (DO) were incubated with grass silage as substrate, at 0 (control; C_0), 5 (C_5), 10 (C_{10}), 50 (C_{50}) and 100 (C_{100}) mg of lyophilized extract per g of feed dry matter, at 39 ºC in a rumen fluid:buffer mixture, together with linseed oil. After different incubation times: 3, 6, 9 and 24 h, the fatty acid (FA) profile present in the fermentation fluid was analyzed by gas chromatography. The extent to which α-linolenic acid (C18:3 *n*-3) was biohydrogenated was retarded in the presence of IE and DO extracts. After 3 hours, the reduction ranged from 23 to 65% with IE and from 15 to 61% with DO. For linoleic acid (C18:2 *n*-6), the reduction in the biohydrogenation efficiency after 3 hours ranged from 19 to 69% and 13 to 56% for IE and DO, respectively. After 6 hours, the concentrations C_{10}, C_{50} and C_{100} also caused significant reductions ($P<0.05$) in the extent of biohydrogenation. Rumenic acid, an important conjugated linoleic acid (CLA) isomer, significantly increased with both extracts for treatment C_{10} in the first 3 hours of incubation, and for most of treatments at

6 hours of incubation. No elevation of vaccenic acid was observed with IE and DO, as compared with the control (P>0.05). A reduction in the accumulation of stearic acid, the end product of biohydrogenation of C18 fatty acids, was observed with the greater concentrations at the end of the incubation period. These results lead us to conclude that phenolic-rich extracts from IE and DO slow down the ruminal fatty acid biohydrogenation during the first hours of incubation and could be used to increase both the bypass of PUFA and accumulation of CLA.
Keywords: Conjugated linoleic acid, fatty acid, phenolic compounds, plant extracts, rumen.

1 **INTRODUCTION**

The production of healthier ruminant food products has been recently an important issue in ruminant nutrition research. The general improvement of the fatty acid profile of meat and milk has concentrated a lot of efforts of both researchers and producers: less atherogenic fatty acids, more unsaturated fatty acids (UFA), more *n*-3 and *n*-6 polyunsaturated fatty acids (PUFA) and conjugated linoleic acids (CLA) A special interest for rumenic acid (*c*9,*t*11-C18:2), has focused on its anti-cancer, anti-atherogenic, immune stimulating and anti-diabetic properties (Whigham et al., 2000; Belury, 2002; Pariza, 2004, Koba; Yanagita, 2013).

Ruminants consume plants or plant-derived feeds where the UFA predominates. However, the biohydrogenation process catalyzed by the rumen microbial population extensively saturates the dietary UFA, thus explaining their low proportions in meat and dairy products. CLA are intermediate in this process. Some of them escape from the rumen, are absorbed in the gut and are transferred to the milk and stored in the tissues. Rumenic acid is the main CLA isomer produced in the rumen. In turn, it represents more than 80% of total CLA found in ruminants products. *Trans* monounsaturated fatty acids are also intermediates of the biohydrogenation process, vaccenic acid (*t*11-C18:1) being the major isomer formed. Vaccenic acid has a specific interest because the part that escapes from the rumen

and reaches the animal cells such as those of the mammary glands is actively desaturated by Δ9-desaturase, leading to more CLA in the milk. Reducing the extent of ruminal biohydrogenation of plant PUFA, in order to increase both their ruminal by-pass and accumulation of biohydrogenation intermediates, especially rumenic acid and vaccenic acid, would allow reaching all the expectations of the consumer of ruminant products, since their fat content would be more unsaturated and would contain more PUFA and CLA.

Phenolic compounds, and more particularly tannins, have been shown to reduce the ruminal biohydrogenation of PUFA at various steps of the pathways (Cabiddu et al., 2010; Jayanegara et al., 2011; Khiaosa-ard et al., 2009; Vasta et al., 2009a and 2009b). This would explain a higher by pass of linolenic and linoleic acids through the rumen, increasing the PUFA concentration in meats and milk, as well as a higher concentration of vaccenic acid serving as the precursor for rumenic acid synthesis within the mammary gland (Jayanegara et al., 2012).

Souza et al. (2008) have shown that leaves from *Inga edulis* are rich in phenolic compounds with a high antioxidant activity. Jackson and Barry (1996) showed that leaves from *Desmodium ovalifolium* present high condensed tannin concentration. The hypothesis tested in the present study was that these two phenolic-rich plant legumes from Amazonian region will interfere with the ruminal biohydrogenation of PUFA, thus improving in turn the fatty acids profile of ruminant-derived food products. In order to verify our hypothesis, we performed *in vitro* incubations of rumen fluid collected from three cannulated cows in the presence of grass silage, linseed oil as PUFA source and phenolic-rich extracts prepared from lyophilized leaves of these plants.

2 **MATERIAL AND METHODS**

2.1 EXPERIMENTAL PLANTS

Leaves of *Inga edulis* (IE) and *Desmodium ovalifolium* (DO) were harvested in January 2012 from two different places: Belem (Capital of the Para state, Brazil) and Igarapé-Açu (121 Km east of Belem). The material was carried to the laboratory in cellulose bags at room temperature within a maximum of 12 h after harvesting. The leaves were cut in small squares of 1–1.5 cm^2 before freezing at -20 ºC. They were freeze-dried to improve the yield of the phenolic compound extraction and avoid their oxidation with air. The freeze-dried leaves were ground until a granulometry below 1 mm using a knife mill (model Pulverisette 14; Fritsch, Idar-Oberstein, Germany) and stored at −20ºC. The samples were sent to Belgium (Université catholique de Louvain, Louvain-la-Neuve) for subsequent extraction, chemical analysis and *in vitro* ruminal incubations.

2.2. CHEMICAL CHARACTERIZATION OF THE PLANT MATERIAL

The two plants used to produce polyphenol-rich extracts as well as the grass silage used as roughage in the *in vitro* ruminal incubations, were analyzed, in duplicate (Table 1), for dry matter (DM) by oven-drying at 105 ºC for 16 h (adapted from methods 967.03 and 930.15; AOAC, 1995), total ash by incineration at 550 ºC for 16 h (adapted from methods 923.03, 967.04 and 942.05; AOAC, 1995), crude protein by the Kjeldahl method (N*6.25) (adapted from methods 981.10 and 991.20; AOAC, 1995), ether extract (adapted from Directive 98/64/EC; Commission of the European Communities, 1998), neutral detergent fiber (NDF) (Van Soest et al., 1991) and acid detergent fiber (ADF) (adapted from method 973.18; AOAC, 1995). NDF and ADF were determined sequentially and were expressed without residual ash. NDF was analyzed with the addition of α-amylase and without sodium sulfite.

Table 1 - Chemical composition of the lyophilized plant material used in the *in vitro* biohydrogenation experiments (grass silage used as roughage and leaves of the legumes *Inga edulis* and *Desmodium ovalifolium*).

	Grass silage	*I. edulis*	*D. ovalifolium*
DM (mg/g)	898.30	924.45	934.71
Crude protein (mg/g DM)	124.90	179.30	125.01
Ether extract (mg/g DM)	113.56	27.49	32.08
NDF (mg/g DM)	565.70	478.48	542.32
ADF (mg/g DM)	332.7	348.95	400.92

DM: dry matter; NDF: neutral detergent fiber; ADF: acid detergent fiber.

2.3 PREPARATION OF EXTRACTS

Dry samples (2.5 g) of IE and DO plants were extracted following the procedure recommended by Makkar (2003a). They were extracted in the dark with 25 mL of a 70:30 acetone:water solution (v:v) in tubes of 50 mL at room temperature for 30 min and then centrifuged at 3000×*g* for 15 min at 5 ºC. The supernatant was recovered and another extraction was done in identical conditions with the same volume of solvent. The two extracts were mixed and evaporated under vacuum at 38 ºC. In order to remove plant pigments and promote a purification of the extract, about 15 mL of diethyl ether were added to the filtrate and the mixture was well shaken. Then the upper (green coloured) organic phase was removed by a suction pump and the procedure was repeated until the liquid was colourless. After evaporation of the remaining organic solvent under vacuum at 38 ºC, the aqueous solution was frozen, freeze-dried and stored at -20 ºC until the *in vitro* tests. During the steps, the extracts were kept saturated with gaseous nitrogen.

2.3.1 **Determination of total phenolics, total flavonols, total flavanols and proanthocyanidins**

The total phenolic content (Table 2) was determined by the Folin–Ciocalteu colorimetric method (Singleton; Rossi, 1965). Results were expressed as mg of gallic acid equivalents (GAE) per g of DM.

The quantification of flavonols was done by means of the aluminium chloride colorimetric method (Chang; Chern, 2002). Results were expressed as mg of rutin equivalents (RE) per g of DM.

The total flavanols were determined using the chromogen, *p*-dimethylaminocinnamaldehyde (DMACA), following the protocol proposed by Delcour and Devarebeke (1985). Results were expressed as mg of catechin equivalents (CAE) per g of DM.

Condensed tannins were determined by the butanol-HCl (95:5, v:v) method as described by Mupangwa et al. (2000). Results are expressed as mg cyanidin equivalents (CE) per g of DM. All determinations were made in duplicate.

2.3.2 **DPPH free radical scavenging assay**

Radical scavenging activity of lyophilized extracts against stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined by the slightly modified method of Brand-Williams et al. (1995). The value of the antioxidant activity was expressed as µM Trolox equivalents (TE) per g of DM.

Table 2 - Total phenolics, flavonols, flavanols, proanthocyanidins and DPPH scavenging activity in lyophilized leaf extracts from *Inga edulis* and *Desmodium ovalifolium*.

	I. edulis	*D. ovalifolium*
Total phenolics (mg GAE/g DM)	450.45	404.77
Total flavonols (mg RE/g DM)	61.21	93.84
Total flavanols (mg CAE/g DM)	136.1	25.89
Proanthocyanidins (mg CE/g DM)	81.57	47.50
DPPH (µM TE/g DM)	2217.21	2695.04

GAE: gallic acid equivalents; RE: rutin equivalents; CAE: catechin equivalents; CE: cyanidin equivalents; TE Trolox equivalents; DPPH : 2,2-diphenyl-1-picrylhydrazyl; DM: dry matter.

2.4 *IN VITRO* INCUBATION WITH RUMINAL FLUID

The rumen fluid used in the *in vitro* experiment was obtained from three rumen-fistulated Holstein cows receiving grass silage *ad libitum*, with a mineral-vitamin supplement and free access to drinking water. Ruminal fluid

from each of the cows was collected manually, just before the morning feeding and then filtered with two metallic filters (a large mesh filter - 1 mm and a minor one - 0.4 mm) and introduced in pre-heated isothermal containers before transportation to the laboratory. The cows were housed in a farm of the Université Catholique de Louvain (Corroy-le-Grand, Belgium). The experiment was conducted from September to December 2012 and was approved by the Commission of Ethics for Animal Experiments of UCL.

A substrate made of 472.5 mg of DM of grass silage added in each of a set of 120 mL capacity glass bottles was used for the *in vitro* incubations. The leaf extracts were added in the proportions of 0 (control), 5, 10, 50 and 100 mg of lyophilized extract per g of feed DM (treatments C_0, C_5, C_{10}, C_{50}, C_{100}, respectively) and subjected to *in vitro* incubation with 50 mL of a rumen fluid:buffer mixture (1:4 v/v) in a water bath at 39ºC for different periods of time, according to Theodorou et al. (1994). The buffer solution used was described by Goering and Van Soest (1970), i.e., a solution containing (g/L) Na_2HPO_4, 1.3571; KH_2PO_4, 1.4752; $MgSO_4$, 0.0698; $NaHCO_3$, 8.3333; $(NH_4)HCO_3$, 0.9524; $CaCl_2.2H_2O$, 0.0157; $MnCl_2.4H_2O$, 0.0119; $CoCl_2.6H_2O$, 0.0019; $FeCl_3.6H_2O$, 0.00952; resazurin, 0.00119; cysteine, 0.2976. The incubation was done after adding 27.5 mg of linseed oil, solubilized in diethyl ether, equivalent to 5.5% of dietary lipid supplementation. All ingredients were mixed under a stream of CO_2, the bottles were sealed with a rubber septum fitted with a crimp seal, placed in a shaking water bath (160 rpm, 39°C) and incubated for 3, 6, 9 and 24 h. This experiment was repeated three subsequent times, each of the three incubations being performed with the rumen fluid from a different cow, involving thus a total of three cows.

At the end of each incubation, the correspond bottles were taken up from the water bath; 5 mL were withdrawn from the incubation mixtures, transferred to 30 mL Pyrex® tubes, which were directly put in a dry ice/ethanol bath to stop the microbial activities and then stored at -20°C for further analyses.

2.5 FATTY ACIDS EXTRACTION AND METHYLATION

The preparation of fatty acid methyl esters (FAME) was carried out as proposed by Park and Goins (1994). C19:0 (1 mg C19:0 in 5 mL of methanol) was added as an extraction standard. The method comprises two main parts: a) the first one consists in an alkaline hydrolysis in order to hydrolyze the triglyceride bonds between the glycerol and the fatty acids. This step was carried out in a water bath at 90 °C for 10 min and relied on the aid of a strong base of NaOH in methanol (0.625 N); b) the next step consists in the methylation of free FA, which took place at 90°C during 10 min with an acid solution of BF3 in methanol (14%). Finally, the FAME were extracted twice with hexane.

2.6 FATTY ACID DETERMINATION

The gas chromatography procedure was carried out as proposed by Dang Van et al. (2011). FAME were separated and quantified with a gas chromatograph (ThermoQuest Trace GC; Thermo Finnigan, Milan, Italy) equipped with a flame ionization detector, an automatic injector, and a fused silica capillary column (100 m × 0.25 mm i.d.), coated with a 0.2-μm film of biscyanopropyl polysiloxane (Rt-2560; Restek Corp., Bellefonte, PA). The system used H_2 as the carrier gas operated at a constant pressure of 200 kPa. The initial oven temperature was 80 °C; it increased at 25 °C/min to 175 °C (held for 25 min), then increased at 10 °C/min to 205 °C (held for 4 min), then increased at 10 °C/min to 225 °C (held for 20 min), and finally decreased at 20 °C/min to 80 °C. The temperature of the flame ionization detector was maintained at 255 °C. Hydrogen flow to the detector was 35 mL/min and air flow was 350 mL/min. Each peak was identified and quantified by comparison of retention times with pure FAME standards and C11:0 as an internal standard of injection (Alltech, Deerfield, IL; except CLA isomers from Nu-Chek Prep, Inc., Elysian, MN). Each FA was expressed as mg/g DM incubated at time 0 (sum of grass silage, linseed oil and lyophilized extract).

2.7 CALCULATION AND STATISTICAL ANALYSIS

The *in vitro* biohydrogenation efficiency in percentage (BH efficiency %) of a given fatty acid (C18:3 *n*-3, C18:2 *n*-6 e C18:1 *n*-9) at incubation time *t* was calculated according to Eq. (1):

$$100 - \frac{Concentration\ of\ FA\ at\ time\ t}{Concentration\ of\ FA\ at\ time\ 0}\ x\ 100 \qquad \text{Eq. (1)}$$

A factorial model of analysis of variance was employed as the statistical model to the data obtained, with main effects of groups (G), extract concentrations (C), incubation time (t), and their interaction (CxG, Cxt, CxG×t). There were two groups (G = IE or DO), five different extract concentrations (C = 0, 5, 10, 50 and 100 mg/g DM) and four different incubation times (t = 3, 6, 9 and 24 h) for each group of plants. Difference between means were further assessed by a post-hoc test Fisher's LSD at $P<0.05$. All these statistical analysis were performed using statistical software STATISTICA version 7.0 (Statsoft, 2004).

3 **RESULTS**

Nutrient composition

The two amazonian legumes, IE and DO, showed similar level of total phenolic compounds in the lyophilized leaf extracts (Table 2), except for flavonol, favanois and condensed tannins composition.

Effects of Amazonian legumes on ruminal biohydrogenation

C18:3 *n*-3

The amount of C18:3 *n*-3 gradually declined ($P<0.05$) with time, at different rates ($P<0.05$) between treatments (Table 3). Compared with the control, the content was higher in IE and DO groups in the first hours of incubation. For example, treatment C_{10} of IE or DO allowed maintain the C18:3 *n*-3 levels 66 and 48% higher after 3 hours of fermentation ($P<0.05$) and for treatment C_{100} of both extracts, the content was approximately 80% higher, compared to control ($P<0.05$). After 6 h, the concentrations C_{10}, C_{50} and C_{100} provided higher C18:3 *n*-3 content. After 9 h, it was observed that the use of treatment C_{50} of DO group caused a significant reduction ($P<0.05$) on the C18:3 *n*-3, whilst using treatment C_{100} provided higher concentrations compared to other treatments including the control. At time 24 h, the use of different concentrations of the two leaf extracts tended to increase compared to the control, with significant difference ($P<0.05$) in the treatment C_{100} of DO group.

C18:2 *n*-6

The amounts of C18:2 *n*-6 were higher with use of C_{10}, C_{50} and C_{100} of IE and DO groups than the control during the first 6 hours. It was observed that treatment C_{50} of DO group showed a reduction in the concentration of C18:2 *n*-6 at time 9 h, similarly found in C18:3 *n*-3 content. The use of C_{100} of DO group kept the concentration higher than the other treatments during all the incubation time.

C18:1 *n*-9

Among the treatments, at time 3 h, the greater amount of C18:1 *n*-9 was found in concentrations of C_{10} and C_{100} of both extracts, when compared to C_0. At time 6 h, there was little variation between the concentrations, however, they were different from the control (P<0.05). At time 9 h, supplementation with IE supplied lower C18:1 *n*-9 content at all concentrations, whereas supplementation with DO showed no significant difference compared to control (P>0.05). After 24 h, there was a significant decrease (during the incubation times) in the C18:1 *n*-9 content on the control treatment and also using the IE and DO extracts at C_5 and DO extract at C_{10} and C_{50}. The concentration C_{100} of DO remained with high levels compared to the other treatments.

*c*9, *t*11-CLA

The *c*9, *t*11-CLA content strongly increased during the incubation time in all the groups from 3 to 6 h (except C_5 of IE), and then slowed down after that. At 3 h, the treatment C_5 (IE and DO) showed an increasing trend compared to C_0, and became significantly higher (P<0.05) in both extract groups with C_{10}. At 6 h, higher concentrations of DO negatively influenced the increase of *c*9, *t*11-CLA content, which was not observed with the IE extract that showed an increase in function of increasing extract concentration. At time 9 h, groups showed no significant difference between them, except the treatment C_{50} of DO extract, that presented *c*9, *t*11-CLA content 10.8 times higher than the control. After 24 h, the difference was not significant (P>0.05) between the treatments except for C_{100} of DO group. The *c*9, *t*11-CLA accumulation was strongly affected by the group effect (P<0.001) as can be seen in Table 4.

*t*11-C18:1

The amounts of *t*11-C18:1 increased significantly (P<0.05) in control group up to 9 h point, decreasing after 24 h. The groups IE and DO at C_5 concentration showed similar behavior with no significant difference between

them. The treatment C_{10} and C_{50} of both extracts IE and DO showed no significant difference at 3 and 24 h (P>0.05) and significant at 6 and 9 h (P<0.05) compared with the control group. The supplementation with C_{100} of both extracts presented lower *t*11-C18:1 content at all-time points when compared with the control group, except for DO group at 24 h.

C18:0

The concentration of C18:0 increased significantly after 24 h in all groups (control; IE and DO) at treatments C_0, C_5, C_{10} and C_{50}. At C_{100} of both extracts, there was no difference (P>0.05) during the incubation times. There was also no influence of the substrate incubated up to 6 h. At 9 h, it was observed a reduction (P<0.05) compared to control group at C_{50} of DO group. At 24 h, the treatment C_{50} of IE group and both extract groups at C_{100} presented lower concentrations (P<0.05) of C18:0.

∑PUFA content

Globally, the ∑PUFA content showed a gradual decrease (P<0.05) according to incubation times in all treatments. After 3 h, compared with the control group, the addition of leaf extracts showed a greater concentration of ∑PUFA content (except for C_5 of DO). At 6 h, at treatments C_{10}, C_{50} and C_{100} (IE and DO) the PUFA content was higher (P<0.05) than for control group. After 9 h of fermentation, only treatments C_{50} and C_{100} of DO extract remained with higher PUFA concentration, and C_{100} of DO extract maintained with higher levels after 24 h of fermentation (P<0.05).

Biohydrogenation efficiency (%) of C18:3 *n*-3, C18:2 *n*-6, C18:1 *n*-9

Figures 1, 2 and 3 illustrate the biohydrogenation efficiency of fatty acids on the present study. After the first 3 hours of incubation, the treatments with leaf extracts showed a significant reduction in the biohydrogenation process of C18:3 *n*-3 without influence of plant group but with a dose dependent response (Figure 1; Table 4). At 6 h, the treatment C_{10} of DO group and the highest concentrations (C_{50} and C_{100}) of both

extracts showed a significant reduction (P<0.05) in the BH efficiency compared to the control group. At time 9 h, there was an increase in the C18:3 *n*-3 BH process at C_{50} of DO group and a significant reduction (P<0.05) at C_{100} treatment compared to control and other treatments. The same decrease was observed after 24 h incubation at this concentration.

Considering the C18:2 *n*-6 biohydrogenation process (Figure 2), at time 3 h only the treatment C_5 of DO group showed no significant difference compared to control; all other treatments showed a significant reduction on biohydrogenation efficiency (P<0.05). At time 6 h, the supplementation with C_{10}, C_{50} and C_{100} of both extracts were effective in reducing the C18:2 *n*-6 biohydrogenation process. At time 9 and 24 h, the process had the same effect as previously seen with C18:3 *n*-3, the biohydrogenation increased with C_{50} of DO group (9 h) and a significant decrease with C_{100} (9 and 24 h) compared to control and other treatments.

For C18:1 *n*-9 biohydrogenation process (Figure 3), at time 3 h concentrations of C_{10}, C_{50} and C_{100} showed a significant reduction compared to control. At time 6 h, treatment C_5 of DO group and all other concentrations (C_{10}, C_{50} and C_{100}) of both extracts were effective in reducing C18:1 *n*-9 biohydrogenation process. After 9 h incubation, the supplementation with IE extract showed an increase in the C18:1 *n*-9 biohydrogenation whatever the concentration used. Nevertheless, the use of DO extract did not affect (P>0.05) the biohydrogenation compared to control. After finalizing the fermentation period, biohydrogenation was completed, differing only in the treatment C_{100} of DO group, which showed a reduction in this process compared to control and other treatments.

4 **DISCUSSION**

Numerous reports have appeared in the literature demonstrating the effects of several compounds on ruminal biohydrogenation, such as fish oil (Shingfield et al., 2006; Hou et al., 2011), essential oils (Durmic et al., 2008), others PUFA sources (Scollan et al., 2001; Sackmann et al., 2003; Kitessa et al., 2010) and plant extracts (Lourenço et al., 2008; Cabiddu et al., 2009;

Jaynegara et al., 2011). Phenolic compounds seem to play a major role in the modulation of the biohydrogenation process (Khiaosa-ard et al., 2009; Vasta et al., 2009a and 2009b; Toral et al., 2011). The present study provides information about influence of phenolic-rich extracts from two amazonian legumes on bypass of PUFA and CLA accumulation.

The two amazonian legumes appeared to be of similar nutritive value, with a comparable level of phenolic compounds. They differed in relation to their CT monomer composition, which each of these monomers and oligomers units have a subset of structures and lead to differences in the chemical structure influencing the reactivity of the CT, which may presents different physiological effects and animal performance. Brunet et al. (2008) showed that presence of prodelphinidins and procyanidins in different proportions in forages, for example, can determine different biological efficacy.

Effects of amazonian leaf extracts on ruminal biohydrogenation of C18:3 *n*-3, C18:2 *n*-6, C18:1 *n*-9

The first hours of incubation were essential to observe the extract effects on ruminal biohydrogenation. After 3 hours, the concentrations of C18:3 *n*-3, C18:2 *n*-6, C18:1 *n*-9 were greater than the control group with treatments C_{10}, C_{50} and C_{100} leaf extracts. This fact was confirmed by evaluating the biohydrogenation efficiency, where the control group presented 58.7, 51.1 and 36.0% for C18:3 *n*-3, C18:2 *n*-6, C18:1 *n*-9 respectively, meanwhile the presence of leaf extracts ranged from 20.3 to 50.2, 15.9 to 42.5 and 16.0 to 26.9%, respectively. After 6 h, the biohydrogenation efficiency was 85.3, 77.9 and 44.8% for the control group and for IE and DO groups ranged from 46.3 to 75.4, 46.6 to 66.6 and 28.9 to 34.0% for C18:3 *n*-3, C18:2 *n*-6, C18:1 *n*-9, respectively. Similarly, Cabiddu et al. (2010) showed that the biohydrogenation of C18:3 *n*-3 and C18:2 *n*-6 ranged from 41.5 to 80.3% and 17.9 to 57.5%, respectively, after 6 h of incubation, of two species (*Vicia sativa* and *Trifolium incarnatum*) at two phenological stages (vegetative and reproductive), using an *in vitro* batch

culture technique. These results demonstrated the higher disappearance of C18:3 *n*-3 compared with that of C18:2 *n*-6, confirming that the disappearance extent is greater when higher the desaturation degrees of C18 fatty acids. In the following hours, only supplementation with highest concentrations of DO extract was able to modify ruminal BH. At time 9 h, it was observed that treatment C_{50} of DO group caused a greater extent of biohydrogenation process of both C18:3 *n*-3 and C18:2 *n*-6.

Appearance of biohydrogenation intermediates

Highest concentrations of *c*9,*t*11-CLA were found in the early stages of incubation. At time 3 h, the treatment C_{10} of IE and DO extracts supplied concentrations 1.9 and 1.6 times higher than the control group, respectively. After 6 h of incubation, the use of C_{10}, C_{50} and C_{100} of IE provided 1.6, 1.9 and 2 times more *c*9,*t*11-CLA, respectively; and using DO extract at C_5 and C_{10} provide levels 1.8 and 2.1 times higher than the control, respectively. After 9 h, only treatment C_{50} of DO group showed to be more effective to increase the concentration of *c*9,*t*11-CLA, supplying 10.8 times higher than control, confirming the findings above in relation to the disappearance of PUFA, since the *c*9,*t*11-CLA is the main intermediate of the biohydrogenation process of C18:2 *n*-6. This is in agreement with Buccioni et al. (2008), which have shown that the rate of appearance of *c*9,*t*11-CLA *in vitro* is higher and occurs in shorter time compared with *t*11-C18:1, before reaching a plateau.

It is well known that the most effective way to enhance the concentration of *c*9,*t*11-CLA in ruminant products is to favor the ruminal production of *t*11-C18:1 (Griinari et al., 2000; Corl et al., 2001; Sackmann et al., 2003). In the muscle (Santora et al., 2000) and in the mammary gland (Griinari et al., 2000), *t*11-C18:1 is partially converted to *c*9,*t*11-CLA by the action of Δ9-desaturase. It was observed that the use of IE and DO extracts were not efficient to increase *t*11-C18:1 content. Low concentrations (C_5 and C_{10}) had a minor influence compared to the highest concentrations. These results suggest that the first step of biohydrogenation, which is the conversion of C18:2 *n*-6 to *c*9,*t*11-CLA was affected by phenolic compounds

from IE e DO, suffering a decreased activity to a larger extent compared with the last step, which leads to the formation of C18:0. This fact was recently observed by Jayanegara et al. (2011) and confirms what has already been noted by Jones et al. (1994), who showed that tannins inhibit *Butyrivibrio fibrisolvens,* the bacterial species known to be a major species involved in ruminal biohydrogenation.

Jayanegara et al. (2011) studied the effects of phenolic compounds in tropical forages, and also did not find a significant relationship between total extractable phenolics and the appearance of the *t*11-C18:1, but a strong positive correlation between condensed tannins (CT) and the appearance of *c*9,*t*11-CLA ($P<0.001$). Cabbidu et al. (2010) affirm that the relationship between phenolic compounds and biohydrogenation is not always linear and this may be attributed to a possible adaptation of rumen microorganisms. Makkar et al. (2003b) reported that microbial populations exhibit a remarkable capacity to adapt to a wide variety of antimicrobial agents. Several mechanisms have been proposed for the resistance mechanisms of the bacteria to tannins: some bacteria grown in the presence of tannin secrete exo-polysaccharides (glycoproteins) that form a protective layer around the cells and thus protect the cells from the action of tannins (Chiquette et al., 1989; Krause et al., 2005); tolerance mechanism of bacteria to tannins may also involve degradation of tannins by bacteria (Odenyo; Osuji, 1998); synthesis of siderophores (Scalbert, 1991) which probably counteract the depletion of iron by tannins; and by secreting increased amounts of proline-rich proteins in the saliva (Mehansho et al., 1983), and these proteins have strong affinity for tannins and may be constitutive or inducible depending upon the animals species.

Considering the final product of the biohydrogenation process, low concentrations of IE and DO extracts had no significant effect on the reduction of C18:0. Only the supplementation with greater concentrations was effective in reducing the final product of the BH after 24 h of incubation. C18:0 was strongly reduced (28.5%) with the use of treatment C_{50} of IE group and above 50% with C_{100} of IE and DO extracts. Vasta et al. (2009a),

using different sources of tannins in similar concentrations to those used in this study (136 mg/g), noted that the concentration of C18:0 was strongly reduced (16%) after 12 h of fermentation.

This lack of effects in the C18:0 content during the incubation may be due to biohydrogenation proceed to a higher extent with high NDF levels in the diet (Sackmann et al., 2003). The microorganisms mainly involved in the BH are cellulolytic strains, which are known to proliferate to a greater extent in the rumen of forage-fed ruminants. More complete biohydrogenation would favor the formation of C18:0. Thus, under the conditions used in this experiment, the environment was more favorable for the development of biohydrogenation, and the phenolic compounds added had a milder effect on ruminal BH at low concentrations. Vasta et al. (2009b), evaluating the effects on biohydrogenation with different substrates (concentrate or herbage) concluded that the effect of tannins on ruminal BH seems to be stronger when they are included into concentrates, rather than with herbage.

Thus, results found in this work demonstrate that in the early stages of incubation, both extracts of IE and DO showed an inhibitory effect on microbial group causing an initial retard on the process, favoring a greater accumulation of the first intermediate of C18:2 *n*-6. Over time, with favorable conditions for the process occurs due to adaptation of the microbial population, biohydrogenation held its complete process.

Significance of phenolic compounds on the biohydrogenation extent

The accumulation of PUFA in the rumen was increased by IE and DO extracts supplementation at the first 6 hours of incubation. This is in agreement with Cabiddu et al. (2010), who observed a negative relationship between tannins from two legumes (*V. sativa* and *T. incarnatum*) and lipolysis and biohydrogenation. Kälber et al. (2011) also hypothesized that buckwheat (*Fagopyrum esculentum*) phenolic compounds were responsible for the increase found in the transfer of C18:2 *n*-6 from feed to milk and Vasta et al. (2009c) concluded that quebracho (*Schinopsis lorentzii*) tannins

(89.3 g/kg of DM) increased the concentration of C18:2 *n*-6 and PUFA in lamb meat. This fact can be supported by the results of the present study.

5 **CONCLUSIONS**

The present results demonstrate that leaf extracts from *Inga edulis* and *Desmodium ovalifolium* have potential to modify *in vitro* ruminal fatty acid biohydrogenation, leading to delay on the extent of ruminal biohydrogenation, increasing *c*9,*t*11-CLA content and causing a bypass of PUFA, suggesting that phenolic compounds affect to a larger extent the conversion of C18:2 *n*-6 to *c*9,*t*11-CLA than the subsequent steps.

Table 3 - C18 fatty acids (mg/g DM) in the fermentation mixture during 24 h of *in vitro* incubation in the presence of *Inga edulis* (IE) or *Desmodium ovalifolium* (DO) leaf extract (means of three replicates). SEM, standard error of the mean.

Fatty acids (mg/g DM)	Group	Treatment (mg/g DM)	Incubation times (h)				
			3	6	9	24	SEM
C18:3 *n*-3	Control	C_0	$8.52^{a}{}_{E}$	$2.82^{b}{}_{C}$	$1.88^{b,c}{}_{A}$	$0.78^{c}{}_{A}$	0.91
	IE	C_5	$12.51^{a}{}_{C}$	$3.38^{b}{}_{C}$	$1.71^{c}{}_{A}$	$0.89^{c}{}_{A}$	1.41
		C_{10}	$14.18^{a}{}_{B}$	$3.97^{b}{}_{C,B}$	$1.55^{c}{}_{A}$	$0.88^{c}{}_{A}$	1.61
		C_{50}	$10.64^{a}{}_{D}$	$4.95^{b}{}_{B}$	$1.90^{c}{}_{A}$	$0.92^{c}{}_{A}$	1.15
		C_{100}	$15.48^{a}{}_{A}$	$6.66^{b}{}_{A}$	$2.67^{c}{}_{A}$	$1.29^{d}{}_{A}$	1.67
	Control	C_0	$8.52^{a}{}_{C}$	$2.82^{b}{}_{D}$	$1.88^{b,c}{}_{B}$	$0.78^{c}{}_{B}$	0.91
	DO	C_5	$9.61^{a}{}_{C}$	$3.83^{b}{}_{C,D}$	$1.58^{c}{}_{B}$	$0.96^{c}{}_{B}$	1.03
		C_{10}	$12.62^{a}{}_{B}$	$4.75^{b}{}_{C}$	$1.75^{c}{}_{B}$	$1.00^{c}{}_{B}$	1.38
		C_{50}	$11.82^{a}{}_{B}$	$6.29^{b}{}_{B}$	$0.22^{c}{}_{C}$	$0.97^{c}{}_{B}$	1.41
		C_{100}	$15.43^{a}{}_{A}$	$10.09^{b}{}_{A}$	$5.17^{c}{}_{A}$	$2.30^{d}{}_{A}$	1.54

Continued

C18:2 *n*-6	**Control**	**C_0**	3.60^{a}_{D}	1.61^{b}_{B}	1.21^{b}_{A}	0.60^{c}_{A}	0.34
	IE	**C_5**	4.90^{a}_{C}	1.59^{b}_{B}	$1.08^{b,c}_{A}$	0.57^{c}_{A}	0.51
		C_{10}	5.82^{a}_{B}	2.48^{b}_{A}	0.95^{c}_{A}	0.58^{c}_{A}	0.63
		C_{50}	4.33^{a}_{C}	2.60^{b}_{A}	1.12^{c}_{A}	0.59^{c}_{A}	0.44
		C_{100}	6.84^{a}_{A}	3.11^{b}_{A}	1.33^{c}_{A}	0.64^{d}_{A}	0.72
	Control	**C_0**	3.60^{a}_{C}	1.61^{b}_{C}	1.21^{b}_{B}	0.60^{c}_{B}	0.34
	DO	**C_5**	$3.97^{a}_{B,C}$	1.73^{b}_{C}	1.06^{c}_{B}	0.61^{c}_{B}	0.51
		C_{10}	5.30^{a}_{A}	2.50^{b}_{B}	1.07^{c}_{B}	0.60^{c}_{B}	0.63
		C_{50}	4.46^{a}_{B}	2.95^{b}_{B}	0.09^{d}_{C}	0.60^{c}_{B}	0.44
		C_{100}	5.71^{a}_{A}	4.15^{b}_{A}	2.76^{c}_{A}	1.20^{d}_{A}	0.72
C18:1 *n*-9	**Control**	**C_0**	3.96^{a}_{C}	2.83^{b}_{C}	2.26^{b}_{A}	1.11^{c}_{A}	0.32
	IE	**C_5**	4.47^{a}_{B}	$3.25^{b}_{B,C}$	1.53^{c}_{B}	0.77^{d}_{A}	0.44
		C_{10}	$5.43^{a}_{A,B}$	$3.72^{b}_{A,B}$	$1.00^{c}_{B,C}$	0.87^{c}_{A}	0.58
		C_{50}	$4.45^{a}_{B,C}$	3.97^{a}_{A}	0.92^{b}_{C}	1.03^{b}_{A}	0.50
		C_{100}	5.53^{a}_{A}	$3.50^{b}_{A,B}$	$1.32^{c}_{B,C}$	1.15^{c}_{A}	0.54
	Control	**C_0**	3.96^{a}_{C}	2.83^{b}_{C}	$2.26^{b}_{A,B}$	1.11^{c}_{B}	0.32
	DO	**C_5**	$4.14^{a}_{B,C}$	3.76^{a}_{B}	$1.89^{b}_{A,B}$	1.01^{c}_{B}	0.38
		C_{10}	5.13^{a}_{A}	4.73^{a}_{A}	2.32^{b}_{A}	0.81^{c}_{B}	0.54
		C_{50}	$4.70^{a}_{A,B}$	3.52^{b}_{B}	1.72^{c}_{B}	0.82^{d}_{B}	0.46
		C_{100}	5.28^{a}_{A}	4.19^{b}_{A}	2.35^{c}_{A}	2.01^{c}_{A}	0.41

Continued

***c*9,*t*11-CLA**	**Control**	**C_0**	0.197^{b}_{B}	0.333^{a}_{C}	0.204^{b}_{A}	0.081^{c}_{A}	0.03
	IE	**C_5**	$0.261^{a}_{A,B}$	0.274^{a}_{C}	0.180^{a}_{A}	0.065^{b}_{A}	0.03
		C_{10}	0.367^{b}_{A}	0.549^{a}_{B}	0.174^{c}_{A}	0.074^{c}_{A}	0.05
		C_{50}	0.195^{c}_{B}	$0.647^{a}_{A,B}$	0.277^{b}_{A}	0.093^{d}_{A}	0.06
		C_{100}	0.115^{c}_{B}	0.692^{a}_{A}	0.232^{b}_{A}	0.099^{d}_{A}	0.07
	Control	**C_0**	0.197^{b}_{B}	0.333^{a}_{C}	0.204^{b}_{B}	0.081^{c}_{B}	0.03
	DO	**C_5**	$0.290^{b}_{A,B}$	0.580^{a}_{B}	0.193^{c}_{B}	0.082^{d}_{B}	0.05
		C_{10}	0.309^{b}_{A}	0.711^{a}_{A}	0.162^{c}_{B}	0.069^{c}_{B}	0.07
		C_{50}	0.170^{c}_{B}	0.410^{b}_{C}	2.272^{a}_{A}	0.083^{d}_{B}	0.27
		C_{100}	0.058^{c}_{C}	0.166^{b}_{D}	0.162^{b}_{B}	0.234^{a}_{A}	0.02
***t*11-C18:1**	**Control**	**C_0**	$2.80^{c}_{A,B}$	5.64^{b}_{A}	7.68^{a}_{A}	5.46^{b}_{A}	0.54
	IE	**C_5**	$2.83^{a}_{A,B}$	5.98^{b}_{A}	7.25^{a}_{A}	5.78^{b}_{A}	0.51
		C_{10}	3.35^{b}_{A}	4.75^{a}_{B}	5.09^{a}_{B}	5.55^{a}_{A}	0.28
		C_{50}	2.25^{b}_{B}	4.87^{a}_{B}	4.85^{a}_{B}	5.35^{a}_{A}	0.38
		C_{100}	1.90^{c}_{C}	3.42^{b}_{C}	$3.72^{a,b}_{C}$	4.38^{a}_{B}	0.29
	Control	**C_0**	$2.80^{c}_{A,B}$	$5.64^{b}_{A,B}$	7.68^{a}_{A}	5.46^{b}_{B}	0.54
	DO	**C_5**	$2.76^{c}_{A,B}$	5.20^{b}_{B}	$7.30^{a}_{A,B}$	5.60^{b}_{B}	0.50
		C_{10}	2.98^{c}_{A}	$6.03^{a,b}_{A}$	6.53^{b}_{B}	5.62^{a}_{B}	0.43
		C_{50}	$2.05^{c}_{B,C}$	3.88^{b}_{C}	5.49^{a}_{C}	$4.71^{a,b}_{B}$	0.40
		C_{100}	1.32^{d}_{C}	2.39^{c}_{D}	3.64^{b}_{D}	7.39^{a}_{A}	0.69

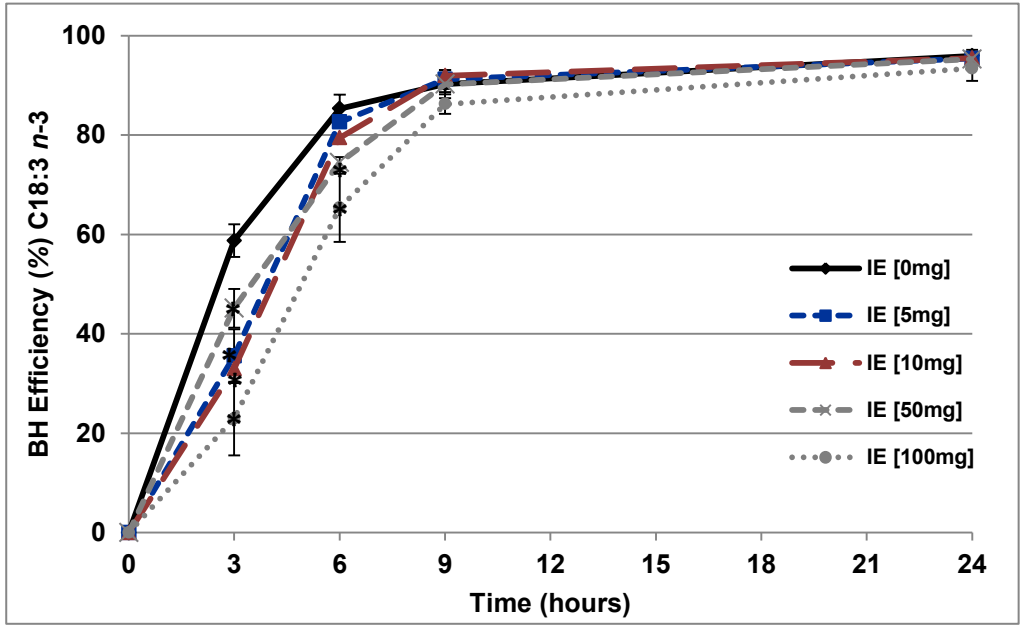
Continued

C18:0	**Control**	**C_0**	4.65^{b}_{A}	5.55^{b}_{A}	5.76^{b}_{A}	10.11^{a}_{A}	1.33
	IE	**C_5**	4.77^{b}_{A}	5.40^{b}_{A}	5.83^{b}_{A}	9.54^{a}_{A}	1.22
		C_{10}	5.31^{b}_{A}	5.45^{b}_{A}	5.53^{b}_{A}	10.64^{a}_{A}	1.41
		C_{50}	4.92^{b}_{A}	5.29^{b}_{A}	4.48^{b}_{A}	7.23^{a}_{B}	0.79
		C_{100}	5.34^{a}_{A}	5.20^{a}_{A}	5.34^{a}_{A}	5.04^{a}_{C}	0.42
	Control	**C_0**	4.65^{b}_{A}	5.55^{b}_{A}	5.76^{b}_{A}	10.11^{a}_{A}	1.33
	DO	**C_5**	4.83^{b}_{A}	5.09^{b}_{A}	$5.10^{b}_{A,B}$	9.27^{a}_{A}	1.20
		C_{10}	5.02^{b}_{A}	5.51^{b}_{A}	$5.66^{b}_{A,B}$	10.79^{a}_{A}	1.51
		C_{50}	5.04^{b}_{A}	5.66^{b}_{A}	4.35^{b}_{B}	10.29^{a}_{A}	1.51
		C_{100}	4.74^{a}_{A}	5.31^{a}_{A}	$5.44^{a}_{A,B}$	4.72^{a}_{B}	0.53
∑PUFA	**Control**	**C_0**	14.00^{a}_{E}	6.71^{b}_{C}	$4.99^{b,c}_{A}$	3.55^{c}_{A}	1.24
	IE	**C_5**	19.48^{a}_{C}	7.16^{b}_{C}	4.77^{c}_{A}	4.24^{c}_{A}	1.88
		C_{10}	22.64^{a}_{B}	$8.81^{b}_{B,C}$	4.24^{c}_{A}	3.82^{c}_{A}	2.33
		C_{50}	17.24^{a}_{D}	$10.10^{b}_{A,B}$	4.87^{c}_{A}	3.34^{c}_{A}	1.65
		C_{100}	24.44^{a}_{A}	12.17^{b}_{A}	5.59^{c}_{A}	3.59^{d}_{A}	2.47
	Control	**C_0**	14.00^{a}_{D}	6.71^{b}_{C}	$4.99^{b,c}_{B}$	3.55^{c}_{B}	1.24
	DO	**C_5**	15.85^{a}_{D}	8.04^{b}_{C}	4.56^{c}_{B}	3.39^{c}_{B}	1.50
		C_{10}	20.17^{a}_{B}	10.25^{b}_{B}	4.89^{c}_{B}	4.16^{c}_{B}	1.94
		C_{50}	18.40^{a}_{C}	11.52^{b}_{B}	7.98^{c}_{A}	3.73^{d}_{B}	1.64
		C_{100}	22.90^{a}_{A}	16.34^{b}_{A}	9.79^{c}_{A}	5.65^{d}_{A}	2.04

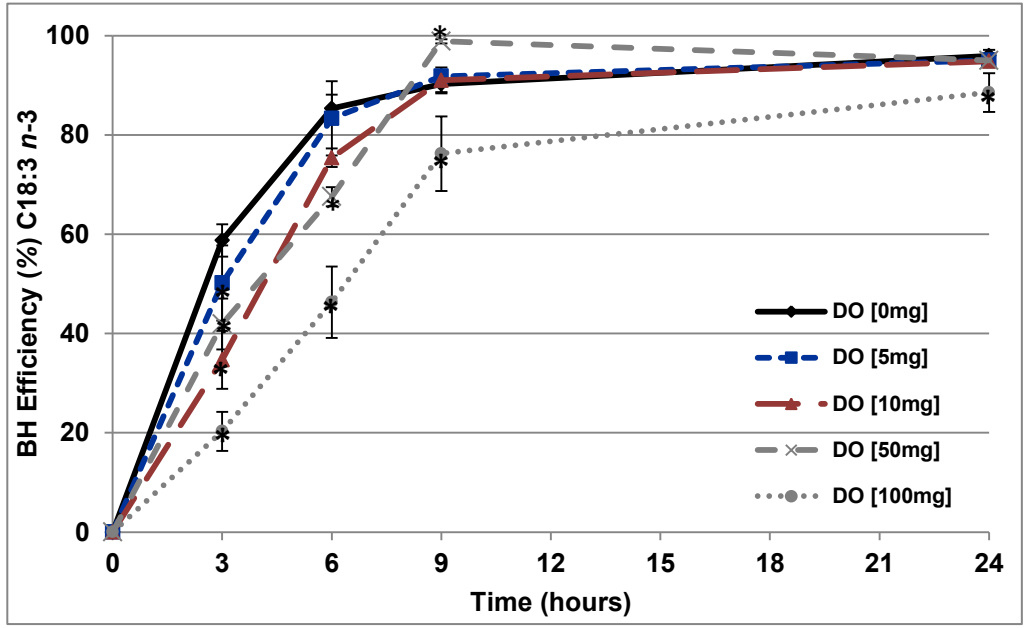
Different subscript letters (A-E) show differences ($P<0.05$) between the treatments for each fatty acid and each plant extract, and different superscript letters (a-e) indicate differences ($P<0.05$) between incubation times (0, 3, 6, 9, 24 h) for each treatment. DM: dry matter.

Table 4 - Univariate tests of significance of main effects of groups (G), extract concentrations (C), incubation times (t) and their interactions. *P<0.05, **P<0.01, ***P<0.001.

Fatty acids	Group (G)	Concentration (C)	Time (t)	G x C	G x t	t x C	G x t x C
C18:3 *n*-3	ns	***	***	***	**	***	**
C18:2 *n*-6	ns	***	***	**	***	***	***
C18:1 *n*-9	***	***	***	*	***	***	*
*c*9, *t*11-CLA	***	***	***	***	***	***	***
*t*11-C18:1	ns	***	***	*	*	***	***
C18:0	ns	***	***	ns	ns	***	ns
PUFA	*	***	***	***	***	***	ns
BH Efficiency							
C18:3 *n*-3	ns	***	***	***	**	***	**
C18:2 *n*-6	ns	***	***	***	*	***	**
C18:1 *n*-9	***	***	***	*	**	***	**

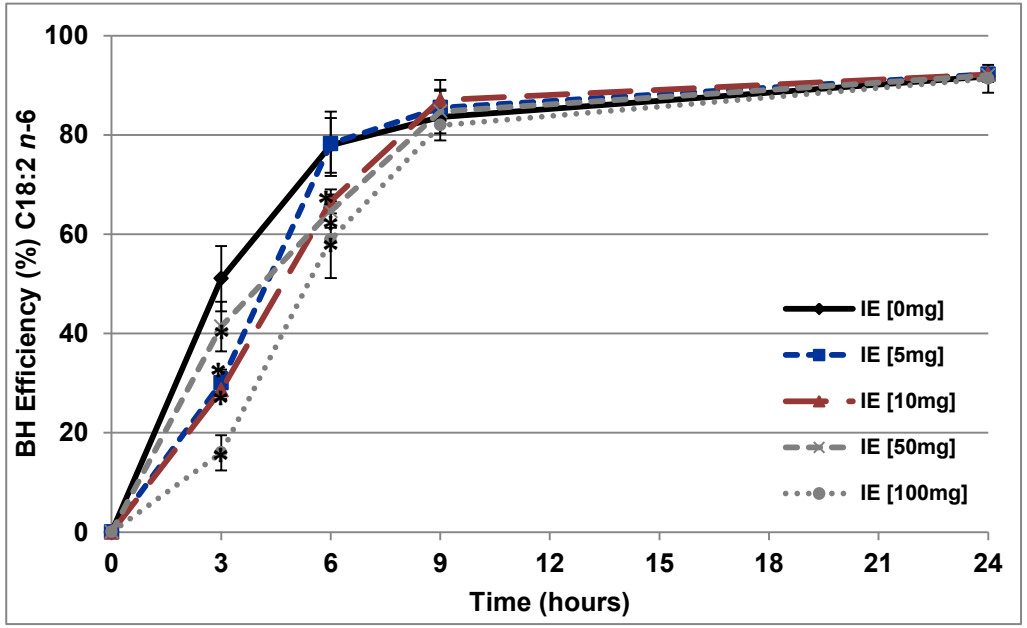


a-1)

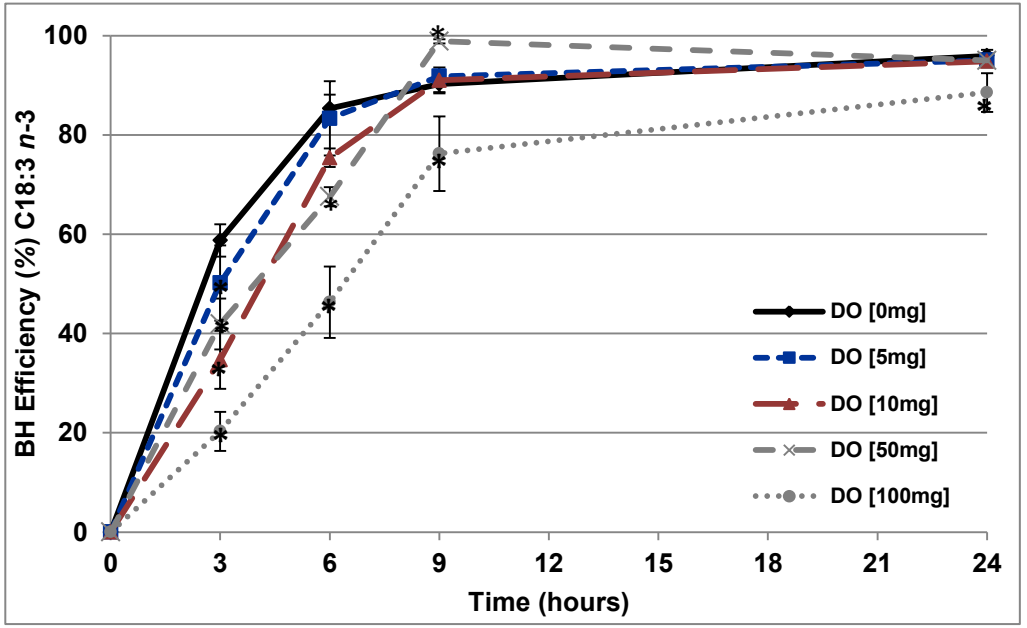


a-2)

Figure 1 - Biohydrogenation (BH) efficiency (%) of C18:3 *n*-3 in the presence of two Amazonian leaf extracts through the incubation of ruminal fluid: a-1) IE: *Inga edulis*; a-2) DO: *Desmodium ovalifolium*; *: significant difference (P<0.05) on the incubation times (t_n) compared to control.

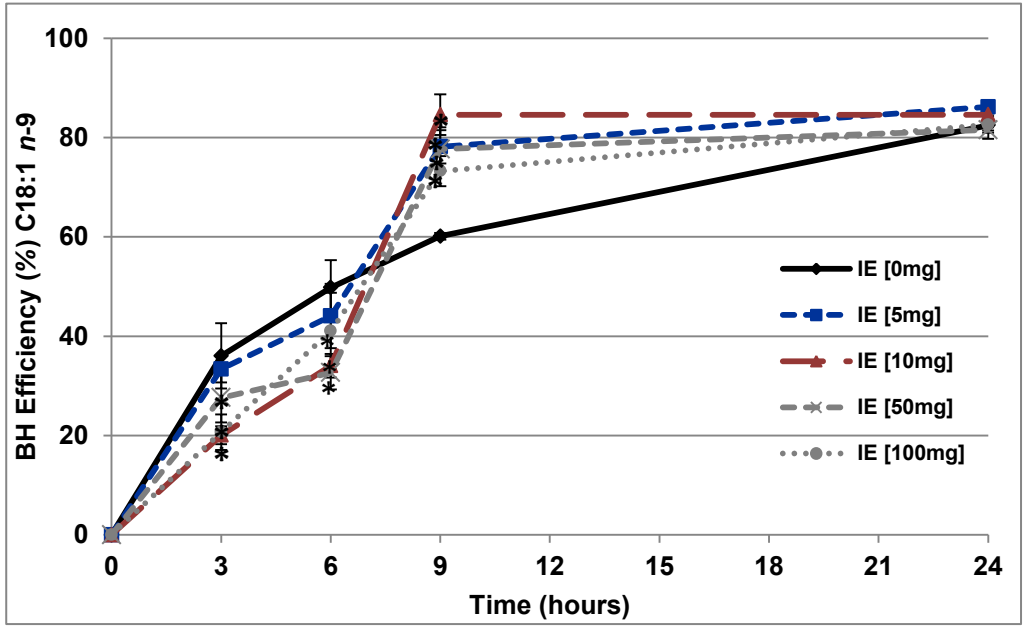


b-1)

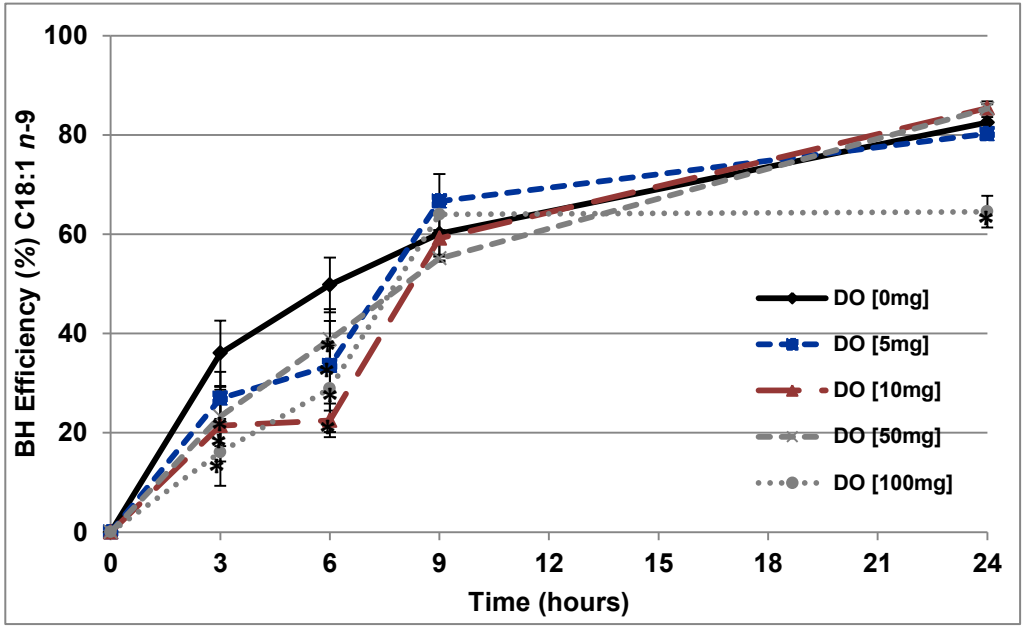


b-2)

Figure 2 - Biohydrogenation (BH) efficiency (%) of C18:2 *n*-6 in the presence of two Amazonian leaf extracts through the incubation of ruminal fluid: b-1) IE: *Inga edulis*; b-2) DO: *Desmodium ovalifolium*; *: significant difference (P<0.05) on the incubation times (t_n) compared to control.



c-1)



c-2)

Figure 3 - Biohydrogenation (BH) efficiency (%) of C18:1 *n*-9 in the presence of two Amazonian leaf extracts through the incubation of ruminal fluid: c-1) IE: *Inga edulis*; c-2) DO: *Desmodium ovalifolium;* *: significant difference (P<0.05) on the incubation times (t_n) compared to control.

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CAPÍTULO IV

INFLUENCE OF *INGA EDULIS* AND *DESMODIUM OVALIFOLIUM* LEAF EXTRACTS ON RUMINAL FERMENTATION CHARACTERISTICS, METHANE AND AMMONIA PRODUCTION *IN VITRO*

INFLUENCE OF *INGA EDULIS* AND *DESMODIUM OVALIFOLIUM* LEAF EXTRACTS ON RUMINAL FERMENTATION CHARACTERISTICS, METHANE AND AMMONIA PRODUCTION *IN VITRO*

C.M.G. Bichara[a], J. Bressan[b], H. Rogez[c]; C. Gardin[d], Q.C. Dang Van[d], E. Mignolet[d], Y. Larondelle[d]

[a]Instituto da Saúde e Produção Animal, Universidade Federal Rural da Amazônia. Avenida Presidente Tancredo Neves, nº 2501 Bairro: Montese, 66.077-830, Belém, Pará, Brasil.

[b]Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, Avenida PH Rolfs s/n, Campus Universitário, 36571-000, Viçosa, Minas Gerais, Brasil.

[c]Faculdade de Engenharia de Alimentos, Universidade Federal do Pará, Avenida Perimetral s/n, 66.095-780, Belém, Pará, Brasil.

[d]Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud, 2/8, B-1348, Louvain-la-Neuve, Belgium.

ABSTRACT

The ruminal fluid from 3 cannulated Holstein dairy cows were used for an *in vitro* study to determine effects of phenolic-rich extracts from two amazonian legumes on rumen microbial fermentation, ammonia and methane production. Treatments were either no supplemental basal diet (control) or supplemental *Inga edulis* (IE) and *Desmodium ovalifolium* (DO) leaf extracts. The added doses were 0 (control; C_0), 5 (C_5), 10 (C_{10}), 50 (C_{50}) and 100 (C_{100}) mg of lyophilized extract per g of feed dry matter (DM) and grass silage used as substrate. After the addition of rumen fluid and buffer mixture, the samples were incubated at 39 ºC at different incubation times (3, 6, 9 and 24 h). Treatment C_{100} of both extracts decreased total volatile fatty acids production at all incubation times. The acetate to propionate ratio was not affected by IE group (P>0.05), with exception of C_{100} treatment at 24 h. For DO group, the concentration C_{50} at 9 h and all times of C_{100} treatment decreased (P<0.05) when compared to control. Relative to the control diet, the phenolic-rich extract reduced the level of ammonia rumen fluid from 8 to 38% and 8.6 to 47% for IE and DO, respectively. Both legumes also presented antimethanogenic effects in the rumen, ranging from 20 to 40% when expressed as mmol CH_4/mmol fermented hexoses. Results suggest that phenolic-rich extracts from *Inga edulis* and *Desmodium ovalifolium* have

potential as feed additives to reduce methane and ammonia production in ruminants.

Keywords: legumes, methanogenesis, phenolic compounds, tannins, rumen.

1 **INTRODUCTION**

The livestock sector represents a significant source of greenhouse gas (GHG) emissions worldwide, generating carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) throughout the production process. CH_4 and CO_2 are natural by-products of anaerobic enteric fermentation of feeds in many animals including ruminants. Methane is produced in strictly anaerobic conditions by highly-specialized methanogenic prokaryotes, all of which are archaea (Hristov et al., 2013).

The inhibition of methanogenesis has long been considered from nutritional aspects, once it can represent an energy loss of 2–12% of the ingested gross energy in ruminants, depending on the type of diet (Johnson and Johnson, 1995) and more recently from the perspective of GHG emissions.

According to USA estimation (EPA, 2011), livestock accounted for about 3.1% of the total GHG emission in 2009, and was the second largest emitter of CH_4 (28% of the total emission). In Brazil, these data are more expressive, with a livestock accounted for about 35% of the total GHG emission in 2010, and the CH_4 emission equivalent to 22.1% (MCTI, 2013). Ruminants are the major contributors to biogenic CH_4 formation, and it has been estimated that preventing CH_4 formation from domesticated ruminants could contribute to stabilizing atmospheric CH_4 concentrations (Crutzen, 1995; Johnson and Johnson, 1995; Wuebbles; Hayhoe, 2000).

Thus, as Brazil has the largest commercial ruminant livestock in the world and feeds the cattle with tropical forage, it has been appointed as the major producer of CH_4, fact that should be used in the future as an argument to embargo Brazilian livestock products (Zotti; Paulino, 2009).

Many chemical feed additives have been tested to decrease the CH_4 production in the rumen; however, they were either toxic to host animals or have transient effects on methanogenesis (Moss et al., 2000; FAO, 2010).

Some phytochemical-rich plant extracts used as natural feed additives have been examined for their effects on ruminal microbial fermentation, including saponins (Goel et al., 2008; Wang et al., 2012), phenolic compounds (Evans; Martin, 2000; Alexander et al., 2008) and essential oils (Cardozo et al., 2004; Busquet et al., 2005). Some plant extracts having high content of monomeric flavonoids may stimulate microbial metabolism and, simultaneously decrease methane production (Broudiscou et al., 2000, 2002), while tannins have also been found to reduce methane production by up to 20% (Woodward et al., 2001; Sliwinski et al., 2002; Waghorn et al., 2002; Min et al., 2005; Jayanegara et al., 2009; Staerfl et al., 2012).

Another goal of ruminant nutrition is the reduction of ruminal protein degradation resulting on a less production of ammonia (NH_3), a potentially toxic chemical detoxified in the liver, decreasing metabolic energy losses and gaseous nitrogen emissions into the environment. NH_3 is not a GHG but has significant environmental implications and therefore the effects of mitigation practices on NH_3 emissions (predominantly from manure storage and land application) were considered (Hristov et al., 2013). Tannins can be used as an additive for protecting rumen-degradable protein in feeds (Getachew et al., 2008; Williams et al., 2010, 2011; Tiemann et al., 2008) representing a useful alternative for improving nitrogen use in ruminants.

In this work, phenolic-rich extracts from two amazonian legumes, *Inga edulis* and *Desmodium ovalifolium*, were used as supplements in rumen inoculum to evaluate their impact on suppression of ruminal protein degradation and methanogenesis *in vitro*.

2 **MATERIAL AND METHODS**

2.1 RUMEN INOCULUM AND DIET

The ruminal fluids were collected before the morning feeding from a rumen-fistulated Holstein cows receiving grass silage *ad libitum,* with a

mineral-vitamin supplement and free access to drinking-water. The ruminal fluids were strained through two metallic filters (a large mesh filter - 1 mm and a minor one - 0.4 mm) and introduced in pre-heated isothermal containers before transportation to the laboratory. The ruminal fluids were provided from 3 cows, each one represented by one incubation unit in each experimental run. The cows were housed in a farm of the Université Catholique de Louvain (UCL) (Corroy-le-Grand, Belgium). The experiment was conducted from September to December 2012 and was approved by the Commission of Ethics for Animal Experiments of UCL.

2.2 PREPARATION OF EXTRACTS

Dry samples (2.5 g) of each plant were extracted following the procedure recommended by Makkar (2003). They were extracted in the dark with 25 mL of a 70:30 acetone:water solution (v:v) in tubes of 50 mL at room temperature for 30 min and then centrifuged at 3000×*g* for 15 min at 5 ºC. The supernatant was recovered and another extraction was done in identical conditions with the same volume of solvent. The two extracts were mixed and evaporated under vacuum at 38 ºC. In order to remove plant pigments and promote a purification of the extract, about 15 mL of diethyl ether were added to the filtrate and the mixture was vigorously shaken. Then the upper (green coloured) organic phase was removed by a suction pump and the procedure was repeated until the liquid was colourless. After evaporation of the organic solvent under vacuum at 38 ºC, the aqueous solution was frozen, freeze-dried and stored at -20 ºC until the *in vitro* tests. During the steps, the extracts were kept saturated with gaseous nitrogen. Chemical characterization of the leaf extracts is shown in Table 1.

Table 1 - Total phenolics, flavonols, flavanois, proanthocyanidins and DPPH scavenging activity in lyophilized leaf extracts from *Inga edulis* and *Desmodium ovalifolium*.

	I. edulis	*D. ovalifolium*
Total phenolics (mg GAE/g DM)	450.45	404.77
Total flavonols (mg RE/g DM)	61.21	93.84
Total flavanois (mg CAE/g DM)	136.1	25.89
Condesed tannins (mg CE/g DM)	81.57	47.50
DPPH (µM TE/g DM)	2217.21	2695.04

GAE: gallic acid equivalents; RE: rutin equivalents; CAE: catechin equivalents; CE: cyanidin equivalents; TE:Trolox equivalents; DPPH: DPPH: 2,2-diphenyl-1-picrylhydrazyl; DM: dry matter.

2.3 *IN VITRO* INCUBATIONS

In each 120 mL capacity glass bottles, amounts of 500 mg of dry matter (DM) of grass silage were used as substrate in each experimental unit. Lyophilized leaf extracts were added in the proportions of zero (control), 5, 10, 50 and 100 mg per g of feed DM (treatments C_0, C_5, C_{10}, C_{50}, C_{100}, respectively) and were incubated with 50 mL of a ruminal fluid:buffer (1:4 v/v) mixture in a water bath at 39 ºC for 24 h, according to Theodorou et al. (1994). For the evaluation of ammonia production, it was supplied a high-protein basal diet of high rumen-degradable protein (diet containing 30% of *Lupinus albinus,* containing approximately 35% DM of protein).

2.4 INCUBATION MEDIA SAMPLING AND MEASUREMENTS

After 3, 6, 9 and 24 h of incubation, the correspond bottles were taken up from the water bath and the accumulated head-space gas volume was measured using a pressure transducer (Sper Scientific – PS100) and it was collected the gas in a calibrated syringe as described by Theodorou et al. (1994) for methane analysis, determined by gas chromatography. After, the bottles were opened to measure pH of the incubation medium. Fermentation media contents were sub-sampled for the analysis of volatile fatty acids (VFA), where a 5 mL sample was stabilized with 1 mL H_2SO_4 0.6 mol/L and

ammonia determination, where a 5 mL sample was added to 0.5 mL H_3PO_4 0.5%. The samples were immediately stored at −20 ºC until analyzis.

Methane concentration was determined by gas chromatography using a GC (Thermo Cientific-Trace GC Ultra, Milan, Italy) equipped with a Carbosphere 80/100 column and thermal conductivity detector (TCD). Carrying gas (He) flow was adjusted to 25 mL/min. The analysis was isothermal for 6.5 min at 65ºC, with detector temperature of 160 ºC. An external standard with known composition of gases was used to identify and quantify gas peaks.

For analysis of VFA, thawed samples were homogenized and centrifuged at 700×*g* for 10 min. The clear supernatant was collected and determined by HPLC method according to Ehrlich et al. (1981) using a column (7.8x300 mm, Alltech IOA-1000 Grace Deerfield, IL, USA) at 60 °C, isocratic elution with 5 mM H_2SO_4, and Refractive Index Detector. A mixture of lactic, acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids was included as a standard in all analyses.

For ammonia determination, thawed samples were homogenized and centrifuged at 700×*g* for 10 min, and the supernatant was analyzed by visible spectrophotometry (Chaney; Marbach, 1962).

2.5 CALCULATIONS AND STATISTICAL ANALYSIS

Parameters studied were pH, total and individual proportions of VFA (expressed in mg/g DM incubated), ammonia (mmol/g DM incubated) and methane production, expressed in mmol/g DM incubated and mmol/mmol fermented hexoses (FH), as suggested by Demeyer and Van Nevel (1975) and Broudiscou et al. (2000). The amount of FH equivalent was estimated by Eq. (1) from outflows of individual VFA:

$$FH\ (mmol) = \frac{(C2+C3)}{2} + C4 \qquad \text{Eq. (1)}$$

In which C2, C3 and C4 are the outflows (mmol) of acetate, propionate and butyrate, respectively. On this study was considered for total VFA the sum of C2, C3, C4, which represents the majority of fermentations occurring in the rumen. The molar concentration of each VFA was calculated by

subtracting the molar proportion at incubation time corresponding (t_n) from the molar proportion in the initial inoculum (buffered rumen fluid).

A factorial model of analysis of variance was employed as the statistical model to the data obtained, with main effects of groups (G), extract concentrations (C), incubation time (t) and their interaction (C×G, C×t, G×t and C×G×t). There were five different extract concentrations (C = 0, 5, 10, 50 and 100 mg/g DM) and four different incubation times (t = 3, 6, 9 and 24) for each group of legumes (IE and DO).

Difference between means were further assessed by a post-hoc test Fisher's LSD at $P<0.05$. All these statistical analysis were performed using statistical software STATISTICA version 7.0 (Statsoft, 2004).

3 **RESULTS**

3.1 PARAMETERS OF RUMINAL FERMENTATION

Supplementation with plant extracts of IE and DO slightly changed pH of ruminal fluid ($P<0.05$) (Table 2), ranging from 6.47 to 6.79. Nevertheless, these variations should not substantively affect fiber digestion.

Total VFA concentrations (Table 2) were also affected by dietary treatment ($P<0.05$), but only at the highest concentrations (C_{50} and C_{100}). Molar proportions of acetate were similar among treatments, whilst for the highest concentration (C_{100}), there was a reduction compared to control and other treatments. For DO group, after 6 and 9 h, C_{50} treatment also showed a decrease on acetate concentration, while for IE group only after 24 h. Molar proportions of propionate were equally affected by both plants: there were no significant differences ($P>0.05$) using C_5, C_{10} and C_{50}, with exception for C_{50} DO extract at time 9 h, which resulted in a significant reduction compared to the other treatments; and a reduction ($P<0.05$) using 100 mg of both extracts. Butyrate was reduced significantly when IE was used at C_{50}, after 24 h and C_{100} treatment after 6, 9 and 24 h of incubation. For DO group, both concentrations (50 and 100 mg) reduced concentration of butyrate after 6, 9 and 24 h. The acetate to propionate ratio (A:P) was not affected by IE group,

with exception of C_{100} treatment at 24 h. For DO group, the C_{50} treatment at 9 h and all-time of C_{100} treatment decreased the A:P compared with control.

3.2 METHANE AND AMMONIA PRODUCTION

Legume extracts affected the ammonia ruminal production (Table 3). For IE extract, lower concentrations (C_5 and C_{10}) showed a reduction in ammonia production after 9 and 24 h of incubation. At concentration C_{50}, this reduction was from 6 h to 24 h and the C_{100} caused a reduction in ammonia production at all incubation times. For DO supplementation, the greatest influence was at 9 h of incubation, where all treatments (C_5, C_{10}, C_{50} and C_{100}) reduced ammonia production (P<0.05). Treatments C_5, C_{10}, C_{50} showed a reduction also at 24 h, and the highest concentration (C_{100}) presented a significant decrease in ammonia production (P<0.05) at all times evaluated.

The effects of IE and DO extracts on *in vitro* methane production are shown in Table 3. C_{100} IE treatment significantly inhibited methane production (mmol CH_4/mmol FH) of 20, 26 and 38% after 6, 9 and 24 h, respectively. For DO extract, treatment C_{100}, inhibited CH_4 production of 26, 20, 33 and 40% after 3, 6, 9 and 24 h, respectively (P<0.05). However, when CH_4 was expressed on dry matter basis (mmol CH_4/g DM), C_{50} treatments have significant effects. IE extract (C_{50}) reduced 23 and 15% the CH_4 production at 9 and 24 h, respectively; DO extract provoked reductions of 26.5, 30.7 and 15% at 6, 9 and 24 h, respectively. For C_{100} treatment, IE extract reduced CH_4 production at 46, 50 and 38%, compared to control, after 6, 9 and 24 h, respectively. Using DO extract, the reduction was 46, 50, 60.7 and 62.3% from 3 up to 24 h of incubation, respectively.

4 **DISCUSSION**

The purpose of this study was an *in vitro* screening of two tropical legumes never studied in relation to their methane and ammonia generating potential in the rumen. Several plant extracts and pure forms of their active compounds were evaluated for their potential application as modifiers of

rumen microbial fermentation (Broudiscou et al., 2000; Kamra et al. 2006; Bodas et al., 2008; Williams et al., 2011; Rodriguez et al., 2011).

VFA are the end products of rumen microbial fermentation, and represent the main supply of metabolizable energy for the ruminant (Van Soest, 1982). Effects of plant extracts on ruminal VFA concentration and composition vary among studies depending on dose and source of extracts. IE and DO extracts did not affect the total VFA at low concentrations (C_5 and C_{10}), but with higher concentrations (mainly C_{100}) there was a reduction on total VFA and molar VFA proportions, suggesting a change in diet fermentability and energy availability. C_{50} DO treatment also reduced concentrations of acetate and butyrate between 6 and 24 h, without significantly affecting the propionate.

This is consistent with other studies that have not found effects of plant extracts at low concentrations, on total VFA concentrations (Hristov et al., 2013; Evans; Martin, 2000; Cardozo et al., 2004). Oh et al. (1968) observed that high doses of different plant essential oils decreased the production of gas and total VFA on *in vitro* fermentations of mixed ruminal microorganisms, suggesting that high doses resulted in a general inhibition of rumen microbial fermentation. Tiemann et al. (2008) observed that the addition of condensed tannins (CT) from four tanniferous tropical shrub legumes reduced VFA with increasing CT level (25 to 100 g of purified CT/kg of forage) and resulted in more acetate and less butyrate and propionate, except *Leucaena leucocephala* in which propionate proportion increased at the expense of butyrate. These authors concluded that species-specific CT affect rumen microbial population differently. Tan et al. (2011) also evaluated different levels of CT from *L. leucocephala* (20 to 60 g of purified CT/kg) and observed that VFA concentrations decreased with increasing CT levels. Molar proportions of acetate and butyrate were not affected by addition of CT, but there was a linear decrease in propionate with increasing CT inclusions. Rodriguez et al. (2011) also found a reduction of total VFA using high concentrations of extracts (300 g/kg DM) from tanniferous browse legumes after 12 h of *in vitro* incubation.

CT containing herbaceous, shrub or tree legumes from temperate and tropical zones have been investigated for their ability to protect proteins from degradation in the rumen, increase the flux of dietary protein to the abomasum and increase the apparent absorption of essential amino acids in the intestines (Ben Salem et al., 2005; Ramírez-Restrepo; Barry, 2005; Hess et al., 2006; Tiemann et al., 2008; Williams et al., 2010, 2011). It is well known that the reduction of protein degradation in the rumen occur due to the formation of tannin–protein complexes (pH range 3.5 - 7.0) and inhibition of the growth and activities of proteolytic bacterial populations. The tannin–protein complex is dissociated in the abomasum at pH <3.5 and in the intestine at pH >7 and protein is available for digestion in the small intestine (Mueller-Harvey, 2006; Patra; Saxena, 2011).

Tiemann et al. (2008) found that the highest CT level (100 mg/g DM) independent of legume species suppressed apparent protein degradation. Two types of *Calliandra calothyrsus* from different provenances, showed this effect also with 75 mg CT/g DM. However, at low-CT levels (25 mg/g DM) there was no difference in the apparent protein degradation between species. Williams et al. (2010) found a 27.8% reduction in ammonia concentrations occurred with *Lotus corniculatus* (20.8 g CT/kg DM) compared to *Medicago sativa* in continuous cultures fed forage diets. Supplementation with *Vaccinium vitis* (2 g of tannins/kg DM) resulted in 45.9% lower total ammonia in dairy cows (Cieslak et al., 2012).

In our experiment, both extracts showed potential to reduce the protein degradation, reaching levels of 38 and 47% of reduction in ammonia production for IE and DO, respectively. The ability of tannins to complex proteins is not only related to the amount of CT in the plant, but mainly with the monomeric composition of the CT (Lascano et al., 2003; Andersson et al., 2006). Lascano et al. (2003) presented evidence suggesting that the monomeric composition of extractable CT in tropical legumes can affect nitrogen utilization by ruminants, which can be hypothesized in this work. The studies of Cortés et al. (2009) also show this proposition, once when they evaluated purified CT extracted from the legumes *Calliandra calothyrsus*,

Flemingia macrophylla and *Leucaena leucocephala* added to soybean meal at similar proportions and concluded that CT from *L. leucocephala* were less effective in protecting proteins from ruminal degradation than CT from either *C. calothyrsus* or *F. macrophylla*.

CT also can react with other sources of protein after chewing by animals, such as enzymes secreted by rumen bacteria and so inhibit rumen carbohydrate fermentation (Barry; Manley, 1986). Barahona et al. (2006) evaluating the effect of CT from six tropical legumes on the activity of fibrolytic enzymes from the anaerobic rumen fungus concluded that the greatest reductions in enzyme activities were observed with tannins purified from *D. ovalifolium* and *Flemingia macrophylla* and the least with tannins from *L. leucocephala*, a well-known tanniferous forages. This result confirms that the reactivity of CT is related to their chemical structure and corroborates with our study that shows *D. ovaliflium* as a source of tannins of great interest in animal nutrition.

Results from several researches suggested that feeding ruminants with polyphenol-rich plant effectively inhibits CH_4 production during the enteric fermentation (Waghorn et al., 2002; Woodward et al., 2004; Huang et al., 2011; Puchala et al., 2012). It is attributed to indirect effects via reduced hydrogen production (and presumably reduced forage digestibility) and via direct inhibitory effects on methanogens (Tavendale et al., 2005). Reductions in methane production has been related to adverse effects on substrate degradation (Beauchemin; McGinn, 2006), but at the same time, some plant species decrease methane production stimulating microbial metabolism (Broudiscou et al., 2000, 2002).

Carulla et al. (2005) reported that feeding *Acacia mearnsii* extract (25 g/kg of DM) to sheep decreased the CH_4 production by approximately 12%. Beauchemin et al. (2007) found that feeding quebracho (*Schinopsis lorentzii*) tannin extract at up to 20 g/kg of DM failed to reduce CH_4 emissions from growing cattle, probably because the threshold required to cause a reduction in CH_4 production should be above 20 g/kg of DM. However, Cieslak et al. (2012) concluded that extracts from *V. vitis* resulted in 8.5% lower CH_4

emissions when cows were feed with 2 g of tannins/kg DM. Tan et al. (2011) found a reduction (33%) on *in vitro* CH_4 production at even the lowest inclusion (20 g/kg DM), but the reductions were more (up to 63%) at higher CT levels. The reason for this discrepancy can be to their relative concentration in the used extracts, and also to the different CT used in the different studies, once the CT from different legumes are not uniform and differ in characteristics such as molecular weight and monomeric composition, which in turn may affect their biological impact, as previously discussed.

Bodas et al. (2008), evaluating the potential of 450 plant species as antimethanogenic additives in ruminant feeds (100 g of each plant/kg DM), identified six plant species as potential antimethanogenic agents, being: *Carduus pycnocephalus*, *Populus tremula*, *Prunus avium*, *Quercus robur*, *Rheum nobile* and *Salix caprea*; all depressed methane production to some degree. However, according to the authors, only *R. nobile* as a plant with the greatest, and most consistent, decrease in methane production, with positive effects on DM digestibility, VFA and propionate production. This is because to propionate formation consumes reducing equivalents, whereas acetate and butyrate formation generate H_2 for methanogenesis. As such, any dietary component or intervention that causes a shift in favor of propionate production will be accompanied by a reduction in CH_4 production per unit of feed fermented (Van Nevel; Demeyer, 1996).

According to Patra and Saxena (2009), tannins exert an anti-microbial action on microbial growth including cellulolytic bacteria and fungi, which may adversely affect fiber utilization. Results from Tan et al. (2011) showed that total methanogens decreased considerably with CT inclusions above 20 mg/kg DM. Tavendale et al. (2005) suggested that the inhibition on growth is due to the bacteriostatic and bactericidal effects of CT. Hess et al. (2003) also reported that CT-containing legumes showed methanogenic toxicity.

In our study, data are insufficient to specify the pathway occurred, but the most likely is that the reduction of methanogenesis was due to indirect

effect, by reduction in feed digestibility, process that result in reducted availability of hydrogen for methanogenesis.

5 **CONCLUSIONS**

Extracts from *Inga edulis* and *Desmodium ovalifolium* showed potential to reduce rumen protein degradation and have antimethanogenic activities, showing great potential as additives in animal nutrition and beneficial effects for the environment.

Table 2 – pH and total volatile fatty acid concentration (VFA, mg/g DM incubated) and acetate, propionate and butyrate proportions after *in vitro* incubation with leaf extracts from *Inga edulis* (IE) or *Desmodium ovalifolium* (DO).

Parameters	Group	Treatments (mg/g DM)	Incubation times (h)				SEM
			3	6	9	24	
pH	Control	C_0	$6.81^{a}{}_{A}$	$6.67^{b}{}_{A}$	$6.62^{b}{}_{A}$	$6.52^{c}{}_{A}$	0.033
	IE	C_5	$6.72^{a}{}_{B}$	$6.69^{a}{}_{A,B}$	$6.58^{b,c}{}_{A}$	$6.54^{c}{}_{A}$	0.025
		C_{10}	$6.73^{a}{}_{B}$	$6.69^{a}{}_{A,B}$	$6.56^{b}{}_{A}$	$6.53^{b}{}_{A}$	0.026
		C_{50}	$6.75^{a}{}_{A,B}$	$6.68^{b}{}_{A}$	$6.63^{b}{}_{A,B}$	$6.53^{c}{}_{A}$	0.026
		C_{100}	$6.70^{a}{}_{B}$	$6.75^{a}{}_{B}$	$6.68^{a}{}_{B}$	$6.54^{b}{}_{A}$	0.027
		C_0	$6.81^{a}{}_{A}$	$6.67^{b}{}_{A}$	$6.62^{b}{}_{A}$	$6.52^{c}{}_{A}$	0.033
	DO	C_5	$6.75^{a}{}_{B}$	$6.70^{a}{}_{A,B}$	$6.57^{b}{}_{A}$	$6.56^{b}{}_{A,B}$	0.026
		C_{10}	$6.76^{a}{}_{A,B}$	$6.70^{a}{}_{A,B}$	$6.58^{b}{}_{A}$	$6.57^{b}{}_{A,B}$	0.025
		C_{50}	$6.79^{a}{}_{A}$	$6.76^{a}{}_{B}$	$6.65^{b}{}_{A,B}$	$6.47^{c}{}_{A}$	0.037
		C_{100}	$6.72^{a}{}_{B}$	$6.76^{a}{}_{B}$	$6.67^{b}{}_{B}$	$6.60^{c}{}_{B}$	0.021
Total VFA	Control	C_0	$2.16^{d}{}_{A}$	$3.10^{c}{}_{A,B}$	$3.83^{b}{}_{A,B}$	$5.74^{a}{}_{A}$	0.402
	IE	C_5	$2.15^{d}{}_{A}$	$3.37^{c}{}_{A}$	$4.12^{b}{}_{A}$	$5.60^{a}{}_{A}$	0.383
		C_{10}	$2.20^{d}{}_{A}$	$3.08^{c}{}_{A,B}$	$4.02^{b}{}_{A}$	$5.75^{a}{}_{A}$	0.399
		C_{50}	$1.99^{d}{}_{A,B}$	$2.81^{c}{}_{B}$	$3.52^{b}{}_{B}$	$5.17^{a}{}_{B}$	0.355
		C_{100}	$1.61^{d}{}_{B}$	$2.16^{c}{}_{C}$	$2.59^{b}{}_{C}$	$3.95^{a}{}_{C}$	0.266
	Control	C_0	$2.16^{d}{}_{A}$	$3.10^{c}{}_{A}$	$3.83^{b}{}_{A}$	$5.74^{a}{}_{A}$	0.402
	DO	C_5	$2.08^{d}{}_{A}$	$3.05^{c}{}_{A}$	$3.99^{b}{}_{A}$	$5.38^{a}{}_{A}$	0.371
		C_{10}	$2.13^{d}{}_{A}$	$3.09^{c}{}_{A}$	$4.14^{b}{}_{A}$	$5.63^{a}{}_{A}$	0.397
		C_{50}	$1.91^{c}{}_{A,B}$	$2.51^{b}{}_{B}$	$2.61^{b}{}_{B}$	$5.34^{a}{}_{A}$	0.406
		C_{100}	$1.52^{d}{}_{B}$	$1.96^{c}{}_{C}$	$2.38^{b}{}_{B}$	$3.68^{a}{}_{B}$	0.251

Continued

Acetate	**Control**	**C_0**	1.21^{d}_{A}	$1.97^{c}_{A,B}$	2.52^{b}_{A}	$3.70^{a}_{A,B}$	0.279
	IE	**C_5**	1.24^{d}_{A}	2.12^{c}_{A}	2.69^{b}_{A}	$3.70^{a}_{A,B}$	0.274
		C_{10}	1.23^{d}_{A}	$1.95^{c}_{A,B}$	2.60^{b}_{A}	3.82^{a}_{A}	0.288
		C_{50}	$1.12^{d}_{A,B}$	1.76^{c}_{B}	2.23^{b}_{A}	3.41^{a}_{B}	0.254
		C_{100}	0.88^{d}_{B}	1.32^{c}_{C}	1.64^{b}_{B}	2.51^{a}_{C}	0.184
	Control	**C_0**	1.21^{d}_{A}	1.97^{c}_{A}	2.52^{b}_{A}	3.70^{a}_{A}	0.279
	DO	**C_5**	1.18^{d}_{A}	1.91^{c}_{A}	2.55^{b}_{A}	3.47^{a}_{A}	0.256
		C_{10}	1.23^{d}_{A}	1.95^{c}_{A}	2.69^{b}_{A}	3.73^{a}_{A}	0.282
		C_{50}	1.06^{c}_{A}	1.56^{b}_{B}	1.51^{b}_{B}	3.51^{a}_{A}	0.290
		C_{100}	0.73^{d}_{B}	1.11^{c}_{C}	1.52^{b}_{B}	2.17^{a}_{B}	0.171
Propionate	**Control**	**C_0**	0.66^{c}_{A}	$0.76^{b,c}_{A,B}$	0.88^{b}_{A}	1.47^{a}_{A}	0.097
	IE	**C_5**	0.63^{c}_{A}	0.86^{b}_{A}	0.98^{b}_{A}	1.31^{a}_{A}	0.080
		C_{10}	0.68^{c}_{A}	$0.78^{b,c}_{A,B}$	0.98^{b}_{A}	1.34^{a}_{A}	0.078
		C_{50}	$0.58^{c}_{A,B}$	$0.70^{c}_{B,C}$	0.91^{b}_{A}	1.33^{a}_{A}	0.089
		C_{100}	0.46^{c}_{B}	$0.56^{b,c}_{C}$	0.61^{b}_{B}	1.06^{a}_{B}	0.072
	Control	**C_0**	0.66^{c}_{A}	$0.76^{b,c}_{A}$	$0.88^{b}_{A,B}$	1.47^{a}_{A}	0.097
	DO	**C_5**	$0.62^{d}_{A,B}$	0.78^{c}_{A}	1.01^{b}_{A}	$1.38^{a}_{A,B}$	0.089
		C_{10}	$0.62^{d}_{A,B}$	0.79^{c}_{A}	0.99^{b}_{A}	$1.33^{a}_{A,B}$	0.082
		C_{50}	$0.59^{c}_{A,B}$	$0.67^{b,c}_{A,B}$	0.79^{b}_{B}	$1.36^{a}_{A,B}$	0.095
		C_{100}	0.50^{c}_{B}	$0.55^{b,c}_{B}$	0.67^{b}_{C}	1.27^{a}_{B}	0.095

Continued

Butyrate	**Control**	**C_0**	0.29^{c}_{A}	0.37^{b}_{A}	0.43^{b}_{A}	0.57^{a}_{A}	0.031
	IE	**C_5**	0.29^{d}_{A}	0.38^{c}_{A}	0.45^{b}_{A}	0.57^{a}_{A}	0.033
		C_{10}	0.28^{d}_{A}	0.35^{c}_{A}	0.44^{b}_{A}	0.59^{a}_{A}	0.034
		C_{50}	0.30^{c}_{A}	$0.35^{b,c}_{A}$	$0.40^{a,b}_{A}$	0.43^{a}_{B}	0.018
		C_{100}	0.27^{b}_{A}	0.29^{b}_{B}	$0.33^{a,b}_{B}$	0.37^{a}_{B}	0.016
	Control	**C_0**	0.29^{c}_{A}	0.37^{b}_{A}	0.43^{b}_{A}	0.57^{a}_{A}	0.031
	DO	**C_5**	0.28^{d}_{A}	$0.35^{c}_{A,B}$	0.43^{b}_{A}	0.53^{a}_{A}	0.029
		C_{10}	0.28^{d}_{A}	$0.36^{c}_{A,B}$	0.45^{b}_{A}	0.57^{a}_{A}	0.034
		C_{50}	0.26^{c}_{A}	$0.30^{b,c}_{B,C}$	0.34^{b}_{B}	0.47^{a}_{B}	0.025
		C_{100}	0.29^{b}_{A}	0.29^{b}_{C}	0.19^{a}_{C}	0.21^{a}_{C}	0.015
A:P ratio	**Control**	**C_0**	1.86^{b}_{A}	2.61^{a}_{A}	2.88^{a}_{A}	$2.52^{a}_{A,B}$	0.133
	IE	**C_5**	1.98^{b}_{A}	2.49^{a}_{A}	2.66^{a}_{A}	2.82^{a}_{A}	0.116
		C_{10}	1.83^{b}_{A}	2.53^{a}_{A}	2.64^{a}_{A}	2.85^{a}_{A}	0.123
		C_{50}	1.94^{b}_{A}	2.52^{a}_{A}	2.49^{a}_{A}	$2.57^{a}_{A,B}$	0.106
		C_{100}	1.98^{b}_{A}	2.36^{a}_{A}	2.68^{a}_{A}	2.37^{a}_{B}	0.097
	Control	**C_0**	1.86^{b}_{A}	2.61^{a}_{A}	2.88^{a}_{A}	2.52^{a}_{A}	0.133
	DO	**C_5**	1.90^{b}_{A}	2.50^{a}_{A}	$2.66^{a}_{A,C}$	2.61^{a}_{A}	0.129
		C_{10}	1.98^{b}_{A}	$2.48^{a}_{A,B}$	$2.75^{a}_{A,C}$	2.82^{a}_{A}	0.118
		C_{50}	1.83^{c}_{A}	$2.42^{a,b}_{A,B}$	$1.97^{b,c}_{B,C}$	2.57^{a}_{A}	0.114
		C_{100}	1.49^{b}_{B}	2.03^{a}_{B}	$2,29^{a}_{C}$	1.73^{b}_{B}	0.124

Different subscript letters (A-C) show differences (P<0.05) between the treatments for each parameter and each plant extract, and different superscript letters (a-d) indicate difference (P<0.05) between incubation times (3, 6, 9, 24 h) for each treatment. DM: dry matter.

Table 3 - Effects of leaf extracts from *Inga edulis* (IE) or *Desmodium ovalifolium* (DO) on the *in vitro* ammonia and methane production.

Parameters	Group	Treatment (mg/g DM)	Incubation times (h)				SEM
			3	6	9	24	
Ammonia (mmol/g DM incubated)	Control	C_0	1.91^{c}_{A}	2.27^{c}_{A}	2.68^{b}_{A}	3.37^{a}_{A}	0.164
	IE	C_5	1.84^{c}_{A}	2.30^{b}_{A}	2.19^{b}_{B}	$2.77^{a}_{B,C}$	0.111
		C_{10}	1.93^{c}_{A}	$2.09^{b,c}_{A,B}$	2.23^{b}_{B}	3.10^{a}_{B}	0.151
		C_{50}	$1.78^{b}_{A,B}$	1.99^{b}_{B}	$1.98^{b}_{B,C}$	2.51^{a}_{C}	0.095
		C_{100}	1.59^{b}_{B}	1.79^{b}_{B}	1.82^{b}_{C}	2.09^{a}_{D}	0.064
	Control	C_0	1.91^{c}_{A}	2.27^{c}_{A}	2.68^{b}_{A}	3.37^{a}_{A}	0.164
	DO	C_5	1.86^{c}_{A}	$2.15^{b,c}_{A}$	2.30^{b}_{B}	3.01^{a}_{B}	0.139
		C_{10}	1.93^{b}_{A}	$2.04^{b}_{A,B}$	$2.22^{b}_{B,C}$	3.08^{a}_{B}	0.148
		C_{50}	$1.83^{b}_{A,B}$	$2.06^{b}_{A,B}$	$1.85^{b}_{C,D}$	2.43^{a}_{B}	0.093
		C_{100}	1.57^{a}_{B}	1.71^{a}_{B}	1.84^{a}_{D}	1.78^{a}_{C}	0.050

Continued

Methane (mmol/mmol fermented hexoses)	**Control**	**C_0**	0.23^{c}_{A}	0.35^{b}_{A}	$0.39^{a,b}_{A}$	0.42^{a}_{A}	0.023
	IE	**C_5**	0.23^{c}_{A}	0.36^{b}_{A}	0.35^{b}_{A}	0.45^{a}_{A}	0.024
		C_{10}	0.22^{c}_{A}	0.35^{b}_{A}	0.38^{b}_{A}	0.45^{a}_{A}	0.026
		C_{50}	0.22^{c}_{A}	0.29^{b}_{B}	$0.33^{b}_{A,B}$	$0.41^{a}_{A,B}$	0.022
		C_{100}	0.19^{c}_{A}	0.28^{b}_{B}	0.29^{b}_{B}	0.26^{a}_{B}	0.019
	Control	**C_0**	0.23^{c}_{A}	0.35^{b}_{A}	$0.39^{a,b}_{A}$	$0.42^{a}_{A,B}$	0.023
	DO	**C_5**	0.25^{c}_{A}	0.34^{b}_{A}	0.37^{b}_{A}	0.45^{a}_{A}	0.023
		C_{10}	0.24^{c}_{A}	$0.33^{b}_{A,B}$	0.36^{b}_{A}	0.45^{a}_{A}	0.025
		C_{50}	$0.21^{c}_{A,B}$	$0.31^{b}_{A,B}$	$0.36^{a,b}_{A}$	0.38^{a}_{B}	0.021
		C_{100}	0.17^{b}_{B}	0.28^{a}_{B}	0.26^{a}_{B}	0.25^{a}_{C}	0.016
Methane (mmol/g DM incubated)	**Control**	**C_0**	0.28^{d}_{A}	0.59^{c}_{A}	0.83^{b}_{A}	1.30^{a}_{A}	0.114
	IE	**C_5**	0.29^{d}_{A}	0.66^{c}_{A}	0.83^{b}_{A}	1.38^{a}_{A}	0.122
		C_{10}	0.28^{d}_{A}	0.60^{c}_{A}	0.84^{b}_{A}	1.43^{a}_{A}	0.127
		C_{50}	0.25^{d}_{A}	$0.46^{c}_{A,B}$	0.64^{b}_{B}	1.11^{a}_{B}	0.096
		C_{100}	0.18^{c}_{A}	0.34^{b}_{B}	0.43^{b}_{C}	0.82^{a}_{C}	0.071
	Control	**C_0**	0.28^{d}_{A}	0.59^{c}_{A}	0.83^{b}_{A}	1.30^{a}_{A}	0.114
	DO	**C_5**	0.29^{d}_{A}	0.60^{c}_{A}	0.84^{b}_{A}	1.32^{a}_{A}	0.116
		C_{10}	0.29^{d}_{A}	0.58^{c}_{A}	0.84^{b}_{A}	1.41^{a}_{A}	0.128
		C_{50}	0.22^{c}_{A}	0.43^{b}_{B}	0.58^{b}_{B}	1.11^{a}_{B}	0.103
		C_{100}	0.15^{c}_{B}	$0.29^{b,c}_{C}$	0.33^{b}_{C}	0.49^{a}_{C}	0.044

Different subscript letters (A-C) show differences ($P<0.05$) between the treatments for each parameter and each plant extract, and different superscript letters (a-d) indicate difference ($P<0.05$) between incubation times (3, 6, 9, 24 h) for each treatment. DM: dry matter.

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2 **CONCLUSÕES GERAIS**

As duas leguminosas, *Inga edulis* e *Desmodium ovalifolium*, apresentam composição nutricional semelhante, assim como em relação ao teor de compostos fenólicos, os quais são importantes nos efeitos *in vitro* aqui estudados. O extrato de *D. ovalifolium* possui menor quantidade de taninos condensados, porém, apresenta efeitos semelhantes ao de *I. edulis*, muito provavelmente devido à estrutura química de sua cadeia polimérica, fato este que influencia diretamente na reatividade biológica desses compostos.

Os extratos de folhas de *I. edulis* e *D. ovalifolium* apresentam efeitos *in vitro* na redução da biohidrogenação dos ácidos α-linolênico e linoleico, aumentando o conteúdo de *c*9,*t*11-CLA ruminal o que pode ocasionar uma maior liberação do rúmen desse intermediário e ácidos graxos insaturados de interesse à saúde humana para serem absorvidos e incorporados à carne e ao leite de ruminantes.

Os extratos também apresentam efeitos satisfatórios em relação à proteção das proteínas da dieta contra a degradação ruminal, o que foi demonstrado pela diminuição da produção de amônia no fluido ruminal. Esse resultado pode ser atribuído à presença dos taninos condensados, devido à elevada capacidade desses de se ligarem às proteínas.

Em relação à metanogênese, resultados satisfatórios também são encontrados, pois a redução da produção de metano ruminal apresenta grandes benefícios para a produtividade animal e para o meio ambiente. Outras fontes de compostos fenólicos de plantas amazônicas podem ser usadas no futuro como estratégias para reduzir a emissão de metano ruminal.

3 **PERSPECTIVAS FUTURAS**

Torna-se de grande importância o desenvolvimento de estudos *in vivo* para a comprovação dos resultados obtidos em condições laboratoriais. A Amazônia apresenta uma capacidade de exploração sustentável satisfatória que permite o desenvolvimento de pesquisas na área de nutrição animal jamais estudadas, permitindo diversos objetivos, dentre elas, a obtenção de alimentos mais saudáveis à saúde humana.

O uso de extratos fracionados é outra linha de pesquisa que merece uma atenção especial, o que tornará possível o isolamento e identificação dos compostos responsáveis por cada ação biológica no modelo animal.

Outra área de grande interesse é o estudo da metagenômica dos microrganismos ruminais, visando assim a busca pelo conhecimento específico das espécies microbianas de animais da região amazônica, outro ponto ainda não explorado na região.