UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

MODULATION OF RUMINAL BIOHYDROGENATION IN SHEEP THROUGH DIETARY
TANNINS OR ENERGY SOURCES

MÓNICA MENDES DA COSTA

Orientador: Professor Doutor Rui José Branquinho de Bessa

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na
Especialidade de Produção Animal

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- Doutora Margarida Rosa Garcez Maia

2017

I dedicate this thesis to

My parents and my sister

My grandparents

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Abstract

Modulation of ruminal biohydrogenation in sheep through dietary tannins or energy sources

In the present thesis, four experiments were conducted to study how ruminal biohydrogenation pathways can be modulated through dietary inclusion of tannin sources and to acquire a better comprehension about the occurence of t10-shifted biohydrogenation pathways. In the first experiment, *in vitro* batch incubations with 100 g/kg dry matter (DM) of extracts of chestnut tannins (mostly hydrolysable tannins) and quebracho, grape seed or rockrose (*Cistus ladanifer)* condensed tannins, as well as a control treatment were incubated for 6 h with ruminal fluid from fistulated sheep and a dehydrated lucerne-based substrate with 60 g/kg DM of sunflower oil. Grape seed and, to a lesser extent, *C. ladanifer* led to a higher disappearance of 18:2n-6 with a consequent higher production of c9,t11-18:2 and t11-18:1 than chestnut, quebracho and control. There was no clear innibition of 18:0 production with any of the extracts comparing with control. In the second experiment, rumen fistulated sheep were fed tannin extracts from mimosa condensed tannins, chestnut hydrolysable tannins or their mixture (100 g/kg DM) in a complete diet with sunflower and linseed oils (40 g/kg DM), following a change-over design (3 treatments, 4 sheep and 4 periods). There was a variable inhibition of ruminal biohydrogenation and a lower "*trans-/cis*-18:1" ratio in bacterial fractions with mimosa than with chestnut. Mimosa led to a lower fermentative activity, as well as a lower abundance of *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio proteoclasticus* and higher abundance of *Selenomonas ruminantium* with a lower bacterial biomass estimate of dimethylacetals than chestnut. In the third experiment, two rumen fistulated rams were housed in metabolic cages and adapted to a wheat-based diet with 41 g/kg DM of sunflower oil. During the first two weeks of trial, the t10-shift occurred temporarily in both animals but in different moments. These results were probably due to individual variability of rumen microbiota, since, for a selected period of the trial, a lower bacterial diversity was found for ram 1 compared to ram 2. Moreover, the t10-shift was associated with an increase of total *trans*-18:1 and a decrease of 18:0. There was no clear association of t10-shift with rumen pH or its expression in blood plasma. In the fourth experiment, 40 lambs were fed, for 6 weeks, with complete diets containing barley or barley completely replaced for dehydrated citrus pulp, dehydrated beet pulp or soybean hulls. All diets were supplemented with an oil blend (soybean:fish oils, 59:10 g/kg DM). Overall, the t10-/t11-18:1 ratio was above 3 in meat and subcutaneous fat, although soybean hulls increased t11-18:1 and c9,t11-18:2 comparing with the other treatments. Citrus pulp led to the lowest gene expression of fatty acid synthase, while that of stearoyl-CoA desaturase was inferior for soybean hulls and beet pulp.

Keywords: Fatty acids, biohydrogenation, rumen bacteria, tannins, starch.

Resumo

Modulação da bioidrogenação ruminal em ovinos através de taninos ou de fontes energéticas da dieta

A dieta dos ruminantes é um dos principais determinantes que influencia a bioidrogenação (BH) ruminal. A inclusão de taninos na dieta, os quais são compostos fenólicos das plantas, pode aumentar a proporção, no rúmen e nos tecidos, de ácidos gordos (AG) bioactivos com efeitos benéficos na saúde humana, tais como os ácidos vacénico (t11-18:1) e ruménico (c9,t11-18:2; conjugado do ácido linoleico - CLA) derivados das vias t11 da BH. A maior quantidade de c9,t11-18:2 presente nos tecidos resulta da dessaturação de t11-18:1 pela estearoil-CoA dessaturase (SCD; Δ9-dessaturase). Contudo, na presença de dietas com alto teor em amido e baixo conteúdo em forragem, com ou sem suplementação com óleos ricos em ácidos gordos polinsaturados, pode ocorrer uma modificação das vias da BH com predomínio das vias t10 relativamente às t11 (o shift-t10) e o concomitante aumento de AG deletérios para a saúde, nomeadamente t10-18:1 e t10,c12-18:2.

Na presente tese, foram realizadas duas experiências com o propósito de estudar o efeito da inclusão de diversos tipos de taninos como moduladores da BH (experiências 1 e 2) e duas experiências para obter uma melhor compreensão dos factores que determinam a ocorrência do shift-t10 (experiências 3 e 4). Em todos os estudos, foram incorporados óleos de origem vegetal ou animal nas dietas para aumentar a formação de intermediários da BH. Na primeira experiência, foi realizado um ensaio *in vitro* com 100 g/kg de matéria seca (MS) de extractos de taninos da castanha (maioritariamente taninos hidrolisáveis) e de extractos de taninos condensados de quebracho, de sementes de uva e de esteva (*Cistus ladanifer)*, bem como um tratamento controlo (sem taninos). As incubações decorreram durante 6 h com fluido de rúmen de ovinos fistulados e um substrato à base de luzerna desidratada com 60 g/kg MS de óleo de girassol (2:1, rácio "foragem/concentrado"). A composição em AG dos tubos de incubação foi obtida por transesterificação combinada, seguida da separação dos ésteres metílicos dos AG por cromatografia gás-líquido e da sua identificação com espectrometria de massa. Determinaram-se também a produção de ácidos gordos voláteis (AGV) e o pH do rúmen, tendo-se verificado apenas pequenas diferenças no pH. Os tratamentos com extractos de uva e, menos marcadamente, de *C. ladanifer* causaram um maior desaparecimento de ácido linoleico (c9,c12-18:2; 18:2n-6) e um consequente aumento do total de *trans*-18:1, nomeadamente de t11-18:1, e de c9,t11-18:2, bem como uma diminuição do total de dimetilacetais (DMA), comparativamente aos extractos de castanha e de quebracho e ao controlo, embora, considerando o total de DMA, esta diferença não tenha sido significativa para o caso do quebracho. Não houve uma clara inibição da produção de ácido esteárico (18:0) com nenhum dos tratamentos em comparação com o controlo, apesar do extracto de uva ter originado uma menor proporção de 18:0 relativamente ao total de

produtos da BH. Na segunda experiência, ovinos fistulados foram alimentados com extractos comerciais de taninos da mimosa (condensados), da castanha (hidrolisáveis) e de uma mistura de ambos (100 g/kg MS) incorporados numa dieta completa (1:1, rácio "foragem/concentrado") suplementada com uma mistura de óleos de girassol e linho (40 g/kg MS), segundo um desenho experimental de "change-over" (3 tratamentos, 4 animais and 4 períodos). Os períodos experimentais tiveram a duração de 3 semanas, includindo 2 semanas de adaptação às dietas e 1 semana de recolha de amostras. As amostras de conteúdo do rúmen foram obtidas antes da refeição da manhã em 2 dias das últimas semanas de cada período com um intervalo de 2 dias entre recolhas. No primeiro dia de amostragem, recolheram-se os conteúdos totais do rúmen para obtenção das bactérias associadas às fracções sólida (SAB) e líquida (LAB), enquanto, no segundo dia, os conteúdos foram usados para avaliação da actividade fermentativa (análise de pH e AGV). Em ambos os dias de recolha, as amostras de conteúdo do rúmen foram utilizadas para a análise de AG, bem como para a extracção de DNA e posterior quantificação do número de cópias de 16S rRNA de bactérias seleccionadas do rúmen. No último dia, recolheram-se amostras de sangue antes e 3 h depois da refeição da manhã. As dietas com extracto de mimosa e com a mistura de extractos causaram uma inibição da BH ruminal em algumas réplicas dos tratamentos e a mimosa originou ainda um menor rácio "*trans*-/*cis*-18:1" nas fracções bacterianas, comparativamente à dieta com extracto de castanha. A dieta com mimosa levou ainda a uma menor concentração do total de AGV, bem como a uma inferior abundância de *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* e *Butyrivibrio proteoclasticus* e a uma maior abundância de *Selenomonas ruminantium*, juntamente com um menor estimativa da biomassa bacteriana por DMA, em comparação com a castanha. Adicionalmente, o tratamento com mimosa originou um aumento do total de oxo-18:0, no plasma sanguíneo e no rúmen, em relação à castanha, enquanto, nas fracções bacterianas, este aumento verificou-se com a mistura de extractos comparativamente à média dos tratamentos com extractos de mimosa e de castanha. Na terceira experiência, dois carneiros fistulados foram colocados em caixas metabólicas e gradualmente adaptados a uma dieta à base de trigo com 41 g/kg MS de óleo de girassol. Durante os 29 dias de ensaio, recolheram-se amostras de conteúdo do rúmen, antes e 3h depois da refeição da manhã. Os conteúdos do rúmen foram também obtidos uma vez por semana a cada 1h30 entre as 9h30 e as 20h00 para a análise da composição em AG e dos grupos taxonómicos bacterianos, juntamente com amostras de sangue recolhidas antes e 3 h depois da refeição da manhã. O shift-*t*10 ocorreu progressiva e temporariamente nas primeiras duas semanas e coincidiu com um acréscimo de ingestão de alimento que se seguiu ao seu decréscimo. O padrão de indução do shift-*t*10 apresentou variabilidade individual, a qual foi provalmente causada por diferenças entre animais a respeito da microbiota do rúmen, na medida em que, num período definido do ensaio, verificou-se uma menor diversidade bacteriana no

animal com maior rácio *t*10-/*t*11-18:1 (carneiro 1) do que no animal com menor rácio (carneiro 2). Considerando os grupos taxonómicos obtidos por pirossequenciação da região 16S rRNA do genoma bacteriano, a abundância dos filos *Actinobacteria* e, em menor extenção, *Spirochaetae* era maior no carneiro 1 em relação ao carneiro 2, contrariamente aos filos *Bacteroidetes* e *Firmicutes*. Para além disso, o shift-*t*10 estava associado ao aumento do total de *trans*-18:1 e à diminuição da produção de 18:0, bem como ao aumento prévio da formação de oxo-18:0 no rúmen. Não se verificou uma clara associação entre o estabelecimento do shift-*t*10 e a sua expressão no plasma sanguíneo e a redução do pH do conteúdo do rúmen. De facto, o aumento do rácio "*t*10-/*t*11-18:1" no rúmen não se encontrava relacionado com um maior rácio "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" no plasma e, apenas num carneiro, ocorreu um aumento pós-prandial do rácio "*t*10-/*t*11-18:1" associado a uma redução do pH. Na quarta experiência, quarenta borregos foram alimentados, durante 6 semanas, com uma de quatro dietas completas (1:4, rácio "foragem/concentrado") suplementadas com uma mistura de óleos de soja e de peixe (59:10 g/kg MS) e contendo, como principal fonte energética, cevada (42% MS) (cereal) ou cevada completamente substituída por polpa de citrinos desidratada, polpa de beterraba desidratada ou cascas de soja. Durante a experência, os parâmetros produtivos foram avaliados. Imediatamente após o abate dos animais, amostras de músculo *Longissimus* foram recolhidas para avaliação da expressão dos genes das enzimas síntase de ácidos gordos (FASN), SCD e acetil-CoA carboxilase (ACACA). Ao terceiro dia após o abate, obtiveram-se amostras de músculo e de gordura subcutânea para análise da composição em AG. A dieta com polpa de citrinos levou a uma redução do ganho de peso diário e a um aumento da probabilidade de desenvolver lesões mais severas de paraqueratose da mucosa do rúmen. As dietas com polpa de citrinos e com cascas de soja foram responsáveis pela diminuição da eficiência alimentar, comparativamente à dieta com cevada. Todos os tratamentos originaram um rácio "*t*10-/*t*11-18:1" acima de 3, na carne e na gordura subcutânea, apesar da dieta com cascas de soja ter causado um aumento de *t*11-18:1 e *c*9,*t*11-18:2 nos tecidos, comparativamente aos outros tratamentos. Adicionalmente verificou-se a menor expressão dos genes da FASN, com a dieta com polpa de citrinos, e da SCD, com as dietas com cascas de soja e polpa de beterraba.

Palavras-chave: Ácidos gordos, bioidrogenação, bactérias do rúmen, taninos, amido.

List of publications

This thesis was based on the following publications or manuscripts:

Mónica Costa, Susana P. Alves, Ângelo Cabo, Olinda Guerreiro, George Stilwell, Maria T. Dentinho, Rui J.B. Bessa (2017). Modulation of *in vitro* rumen biohydrogenation by *Cistus ladanifer* tannins compared with other tannin sources. *Journal of the Science of Food and Agriculture*, 97(2), 629-635.

Mónica Costa, Susana P. Alves, Alice Cappucci, Shaun R. Cook, Ana Duarte, Rui Caldeira, Tim A. McAllister, Rui J.B. Bessa (2017). Mimosa condensed tannins induce higher variability of ruminal biohydrogenation than chestnut hydrolysable tannins. (In advanced preparation to be submitted to *Journal of Agriculture and Food Chemistry*).

Mónica Costa, Susana P. Alves, Ângelo Cabo, George Stilwell, A. Troegeler-Meynadier, F. Enjalbert, Rui Caldeira & Rui J.B. Bessa (2017). The induction of *trans*-10 shift in sheep fed with a concentrate-based diet supplemented with sunflower oil. (In preparation).

Mónica Costa, Susana P. Alves, Alexandra Francisco, João Almeida, Cristina M. Alfaia, Susana V. Martins, José A. M. Prates, José Santos-Silva, Olena Doran, Rui J. B. Bessa (2017). The reduction of starch in finishing diets supplemented with oil does not prevent the accumulation of *trans*-10 18:1 in lamb meat. *Journal of Animal Science*, 95(8), 3745-3761.

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List of abbreviations

a*	Redness (colour dimension)
AA	Arachidonic acid
ACACA	Acetyl-CoA carboxylase α (isoform)
ACACB	Acetyl-CoA carboxylase β (isoform)
ACC	Acetyl-CoA carboxylase
ACTB	β-actin (housekeeping gene)
ADF	Acid detergent fibre
ADG	Average daily weight gain
Ag+ - HPLC	Silver high performace liquid chromatography
Apo	Apoprotein
b*	Yellowness (colour dimension)
BCFA	Branched chain fatty acids
BH	Ruminal biohydrogenation
BI	Biohydrogenation intermediates
BW	Body weight
C	Cereal
_c_	_Cis_
C*	Chroma (colour saturation)
CAD	Coronary artery disease
CAP	Common Agricultural Policy
CC	Commercial compound
cDNA	Complementary deoxyribonucleic acid
CH	Chestnut tannin extract
CHD	Coronary heart disease
CIE	Comission Internationale de l´Eclairage
CIISA	Centro de Investigação Interdisciplinar em Sanidade Animal
CL	Rockrose (_C. ladanifer_) tannin extract
CLA	Conjugated linoleic acid
CLNA	Conjugated linolenic acid
CO₂	Carbon dioxide
CoA	Coenzyme-A
COX	Cyclooxygenase
CP	Crude protein
CT	Condensed tannins
CVD	Cardiovascular disease
d	Day

DBP	Dehydrated beet pulp
DCP	Dehydrated citrus pulp
DHA	Docosahexaenoic acid
DM	Dry matter
DMA	Dimethylacetals
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
E	Total energy intake
EC	European Comission
ECFA	Even- and linear-chain fatty acids
EDTA	Ethylenediamine tetraacetic acid
ELOVL	Fatty acid elongases
EPA	Eicosapentanoic acid
EU	European Union
EUROP	Carcass classification system (5 classes)
FA	Fatty acids
FADS	Fatty acid desaturase
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FASN	Fatty acid synthase
FDA	Food and Drug Administration
FFA	Free fatty acids
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GeT	Genomic and Transcriptomic platform
GS	Grape seed tannin extract
H$_2$	Hydrogen
HCW	Hot carcass weight
HDL-C	High density lipoproteins-cholesterol
HETE	Hydroxyeicosatetraenoic acid
HT	Hydrolysable tannins
IMF	Intramuscular fat
INE	Instituto Nacional de Estatística
INIAV	Instituto Nacional de Investigação Veterinária e Agrária
INRA	National Institute for Agricultural Research
iTFA	*Trans* fatty acids from industrial sources
KKCF	Kidney knob channel fat

L*	Lightness (colour dimension)
LAB	Liquid associated bacteria
LA-I	Linoleic acid isomerase
LC-PUFA	Long chain polyunsaturated fatty acids
LC-SFA	Linear chain saturated fatty acids
LDL-C	Low density lipoproteins-cholesterol
LM	*Longissimus* muscle
LOX	Lipoxygenase
Lp	Lipoproteins
LT	Leukotrienes
M	Mimosa tannin extract
MC	Mimosa plus chestnut tannin extracts
ME	Metabolizable energy
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
MW	Molecular weight
n-3	Fatty acids of the n-3 serie
n-6	Fatty acids of the n-6 serie
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acids
NF-Y	Nuclear factor Y
NL	Neutral lipids
NT-BCFA	Non-terminal branched chain fatty acids
O_2	Oxygen
OBCFA	Odd- and branched-chain fatty acids
OCFA	Odd- and linear-chain fatty acids
OH	Hydroxyl group
PG	Prostaglandins
pH	Potential of hydrogen
PL	Polar lipids
PP	Peroxisome proliferator
PPARα	Peroxisome proliferator-activated receptor alpha
PPO	Polyphenol oxidase
PUFA	Polyunsaturated fatty acids
QB	Quebracho tannin extract
RNA	Ribonucleic acid
RPLP0	Ribosomal phosphoprotein P0 (housekeeping gene)

rRNA	Ribosomal ribonucleic acid
rTFA	*Trans* fatty acids from ruminal biohydrogenation
SAB	Solid associated bacteria
SCD	Δ-9 desaturase or stearoyl-CoA desaturase
SCDi-17	Stearoyl-CoA desaturase activity index
SEM	Standard error of mean
SFA	Saturated fatty acids
SH	Soybean hulls
sn	Stereospecific numbering
spp.	More than one species
SREBP	Sterol regulatory element-binding protein
STAT	Statistics Division
t	*Trans*
T10C	*t*10-shift inducing concentrate
TAG	Triacylglycerols
T-BCFA	Terminal branched chain fatty acids
TC	Total cholesterol
TFA	*Trans* fatty acids
TX	Thromboxane
UFA	Unsaturated fatty acids
UK	United Kingdom
VFA	Volatile fatty acids
VLDL	Very low density lipoproteins
WBS	Warner-Bratzler shear force
WHO	World Health Organization

List of units and symbols

<	Lower than
%	Percentage
>	Higher than
±	Standard error deviation
∑	Sum
µg	Microgram
16S	16 Svedberg units
cm	Centimetre
cm^2	Square centimeters
Da	Dalton
F	Force unit
g	Gram
h	Hour
kcal	Kilocalorie
kg	Kilogram
L	Litre
mg	Miligram
mL	Mililitre
mmol	Milimol
ºC	Degree Celsius
s	Second
vol	Volume
wt	Weight
xg	Times gravity
α	Alpha
β	Beta
Δ	Delta

Introduction

Ruminant edible fats are rich in saturated fatty acids (SFA) but their content of polyunsaturated fatty acids (PUFA) is low and of *trans*-fatty acids (TFA) is variable. These features are directly related to ruminal biohydrogenation (BH), which consists of isomerization, hydrogenation or hydration of dietary non-esterified unsaturated fatty acids (UFA) by rumen microbiota (Bessa, Alves & Santos-Silva, 2015). The human intake of SFA and TFA has been related to an increased risk of cardiovascular disease (CVD) (Givens, 2009; Mozaffarian, Aro & Willett, 2009). However, not all TFA are deleterious to human health, as it is the case of vaccenic acid (*t*11-18:1) that can lead to a decrease of atherosclerosis development (Aldai, de Renobales, Barron & Kramer, 2013). Moreover, endogenous conversion of *t*11-18:1 by Δ^9-desaturase or stearoyl-CoA desaturase (SCD) is responsible for up to 87% of rumenic acid (*c*9,*t*11-18:2) deposited in tissues (Palmquist, St-Pierre & McClure, 2004), which is the major conjugated linoleic acid (CLA) in the meat (Parodi, 2003; Khanal & Dhiman, 2004). The *c*9,*t*11-18:2 is known to prevent CVD and tumorigenesis (Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006). Additionally, PUFA, particularly n-3 PUFA, have anti-inflammatory effects that can lead to the suppression of chronic diseases, such as CVD (Calder, 2006; Givens, 2009).

Several studies have attempted to explore the effects of dietary compounds on ruminal BH with the aim of increasing the amount of PUFA, *t*11-18:1 and *c*9,*t*11-18:2 and decreasing SFA content in meat and milk. However, there is a lack of knowledge about the main purposes of the occurrence of BH with a report by Bessa, Santos-Silva, Ribeiro and Portugal (2000) proposing that BH would be a response of rumen ecosystem to stress stimuli induced by lipid overload with a hydrogenation of PUFA and a consequent production of TFA. The effect of tannins, which are polyphenolic compounds that constitute secondary metabolites of plants, on ruminal BH have been described in the literature (Vasta & Bessa, 2012). In the majority of studies, tannins were incorporated in a diet or dietary substrate supplemented with oils rich in PUFA, which were found to exacerbate the ruminal production of BH intermediates (BI), such as *t*11-18:1 and *c*9,*t*11-18:2 (Bessa, Portugal, Mendes & Santos-Silva, 2005). The dietary incorporation of tannins was shown to inhibit the last step of BH, reducing the production of stearic acid (18:0) and consequently promoting the accumulation of *t*11-18:1 and, in some reports, also of *c*9,*t*11-18:2 in the rumen (Vasta & Bessa, 2012). However, other studies suggested a depression of the first steps of BH that led to an enhancement of 18:2 and 18:3 PUFA, with tannins (Roy *et al.*, 2002; Kronberg, Scholljegerdes, Barceló-Coblijn & Murphy, 2007; Cabiddu *et al.*, 2009). The inconsistency of tannins' effects might be related to the molecular type and source of these compounds, dose

and time of administration and the animal species involved (Frutos, Hervás, Giráldez & Mantecón, 2004; Toral, Hervas, Belenguer, Bichi & Frutos, 2013).

Moreover, the accumulation of beneficial fatty acids (FA) is constrained if an alteration of ruminal BH, termed t10-shift, is present. In fact, t10-shifted BH pathways are associated with a predominance of t10-18:1 to the detriment of t11-18:1, as the main *trans* monoene isomer in the rumen and tissues (Aldai *et al*., 2013; Bessa *et al*., 2015). Besides the deleterious effects to human health promoted by t10-18:1, such as an increased risk of CVD (Hodgson, Wahlqvist, Boxall & Balazs, 1996; Aldai *et al*., 2013), this FA cannot be converted into c9,t11-18:2. Since high-starch low-forage diets with or without PUFA supplementation fed to ruminants in the fattening phase were reported as being responsible for the occurrence of the shift (Bessa *et al*., 2005; Alfaia *et al*., 2009; Rosa *et al*., 2014), the replacement of cereals with low-starch feeds might be an option to prevent the establishment of t10-shift. These alternative energy sources include industrial by-products, such as citrus and beet pulps and soybean hulls, and, in general, their incorporation in diets does not impair animal productive performance (Bampidis & Robinson, 2006; Vasta, Nudda, Cannas, Lanza & Priolo, 2008). The stimulation of t11 BH pathways was verified when cereals were replaced for citrus pulp causing an increased ruminal production of c9,t11-18:2 and t11-18:1. The accumulation of these BI was either associated with the presence of phenolic compounds in the by-product (Lanza *et al*., 2015) or the suppression of t10 BH pathways (Santos-Silva *et al*., 2016). However, in general, even with a stimulation of t11 BH pathways, the replacement of cereals with citrus pulp did not lead to a general prevention of t10-shift, which was probably caused by a high animal variability in its occurrence (Bessa *et al*., 2015; Santos-Silva *et al*., 2016).

In the present thesis, four experiments were conducted to study how ruminal biohydrogenation (BH) pathways can be modulated through dietary inclusion of tannin sources (experiments 1 and 2) and to acquire a better knowledge about the induction and development of t10-shifted BH pathways (experiment 3 and 4). Those methodological approachs were: a) the inclusion of condensed and hydrolysable tannin extracts in a dietary substrate (*in vitro* - experiment 1; *in vivo* - experiment 2) and b) the alteration of the dietary starch level, such as the incorporation of a high starch level in a diet for rumen fistulated sheep (experiment 3) and the replacement of cereal with alternative energy sources (dehydrated citrus pulp, dehydrated beet pulp or soybean hulls) in lambs' finishing diets (experiment 4). Both experimental models included the supplementation with oils rich in PUFA. Considering an integrative approach, the impact of each strategy on ruminal BH was analysed, although, in experiment 4, BH was only indirectly evaluated by the FA composition of meat and subcutaneous fat. Also, in the lattest trial, it was studied the influence of the energy sources on productive performance, carcass and meat quality traits and on the expression of genes in *Longissimus* muscle that codify for SCD, acetyl-CoA carboxylase α

(ACACA) and fatty acid synthase (FASN) enzymes, which are associated with endogenous synthesis of $c9,t11$-18:2 and *de novo* FA synthesis, respectively.

This document is organized in 7 Chapters, which include the bibliographic review and objectives (Chapter 1), followed by the results obtained from each experiment in the form of scientific publications (Chapter 2 to 5). Afterwards, a general discussion of results achieved with the experimental models was done (Chapter 6), as well as the main conclusions and implications of the present work (Chapter 7).

CHAPTER 1 – Scientific background and objectives

1.1. Ovine production and meat consumption

1.1.1. Livestock and meat

The world ovine population is around one billion and, in 2014, it amounted to 1 209 908 142 animals. The main producers of sheep are China and Australia. In fact, from 2003 to 2014, Asia accounted for 43.3% of total ovine population with only 11.8% from Europe (Food and Agriculture Organization of the United Nations Statistics Division [FAOSTAT], 2015). The world sheep meat production in 2013 was 8 702 257 tonnes of carcass weight and, although a decrease on its amount was verified between 2007 and 2010, there has been an increase afterwards.

In 2014, the amount of sheep in the European Union (EU-28) was 97 665 170 and so the EU is far from being self-sufficient in the ovine meat sector and, consequently, imports considerable quantities, mainly from New Zeeland and Australia (FAOSTAT, 2015; European Comission [EC], 2016). After the mid-term review of the Common Agricultural Policy (CAP) that led to the decoupling of subsidies in 2003, a reduction of the total livestock was reported for various species. Regarding sheep livestock, the most pronounced fall (12.1%) for 14 EU Member States with more than 500 000 sheep occurred between 2005 and 2014. During this period, the sheep flocks decreased 30.0% (2 903 to 2 032.62 million heads) in Portugal but from 2014 to 2015 an increase of 0.5% was reported. That increase was not only verified in Portugal but also in countries of EU that are major producers of ruminants, such as United Kingdom (UK) and Spain (EC, Eurostat, 2015a). In fact, during 2015, 23 103 and 16 522.96 million heads were found in UK and Spain, respectively and, in 2014, the UK (39.7%) and Spain (16.4%) contributed with 56.1% of the total small ruminant meat produced in EU-28.

Similar to the livestock, a diminishment of sheep meat production (1 065 456 to 707 000 tonnes) was verified in EU-28 between 2005 and 2014 and, particularly in Portugal, it declined around 14 000 tonnes, reaching the 10 222 tonnes (887 619 heads) (EC, Eurostat, 2015b; FAOSTAT, 2015; Instituto Nacional de Estatística [INE], 2015). However, the number of sheep slaughtered in slaughterhouses increased between 2014 and 2015 and, in Portugal, it reached 893 802 heads that corresponds to 10 472 tonnes, consisting, the majority of these animals (57% of total slaughters), of lambs with more than 10 kg of carcass weight (INE, 2015).

Concerning the composition of sheep flock in Portugal during 2014, dairy ewes corresponded to only 20.6% of the national sheep number and so there was a predominance of meat production (EC, Eurostat, 2015a). Moreover, a significant concentration at regional level can be found with Alentejo presenting more than half of the livestock, accounting for a total of 1 172 000 animals in 2015 (INE, 2015).

The lamb meat consumption in the world averaged 1.7 kg/year per capita during the period of 2013 to 2015 with an important contribution from EU-28 (1.8 kg/year per capita). Considering sheep and goat meats, the degree of self-sufficiency in Portugal increased between 2014 and 2015, reaching 82.6% and the consumption was 2.2 kg/year per capita (INE, 2015). In Portugal, Merino Branco is the most used lambs' breed for meat production, corresponding to a slaughter body weight (BW) up to 25 kg and HCW between 10 and 13 kg.

In the world, a further increment in the production of ovine meat (15.3 to 20.1 million tonnes) was projected, but at a declining rate in developed countries that will probably only contribute to an increase from 3.1 to 3.5 million tonnes, considering the period between 2015 and 2030. The sheep and goat livestock were also predicted to increase worldwide from 1997/99 to 2030 (1749 to 2309 million heads) with a low contribution of developed countries (341 to 358 million heads) (FAO, 2003). The projections for Europe and Central Asia followed these trends with an enhancement of ovine meat from 2005/07 to 2030 (2.2. to 2.5 million tonnes), although at a decreased annual growth rate (0.62 to 0.21%) (FAO, 2012).

1.1.2. Production systems

In Southern countries of EU-28, ovine dairy production is significant and, consequently, carcass weights are lower than in Northern countries, which have a predominant meat production system. This fact, together with the lower prolificacy of Southern sheep breeds, gives rise to less productivity in Southern countries than in the others (Sañudo, Sanchez & Alfonso, 1998).

Production systems from Spain were described as representatives of Southern countries by Sañudo *et al*. (1998), which are similar to the ones from Portugal and consist of meat production from milk or suckling lambs, light or fattening lambs and adult sheep. Milk lambs come from the extensive dairy production system. They are slaughtered at 25 to 45 days in order to milk the ewes, originating carcasses with an average of 5.26 kg of hot carcass weight (HCW). However, if animals are supplemented with milk-concentrate, a later slaughtering can be executed. Light lambs are reared with ewes' milk and supplemented with pasture or finished with concentrate, being slaughtered at 4 to 6 months of age obtaining 8.5 to 14 kg of HCW. This semi-extensive system is the more frequent in Iberian countries and the Merino breed and its cross-breeding are the most used type of lambs. Fattening lambs come from an intensive production system, since, after weaning, they are kept indoors until reaching 90 to 100 days of age and HCW around 13 to 15 kg. Adult sheep comprise animals in the end of their productive cycle that come from milk and meat production systems.

Carcass traits, meat quality and lipid composition

Feeding systems can influence carcass traits and meat quality of sheep. The muscle proportion has been reported to be higher with very lean carcasses for lambs fed with pasture, which is related to a lower average daily weight gain (ADG) of these animals, than the ones supplemented with concentrate (Santos-Silva, Mendes & Bessa, 2002b). In fact, the concentrate feeding generally leads to increased intramuscular fat (IMF) deposition in ruminants compared to pasture, which has been associated with a higher availability of glucogenic precursors derived from starch feedstuffs fermentation that increases the concentration of insulin in plasma stimulating a lipogenic response (Bessa *et al.*, 2015).

The IMF can be an important meat quality trait as it is a reservoir of FA that might have an important influence on human health and also due to its relation with meat sensorial characteristics (Daley, Abbott, Doyle, Nader & Larson, 2010; Bessa *et al.*, 2015). The level of IMF of lamb meat that contributes for its good quality was considered to be 5% (Hopkins, Fogarty & Mortimer, 2011), which corresponds to a ''low in fat" meat (Scollan *et al.*, 2006). The IMF is composed of two fractions that correspond to neutral (NL) and polar (PL) lipids, which include FA present in intramuscular adipocytes and muscle fibres, respectively (Raes, De Smet & Demeyer, 2004; Bessa *et al.*, 2015). The intramuscular adipose tissue is also known as "marbling fat" (Wood *et al.*, 2008) and comprises isolated or clustered adipocytes located along the fibres and in the interfascicular area. This fat portion mainly consists of triacylglycerols (TAG), while lipids in fibres have a high proportion of phospholipids, placed in cell membranes, and a smaller amount of TAG (cytosolic droplets) and cholesterol. The phospholipid content is relatively constant, although it depends on the metabolic type of the muscle fibre, being higher in more oxidative muscles (Raes *et al.*, 2004). The ruminant species and their stage of growth can play a minor role on the steadiness of the PL fraction. In fact, this constancy seems to be stricter in bovine than in ovine and other farm animal species muscles and an initial increase of PL in the first stages of IMF deposition was reported, which might be associated with a greater amount of cellular membranes due to a higher metabolic activity and adipocytes hyperplasia (Bessa *et al.*, 2015). Conversely, the amount of TAG varies widely (0.2 to 5 g/100 g of fresh tissue) (Raes *et al.*, 2004) and it depends essentially on the muscle type, overall body fatness degree and animal breed. Therefore, the composition of IMF results from the balance between uptake, synthesis and degradation of TAG. So, modifications of IMF induced by the diet are mainly due to an impact on the amount of FA present in TAG. In fact, IMF contains an average of 45 to 48, 35 to 45 and up to 5 g/100 g of total FA as SFA, monounsaturated FA (MUFA) and PUFA, respectively (Scollan *et al.*, 2006) but, if just the phospholipid portion was considered, a predominance of PUFA would be obtained. Indeed, phospholipids contain PUFA at 20 to 50 g/100 g of total FA, whereas their content in TAG has been described as 2 to 30 g/100 g of

total FA. In bovine muscles, a considerable low proportion of PUFA in TAG can be found (2 to 3 g/100 g of total FA) (Raes *et al*., 2004). The incorporation PUFA in PL is limited and mostly includes 18:3n-3 and 18:2n-6 with even a higher selectivity for their corresponding derivatives n-3 and n-6 long chain (LC)-PUFA (Wood *et al*., 2008; Jerónimo, Alves, Prates, Santos-Silva & Bessa, 2009; Jerónimo *et al*., 2011). A greater affinity has been described for 18:2n-6 than for 18:3n-3 deposition (De Smet, Raes & Demeyer, 2004; Sinclair, 2007; Wood *et al*., 2008), although 18:3n-3 is the preferred substrate for enlogases and desaturases enzymatic complex. Moreover, increased levels of 18:2n-6 comparing with 18:3n-3 in IMF might also be due to a lower rumen BH of 18:2n-6 (Wood *et al*., 2008). In the study reported by Jerónimo *et al*. (2011), a preferential incorporation of *cis*-isomers in PL was verified, such as the majority of *cis*-18:1 originated from oleic acid (*c*9-18:1), even though *c*9-18:1 predominated in NL. However, the majority of trans C18 FA derived from BH were accumulated in NL, including *c*9,*t*11-18:2 and *t*11-18:1. In fact, other studies reported that more than 80% and up to 99% of the two BI were present in TAG of very lean and fatter meats, respectively (Bessa *et al*., 2015). These results indicate that an increase of *trans*-FA in muscle probably has low potential to change the membrane FA composition and structure and, consequently, the cellular function.

The production and feeding systems were shown to affect the FA composition of meat and, consequently, its quality and nutritional value. In fact, in one study by Sañudo *et al*. (2000), the meat from British lamb types fed with forage-based diet had higher 18:0, 18:3n-3 and n-3 LC-PUFA and lower 18:2n-6 than the one from concentrate-fed Spanish breeds. The increase of 18:3 in British types was related to higher odour and flavour intensity scores comparing with the other animals. Santos-Silva, Bessa and Santos-Silva (2002a) also described that the FA profile of lambs' muscle was effective in the identification of feeding systems, since there was an increase of 18:3n-3; n-3 LC-PUFA; CLA (mainly *c*9,*t*11-18:2) and *trans*-18:1, as well as a decrease of "n-6/n-3 PUFA" ratio when lambs were reared with their dams on pasture comparing with the ones fed with concentrate. The lower value of the ratio indicates a higher nutritional value of meat promoted by the corresponding system (Wood *et al*., 2004).

The modification of meat FA profile by feeding systems can also affect meat sensory characteristics. Indeed, ruminants' diets can modulate the lipid content of IMF. There is a strong relationship between IMF and the amount of TAG. Both of them increase when animals are fed a concentrate- in opposition to a forage-based diet and depend on the degree of overall body fatness, breed and muscle type (Scollan *et al*., 2006; Bessa *et al*., 2015). A higher IMF content may raise meat tenderness and juiciness, although the strength of their correlation varies between studies. The disposition of IMF allows for a more easily breakdown of muscle structure in the mouth contributing for an increased tenderness.

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Besides, the concentrate based-diets might lead not only to an increment of IMF but also of some specific SFA named non-terminal branched chain FA (NT-BCFA) mostly present in subcutaneous fat, which may allow for a decreased firmness of lambs' fat due to their low melting points. A greater retention of water in meat during cooking promoted by IMF deposition may lead to a higher juiciness (Wood *et al*., 2008). Additionally, Angood *et al*. (2008) found that the two sensory characteristics were affected by levels of IMF but juiciness was the mostly influenced.

Moreover, differences in IMF and subcutaneous fat content were suggested to be related to meat odours and flavours (Resconi, Campo, Furnols, Montossi & Sanudo, 2009). Meat flavour is derived from the Maillard reaction between amino acids and reducing sugars and the thermal degradation of lipids (Scollan *et al*., 2006). This attribute is important for meat appreciation by consumers, particularly considering lamb meat (Young, Reid, Smith & Braggins, 1994). The species-specific aromas result from fat-soluble volatile compounds and aromatic compounds derived from lipid oxidation (Mottram, 1998). The increase of IMF with concentrate compared to forage feed can be responsible for higher "fatty" flavour and "fatty/oily" odour scores (Resconi *et al*., 2009). Moreover, feeding considerable amounts of forage to ruminants has been associated with ''fishy'' (Scollan *et al*., 2006) and "pastoral" (Sinclair, 2007) flavours and with a ''fishy'' odour (Resconi *et al*., 2009) of meat, mainly due to increased n-3 PUFA in this type of feed (Scollan *et al*., 2006; Sinclair, 2007). Considering that PUFA are liable to oxidative breakdown, an increased lipid oxidation can occur with forage based-diets leading to higher rancid or acid flavours and rancid odour scores. Conversely, the concentrate feed was related to increased intensity of typical lamb aroma and flavour (Resconi *et al*., 2009). However, even though it was more extensively reported in steers, when a concomitant enhancement of forage and vitamin E intake occurs, the antioxidant properties of this compound helps to raise the oxidative stability of PUFA and, consequently, can be accompanied by an improvement of meat flavour and a longer maintenance of meat red colour saturation (chroma, *C) (Scollan *et al*., 2006; Wood *et al*., 2008).

The meat colour can also be influenced by feeding systems and might be associated with meat quality in ruminants. In fact, Bessa, Lourenço, Portugal and Santos-Silva (2008) reported a darker meat with lower L* (lightness) and higher a* (redness) (Comission Internationale de l´Eclairage, CIE L*, a*, b* system) values with concentrate than with forage diets due to an increased HCW with concentrate when all lambs were slaughtered at the same age, although, in some studies of bovine meat (Vestergaard, Oksbjerg & Henckel, 2000; Raes *et al*., 2003; Nuernberg *et al*., 2005), the opposite was found, which may be explained by a higher proportion of muscle oxidative fibres presenting an increased content of myoglobin with grass feeding (Vestergaard *et al*., 2000). However, these effects of the

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basal diet on meat colour may not be detrimental and the slaughter of heavier animals can increase the productivity and flexibility of production systems without compromising meat quality and acceptability. Indeed, Santos-Silva *et al*. (2002b) even verified that lamb meat colour did not considerably change with feeding systems, since the concomitant increase of a* and b* (yellowness) estimates with concentrate compared with pasture treatments led to a relatively constant "a*/b*" ratio. Also, although the latter authors observed a decrease of L* and b* colour estimates when slaughter weight was incremented from 24 to 30 kg, this effect was not detrimental considering that meat was within the range of light pink meats preferred by consumers, which is characterized by L* > 45, a* between 15 and 18 and a*/b*≈2 (Sanudo *et al*., 1992) or, more recently, by L* ≥ 44 and a* ≥ 14.5 (Khliji, van de Ven, Lamb, Lanza & Hopkins, 2010). Noticeable, the a* value was slightly lower than those recommended but not enough to affect the meat acceptance. Moreover, a minimal impact of slaughter BW on meat sensory characteristics has been reported with only an increase of juiciness (Santos-Silva & Portugal, 2001) and flavour intensity (Teixeira, Batista, Delfa & Cadavez, 2005).

Considering meat tenderness, a relation between this sensory attribute and Warner-Bratzler shear force (WBS) was proposed, consisting on the following classification: very tender, WBS < 3.2 kg; tender, 3.2 kg < WBS < 3.9 kg; medium tender, 3.9 kg < WBS < 4.6 kg and tough, WBS > 4.6 kg (Shackelford, Morgan, Cross & Savell, 1991). In the study from Teixeira *et al*. (2005), an increase of shear force with higher slaughter BW had no influence on meat toughness and consequently on its tenderness. In general, lamb meat was found to be tender or very tender regardless of the treatment (Santos-Silva & Portugal, 2001; Santos-Silva *et al*., 2002b; Francisco *et al*., 2015). However, higher WBS values, corresponding to medium tender or tough meat, were reported by Bessa *et al*. (2008) but these results were probably due to an inferior ageing period of the meat, which may lead to a decreased collagen solubility and fragmentation (Sullivan & Calkins, 2011).

1.2. Effects of dietary fat on human health

1.2.1. Saturated fatty acids

Ruminant edible fats are rich in SFA (Bessa *et al*., 2015) and the ruminant milk is the largest source of SFA, which often contributes to 41% of all SFA of Human diet (Givens, 2009). The intake of SFA has been related to an increased risk of CVD and, although less documented, also to a reduction of insulin sensitivity and consequent predisposition to the onset of diabetes (Givens, 2009; FAO, 2010). Considering this, an attempt to decrease the intake of SFA has been made and so the SFA daily intake was recommended to be lower than 10% of total energy (E), which corresponds to amounts inferior to 33 g for male and to 26 g for female adults with moderate activity (FAO, 2001, 2010). However, not all SFA have similar metabolic influence, since those from C12 to C16 have a greater effect on raising low density lipoproteins-cholesterol (LDL-C) and lowering the "total cholesterol (TC)/high density lipoproteins-cholesterol (HDL-C)" ratio than the others. Even among these FA, the lauric acid (12:0) was suggested to be less detrimental than myristic (14:0) and palmitic (16:0) acids (Mensink, Zock, Kester & Katan, 2003). Oppositely, the 18:0, which is generally the major SFA in ruminant meat together with 16:0, was shown to be beneficial or neutral to human health (FAO, 2010). In one study, this FA reduced LDL-C and "total cholesterol (TC)/HDL-C" ratio and had a neutral effect with respect to HDL-C comparing with the remaining SFA (Hunter, Zhang & Kris-Etherton, 2010). In fact, Hunter *et al*. (2010) reported an intermediate influence of 18:0 between that of healthier UFA, such as *c*9-18:1 and 18:2n-6, and other SFA. The *c*9-18:1 is the predominant *cis*-MUFA in the meat, being the main product of 18:0 desaturation. So, an increase of 18:0 may also contribute for beneficial effects of *c*9-18:1, such as the reduction of "TC/HDL-C" ratio (FAO, 2010). Although a general acceptance that SFA consumption is associated with an adverse influence on human health is present, there is controversy about this issue, since some studies revealed that SFA are not or are weakly associated with CVD, particularly with coronary artery disease (CAD) (Lawrence, 2013). Additionally, iso and anteiso branched chain FA (BCFA) were reported to have health promoting properties, which include decreased FA biosynthesis (Wongtangtintharn, Oku, Iwasaki & Toda, 2004) and induction of apoptosis of cancer cells (Yang *et al*., 2000) with a consequent inhibition of tumoral growth. The main BCFA associated with these effects was the 13-methyltetradecanoic acid (iso-15:0) (Yang *et al*., 2000; Wongtangtintharn *et al*., 2004) but other FA, such as 12-methyltetradecanoic acid (anteiso-15:0), 14-methylpentadecanoic acid (iso-16:0) and, in a lesser extent, the straight-chain FA pentadecanoic acid (15:0) and 14:0 also presented cytotoxicity effects (Wongtangtintharn *et al*., 2004). Moreover, these BCFA were shown to reduce the incidence of necrotizing enterocolitis and to modify ileal microbiota composition in a rat model (Ran-Ressler *et al*., 2011) and that can be important

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as these FA are constituents of the healthy human newborn gastrointestinal tract (Ran-Ressler, Devapatla, Lawrence & Brenna, 2008).

1.2.2. *Trans* fatty acids

The TFA are UFA that contain at least one double bond in the *trans* configuration instead of the usual *cis*. In double bonds with *trans* configuration, the hydrogen atoms are located on the opposite sides of the carbon chain (Dhaka, Gulia, Ahlawat & Khatkar, 2011; Tardy, Morio, Chardigny & Malpuech-Brugere, 2011). These isomers might come from industrial sources (iTFA) by partial hydrogenation of vegetable oils or from ruminal BH (rTFA). Generally, t11-18:1 is the major rTFA, while elaidic acid (t9-18:1) is the main iTFA (Dhaka *et al*., 2011). The human dietary consumption of TFA has been associated with the development of CVD, particularly myocardial infarction and coronary heart disease (CHD), due to endothelial dysfunction, systemic inflammation and adverse influence on the concentration of serum lipoproteins (Lp) and lipids. The effects on Lp and lipids include an increase of LDL-C, apoprotein (Apo) B, Lp (a) and fasting TAG; a decrease of HDL-C and Apo A-I and a higher "TC/HDL-C" ratio in comparison with other FA, specially MUFA and PUFA. Some studies reported a relation between TFA and tumorigenesis possibly associated with the proinflammatory effects (Mozaffarian *et al*., 2009; Dhaka *et al*., 2011; Aldai *et al*., 2013). There is also inconsistent evidence of predisposition for a decreased insulin sensitivity and consequently diabetes and for gain weight with TFA (Dhaka *et al*., 2011; Tardy *et al*., 2011). Moreover, a higher risk of myocardial infarction and CHD when TFA (2% of E) replaced SFA was reported and this effect was gradually more pronounced with the substitution of *cis*-MUFA and *cis*-PUFA (Mozaffarian *et al*., 2009). In order to reduce the incidence of these adverse effects, the TFA daily intake was recommended to be lower than 1% of E, which corresponds to amounts inferior to 3.3 g for male and to 2.6 g for female adults with moderate activity (2987 and 2355 kcal ingested per day, respectively) (FAO, 2001, 2010). The consumption of rTFA at current levels should not raise health concerns, since they account for a small part of total FA in milk (2-5%) and meat (3-9%) (Wang, Jacome-Sosa & Proctor, 2012). Conversely, iTFA may reach 50% in hydrogenated fats and so more attention has been given to iTFA instead of rTFA (Weggemans, Rudrum & Trautwein, 2004; Aldai *et al*., 2013). Indeed, national policies in order to eliminate iTFA from dietary sources have been adopted (World Health Organization [WHO], 2012), including the specification of the TFA content in the label of every packaged food (Food and Drug Administration [FDA], 2013). The fact that these regulations have been successful in many countries, together with an increase of total rTFA in products derived from ruminants fed with concentrate-based diets supplemented with oils, have contributed to an enhanced

importance of the consumption of rTFA. The definition of TFA for regulation purposes includes all FA with isolated *trans* non-conjugated double bonds and does not consider that specific isomers may have beneficial or deleterious impact on human health. Noticeable, not all conjugated isomers are beneficial and some non-conjugated ones are even desirable (Aldai *et al*., 2013). Indeed, some studies with animal models have demonstrated that *t*11-18:1 can lead to a decrease of atherosclerosis development in opposition to *t*10-18:1 and *t*9-18:1 (Bauchart *et al*., 2007; Dupasquier *et al*., 2007; Aldai *et al*., 2013). Also, Hodgson *et al*. (1996) reported a positive association of *t*10-18:1 and *t*9-18:1 with the degree of CAD. Moreover, *t*11-18:1 is endogenously converted into *c*9,*t*11-18:2 (Bessa *et al*., 2015) that is known to improve immune function and to prevent atherosclerosis, CVD, hypertension and different types of cancer (Bhattacharya *et al*., 2006; Gebauer *et al*., 2011; Wang *et al*., 2012). The predominant conjugated FA in ruminant-derived products is usually the *c*9,*t*11-18:2 but, in certain conditions as in the presence of *t*10-shift, an increase of *t*10,*c*12-18:2 to the detriment of *c*9,*t*11-18:2 might happen (Aldai *et al*., 2013). Although some studies suggested that the two CLA isomers may act similarly in inhibiting atherosclerosis (Bhattacharya *et al*., 2006), there is more evidence about an antagonist action. In fact, *t*10,*c*12-18:2 was shown to have deleterious effects on human health, increasing the risk of atherosclerosis (Tricon *et al*., 2004; Kostogrys, Maslak, Franczyk-Zarow, Gajda & Chlopicki, 2011) and stimulating adenoma growth (Rajakangas, Basu, Salminen & Mutanen, 2003), even with the beneficial anti-adipogenic role of this isomer described by Bhattacharya *et al*. (2006). Also, some conjugated linolenic acid (CLNA) isomers, such as *c*9,*t*11,*t*13-18:3 and *c*9,*t*11,*c*13-18:3, were identified as having bioactive properties, contributing to an inhibition of atherosclerosis, tumorigenesis, inflammation and to an improvement of immune response (Hennessy, Ross, Devery & Stanton, 2011). Differences between TFA related to human health might even occur with FA presenting structural similarity. In fact, although both *t*10,*c*15-18:2 and *t*10,*c*12-18:2 have a *t*10 double bond, the anti-adipogenic effects of *t*10,*c*12-18:2 were not observed with *t*10,*c*15-18:2 (Vahmani, Meadus, Rolland, Duff & Dugan, 2016). However, in general, *cis,trans*-18:2 were associated with a higher predisposition to develop CVD than *trans*-18:1 (Aldai *et al*., 2013).

1.2.3. Polyunsaturated fatty acids

Linolenic (18:3n-3) and linoleic acids (18:2n-6) are PUFA that are considered as essential FA, since they cannot be synthesized *de novo* by mammals and, consequently, they have to be present in the diet. Both of them have methylene interrupted double bonds and, for 18:3n-3, the last double bond is located in the third carbon counting from methyl end whereas, for 18:2n-6, it is placed in the sixth carbon. The 18:3n-3 and 18:2n-6 are converted into n-3 LC-PUFA and n-6 LC-PUFA, respectively. The fact that the efficiency of these two pathways is

very low, which is exacerbated by a competition between the two substrates to utilize the same enzymes, stands for the supplementation of human diets with LC-PUFA. Although 18:3n-3 has been described as a much stronger suppressor of 18:2n-6 conversion than the opposite, the incorporation of n-3 LC-PUFA is of particular importance, since the intake of n-3 PUFA is lower than that of n-6 PUFA (Barceló-Coblijn & Murphy, 2009; FAO, 2010). Besides, n-3 LC-PUFA have been associated with a more beneficial impact on human health compared to n-6 LC-PUFA. In fact, they were positively related to cognitive ability and present anti-inflammatory potential that can lead to the suppression of chronic diseases, such as rheumatoid arthritis, atherosclerosis and CHD (Calder, 2006; Givens, 2009). The n-3 LC-PUFA, mainly eicosapentanoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, can replace arachidonic (AA, 20:4n-6) acid, which is the main n-6 LC-PUFA located in cell membrane phospholipids, or inhibit its metabolism. The AA is the precursor of eicosanoids including 2-series prostaglandins (PGs) and thromboxane A2 (TXA2) originated by cyclooxygenase-2 (COX-2), as well as 5-hydroxyeicosatetraenoic acid (5-HETE) and 4-series leukotrienes (LT) produced by 5-lipoxygenase (5-LOX). These compounds have a pro-inflammatory role and, although EPA can also act as a substrate for these enzymes, this FA gives rise to eicosanoids that present a slightly different structure and, consequently, a lower potency compared to the ones formed from AA, such as 3-series PGs and TX and 5-series LTs. The inhibition of AA metabolism might consist on the suppression of COX-2 and 5-LOX by EPA or of COX-2 by DHA. Moreover, EPA can inhibit AA release from phospholipids by phospholipase A2 (Calder, 2002, 2006, 2010). These two PUFA may have other anti-inflammatory actions that are independent from their direct influence on eicosanoid formation and result in decreased leukocyte chemotaxis and reactive oxygen species, cytokines and adhesion molecules productions (Calder, 2006). The effects on cytokines and adhesion molecules mostly occur at the level of altered gene expression though an impact on the activity of transcription factors (Calder, 2002, 2006). Also, a group of anti-inflammatory and inflammation resolving mediators might be formed from EPA (E-series resolvins) and from DHA (D-series resolvins, docosatrienes and neuroprotectins). The corresponding reaction pathways involve COX and LOX enzymes (Calder, 2006, 2010). Considering PUFA intake, previous studies were focused on n-6 to n-3 PUFA ratio in meat, which should be less than 4 (Wood *et al.*, 2004), but, more recently, a balanced intake of n-6 and n-3 PUFA was recommended (FAO, 2010) because not only n-3 PUFA but also n-6 PUFA have been associated with beneficial effects. In fact, n-6 LC-PUFA were shown to have anti-inflammatory properties, mainly through the production of PGE2 (Calder, 2006). The adequate daily intake of total PUFA for an adult was proposed to be 6-11% of E (FAO, 2010) with n-6 and n-3 PUFA corresponding to 5-8% and 1-2%, respectively (WHO, 2003). So, the ingestion of total PUFA should be 19.9 to 36.5 g for male and 15.7 to 28.8 g for female adults

with moderate activity. Attending to essential FA, the minimum intake values to prevent deficiency symptoms were estimated to be 2.5% of E for 18:2n-6 and 0.5% of E for 18:3n-3. The reference intake for EPA plus DHA, mainly found in oily fish and fish oils (Calder, 2006), was established to be 250 mg/day, although a variation between countries, depending on the sustainability of fish supply, might occur (FAO, 2010), as it is the case of UK where this value is 450 mg/day (d) (Givens, 2009).

1.3. Lipid metabolism in the rumen

The ruminal lipid metabolism is responsible for an extensive transformation of dietary lipids, consisting in two major processes: lipolysis and BH. It allows the conversion of PUFA into UFA and SFA, which are known to be more predominant in ruminant dairy products and meat comparing with the non-ruminant ones (Jenkins, Wallace, Moate & Mosley, 2008). However, there is a proportion of PUFA that is not biohydrogenated and bypasses the rumen intact, being absorbed and deposited in body fat, contributing to the enrichment of meat on PUFA (Scollan *et al*., 2006).

1.3.1. Lipolysis

The dietary lipids that enter the rumen, including TAG (predominating in cereals and plant oils) and sulfo-, galacto- and phospholipids (mostly present in forages) are converted into non-esterified free FA (FFA) through a process denominated lipolysis, which consists in the hydrolysis of the ester linkages of dietary acyl lipids. This process allows for the presence of a free carboxyl group that is essential for the BH to proceed. The lipolysis is mainly due to lipases produced by rumen microorganisms, although plant endogenous lipases might play a minor role. Generally this metabolic pathway occurs rapidly but its extent might be partial inhibited by certain conditions, such as the presence of low rumen pH (Jenkins *et al*., 2008; Lourenço, Ramos-Morales & Wallace, 2010).

1.3.2. Biohydrogenation

The first evidence of ruminal BH was obtained with a decrease of 18:3n-3 present in linseed oil, when the oil was incubated with sheep rumen contents (Reiser, 1951). After that, Shorland, Weenink and Johns (1955) described that the BH of 18:3n-3 in the rumen of sheep orginates 18:2 and 18:1 isomers, including *trans*-18:1, and 18:0. In the *in vitro* study reported by Shorland, Weenink, Johns and McDonald (1957), the BH of 18:3n-3 was demonstrated in more detail, together with the conversion of 18:2n-6 and *c*9-18:1 into BI and 18:0. Later,

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Ward, Scott and Dawson (1964) incubated ^{14}C-labeled 18:3n-3, 18:2n-6 and c9-18:1 with ovine rumen contents and found similar results for the BH of 18:3n-3 to the ones reported by Shorland *et al.* (1955) and Shorland *et al.* (1957), with 18:2n-6 and c9-18:1 being mostly converted into 18:0 and small amounts of *trans*-18:1. Subsequently, Wilde and Dawson (1966) proposed a scheme for the BH pathways of ^{14}C-labeled 18:3n-3. More recently, the complexity of BH pathways of ^{13}C-labeled 18:3n-3 (Lee & Jenkins, 2011) and c9-18:1 and ^{14}C-labeled 18:2n-6 (Jenkins *et al.*, 2008) was analysed in some *in vitro* and *in vivo* studies.

The BH of 18:2n-6 and 18:3n-3 consist of a first step of c12 double bonds isomerization proceeded by the reduction of the respective products into several 18:2 and 18:1 BI until the formation of 18:0. The most common 18:1 BI produced are *trans*-18:1, mainly the t11-18:1 (Harfoot & Hazlewood, 1997; Bessa *et al.*, 2000). Generally, the main FA leaving the rumen is 18:0 but the incompleteness of BH, due to the fact that the conversion of 18:1 into 18:0 is a rate limiting step, leads to a high amount of 18:1 BI available for intestinal absorption (Shingfield & Wallace, 2014). Considering the majority of diets, the proportional BH of 18:2n-6 and 18:3n-3 ranges from 70% to 95% and 85% to 100%, respectively (Shingfield, Bonnet & Scollan, 2013).

Although the initial isomerization of 18:2n-6 BH mostly originates c9,t11-18:2, several other CLA isomers have been identified with double bond positions varying between C7,9 and C12,14, such as t10,c12-18:2 and t9,t11-18:2. The production of t10,c12-18:2 is more relevant when animals are fed with concentrate-based diets. Moreover, the 18:2n-6 can be subsequently converted into non-conjugated t9,c12-18:2 or the oxygenated FA 13-OH-c9-18:1, 13-OH-18:0 and 13-oxo-18:0 (Jenkins *et al.*, 2008; Shingfield, Bernard, Leroux & Chilliard, 2010a). Also, one study reported the formation of 10-OH-c12-18:1, resulting from the BH of this PUFA, that was suggested to be oxidized into c9,t11-18:2, although that was only carried out by washed cells of cultured *Lactobacillus acidophilus* (Ogawa, Matsumura, Kishino, Omura & Shimizu, 2001).

The isomerization of 18:3n-3 originates conjugated triene isomers, mainly c9,t11,c15-18:3 but also t9,t11,c15-18:3 and c9,t13,c15-18:3 (Shingfield *et al.*, 2010a). Additionally, other 18:3 isomers (c9,t12,c15-18:3; c9,t12,t15-18:3; t9,t12,t15-18:3) were observed in the rumen of dairy cows by Loor, Ueda, Ferlay, Chilliard and Doreau (2004).The sequential reduction of 18:3 BI leads to the formation of non-conjugated dienes, such as t11,c15-18:2 and c9,t13-18:2 (Destaillats, Trottier, Galvez & Angers, 2005) and the conjugated t13,c15-18:2. Moreover, Lee and Jenkins (2011) reported the conversion of 18:3n-3 into c9,t11-18:2, which was previously found to be only produced from 18:2n-6. Then, the reduction of 18:2 isomers originates 18:1 isomers including t11-18:1 and minor amounts of c15-18:1. It is to notice that, before t11,c15-18:2 is reduced, it can be isomerised to form t11,c13-18:2 (Vasta & Bessa,

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2012). Other studies have demonstrated an even more complexity of 18:3n-3 BH pathways compared with the initially described simplicity (Jenkins *et al*., 2008). In fact, new BI were proposed, such as *c*12,*c*15-18:2 (Bessa *et al*., 2007) and *t*10,*c*15-18:2 (Alves & Bessa, 2014). Both these non-conjugated 18:2 were found in the muscle of lambs and *t*10,*c*15-18:2 was also observed in their digestive contents. However, the basal diet fed to the animals varied between studies, since *c*12,*c*15-18:2 was present with dehydrated lucerne and 7.4% of linseed oil and *t*10,*c*15-18:2 was produced with a complete diet supplemented with 8% of an oil blend (soybean oil plus linseed oil, 1:2 vol/vol). The *t*10,*c*15-18:2 was suggested to be a BI of *t*10-shifted BH pathways. Recently, Honkanen *et al*. (2016) reported the formation of other 18:3n-3 BI, in incubated rumen contents, such as *c*7,*c*12,*c*15-18:3 and *c*8,*c*12,*c*15-18:3, which provides an evidence that the isomerization of 18:3n-3 may involve migration of *c*9, and not only of *c*12, double bond. In this study, small amounts of *t*9,*t*11,*c*13-18:3 and the conjugated *t*11,*c*13-18:2; *t*11,*t*13-18:2 and *t*11,*t*14-18:2 were also identified as a result of 18:3n-3 BH.

The extent of *c*9-18:1 BH is lower than that for 18:2n-6 and 18:3n-3 and generally varies between 58% and 87% (Shingfield *et al*., 2013). The *c*9-18:1 has been described as being directly reduced into 18:0 but a previous isomerization of this FA into numerous *trans*-18:1 isomers with double bond positions from C6 to C16 might also occur. Moreover, other BI of *c*9-18:1 may be present including derivatives of 18:0, such as the hydroxystearic acid 10-OH-18:0, which is further oxidized into the ketostearic acid 10-oxo-18:0 (Jenkins *et al*., 2008; Shingfield *et al*., 2010a).

In order to explain the occurrence of ruminal BH, some hypotheses have been proposed. The first one evaluated, consisted on the fact that the BH would be a mechanism for reducing power disposal, serving as hydrogen acceptor pathways. Nevertheless, this explanation does not seem probable, since a small proportion of hydrogen equivalents are removed by BH. Other more accurate hypotheses considered that BH could serve the purpose of PUFA detoxification associated or not with *trans*-18:1 production as an adaptive response to stress stimuli (Bessa *et al*., 2000). In fact, the toxic effects of PUFA to rumen bacteria have been demonstrated with a higher sensitivity of 18:0 producers (Maia, Chaudhary, Figueres & Wallace, 2007; Paillard *et al*., 2007). The incorporation of *trans*-18:1 into cell membranes may increase their stability and so, it was suggested as being a protective mechanism against environmental conditions including rumen lipid overload (Bessa *et al*., 2000; Vasta & Bessa, 2012).

1.3.3. The role of rumen microorganisms on lipolysis and biohydrogenation

The lipolytic activity has been reported in rumen bacteria and protozoa. In some metabolic studies concerning bacteria, *Anaerovibrio lipolytica* was able to hydrolyze diglycerides and triglycerides but not phospholipids and galactolipids. The lattest were shown to be hydrolysed by *Butyrivibrio* spp. and, posteriorly, the presence of esterase activity was even observed in *Butyrivibrio fibrisolvens*. So, *A. lipolytica* and *Butyrivibrio* spp. probably dominate the ruminal lipase activity in ruminants fed concentrate- and forage-based diets, respectively (Jenkins *et al*., 2008; Lourenço *et al*., 2010; Vasta & Bessa, 2012). There is a lack of knowledge about the role of the protozoa in lipolysis. Early studies suggested that 30% to 40% of the rumen lipolytic activity was caused by *Epidinium* spp. (Wright, 1961). However, it is possible that bacteria or feed chloroplasts ingested by protozoa were the main responsible for this activity (Harfoot & Hazlewood, 1997; Lourenço *et al*., 2010).

The principal bacteria described as being involved in rumen BH are cellulolytic bacteria belonging to the *Butyrivibrio* group, including the genera *Butyrivibrio* and *Pseudobutyrivibrio* (Vasta & Bessa, 2012). The importance of these bacteria in BH was initially verified by microbiological studies, which allowed the identification of *B. fibrisolvens* as responsible for the production of *c*9,*t*11-18:2 and *t*11-18:1 (Polan, Tove & Mcneill, 1964; Kepler, Hirons, Mcneill & Tove, 1966).

Afterwards, a phenotypical and functional classification of bacteria was proposed as follows: group A bacteria that hydrogenate 18:2n-6 and 18:3n-3 to 18:1 isomers, mainly *t*11-18:1, and group B bacteria that hydrogenate the same PUFA to 18:0. The *B. fibrisolvens* was considered as belonging to group A, while group B bacteria were initially classified as *Fusocillus* spp. (Kemp, White & Lander, 1975; Kemp & Lander, 1984). Nevertheless, in a later study by van de Vossenberg and Joblin (2003), a strain of a bacterium phenotypically similar to *Fusocillus* spp. that was able to produce 18:0 was described as phylogenetically close to *Butyrivibrio hungatei*. However, the classification of group B bacteria as *Fusocillus* spp. or *B. hungatei* was incorrect. In fact, phylogenetic analyses using 16S rRNA sequences of rumen bacterial strains identified the 18:0 producers as *Clostridium proteoclasticum* (Wallace *et al*., 2006; Paillard *et al*., 2007), which was reclassified as *Butyrivibrio proteoclasticus* by Moon *et al*. (2008). Interestingly, some *in vivo* studies on rumen bacterial diversity in cows and ewes suggested that *B. fibrisolvens* and *B. proteoclasticus* might not play a major role in BH, considering that other uncultured bacteria phylogenetically classified as *Prevotella* spp. and *Lachnospiraceae incertae sedis*, as well as some unclassified *Bacteroidales*, *Clostridiales*, and *Ruminococcaceae* are probably more relevant in these metabolic pathways (Belenguer, Toral, Frutos & Hervas, 2010; Huws *et al*., 2011; Castro-Carrera *et al*., 2014). In fact, according to Huws *et al*. (2011), *Prevotella* genus and

Lachnospiraceae and *Ruminococcaceae* families might be involved in the ruminal production of *c*9,*t*11-18:2, *t*11-18:1 and 18:0. Also, bacterial species belonging to the genera *Treponema-Borrelia*, *Micrococcus*, *Megasphaera* and *Eubacterium* have been associated with BH (Patra & Saxena, 2011). These recent advances are only possible due to the development of 16S rRNA based molecular analysing methods that allow for a more accurate estimation of bacteria diversity comparing with the traditional microbiological methods (Deng, Xi, Mao & Wanapat, 2008).

The structural modification of bacterial populations might be indirectly evaluated by the amount of odd- and branched-chain fatty acids (OBCFA) (Bessa *et al*., 2009; Fievez, Colman, Castro-Montoya, Stefanov & Vlaeminck, 2012) and dimethylacetals (DMA) (Saluzzi, Colin, Flint & Smith, 1995) present in the rumen. In fact, both DMA and OBCFA have the potential to be internal microbial markers, although DMA might even have a greater potential than OBCFA, since DMA are exclusively formed from structural lipids of bacteria, while OBCFA can result not only from *de novo* bacterial synthesis for incorporation into cell membranes (Fievez *et al*., 2012) but also from feedstuffs (Alves, Cabrita, Jeronimo, Bessa & Fonseca, 2011). In fact, DMA are originated from the acid catalysis of the vinyl-ether (alk-1´-enyl) chain present in plasmalogens, which constitute a special class of phospholipids characterized by the presence of a vinyl-ether bond at the sn-1 position of glycerol. The plasmalogens are also composed by aliphatic moieties at sn-1 and sn-2 positions (Brites, Waterham & Wanders, 2004), which consist of OBCFA with 15 to 18 carbons or even- and linear- chain FA (ECFA) (Miyagawa, 1982; Alves, Santos-Silva, Cabrita, Fonseca & Bessa, 2013b). Also, some BI, such as *trans*-18:1, can be incorporated in plasmalogens. These ether-phospholipids may have the role of providing plasticity to bacterial cell membranes, regulating their permeability (Goldfine, 2010). Several rumen bacteria were reported as containing great amounts of DMA, particularly strains of *B. fibrisolvens*, *Streptococcus bovis* and *Bifidobacterium thermophilus*, but also *Ruminococcus flavefaciens*, *Eubacterium ruminantium* and *Ruminococcus albus*. Moreover, Miyagawa (1982) reported the presence of anteiso-15:0, as the main DMA, in bacteria belonging to *Butyrivibrio* genus. The dietary substrate may alter the DMA composition of rumen contents (Saluzzi *et al*., 1995; Alves *et al*., 2013b) and, in one study, only modifications on the amount of individual DMA were found with the total of DMA remaining constant (Alves *et al*., 2013b). Considering the OBCFA, there are other difficulties when using these FA in the evaluation of the bacterial composition of rumen contents, especially considering the "iso-FA/anteiso-FA" ratio. In fact, although iso-FA, such as iso-17:0, were mostly associated with cellulolytic bacteria and anteiso-FA, mainly anteiso-15:0, were related to amylolytic bacteria (Fievez *et al*., 2012), in a report from Vlaeminck, Fievez, van Laar and Demeyer (2004), anteiso-15:0 and anteiso-17:0 were

produced by cellulolytic and odd- and linear-chain FA (OCFA) (15:0 and heptadecanoic acid, 17:0) by amylolytic bacteria.

The role of protozoa on BH is not completely understood due to the lack of studies concerning this issue comparing with the ones related to bacteria. However, these microorganisms are important sources of UFA to the host, accounting for 30 to 43% of the CLA and 40% of the *t*11-18:1 reaching the duodenum (Yáñez-Ruiz, Scollan, Merry & Newbold, 2006). In fact, protozoa were described as containing at least two to three times more UFA than bacteria (Devillard, McIntosh, Newbold & Wallace, 2006). Although one study verified that mixed rumen protozoa were able to convert 18:2n-6 into *c*9,*t*11-18:2 (Or-Rashid, AlZahal & McBride, 2008), other reports did not detect a participation of protozoa in BH (Devillard *et al*., 2006; Boeckaert *et al*., 2009). So, the most acceptable explanation for the high content of BI in protozoa consists in the association of these microorganisms with bacteria (endo- and ectosymbionts) or on the ingestion of bacteria by protozoa (Williams & Coleman, 1992; Vasta & Bessa, 2012). Some protozoan species were identified as being involved in BH, such as *Ophryoscolex caudatus*, *Entodinium nannelum* and the holotrich *Isotricha prostoma*, although the latter was described as only containing low concentrations of CLA and *t*11-18:1 (Devillard *et al*., 2006). Indeed, bacteria associated with *I. prostoma* were reported as responsible for the conversion of a limited amount of 18:2n-6 and *c*9,*t*11-18:2 into *t*11-18:1 (Boeckaert *et al*., 2009).

Reports concerning the involvement of fungi in BH are scarce. According to Nam and Garnsworthy (2007), one isolate belonging to *Orpinomyces* genus presented the highest rate of BH compared to other fungi isolates, playing a role in the formation of *c*9,*t*11-18:2 and *t*11-18:1. Moreover, Maia *et al*. (2007) considered the participation of *Neocallimastix frontalis* and *Piromyces communis* in these metabolic pathways but found that only *N. frontalis* could convert 18:2n-6 into *c*9,*t*11-18:2.

Another important group of microorganisms present in rumen microbiota are the methanogens that belong to *Archaea* domain and were described as responsible for ruminal methane production using CO_2 and H_2 as substrates (Johnson & Johnson, 1995). Although their involvement in BH is unknown, a modification of the abundance of methanogens might affect protozoa (Guo *et al*., 2008) and bacteria (Goel, Makkar & Becker, 2009). Indeed, Guo *et al*. (2008) verified a decrease of methane production together with a reduction of protozoa but without an inhibition of archaeal microorganisms in *in vitro* incubated rumen fluid, which evidences an association between methanogens and protozoa. As a matter of fact, the attachment of *Archaea* in the exterior surface of ciliate protozoa (Vogels, Hoppe & Stumm, 1980) and the endosymbiotic relation between the two groups of microorganisms (Finlay *et al*., 1994) were reported with methanogens from the most abundant methanogenic family in

the rumen (*Methanobacteriaceae*) appearing to be free-living or associated with protozoa (Sharp, Ziemer, Stern & Stahl, 1998). Moreover, Goel *et al*. (2009) observed a diminishment of the abundance of *R. flavefaciens* when methanogens were decreased in rumen contents from fermentation systems, which might be explained by an influence of a higher partial pressure of hydrogen on the metabolism of bacterial species caused by the reduction of *Archaea* (Wolin, Miller & Stewart, 1997).

1.3.4. The *t*10-shifted biohydrogenation pathways

Feeding ruminants in the fattening phase with high-starch and low-forage diets has been reported to lead to a shift from *t*11 to *t*10 BH pathways that is known as the *t*10-shift. The major consequence of the shift is the predominance of *t*10-18:1 to the detriment of *t*11-18:1, as the main *trans* monoene isomer in the rumen and tissues (Aldai *et al*., 2013; Bessa *et al*., 2015). The dietary supplementation with PUFA was described as not essential for the establishment of the shift (Bessa *et al*., 2005), although it was shown to exacerbate the accumulation of *t*10-18:1 that can reach up to 10% of meat FA (Bessa *et al*., 2005; Oliveira, Alves, Santos-Silva & Bessa, 2014). To date, there are three proposed *t*10-shifted BH pathways. Initially, Griinari and Bauman (1999) considered that both 18:2n-6 and 18:3n-3 could be converted into *t*10-18:1 as a final product. Before the formation of *t*10-18:1, these FA would be isomerized into *t*10,*c*12-18:2 and *t*10,*c*12,*c*15-18:3, respectively, with an additional reduction of *t*10,*c*12,*c*15-18:3 into *t*10,*c*15-18:2 (Figure 1). Lately, Mosley, Powell, Riley and Jenkins (2002) and Proell, Mosley, Powell and Jenkins (2002) suggested that *t*10-18:1 could be originated by an extensive double bond migration and *cis/trans* isomerization of 18:1 isomers, mainly *t*11-18:1 and *c*9-18:1. More recently, Kishino, Ogawa, Yokozeki and Shimizu (2009) and Kishino *et al*. (2013) described a multi-component enzyme system composed of four enzymes (hydratase, dehydrogenase, isomerase and saturase) from *Lactobacillus plantarum* that could produce *t*10,*c*12-18:2 and *t*10-18:1 from 18:2n-6 and *t*10,*c*15-18:2 from 18:3n-3, although no reduction of *t*10,*c*15-18:2 into *t*10-18:1 was verified. Also, 10-OH,*c*12-18:1 and 10-OH-18:0 would be hydrated into *t*10,*c*12-18:2 and *t*10-18:1, respectively. The BH pathways that allow for the conversion of 18:3n-3 into *t*10-18:1 are not clear and Zened, Enjalbert, Nicot and Troegeler-Meynadier (2013b) even reported their absence. Also, the presence of *t*10,*c*12,*c*15-18:3 in pure cultures, digesta or tissues was never verified (Bessa *et al*., 2015). However, Kemp *et al*. (1975) described the production of *t*10-18:1; *t*10,*c*15-18:2 and *c*10,*c*15-18:2 in a medium with 18:3n-3 and, in a study by Alves and Bessa (2014), the *t*10,*c*15-18:2 was found in digestive contents and meat of lambs expressing *t*10-shifted BH pathways.

The *t*10-shift can be evaluated by the "*t*10-/*t*11-18:1" ratio in tissues or more appropriately in abomasum or rumen contents. Nevertheless, a high variability of ratio values can be found among animals due to a variation in susceptibility to *t*10-shift induction, even with the same dietary conditions (Bessa *et al*., 2015). This fact might be caused by differences on individual rumen microbiota (Rosa *et al*., 2014; Santos-Silva *et al*., 2016) and breeds (Costa *et al*., 2013). Moreover, some studies have attempted to establish an association between the occurrence of *t*10-shift and a reduction of rumen pH but the results obtained were inconsistent (Piperova *et al*., 2002; Troegeler-Meynadier, Nicot & Enjalbert, 2007; Colman, Tas, Waegeman, De Baets & Fievez, 2012; Zened *et al*., 2013b). The relevance of these reports consists of the fact that *t*10-shift leads to milk fat depression syndrome in dairy ruminants (Griinari *et al*., 1998; Bauman & Griinari, 2003) and can be related other metabolic disorders, such as ruminal acidosis (Bessa *et al*., 2015). Although, it seems that a largely fluctuating low pH is necessary to promote the establishment of the shift (Bauman & Griinari, 2003; Colman *et al*., 2012), fast changes of pH in opposition to the progressive development of the shift makes difficult to relate pH and *t*10-shift (Zened *et al*., 2013b; Bessa *et al*., 2015). Also, a predominance of *t*11 BH pathways might occur at low pH, considering that the activity of linoleate isomerase that converts 18:2n-6 into *c*9,*t*11-18:2 and is produced by *B. fibrisolvens* has been described at a wide range of pH (5.5 to 8.7) (Kim, Liu, Bond & Russell, 2000). The time required for the occurrence of *t*10-shift was reported to be about 8 to 18 days, after starting to feed the animals with a high-starch plus sunflower oil diet, and then it remains constant (Roy, Ferlay, Shingfield & Chilliard, 2006; Zened *et al*., 2013b). Moreover, Zened *et al*. (2013b) verified a gradual increase of *t*10-18:1 until day 8 and an abrupt decrease of *t*11-18:1 on day 2, which probably indicates an adaptation of rumen microbiota in order to establish the *t*10-shift.

The rumen microbiota responsible for the *t*10-shift has been scarcely studied. However, some strains of *Megasphaera elsdenii*, mostly *M. elsdenii* YJ-4, were found to produce *t*10,*c*12-18:2 from 18:2n-6, when non-lactating dairy cows were fed a diet with grain at 90% dry matter (DM) (Kim, Liu, Rychlik & Russell, 2002). Nevertheless, in a study by McKain, Shingfield and Wallace (2010), *Propionibacterium acnes* was the only bacterial species that could form *t*10,*c*12-18:2 , being also able to convert *t*10-18:1 into 10-OH-18:0 and to dehydrate 10-OH-18:0 into 10-oxo-18:0. In fact, Verhulst, Janssen, Parmentier and Eyssen (1987) and Wallace, McKain, Shingfield and Devillard (2007) had already reported the production of *t*10,*c*12-18:2 from pure rumen cultures of *P. acnes* and Liavonchanka, Hornung, Feussner and Rudolph (2006) even described the structure and mechanism of a isomerase from *P. acnes* that catalyses the conversion of 18:2n-6 into *t*10,*c*12-18:2. Moreover, the involvement of other species of *Propionibacterium*, *P. freudenreichii*, in this BH step was verified by Jiang, Bjorck and Fonden (1998). Also, *Lactobacillus* genus, including *L.*

bulgaricus and eight strains of *Lactobacillus plantarum* were identified as producers of *t*10,*c*12-18:2, although these bacteria mainly formed *c*9,*t*11-18:2 and *t*9,*t*11-18:2, in the report by Yang *et al*. (2014). Similarly, Kishino *et al*. (2009) and Kishino *et al*. (2013) found that *L. plantarum* could be involved in the production of those BI and of *t*10-18:1, even though this bacterium has not been considered as major and specialized in the rumen. However, a great abundance of *Lactobacillus* spp. was verified when animals were fed high grain diets, as reviewed by Bessa *et al*. (2015). Little is known about bacteria responsible for the reduction of *t*10,*c*12-18:2 into *t*10-18:1 but McKain *et al*. (2010) identified *B. fibrisolvens* as associated with this BH step and also as a producer of small amounts of *t*12-18:1 and *c*12-18:1. Moreover, the unclear *t*10 BH pathways of 18:3n-3 might be established by *Ruminococcus albus* F2/6 (Kemp *et al*., 1975) or *Lactobacillus plantarum* (Kishino *et al*., 2009; Kishino *et al*., 2013). Bacteria belonging to *Ruminococcaceae* and *Lachnospiraceae* families and to *Prevotella* genus were found in the rumen of animals fed concentrate based-diets. Considering that *Prevotella* and *Lachnospiraceae* were described as possibly involved in *t*10 BH pathways (Toral *et al*., 2016), other bacteria present in high grain feeds may also play a role in the *t*10-shift.

Figure 1. Major (left side) and *t*10-shift (right side) biohydrogenation pathways of 18:2n-6 and 18:3n-3. Adapted from Bessa *et al*. (2015).
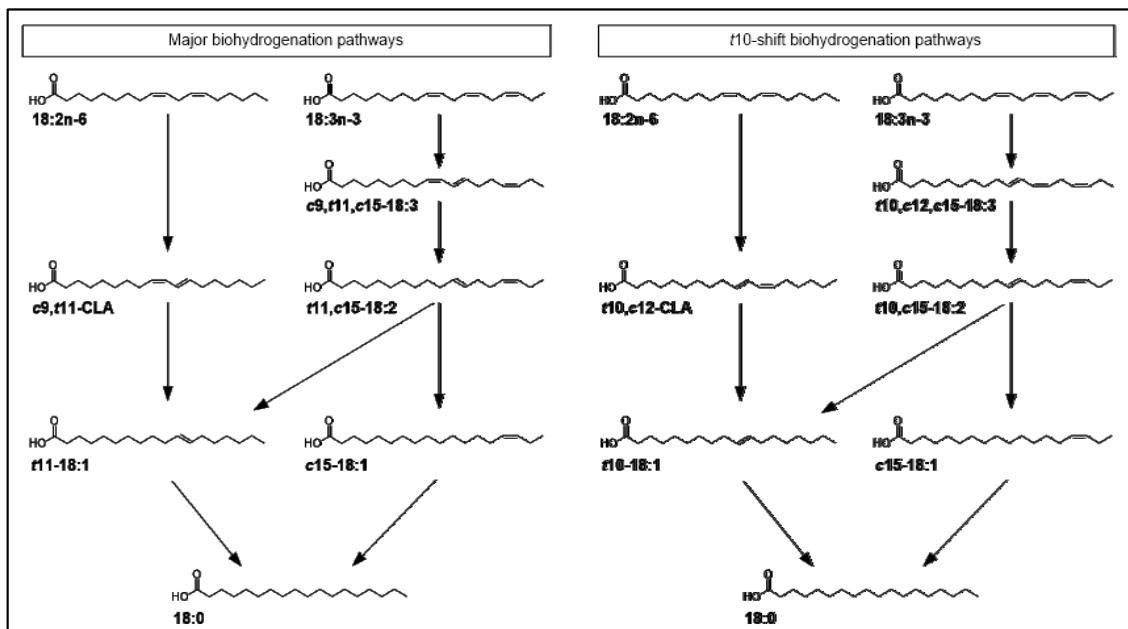
1.4. Fatty acid uptake, metabolism and deposition in tissues

1.4.1. Fatty acid uptake

Fatty acids leave the rumen as non-esterified FA (NEFA) and small amounts of 1,2-diacylglycerols and monoacylglycerols. After adsorption in the small intestine, most of them are esterified, in the enterocytes, into TAG, phospholipids and cholesterol esters, which are transported in the lymph as chylomicrons and very low density lipoproteins (VLDL) and further in the blood plasma as LDL and HDL (Vernon & Flint, 1988; Demeyer & Doreau, 1999; Shingfield *et al*., 2013). The majority of FA circulating in the plasma consists of cholesterol esters and phospholipids within HDL with NEFA generally accounting for only 3.5 to 5.8 mg/100 mL of plasma (Moore & Christie, 1979). However, TAG is the predominant lipid in the lymphatic system, comprising 70-80% of total lipid in sheep and cattle, and is the main source of FA for tissues (Vernon & Flint, 1988).

1.4.2. Fatty acid *de novo* synthesis

Fatty acids incorporated into the adipose tissue, such as the subcutaneous, intramuscular and mammary fat, are not only a result of NEFA and TAG uptake from plasma but also of *de novo* FA synthesis in the tissue (Shingfield *et al*., 2013).

The adipose tissue is the predominant site of *de novo* FA synthesis in ruminants but, during lactation, the mammary gland assumes more importance. This metabolic pathway occurs in the cytoplasm and consists in a series of decarboxylative condensation reactions with NADPH as a hydrogen donor. Initially, acetyl units derived mostly from acetate but also from glucose or lactate are added to acetyl-CoA that is transformed into malonyl-CoA by the acetyl-CoA carboxylase (ACC). Then, the FASN converts the malonyl-CoA into 16:0 and, to a minor extent, into 14:0 with the involvement of glucose-6-phosphate dehydrogenase or malic enzyme. The final products can serve as a substrate for further elongation and desaturation into LC-PUFA and desaturation into MUFA (Demeyer & Doreau, 1999; Nguyen *et al*., 2008; Shingfield *et al*., 2013). Two isoforms of ACC, ACCα or ACACA and ACCβ or ACACB, were reported in sheep with the first originating malonyl-CoA (Alvarenga, Chen, Furusho-Garcia, Perez & Hopkins, 2015).

1.4.3. Desaturation of monounsaturated and saturated fatty acids

De novo synthesized SFA and, probably in a lesser extent, exogenous SFA are desaturated in the muscle and mammary gland but mainly in the adipose tissue by SCD. This endoplasmic reticulum membrane bound enzyme (Shingfield & Wallace, 2014) is considered a marker of adipocyte differentiation (Graugnard, Berger, Faulkner & Loor, 2010) and is responsible for the desaturation of saturated or *trans*-monounsaturated fatty acyl-CoA substrates by introducing a *cis* double bond at carbon 9 position of the acyl chain, originating *cis*-9 MUFA and *cis*-9,*trans*-x dienes. The catalysis by SCD requires O_2, cytochrome b5 reductase and the electron acceptor cytochrome b5. The substrates include mainly 14:0, 16:0, 18:0 and *t*11-18:1 but also 17:0 and *t*7-18:1 and presumably *t*13-, *t*14-, and *t*15-18:1 (Shingfield *et al.*, 2013; Shingfield & Wallace, 2014; Bessa *et al.*, 2015). The *t*7,*c*9-18:2 (CLA) and *c*9,*t*11-18:2 are produced from *t*7-18:1 and *t*11-18:1, respectively. In fact, it was reported that about 20 to 30% of the *t*11-18:1 absorbed is desaturated into *c*9,*t*11-18:2 (Bessa *et al.*, 2015) and that the endogenous conversion is responsible for up to 87% of this CLA in tissues (Palmquist *et al.*, 2004). However, the major product of the SCD enzyme is *c*9-18:1, which contributes to the maintenance of membrane fluidity when incorporated into phospholipids (Shingfield & Wallace, 2014). Moreover, it was proposed that *t*9-16:1 can also be a substrate to produce *c*9,*t*11-18:2 through an elongation process due to FA elongases 5 (ELOVL5) and 6 (ELOVL6) followed by desaturation (Kadegowda, Burns, Miller & Duckett, 2013). Recently, Vahmani, Rolland, Gzyl and Dugan (2016) reported several other products of the SCD enzyme in the adipose tissue of steers, including *c*9,*t*12-18:2 and *c*9,*t*13-18:2, as well as some 18:2 isomers (*c*9,*t*15-18:2, *c*9,*t*14-18:2) that have been previously tentatively identified by Pollard, Gunstone, James and Morris (1980) and the novel *c*9,*t*16-18:2 and *t*6,*c*9-18:2.

In ruminants, two isoforms of SCD gene (SCD1 and SCD5) are present but the contribution of SCD5 to the desaturation of FA remains unclear. The expression of SCD1 gene is higher in the adipose tissue than in the muscle, being that the adipose tissue is the major site of desaturase activity in growing ruminants (Shingfield & Wallace, 2014; Bessa *et al.*, 2015).

Measurement of stearoyl-CoA desaturase activity and expression

Some assays have been performed in order to directly measure SCD protein activity using labelled radioactive subtracts. These assays consisted in the exposure of adipose tissue homogenates from mice to [14]C-18:0 (Enser, 1975) and from steers to ^{14}C-palmitoyl-CoA; (Archibeque, Lunt, Gilbert, Tume & Smith, 2005). However, the direct evaluation of SCD activity is difficult to conduct due to the fragile nature and close association with the endoplasmic reticulum membrane of this enzyme. So, the complex procedures for the measurement of activity must be conducted very quickly after tissue sampling. Moreover, the

lability of SCD might be enhanced by its denaturation caused by the successive detergent extractions that should be performed in order to isolate SCD from microsomes. Also, a microsomal protease may be responsible for a decline in SCD activity making its evaluation even more difficult (Hodson & Fielding, 2013). For these reasons, the quantification of SCD mRNA expression has been more extensively applied than the direct evaluation of SCD activity. However, the circadian nutrient absorption cycles with postprandial stimulation followed by under-expression of the gene makes a single measurement of SCD expression, as it is obtained with samples collected from ruminants slaughtered in standard commercial conditions that usually are submitted to a fasting period up to 24 h, hardly representative of the overall finishing period. So, a better alternative consists in the use of SCD product/substrate ratios, which allows an evaluation of SCD activity during the whole period of fat deposition. The "c9-17:1/(17:0 + c9-17:1)" ratio, named SCDi17, was suggested to be a suitable index to evaluate SCD activity, because it is mostly free of confounding effects. In fact, c9-17:1 is virtually absent in feedstuffs and is not detectable or is present in trace amounts in rumen and abomasum contents. Moreover, SCDi17 can explain about 80-90% of the "c9,t11-18:2/(t11-18:1 + c9,t11-18:2)" ratio, even considering that a low supply of t11-18:1 with an increased SCD activity is common in finishing ruminants fed with concentrate based-diets. Attending to the fact that a higher response of SCD activity to dietary treatments is present in NL than in PL fractions, the application of SCDi17 in relation to FA present in adipose tissue or muscle TAG is more accurate compared to FA in total meat (Bessa *et al*., 2015). Noticeable, it is possible to calculate isotopic desaturation indices. Indeed, Chong *et al*. (2008) proceeded to the intravenous administration of the stable isotope [^{2}H$_2$-16:0] in humans and calculated the "[^{2}H$_2$] c9-16:1/[^{2}H$_2$]-16:0" ratio. Nevertheless, the ratio values obtained by this method were considerably lower than the ones calculated from the non-isotopic measurement, because the isotopic index considers the fasting rather than the average SCD activity and does not reflect the desaturation of *de novo* synthesized 16:0. Consequently, although less confounding effects are present in the isotopic evaluation, the non-isotopic measurement seems to be a better approach to determine SCD activity (Hodson & Fielding, 2013).

1.4.4. Synthesis of long chain polyunsaturated fatty acids

The main focus of FA elongation and desaturation pathways has been on the conversion of dietary n-3 and n-6 PUFA, mostly 18:3n-3 and 18:2n-6, into LC-PUFA. These reactions are catalyzed by an enzymatic system consisting in fatty acyl-CoA synthetases, Δ6 and Δ5 desaturases and elongases (Barceló-Coblijn & Murphy, 2009). Contrarily to *de novo* FA synthesis, some studies indicated that the liver is the predominant site of LC-PUFA formation instead of the adipose tissue (Gruffat, Gobert, Durand & Bauchart, 2011; Cherfaoui *et al*.,

2012). In fact, Cherfaoui *et al*. (2012) reported that several proteins involved in the synthesis of n-3 LC-PUFA, such as elongases 2 and 5, were present in the intermuscular fat at a 10-fold lower abundance than in the liver.

For the conversion of 18:3n-3 and 18:2n-6, there is a competition for the same enzymes, although 18:3n-3 appears to be a stronger suppressor of n-6 LC-PUFA than 18:2n-6 of n-3 LC-PUFA formation. First, both 18:3n-3 and 18:2n-6 are desaturated into 18:4n-3 and 18:3n-6 by Δ6 desaturase, following a chain-elongation into 20:4n-3 and 20:3n-6 and desaturation by Δ5 desaturase into EPA and AA, respectively. Then, two elongation, one desaturation and one β-oxidation steps originate DHA and 22:5n-6 as final products corresponding to n-3 and n-6 series reactions, respectively. The elongation of 20:5n-3 is catalysed by elongase-2, forming docosapentaenoic acid (DPA; 22:5n-3) (Barceló-Coblijn & Murphy, 2009) (Figure 2). The elongation and desaturation pathways occur in two cellular compartments, such as the mitochondria, for FA synthesized *de novo*, and the endoplasmic reticulum (microsomes), for FA with at least 18 carbons. The β-oxidation step is present in the peroxisomes (Demeyer & Doreau, 1999; Barceló-Coblijn & Murphy, 2009). Several studies reported a low efficiency of conversion of 18:3n-3 into n-3 LC-PUFA, which contributes to the relevance of supplementing ruminants' diet with sources of n-3 LC-PUFA and not only of 18:3n-3. In fact, Scollan *et al*. (2001) described that, comparing with the incorporation of linseed oil as a source of 18:3n-3, the dietary inclusion of algae that are rich in DHA led to a 5-fold increase of DHA in the phospholipid fraction of *Longissimus* muscle of lambs, while a more modest enhancement of EPA (from 3.84 to 8.73% of total FA) was verified with a combination of fish oil and algae containing EPA in their compositions. Moreover, Scollan *et al*. (2001) observed that, in the phospholipids of muscle and compared to control treatment, the proportion of DHA was not modified by feeding steers with linseed oil, whereas fish oil caused a 2-fold increment of this FA. Additionally, a greater increase of EPA was reported for fish oil (2.31 to 4.87% of total FA; 10 to 24 mg/100g muscle), although an EPA proportion of 3.6% of total FA (15 mg/100g muscle) was still verified with linseed oil.

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Figure 2. Pathways of n-6 and n-3 LC-PUFA biosynthesis. Adapted from Barceló-Coblijn and Murphy (2009).
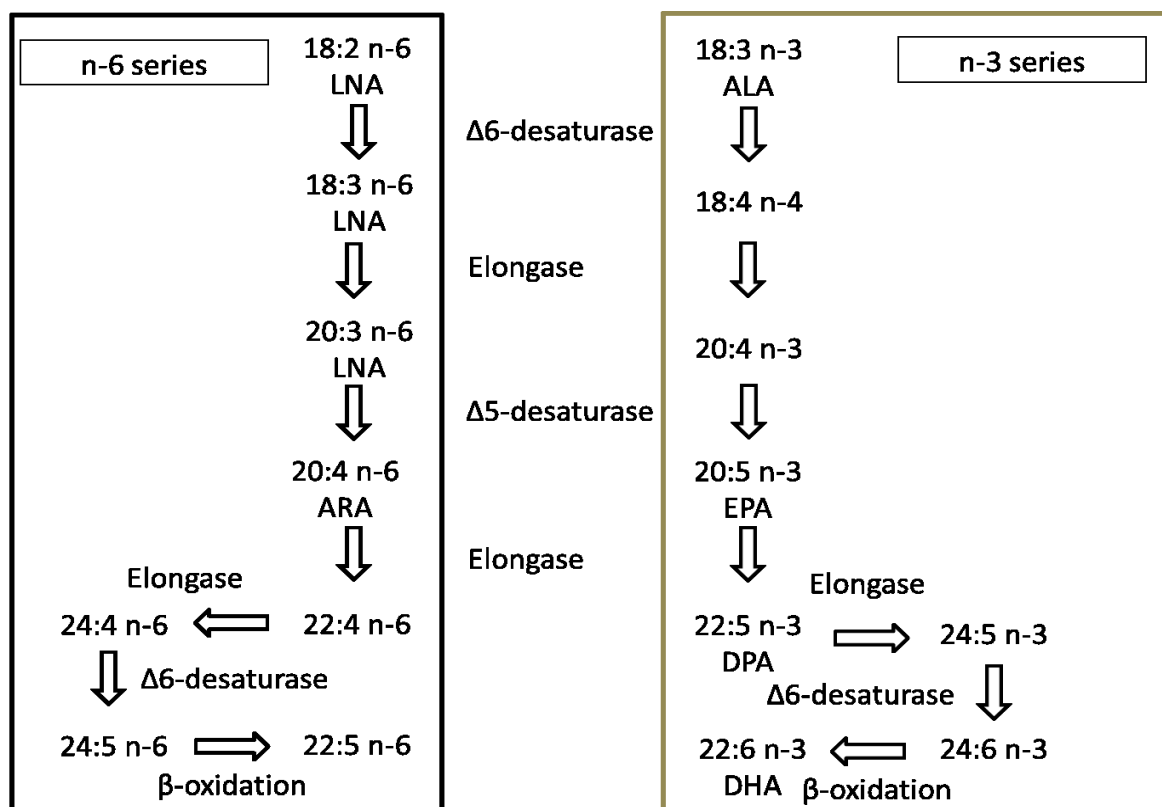
1.4.5. Factors affecting the fatty acid metabolism in tissues

The FA synthesis, desaturation and elongation can be affected by dietary and hormonal factors. Attending to FA desaturation of SFA and MUFA, concentrate feeds have been described as responsible for an increased expression of SCD gene, contrarily to forage diets (Duckett, Pratt & Pavan, 2009; Joseph *et al*., 2010; Buchanan *et al*., 2013; Costa *et al*., 2013; Bessa *et al*., 2015). This fact is mainly due to an insulinemic effect caused by a higher glucose availability promoted by the starch rich content of the concentrates. The influence of insulin is expected, considering its powerful activator effect on transcription factors that regulate SCD1 expression, mostly the sterol regulatory element-binding protein (SREBP)-1c and the nuclear factor Y (NF-Y). Interestingly, glucose and fructose may directly activate SCD1 transcription but the mechanism is not clear. The SCD enzyme has an adipogenic activity increasing TAG deposition in tissues, which leads to an increment of IMF content with the concentrate feed. This can be associated with a higher deposition of *c*9,*t*11-18:2 in this fat reservoir when the ruminal production of *t*11-18:1 is not compromised by the occurrence of *t*10-shifted BH pathways (Bessa *et al*., 2015). Indeed, Daniel, Wynn, Salter and Buttery (2004) verified a decrease of *c*9,*t*11-18:2 in the muscle and adipose tissue depots of

concentrate comparing with forage-fed lambs due to a reduced availability of *t*11-18:1 in the abomasum with the concentrate diet. Moreover, a lower insulinemia may not be the only reason for the decreased expression and activity of SCD with forage diets. In fact, the high content of PUFA, mainly n-3 PUFA such as 18:3n-3, might also act as an inhibitor (Waters, Kelly, O'Boyle, Moloney & Kenny, 2009; Herdmann, Nuernberg, Martin, Nuernberg & Doran, 2010; Corazzin, Bovolenta, Sacca, Bianchi & Piasentier, 2013), as well as the increase of *t*10,*c*12-18:2 (Chung, Choi, Kawachi, Yano & Smith, 2006; Shingfield & Wallace, 2014) and *c*9-18:1, the dietary addition of plant oils, the administration of cobalt EDTA or cobalt acetate and the influence of glucagon and thyroid hormone (Shingfield & Wallace, 2014). However, in some studies, the impact of these factors cannot be verified, as it is the case of n-6 PUFA (Shingfield *et al.*, 2013), *c*9-18:1 (Shingfield *et al.*, 2013; Choi *et al.*, 2014), *t*10,*c*12-18:2 and even n-3 PUFA (Choi *et al.*, 2014). Although SCD is mainly influenced by modifications of the diet, the ruminants' age and breed have also been reported to exert effects on the expression of this gene (Shingfield *et al.*, 2013). In fact, in one study that evaluated the distinction between two Portuguese bovine breeds (Barrosã and Alentejana), a higher SCD expression in the subcutaneous adipose tissue of Barrosã was verified probably due to a higher adipocyte maturity found in this breed (Costa *et al.*, 2013). Considering the age, a 6-fold higher SCD1 gene expression at sixteen than at twelve months of age in steers was described in literature (Choi *et al.*, 2014). Indeed, the greatest SCD1 gene expression observed with older ages has been also associated with an increased differentiation and volume of adipocytes (Chung, Lunt, Kawachi, Yano & Smith, 2007). The factors that affect FA synthesis are mostly the same that alter FA desaturation (Nguyen *et al.*, 2008). In fact, similarly to SCD, a higher ACACA (da Costa, Pires, Fontes & Mestre Prates, 2013) and FASN (Nguyen *et al.*, 2008; Joseph *et al.*, 2010) expression has been described with an increase of concentrate level in the diet, although, in one study, the ACACA gene expression was not affected by differences of the energy source and level in the feeds (Joseph *et al.*, 2010). Also, the ACACA activity is not always influenced by dietary n-3 PUFA (Herdmann *et al.*, 2010). Moreover, Kadegowda *et al.* (2013) and Chung *et al.* (2007) reported that the inhibition of SCD1 activity might be associated with reduced activity of enzymes responsible for FA de novo synthesis, but, in the study by Chung *et al.* (2007), this phenomenon was only found in forage fed animals. Additionally, genetic factors may alter the expression of ACACA and FASN and the animal gender can even play a role as it was shown a higher expression of these genes in females compared to males (Alvarenga *et al.*, 2015).

In some studies, a mechanism of negative feedback seems to regulate the expression and activity of SCD, ACACA and FASN. Indeed, a decrease of the endogenous *c*9,*t*11-18:2 synthesis by SCD was verified when the exogenous supply of CLA increased (Palmquist *et al.*, 2004). Also, a negative effect of malonyl-CoA on the expression of ACACA has been

reported (Bernard, Leroux & Chilliard, 2008) and, in one study, a lower amount of 16:0 and 14:0 caused an increase of FASN. The fact that the latter results were observed with a forage based diet and not with a concentrate feed is understandable, if the occurrence of a plateau in FASN expression originated by the high starch level feeding is considered (Buchanan *et al*., 2013).

Similarly to FA synthesis and desaturation, the LC-PUFA formation can be affected by many genetic and environmental factors, even though the FA composition of the diet is considered to be the most important one. Identically to SCD, ACACA and FASN, the expression of Δ5 (FADS1) and Δ6 (FADS2) FA desaturases genes was shown to be decreased by dietary PUFA, mainly due to a suppression of SREBP-1c (Nakamura & Nara, 2002; Herdmann *et al*., 2010). Also, peroxisome proliferators (PPs) might induce the expression of these genes, although this effect is enigmatic because the major role of PPs is to stimulate FA oxidation enzymes by activating the transcription factor PP-activated receptor-α (PPARα) (Nakamura & Nara, 2002). In addition, FA elongases and desaturases may be subjected to negative feedback by their end products. In fact, both AA and DHA were reported to suppress the conversion of 18:2n-6 and 18:3n-3 into n-6 and n-3 LC-PUFA, respectively (Shingfield *et al*., 2013).

1.5. Modulation of ruminal biohydrogenation and muscle fatty acid profile

Several nutritional strategies have been applied to improve the FA composition of ruminants' meat (Sinclair, 2007; Vasta & Bessa, 2012). Dietary manipulation can modify ruminal BH, allowing for an accumulation in the muscle of bioactive FA with health promoting functions, such as *t*11-18:1 and *c*9,*t*11-18:2 to the detriment of *t*10-18:1 and *t*10,*c*12-18:2, without compromising animal performance (Aldai *et al*., 2013). Also, some strategies have been used in order to increase n-3 PUFA deposition in tissues, contributing for the enhancement of meat nutritional value (Shingfield *et al*., 2013).

1.5.1. The type and proportion of dietary forage and concentrate

The modulation of BH and muscle FA composition in ruminants by modifying the proportion of forage and concentrate in the diet has been described in literature. Forages and pasture generally have a high content of 18:3n-3 in contrast with concentrate feeds, which are rich in 18:2n-6 (Shingfield *et al*., 2013). This fact can explain the increase of 18:3n-3 (Santos-Silva *et al*., 2002a; Bessa *et al*., 2005) and, in some studies, also the reduction of 18:2n-6 (Sinclair, 2007; Wood *et al*., 2008; Shingfield *et al*., 2013) found in the muscle of lambs reared and finished on grass comparing with the ones on concentrate, respectively. Different

forage species may also distinctively influence the proportion of these PUFA. In fact, it was reported that grazing legumes, such as lucerne or red clover, led to an enhancement of 18:3n-3 and particularly of 18:2n-6 in the muscle, compared to perennial ryegrass. Moreover, forage feeding has been associated with a decrease of 16:0 and c9-18:1 and with a higher proportion of 18:0 in the muscle (Sinclair, 2007). This modification of c9-18:1 and 18:0 might be related to a lower activity of SCD with forage diets than with concentrates. So, an increased t11-18:1 and a decreased c9,t11-18:2 deposition in the muscle might occur with forage-based diets due to a reduced endogenous conversion of t11-18:1 into c9,t11-18:2. The inferior amount of c9,t11-18:2 may also be caused by its lower ruminal production from 18:2n-6, the main PUFA converted into this CLA (Bessa et al., 2015). However, it was reported a higher proportion of both t11-18:1 and c9,t11-18:2 in the muscle of lambs fed ground and pelleted lucerne comparing with the ones fed concentrate (Bessa et al., 2005). This fact can be explained by the occurrence of t10-shifted BH pathways with high starch diets that leads to a substitution of t11-18:1 by t10-18:1. Even with a higher activity of SCD promoted by concentrate, the t10-18:1 cannot be converted into any CLA and simply accumulates in tissues as the main $trans$ monoene isomer. The greater deposition of t10-18:1 might be accompanied by an increase of t10,c12-18:2 (Bessa et al., 2005; Aldai et al., 2013; Bessa et al., 2015). In some studies, the impact of modifications of dietary "forage/concentrate" ratio on ruminal BH and muscle FA composition was analysed. For instance, Lee, Tweed, Dewhurst and Scollan (2006) verified no effect of a diminishment of the ratio (80/20 to 20/80) on the duodenal flow of total $trans$-18:1 with only a tendency for an enhancement of t10-18:1. Moreover, Vlaeminck, Fievez, Demeyer and Dewhurst (2006) reported an increase of t10-18:1, from 4.1 to 22.9 mg/g of total FA, in liquid associated bacteria (LAB) in the rumen of dairy cows when the ratio was decreased from 80/20 to 35/65 but, in the duodenal content and solid associated bacteria (SAB), t10-18:1 was only 2-fold higher with the concentrate-based diet comparing with the highest ratio treatment. Similarly to Lee et al. (2006) and Vlaeminck et al. (2006), an absence of effect of the proportion of forage on the amount of t11-18:1 was observed by Alfaia et al. (2009), together with an increase of t10-18:1 in the $Longissimus$ muscle of bulls fed exclusively or finished with concentrate (2 or 4 months) related to that of animals from pasture feeding. Also, Alfaia et al. (2009) reported a decrease of c9,t11-18:2 with forage feed and forage added with concentrate for 2 months comparing with the other feeding regimes (feedlot or 4 months of concentrate). Moreover, Rosa et al. (2014) verified a higher proportion of t10-18:1 in the muscle with the addition of ground maize at 4 kg/day to a forage-based diet compared to no addition (5.5 and 2.5 mg/g of total FA, respectively) and that was even more pronounced with increasing levels of incorporation (4 to 8 kg/day) (5.5 and 14.8 mg/g of total FA, respectively). Although t11-18:1 only decreased with the absence of maize, the c9,t11-18:2 tended to be

lower with the higher compared to the lower level of maize. Concerning the amount of *t*10,*c*12-18:2, no differences were found with modifications of "forage/concentrate" ratio in the studies by Lee *et al*. (2006), Alfaia *et al*. (2009) and Rosa *et al*. (2014).

Ruminants fed with forage- and concentrate-based diets are characterized by different rumen microbiota populations and the first normally have a higher bacterial diversity (Fernando *et al*., 2010). Fernando *et al*. (2010) reported a decrease of bacteria from *Fibrobacteres* and *Firmicutes* phyla and an increase of the ones belonging to *Bacteroidetes* and *Proteobacteria* in the rumen of steers fed with incremental levels of grain (20% to 80% DM). In fact, it was found a reduction of *B. fibrisolvens* (*Firmicutes*) and *Fibrobacter succinogenes* (*Fibrobacteres*). Conversely, *Prevotella bryantii* (*Bacteroidetes*), *M. elsdenii*, *Selenomonas ruminantium* were increased, although the last ones are included in *Firmicutes* phylum. The amylolytic *Streptococcus bovis* did not significantly change with the gradual increase of grain level in the diet and, in a study conducted by Klieve *et al*. (2003), its abundance remained constant when steers were fed with grain at 75% DM, which demonstrates the importance of this bacteria in both forage- and concentrate-based diets. Also, Klieve *et al*. (2003) reported an increase of *M. elsdenii* and a decline of *B. fibrisolvens* with the grain-based diet. Similarly to Fernando *et al*. (2010), Tajima *et al*. (2001) verified an increase of *S. ruminantium* and *P. bryantii* and a decrease of *F. succinogenes* when forage- was changed to concentrate-based diet. Noticeably, Castro-Carrera *et al*. (2014) suggested that the amylolitic *S. ruminantium* might occupy a special ecological niche in the rumen and, although its participation in BH is not clear, this bacterium is known to be involved in FA hydration, particularly, in the conversion of *c*9-18:1 into 10-OH-18:0 (Hudson, MacKenzie & Joblin, 1995). Moreover, a reduction of bacteria belonging to *Fibrobacteres* phylum was also found by Zened *et al*. (2013a), when starch was added to cows' diet from 22% to 33% DM. Additionally, Tajima *et al*. (2001) verified a decrease of *R. flavefaciens* and Zened *et al*. (2013a) described a general decline of *Ruminococcaceae*, together with a decrease of other cellulolytic bacteria, including *Butyrivibrio-Pseudobutyrivibrio* genera from *Lachnospiraceae* family. A reduction of unclassified *Clostridiales* and *Rikenellaceae*_RC9 with an increase of *Bifidobacterium* was also reported by Zened *et al*. (2013a). Besides *Clostridiales* and *Ruminococcaceae*, bacteria from *Bifidobacterium* genus were recently suggested to play a role on BH, since they were implicated in the production of CLA and CLNA isomers (Gorissen *et al*., 2010; Park *et al*., 2011).

1.5.2. Dietary lipid supplementation

Besides modifications of the proportion of forage and concentrate in the diet, dietary supplementation with plant oils or seeds rich in n-3 or n-6 PUFA has the potencial to allow for a higher concentration of these FA in tissues. However, the inclusion of FA rich supplements is limited to a maximum of around 60 g/kg DM, in order to avoid impairment of rumen function (Scollan *et al.*, 2006). Lipid sources presenting high amounts of 18:2n-6 include safflower, sunflower, canola or rapeseed and soybean oils, while 18:3n-3 is only commercially available in linseed or flaxseed, rapeseed and soybean oils. The linseed oil has the greatest content of 18:3n-3. These dietary PUFA supplements can be protected or unprotected from ruminal BH. Although protected sources are more efficient in promoting PUFA accumulation in tissues, the majority of studies evaluated the effect of the unprotected ones. In some of these reports, the supplementation with oils rich in 18:2n-6 has been associated with an increase of this FA in the muscle by approximately 0.5-fold (Sinclair, 2007) but the incorporation of whole linseed was shown to double the proportion of 18:3n-3 in the muscle and subcutaneous adipose tissue (Wachira *et al.*, 2002). The higher variation of 18:3n-3 might be due to the more selective incorporation of 18:2n-6 in phospholipids (De Smet *et al.*, 2004; Sinclair, 2007; Wood *et al.*, 2008). Moreover, the replacement of sunflower oil by linseed oil was related to an increase of 18:3n-3, *c*9-18:1 and n-3 LC-PUFA and a concomitant decrease of 18:2n-6 and n-6 LC-PUFA in the muscle, in order to maintain the degree of unsaturation of C18 FA in cell membranes (Jerónimo *et al.*, 2009), following the homeoviscous adaptation mechanism suggested by Scislowski, Durand, Gruffat-Mouty, Motta and Bauchart (2004). Also, there was a diminishment of *c*9,*t*11-18:2 with this substitution, which is probably caused by the decrease of 18:2n-6 (Jerónimo *et al.*, 2009). However, the supplementation with sources rich in 18:3n-3 is not always responsible for an enhancement of n-3 LC-PUFA in tissues and it can even be coupled with a decrease of n-3 LC-PUFA, which suggests an inhibition of 18:3n-3 metabolism, as demonstrated by Bessa *et al*. (2007) when linseed oil at 7.4% DM was added to a lucerne-based diet. So, the dietary incorporation of n-3 LC-PUFA sources is a more effective manner of increasing these FA (Sinclair, 2007), considering the extensive ruminal BH of n-3 PUFA and the low efficiency of elongation and desaturation enzymatic systems (Bessa *et al.*, 2015). The n-3 LC-PUFA supplements include marine products, mostly fish oils and meals and microalgae, with microalgae leading to considerable increases of n-3 LC-PUFA in the muscle of ruminants (Alvarenga *et al.*, 2015; Bessa *et al.*, 2015). In fact, DHA and EPA present in microalgae and also in encapsulated fat from fish oil were shown to have a lower rate of BH than the ones found in fish oil (Sinclair *et al.*, 2005). However, the addition of fish oil to a forage feed was reported to increase EPA and DHA from 0.7 to 2.3 and 0.3 to 0.8, respectively, in the muscle of lambs (Wachira *et al.*, 2002). Even though DHA is usually present in much lower

concentrations in ruminant meat than other C20 and C22 FA (Bessa *et al.*, 2015), its increase was reported to be up to 3.7-fold with fish oil and to 10.6-fold with fish oil plus marine algae in the muscle (Sinclair, 2007). Noticeably, the dietary supplementation with fish oils may also modify ruminal BH, since they have been considered as potent inhibitors of 18:0 production, leading to an increased flow of *trans*-18:1 and *trans*-18:2 leaving the rumen (Shingfield *et al.*, 2003; Lee *et al.*, 2008; Shingfield *et al.*, 2010b; Shingfield *et al.*, 2012). Moreover, Shingfield *et al.* (2012) reported an enhancement of C20 and C22 FA in the omasal digesta with the addition of fish oil, which was due to an extensive BH of DHA and EPA. The supplement even promoted an increase of 10-OH-18:0 + 9-oxo-18:0, 10-oxo-18:0, *trans*-16:1 and *trans/trans*-16:2. The effect on C16 FA was probably caused by an inhibition of their ruminal reduction into 16:0 (Shingfield *et al.*, 2010b; Shingfield *et al.*, 2011). Moreover, another methodological approach to increase the metabolic availability of n-3 LC-PUFA in tissues consists in the direct infusion of these FA in the abomasum, since it allows for n-3 LC-PUFA to bypass ruminal BH (Fortin *et al.*, 2010; Bessa *et al.* 2015).

The dietary lipid supplementation was reported to interact with the basal diet and induce changes in rumen bacterial populations possibly involved in BH. Attending to plant oils, Zened *et al.* (2013a) verified that the interaction between starch (33% DM) and sunflower oil (5% DM) caused an increase of *Lachnospiraceae incertae sedis* and a tendency for a higher abundance of *Prevotella*, as well as a decrease of unclassified or uncultured *Ruminococcaceae*, uncultured *Lachnospiraceae* and unclassified *Firmicutes*.

Moreover, a higher abundance of *Prevotella* in goats and *Lachnospiraceae* in cows was also found with a diet containing a high starch level (32.5% DM; cereal grain at 75.3% DM) plus sunflower oil (9% DM) and it was suggested that these bacteria were related to *t*10-shifted BH pathways, leading to an enhancement of *t*10-18:1 in the rumen, in relation with the control diet (without oil supplementation) (Toral *et al.*, 2016). The concomitant addition of plant oil (sunflower oil) (2.5 % DM) and marine algae (16% and 24% DM) in a basal diet with 48.5% of forage and 51.5% of concentrate fed to ewes was reported by Toral *et al.* (2012) as causing an increase of uncultured *Lachnospiraceae* (Butyrivibrio-related bacteria) and *Veillonellaceae* (*Quinella*-related bacteria) that might have been responsible, respectively, for an enhancement of *t*10-18:1 (McKain *et al.*, 2010) and, considering the phylogenetic similarity between *Quinella ovalis* and *S. ruminantium*, 10-oxo-18:0 (Krumholz *et al.*, 1993; Hudson *et al.*, 1995) in the rumen. Concerning fish oils, a gradual decrease of the abundance of *B. fibrisolvens* and *Pseudobutyrivibrio* in the omasal digesta of cows was related to an increase of fish oil level from 75 to 300 g/d added to a 58:42 forage-to-concentrate basal diet (Shingfield *et al.*, 2012). Conversely, there was a tendency for a dose-dependent increase of *P. acnes* and this bacterium might have been related to the occurrence of *t*10-shift (Bessa *et al.*, 2015) in the study by Shingfield *et al.* (2012), although

the ability of *P. acnes* to produce *t*10-18:1 was never clearly demonstrated. In the report by Toral *et al.* (2016), a concentrate-based diet (starch level of 23.9% DM; cereal grain at 54.9% DM) plus fish oil (3.6% DM) led to a decrease of some bacterial species, such as bacteria related to *Ruminococcaceae* family in cows and to *Pseudobutryrivibrio* genus in goats, comparing with the control treatment.

1.5.3. Plant secondary compounds – Tannins

Characterization, localization and nutritional effects of tannins

Tannins are polyphenolic compounds that constitute secondary metabolites of plants with variable but relatively high molecular weights. They have the ability to form complexes with proteins and to a lesser extent with carbohydrates. Based on the molecular structure, tannins are classified into hydrolysable (HT) and condensed tannins (CT). The HT include gallotannins and ellagitannins that result from the esterification of a central core (polyol) with phenolic groups (gallic acid and hexahydroxydiphenic acid, respectively), while the CT consist of proanthocyanidins and are polymers of flavan-3-ol units ((epi)catechin and (epi)gallocatechin). Tannins may be present in forage trees, shrubs and legumes, cereals and grains and their concentrations are normally higher in new leaves and flowers. Beneficial or detrimental nutritional effects can be promoted by the presence of tannins in ruminants' diets. The benefits include increased milk yields and fertility, higher growth rates due to an enhanced amount of protein that is available for digestion in the small intestine, increased wool growth, improved animal welfare through prevention of bloat and parasitism and inhibition of methanogenesis (Waghorn & McNabb, 2003; Mueller-Harvey, 2006; Patra & Saxena, 2011). The deleterious effects consist of lower digestibility of protein and dry matter with carbohydrate, starch and plant cell wall being less affected. The decrease of diet´s digestibility with polyphenolic compounds can be caused by their binding to dietary substrates and digestive enzymes and also by their toxicity towards intestinal and rumen microorganisms (Mueller-Harvey, 2006; Patra & Saxena, 2009). In particular, the depression of fibre digestion by tannins might lead to a decrease of rumen fermentative activity, including a lower total volatile fatty acid (VFA) production (Patra & Saxena, 2011), while that effect on protein may originate a reduction of branched-chain VFA (Bhatta *et al.*, 2009; Hassanat & Benchaar, 2013) as these FA result from the breakdown of the carbon skeleton of amino acids (Van Soest, 1994). Overall, the influence of tannins on digestibility of dietary compounds can cause an impaired animal productive performance characterized by lower live weight gains, milk yield and wool growth (Mueller-Harvey, 2006). Hydrolysable tannins can be hydrolysed by microbial cleavage of ester bonds and depside linkages but no

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depolymerisation of CT has been demonstrated under anaerobic conditions (McSweeney, Palmer, McNeill & Krause, 2001b). So, HT were considered to have a higher toxicity than CT, since products resulting from their hydrolysis might be toxic to ruminants once they are absorbed (Reed, 1995). The occurrence of beneficial or detrimental effects of tannins depends on the molecular type, dose and time of administration and the animal species involved (Frutos *et al*., 2004; Toral *et al*., 2013).

Influence of tannins on biohydrogenation

The inclusion of tannins in ruminants' diets may be responsible for a modulation of BH. In fact, these polyphenolic compounds have been shown to inhibit the last step of BH, reducing the production of 18:0 and consequently promoting the accumulation, in the rumen, of *trans*-18:1, mostly of *t*11-18:1 *in vitro* (Khiaosa-Ard *et al*., 2009; Vasta, Makkar, Mele & Priolo, 2009a; Buccioni, Minieri, Rapaccini, Antongiovanni & Mele, 2011; Carreño, Hervas, Toral, Belenguer & Frutos, 2015) and *in vivo* (Vasta *et al*., 2009b; Vasta *et al*., 2010a) but also of *t*10-18:1, in some *in vivo* reports (Vasta *et al*., 2009b; Alves, Francisco, Costa, Santos-Silva & Bessa, 2017), when tannins were added to a concentrate-based diet. However, these results are inconsistent, considering that other studies suggested a depression of the first steps of BH that led to an enhancement of 18:3n-3 in the ruminal fluid (Kronberg *et al*., 2007) and an accumulation of 18:2 and 18:3 in the milk of dairy ewes fed with sulla (*Hedysarum coronarium*) (Roy *et al*., 2002; Cabiddu *et al*., 2009) or even a stimulation of BH with decreased 18:2n-6 and 18:3n-3 and increased *t*11-18:1; *c*9,*t*11-18:2 and 18:0 in the rumen content of lactating ewes, when grape seed was incorporated in the diet (Correddu *et al*., 2015). The influence of tannins on BH can be conditioned by the same factors that determine the occurrence of beneficial or detrimental effects caused by these compounds. Indeed, in an *in vitro* study by Carreño *et al*. (2015), different doses of condensed (*Schinopsis lorentzii* - quebracho and *Vitis vinifera* - grape) and hydrolysable (*Castanea sativa* - chestnut and *Quercus* spp. - oak) tannin extracts were analysed (20 to 80 g/kg DM). Curiously, although both tannin types promoted an accumulation of 18:2n-6, 18:3n-3 and a decrease of 18:0 with a tendency for an increase of *t*11-18:1, these effects were more pronounced with the lowest doses of tannins, particularly with oak extract at 20 g/kg DM. Similarly, Buccioni *et al*. (2011) found an increment of *t*11-18:1 and *c*9,*t*11-18:2 in SAB present in rumen contents with tannin extracts from chestnut and quebracho at 49 g/kg DM comparing with the same compounds at 82 g/kg DM, except for an increased accumulation of *c*9,*t*11-18:2 with CT at 82 g/kg DM after 18h of incubation. A reduction of 18:0 production was also verified with both tannin types until 18h but this effect was more evident with the higher concentration of HT. So, in general, only marginal differences were found between the two types of tannins, regardless

their doses in the diet. However, in the report by Vasta *et al*. (2009a), the higher dose (1 mg/mL) of CT from carob (*Ceratonia siliqua*), acacia (*Acacia cyanophylla*) and quebracho led to a greater inhibition of the last BH step than the lower one (0.6 mg/mL). Moreover, Buccioni *et al*. (2011) verified a more pronounced effect of tannins with an increase of the incubation time from 6h to 18h. Conversely, in an *in vivo* experiment by Toral, Hervas, Bichi, Belenguer and Frutos (2011), a 1:1 (wt/wt) mixture of quebracho and chestnut tannin extracts did not considerably influence the FA profile in milk and no differences between times of exposition of dairy ewes to these compounds were observed. Nevertheless, the markedly low dose of tannins (10 g/kg DM) or the combination of two sources used by Toral *et al*. (2011) might be the reason for the discrepancies of these results. So, the combined influence of dose and time should be considered when evaluating the effect of tannins on BH, since a gradual adaptation of rumen microbiota to the presence of polyphenolic compounds can occur (Makkar, 2003). Noticeably, the diet´s composition can also modulate the action of tannins. In fact, in one *in vivo* study, only when tannins were added to a concentrate-based diet and not to herbage feed, an inhibition of 18:0 production with a consequent accumulation of *trans*-18:1 occurred in the rumen. The lower effects of tannins on ruminal BH observed in forage-fed lambs were probably caused by the higher neutral detergent fibre (NDF) and PUFA intakes promoting a more favourable environment for the development of BH with the treatment, although feed selection and a consequent inferior ingestion of tannins could not be exclude for that diet (Vasta *et al*., 2009b).

One of the factors that might be responsible for the modulation of BH by tannins consists of a modification of rumen bacterial growth and activity. This mechanism was suggested to be species-specific with a maintenance of total microbial protein (Min *et al*., 2002) and bacterial abundance (Vasta *et al*., 2010a), although Khiaosa-Ard *et al*. (2009) described an enhancement of bacterial counts in rumen contents incubated with *Acacia mearnsii* extract (79 g/kg DM of CT). In the study by Vasta *et al*. (2010a), feeding lambs with 95.7 g/kg DM of quebracho tannin extract increased the relative quantity of *B. fibrisolvens* and tended to decrease the one of *B. proteoclasticus* in the rumen. However, a reduction of *B. fibrisolvens* C211a and also of *B. proteoclasticus* B316, *Eubacterium* sp. C12b and *S. bovis* B315 was reported when *Lotus corniculatus* (CT at 32 g/kg DM) was incorporated in sheep´s diet (Min *et al*., 2002). Additionally, an inhibition of *B. fibrisolvens* growth was found by Jones, Mcallister, Muir and Cheng (1994) with sainfoin (*Onobrychis viciifolia*) containing CT from 200 to 600 µg/mL. Moreover, the latter authors verified a floculation of *S. bovis* (CT≥100 µg/mL) and a little effect of tannins on the abundance of *Prevotella ruminicola* and *Ruminobacter amylophilus*. Moreover, Wang, Alexander and McAllister (2009) reported an increase of the non-cellulolytic *S. ruminantium*, *P. bryantii* and *R. amylophilus*, a decrease of the cellulolytic *F. succinogenes* and *R. albus* and no effect on *R. flavefaciens* during 24h of

incubation with phlorotannins (500 µg/mL) from the algae *Ascophyllum nodosum*. The effect of tannins on cellulolytic bacteria is in agreement with their predominant influence on SAB, which include the majority of these microorganisms (Michalet-Doreau, Fernandez, Peyron, Millet & Fonty, 2001) and have been characterized by a high proportion of BI (Vlaeminck *et al*., 2006; Bessa *et al*., 2009), compared to LAB (Buccioni *et al*., 2011; Minieri *et al*., 2014).

The structure of tannins and in particular their degree of polymerization may influence the effect of these compounds on rumen bacteria. Indeed, Sivakumaran *et al*. (2004) described a higher inhibition of the abundance of *B. fibrisolvens* CF3 with low and medium molecular weight (MW) proanthocyanidins fractions from *Dorycnium rectum* at 100 µg/mL of medium than with the high MW fraction. The last one even stimulated the growth of this bacterium during the first 6h of incubation. The greater inhibition of bacterial growth with low and medium MW fractions was also reported for *B. proteoclasticus* B316^T with proanthocyanidins at 200 µg/mL. Conversely, Durmic *et al*. (2008) found a higher susceptibility of *B. proteoclasticus* P 18 to tannins than that of *B. fibrisolvens* JW 11, but the discrepancies between results might be due to the use of different bacterial strains. Other bacteria possibly involved in BH have shown resistance to tannins. Indeed, *Selenomonas* species, particularly *S. ruminantium*, were found to be tolerant to CT present in *Acacia angustissima* (Odenyo *et al*., 2001; Krause, Smith & McSweeney, 2004) and *Calliandra calothyrsus* (Odenyo *et al*., 2001). Moreover, Odenyo *et al*. (2001) identified two isolates that were resistant to these tannin sources, one was classified as Streptococcus spp., with the same profile as *S. caprinus*, and the other was indicated as *B. fibrisolvens*. Oppositely, strains of *B. fibrisolvens* analysed by Krause *et al*. (2004) were sensitive to tannins. Still, the latter authors verified that *Streptococcus gallolyticus*, a *S. bovis*-related species, was tolerant to these compounds. Additionally, Brooker *et al*. (1994) described the resistance of *S. caprinus* in the rumen of feral goats browsing tannin-rich *Acacia aneura*.

Besides the influence of tannins on bacterial abundance, the action of these compounds on enzymes responsible for FA production has been evaluated. In fact, tannins might not only induce morphological changes on the cell wall and consequently alter the growth of some bacteria but they have also the ability to adhere to bacterial cell wall binding extracellular enzymes and to enzymes secreted by microorganisms (Makkar, 2003; Patra & Saxena, 2011). Additionally, the phenolic compounds may inhibit lipolysis and PUFA BH by entrapping lipids within protein-phenol complexes. This mechanism was proposed for the oxidation products of these compounds (quinones) present in red clover (*Trifolium pratense*) and produced through the action of polyphenol oxidase (PPO) (Lee, Tweed, Cookson & Sullivan, 2010; Van Ranst, Lee & Fievez, 2011). Moreover, Cabiddu *et al*. (2010) described a stronger inhibition of lipolysis and BH with tannic polyphenols from vetch (*Vicia sativa*) and especially from crimson clover (*Trifolium incarnatum*) than with quinones. Considering the

linoleic acid isomerase (LA-I), Vasta *et al.* (2009a) found that the activity of this enzyme was not affected by tannins, although there was a decrease of total CLA produced by LA-I. However, Vasta *et al.* (2010a) reported a decrease of LA-I activity but the presence of tannins did not influence the production of CLA and even caused an accumulation of *c*9,*t*11-18:2. This lack of relation between LA-I activity and CLA produced in the rumen, together with an effect of tannins on VFA production (Vasta *et al.*, 2009a) and on the concentration of microbial protein (Vasta *et al.*, 2009a; Vasta *et al.*, 2010a) probably indicate that tannins interfered with bacterial activity and not with LA-I *per se*. The influence of polyphenolic compounds on VFA production described by Vasta *et al.* (2009a) consisted of a diminishment of iso-butyric (iso-4:0) and iso-valeric (iso-5:0) acids, which are precursors of BCFA (Fievez *et al.*, 2012), and of "acetate (2:0)/propionate (3:0)" ratio that might be associated with a reduction of cellulolytic bacteria (Vasta & Bessa, 2012). Attending to SCD, in the *in vivo* study by Vasta *et al.* (2009c), there was a higher expression of the enzyme when lambs were fed a forage based diet supplemented with quebracho tannin extract at 89.3 g/kg DM. This response was verified with forage but not with concentrate feed, which can be due to a different effect of tannins on the FA (Vasta *et al.*, 2009b) and protein (Priolo, Micol & Agabriel, 2001) absorbed depending on the type of diet. Also, an increase of SCD activity (Rana, Tyagi, Hossain & Tyagi, 2012) and indices (Whitney, Lupton & Smith, 2011; Rana *et al.*, 2012) under the influence of tannins was reported.

Influence of tannins on muscle fatty acid profile

The modulation of ruminal BH by tannins affects the muscle FA profile. In fact, the supplementation of male goat kids' diet with extract of *Terminalia chebula* containing 497.1 g/kg of total tannins led to an increase of total CLA, particularly of *c*9,*t*11-18:2, and a decrease of 18:0 in the *Longissimus* muscle. The effect of the phenolic compounds on BH was more pronounced with the higher (3.18 g/kg of BW) than with the lower (1.06 g/kg BW) dose of plant extract. These results are probably due to an inhibition of the last step of BH, since the same pattern was observed in the ruminal fluid with even an enhancement of ruminal *t*11-18:1, and the increased activity of SCD. An improvement of the nutritional value of meat was also obtained when tannins were added to the diet, with a greater deposition of total MUFA and PUFA and a decrease of SFA content in the muscle (Rana *et al.*, 2012). Similar results were reported by Whitney *et al.* (2011) when cottonseed hulls replaced redberry juniper (*Juniperus pinchotii*) leaves in lambs' diet. These authors verified an increase of *c*9,*t*11-18:2 and a tendency for a reduction of 18:0 in the muscle with the increment of the total CT incorporated (31 to 44 g/kg DM). Previously, an enhancement of *c*9,*t*11-18:2 was also observed by Jerónimo *et al.* (2010), together with an increase of *t*11-18:1 in the muscle, after feeding lambs with rockrose (*C. ladanifer*) at 250 g/kg of DM (21 g

of CT/kg DM) in combination with 6% of sunflower and linseed oils (1:2, vol/vol). However, a decrease of 18:0 was only present in abomasal digesta. In the latter study, the inclusion of grape seed extract at 25 g/kg of DM (averaging 14 g of CT/kg DM) in the diet did not cause major effects on the FA profile of abomasum and muscle. The distinct influence of the two tannin sources on the BI pattern was probably due to differences of CT structure and dose or the presence of other secondary compounds in leaves and soft stems of *C. ladanifer* (Gomes, Mata & Rodrigues, 2005; Sosa, Alias, Escudero & Chaves, 2005). In a recent study, an interaction between increasing levels of *C. ladanifer* and oil blend (soybean and linseed oils; 1:2, vol/vol) was also described but, instead of promoting the accumulation of *c*9,*t*11-18:2 and *t*11-18:1 in the muscle, it induced an increment of *t*10-18:1; *t*10,*t*12-18:2; *t*10,*c*12-18:2 and *t*7,*c*9-18:2 deposition, which can be probably explained by the establishment of *t*10-shift caused by a lower forage:concentrate proportion (1:1) (Francisco *et al*., 2016) than the one (9:1) considered in the report by Jerónimo *et al*. (2010).

1.5.4. Alternative energy sources – Agro-industrial by-products

The use of agro-industrial by-products in ruminants' nutrition has been recently adopted as an alternative to cereals, in order to reduce feeding costs and to recycle waste material without the need for costly waste disposal, as well as to diminish the dependence of animal diets on grains that can be consumed by humans. These compounds, including citrus pulp, sugar beet pulp and soybean hulls, may replace the cereal without compromising animal performance (Bampidis & Robinson, 2006; Vasta *et al*., 2008). Citrus and sugar beet pulps have a high content of pectic substances, which are part of the soluble fibre, and sugars (Fegeros, Zervas, Stamouli & Apostolaki, 1995; Leiva, Hall & Van Horn, 2000; Bampidis & Robinson, 2006). The sugar beet pulp also presents great amounts of structural fibre, mainly NDF composed of readily fermentable polysaccharides (Vasta *et al*., 2008), but soybean hulls are richer in this type of fibre (Garleb, Fahey, Lewis, Kerley & Montgomery, 1988) (Table 1).

Table 1. Chemical composition of agro-industrial by-products (g/kg dry matter). Adapted from Feedipedia (2017).

Chemical composition	Dehydrated citrus pulp	Dehydrated beet pulp	Soybean hulls
Dry matter[1]	896	892	891
Crude protein	70	93	131
NDF[2]	211	481	644
ADF[3]	154	241	462
Ether extract	24	9	22
Starch	75	5	52
Sugar	245	76	16
Ash	69	77	52

[1]g/kg feed; [2]neutral detergent fibre; [3]acid detergent fibre

Influence of alternative energy sources on animal productivity and meat quality

The replacement of cereal by 24% to 35% of citrus pulp (Caparra, Foti, Scerra, Sinatra & Scerra, 2007; Rodrigues *et al*., 2008; Inserra *et al*., 2014) or the concomitant substitution of forage and 30% of concentrate in the diet (Scerra, Caparra, Foti, Lanza & Priolo, 2001) by this by-product showed no adverse effects on lambs' productivity and meat quality. However, a higher incorporation of citrus pulp (45%) caused impaired feed conversion efficiency and lower carcass weights and dressing percentage (Caparra *et al*., 2007), even considering that, in a study by Bueno, Dos Santos, Da Cunha, Neto and Veríssimo (2004), the total replacement of maize by the alternative energy source did not affect animal performance. Moreover, there was no effect of citrus pulp on meat colour in the report by Scerra *et al*. (2001), but Caparra *et al*. (2007) described a decrease of a* and C* values in the meat with the by-product. A similar modification of colour with a reduction of b* was reported by Inserra *et al*. (2014), although the meat colour stability was maintained over storage time. This fact might be associated with a decrease of lipid oxidation (Faustman, Sun, Mancini & Suman, 2010) promoted by high levels of bioactive compounds with antioxidant properties, such as polyphenols and flavonoids, found in citrus pulp (Abeysinghe *et al*., 2007; Tripoli, La Guardia, Giammanco, Di Majo & Giammanco, 2007). Moreover, no effect on L* value was observed by Inserra *et al*. (2014). Conversely, Lanza, Priolo, Biondi, Bella and Salem (2001) verified an increase of this value with the dietary incorporation of carob and orange pulps (10% of each), which was probably due to the presence of CT in citrus pulp (Bampidis & Robinson, 2006). Additionally, meat sensorial characteristics were not affected in the studies by Scerra *et al*. (2001) and Caparra *et al*. (2007), even though Scerra *et al*. (2001) suggested that there was probably a slightly increase of meat tenderness, since shear force tended to be lower with citrus pulp (Shackelford *et al*., 1991; Shackelford, Wheeler & Koohmaraie, 1995).

There was no effect of beet pulp incorporation in lambs' diet (70% DM) on subcutaneous fat colour and productive performance (Normand *et al*., 2001). Also, Olfaz, Ocak, Erener, Cam and Garipoglu (2005) found no impairment of sheep's productivity and carcass traits with the replacement of forage by 40% and 60% of the by-product. In fact, a beneficial effect of beet pulp was even observed by Olfaz *et al*. (2005), since higher feed efficiency and lower "DM intake (DMI)/ average daily gain (ADG)" ratio values were obtained with both dietary inclusion levels. Moreover, meat colour and sensorial and physical characteristics were not influenced by the incorporation of the by-product, except for a decreased pH with 40% and 60% of replacement and an increased L* value with the higher level. However, these results might be due to stress conditions before slaughter, chilling regime or carcass processing and not caused by a direct influence of beet pulp (Olfaz *et al*., 2005).

The effect of replacing maize with soybean hulls (up to 15%) was evaluated by Santos *et al*. (2010) and a higher NDF intake with the by-product without any other effect on intake and digestibility were described. Moreover, Zervas, Fegeros, Koytsotolis, Goulas and Mantzios (1998) and Ludden, Cecava and Hendrix (1995) reported that the inclusion of soybean hulls at 20 to 60% DM caused an increase of NDF digestibility. Additionally, Ipharraguerre, Shabi, Clark and Freeman (2002) observed an enhancement of NDF and acid detergent fibre (ADF) intake and digestibility, together with lower non-structural carbohydrates digestibility, when the by-product replaced maize from 10 to 40% DM and these findings were even more evident with the highest level of substitution. The results described are mainly due to the distinct composition of soybean hulls and cereal (Vasta *et al*., 2008), which can also affect the productive performance of ruminants. In fact, soybean hulls present lower energy content (National Research Council [NRC], 2007), as well as smaller size and higher specific gravity of particles than cereals (Ipharraguerre & Clark, 2003), and that can contribute a different influence of these energy sources on animal growth. Hsu *et al*. (1987) reported a decrease of ADG and feed efficiency of lambs with soybean hulls at 50% of DM in a concentrate-based diet comparing with the same amount of maize, but there was no difference between energy sources when they were used at higher levels (70% of DM), except for an enhancement of DMI. Posteriorly, Ludden *et al*. (1995) observed an association between increasing levels of soybean hulls (up to 60% of DM) and lower ADG and feed efficiency and higher DMI in steers. Similar results were found by Ferreira *et al*. (2011a), although no effect of the energy source on ADG of lambs were presented when soybean hulls were gradually incorporated to 32.4% of DM. The discrepancies among results might be due to distinct levels of dietary supplementation with soybean hulls or even to differences between animal species. The composition of the basal diet may also constrain the impact of soybean hulls on productive performance, considering that, in general, no effect of the by-product on ADG (Anderson, Merrill & Klopfenstein, 1988a; Anderson, Merrill, Mcdonnell & Klopfenstein, 1988b), DMI and

feed to gain ratio (Anderson *et al.*, 1988b) was found in steers fed forage based-diets, although higher DMI and lower feed efficiency with ground soybean hulls at the rate of 2.1 kg DM/hd/d than maize were reported in the study by Anderson *et al.* (1988b). Considering meat quality, Rossi *et al.* (2016) described no modification of this factor when maize was replaced for an average of 23% of soybean hulls, except for a decreased meat shear force that might indicate an increase of meat tenderness (Shackelford *et al.*, 1991; Shackelford *et al.*, 1995).

Influence of alternative energy sources on biohydrogenation and tissue fatty acid profile

The partial replacement of barley by 24% and 35% of citrus pulp was responsible for an increase of *c*9,*t*11-18:2 in the *Longissimus* muscle and of *t*11-18:1 in the plasma of lambs, according to Lanza *et al.* (2015). Additionally, the higher level of incorporation led to an enhancement of *c*9,*t*11-18:2, together with a decrease of 18:0, in the plasma. A possible explanation for these results consists in the presence of phenolic compounds in citrus pulp (Abeysinghe *et al.*, 2007) that might include tannins with an influence on ruminal BH (Vasta & Luciano, 2011). The increase of *c*9,*t*11-18:2 in the muscle was mostly originated by a higher activity of SCD, since the desaturation-CLA index (Aldai *et al.*, 2006) was greater with 24% of citrus pulp. Moreover, EPA and DHA were increased in the muscle with 35% and 24% of cereal replacement, respectively. Also, an increment of "PUFA/SFA" ratio with both levels was found probably due to a higher intake of PUFA, particularly of 18:3n-3, with the by-product (Lanza *et al.*, 2015).

The reduction of dietary starch content with the replacement of cereal by alternative energy sources might prevent the occurrence of the *t*10-shift. In fact, the incorporation of citrus pulp at 24% DM instead of barley and maize in dairy ewes' diet led to a stimulation of *t*11 and, consequently, to a suppression of the *t*10 BH pathways. However, the high animal variability in the establishment of the *t*10-shift did not allow for an average of "*t*10-18:1/*t*11-18:1" ratio value below one with the by-product (Santos-Silva *et al.*, 2016).

The partial replacement of barley and wheat by 70% (Normand, Bas, Berthelot & Sauvant, 2005) and barley by 12% (Bodas *et al.*, 2007) of beet pulp was shown to decrease 18:2n-6 and increase 18:0 in the caudal adipose tissue and muscle of lambs, respectively. In the study by Normand *et al.* (2005), there was also a reduction of 18:3n-3 and 18:1 isomers with the by-product. These results may be due to a higher stimulation of BH with beet pulp compared to cereals, since the alternative energy source has a greater content of NDF (Vasta *et al.*, 2008), which might promote the activity of cellulolytic bacteria involved in BH (Vasta & Bessa, 2012). Moreover, Normand *et al.* (2005) and Bodas *et al.* (2007) described

an increase of 16:0 with beet pulp. This result may be explained by an enhancement of "acetate/propionate" ratio, although that was only evaluated by Bodas *et al*. (2007). In fact, the highest acetate and the lowest propionate productions obtained with the replacement of cereal by alternative energy sources were reported by Ipharraguerre *et al*. (2002) and Bampidis and Robinson (2006). Oppositely to Normand *et al*. (2005) and Bodas *et al*. (2007), Olfaz *et al*. (2005) verified an increase of 18:2n-6 and a decrease of 18:0 in the muscle of rams. However, these discrepancies might be due to a substitution of forage instead of cereal by beet pulp (40 and 60%) in the study by Olfaz *et al*. (2005). Also, Olfaz *et al*. (2005) found a reduction of *c*9-18:1 with 60% and of AA with both levels of the by-product. Although, to date, there are no studies evaluating the influence of beet pulp on the establishment of *t*10-shift, Renna, Collomb, Munger and Wyss (2010) described lower *t*7,*c*9-18:2; *t*10,*c*12-18:2 and *t*10,*t*12-18:2 and higher *t*11,*c*13-18:2; *t*9,*t*11-18:2 and *t*7,*t*9-18:2 in the milk of beet pulp- than cereal-supplemented cows but no differences of *t*11-18:1 and *c*9,*t*11-18:2 were observed between dietary treatments.

Recently, Rossi *et al*. (2016) reported an increase of SFA and a decrease of UFA in the muscle with the replacement of cereal by soybean hulls, similarly to that obtained with beet pulp in the study by Bodas *et al*. (2007). Also, an enhancement of 16:0, *t*9-18:1 and 18:3n-3 and a reduction of *c*9-18:1 was observed with soybean hulls diet.

1.6. Objectives

The general objective of the present study was to acquire insight about ruminal BH and how it can be modulated by stress stimuli promoted by tannins and high starch content in the diets. The two main specific objectives consisted in evaluating the modulation of BH by tannin extracts, particularly the stimulation of t11-18:1 production in the rumen (*in vitro* and *in vivo* experiments), and understanding the biological process that leads to the establishment and development of t10-shift (two *in vivo* trials). So, the following purposes were accomplished:

Experiment 1 – *In vitro* batch incubation of fistulated sheep's rumen content with condensed (quebracho, grape seed and *C. ladanifer*) and hydrolysable (chestnut) tannin extracts at 100 g/kg DM (Chapter 2):

- Evaluate the hypothesis of a more effective modulation of BH by *C.ladanifer* than by the other most common sources of tannins in stimulating t11-18:1 production.

- Compare modifications on ruminal BH caused by the addition of the four tannin extracts, giving emphasis to *C. ladanifer*.

Experiment 2 – Supplementation of fistulated sheep's diet with extracts of hydrolysable (chestnut) and condensed (mimosa) tannins and a mixture of the two sources at 100 g/kg DM (Chapter 3):

- Compare the influence of the two types of tannins on ruminal BH.

- Evaluate if the mixture of both tannin sources was more effective in modulating BH than their isolated use.

- Analyse the impact of the tannin extracts in specific rumen bacteria possibly involved in BH.

- Evaluate the impact of the tannin extracts on rumen bacterial biomass.

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Experiment 3 – Feeding fistulated sheep with a wheat grain-based diet (723 g/kg DM) supplemented with sunflower oil at 41 g/kg DM, in order to analyse the establishment and progression of *t*10-shift and, consequently, to have a better knowledge of how to prevent its occurrence (Chapter 4):

- Evaluate the time necessary for the onset of *t*10-shift and the constancy of the *t*10-shifted metabolic pathways.

- Relate the animal feed intake and rumen pH with the progression of *t*10-shift

Experiment 4 – Replacement of cereal with alternative energy sources (dehydrated citrus pulp, dehydrated beet pulp or soybean hulls) in lambs' finishing diets incorporated with a blend of soybean (59 g/kg DM) and fish (10 g/kg DM) oils (Chapter 4):

- Evaluate if the substitution of cereal with low starch energy sources prevents the establishment of *t*10-shift, leading to a reduction of *t*10-18:1 and a concomitant increase of *t*11-18:1 and *c*9,*t*11-18:2 in the meat and subcutaneous fat.

- Analyse the effect of alternative energy sources on animal productive performance and meat quality traits.

CHAPTER 2 – Modulation of *in vitro* rumen biohydrogenation by *Cistus ladanifer* tannins compared with other tannin sources

Modulation of *in vitro* rumen biohydrogenation by *Cistus ladanifer* tannins compared with other tannin sources

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Contribution of Mónica Costa to this article:

Mónica Costa participated in the animal experiment, the collection of rumen content and animal management. Also, she determined the FA content and performed the data processing and statistical analysis. Furthermore, Mónica Costa participated in the interpretation and discussion of the results, as well as in the writing of the manuscript.

ABSTRACT

Tannins are polyphenolic compounds able to modify the ruminal BH of unsaturated fatty acids, but their activity may vary among different tannin sources. The effect of rockrose (*Cistus ladanifer*) on BH has never been compared with other more common tannin sources. Tannin extracts (100 g/kg substrate DM) from chestnut (CH, *Castanea sativa*) (750 g/kg of mostly hydrolysable tannins), quebracho (QB, *Schinopsis* spp.) (720 g/kg of condensed tannins), grape seed (GS, *Vitis vinifera*) and rockrose (CL, *Cistus ladanifer*) (950 g/kg of condensed tannins) were incubated *in vitro* for 6 h with ruminal fluid using as substrate a feed containing 60 g/kg of sunflower oil. A control treatment with no added tannins was also included. Compared to control, GS and CL, but not CH and QB, increased ($P < 0.05$) the disappearance of 18:2n-6 with a consequent higher production of *c*9,*t*11-18:2 and *t*11-18:1. However, no differences among treatments ($P > 0.05$) were observed for the disappearance of *c*9-18:1 and 18:3n-3. The production of 18:0 was not different ($P > 0.05$) among treatments, although its proportion in the total BH products was lower ($P < 0.05$) for GS than for the other treatments. Condensed tannins from GS and, in less extent, from CL stimulates the first steps of BH, without a clear inhibition of 18:0 production.

Keywords: Biohydrogenation; tannins; fatty acids; rumen; *Cistus ladanifer*

2.1. Introduction

Tannins are phenolic compounds that represent an important class of plant secondary metabolites, presenting a variety of molecular weights and structures. Based on their molecular structure, tannins are classified as hydrolysable, condensed or a combination thereof (Schofield, Mbugua & Pell, 2001). It has been suggested that these compounds modify rumen microbial activity (McSweeney *et al*., 2001b), including the BH of dietary UFA (Vasta & Bessa, 2012). Ruminal BH involves an extensive metabolization of dietary UFA by sequential isomerizations, hydrogenations, as well as hydrations and dehydrations, originating numerous BH products, including CLA isomers, *trans*-octadecenoates, OH- and oxo-C18 FA and, as the major end product, the 18:0 (Shingfield & Wallace, 2014; Bessa *et al*., 2015). Therefore, BH is the main determinant of the occurrence of *trans*-FA, conjugated FA, and of the highly saturated nature of ruminant edible fats. The potential of tannins to modulate BH has recently received considerable attention, but inconsistent results have been reported (Kronberg *et al*., 2007; Benchaar & Chouinard, 2009; Cabiddu *et al*., 2009; Jayanegara, Kreuzer, Wina & Leiber, 2011). Factors that may be responsible for the

variability of tannin effects on BH include their molecular nature and quantity in the diet, the basal diet composition and the duration of utilization (Toral *et al.*, 2011; Toral *et al.*, 2013; Carreño *et al.*, 2015). Quebracho (*Schinopsis spp.*) and chestnut (*Castanea sativa*) tannin extracts are commercially available and the most studied sources of condensed and hydrolysable tannins, respectively (Vasta *et al.*, 2009a; Vasta *et al.*, 2010a; Buccioni *et al.*, 2011). Despite their commercial availability, grape seed (*Vitis vinifera*) tannin extracts or ground grape seeds have been scarcely studied *in vivo* (Jerónimo *et al.*, 2010; Correddu *et al.*, 2015; Correddu, Gaspa, Pulina & Nudda, 2016) and only one *in vitro* study was published recently (Carreño *et al.*, 2015). Rockrose (*Cistus ladanifer*) is an evergreen spontaneous Mediterranean tanniferous shrub reported to modulate ruminal BH and to modify the FA composition of lamb meat when incorporated in oil supplemented diets (Jerónimo *et al.*, 2010; Jerónimo *et al.*, 2012; Francisco *et al.*, 2015). The effects of *C. ladanifer* plant on FA metabolism are mostly due to its high content of condensed tannins, which range from 40 to 160 g/kg DM (Guerreiro *et al.*, 2016b). Considering the influence of *C. ladanifer* on ruminal lipid metabolism, we hypothesize that its tannins will be more effective in modulating the BH than the most common sources of tannins. Attending to the fact that *C. ladanifer* tannins are not commercially available and that their effects on BH have never been directly compared with other tannin sources, the present study aimed to compare the modifications on ruminal BH evaluated *in vitro* caused by the addition of four tannin extracts, including that from *C. ladanifer*.

2.2. Material and methods

2.2.1. Animal handling and management

Animal handling followed EU Council Directive 2010/63/EU (EC, 2010) concerning animal care and rumen-fistulated animals were used after approval of the ethical committee of the Faculty of Veterinary Medicine, University of Lisbon. Two sheep approximately 1 year old and of 40 kg live weight were used as rumen content donors. The animals were fed 500 g of a commercial compound feed and 800 g of grass hay, both divided into two equal meals per day (9h30 and 17h00). The compound feed comprised maize, soybean, sunflower, wheat, wheat bran and rape and contained 219 g/kg DM of crude protein; 95 g/kg DM of crude fibre; 67 g/kg DM of ash; 35 g/kg DM of ether extract; 4 g/kg DM of sodium and a vitamin pre-mix providing 7500 IU/kg of vitamin A; 1,500 IU/kg of vitamin D3 and 7.5 mg/kg of vitamin E. The grass hay contained 35 g/kg DM of crude protein and 764 g/kg DM of neutral detergent fibre.

2.2.2. Tannin extracts

We used commercial extracts including a crude extract of quebracho (Unitán, Argentina) declared to contain 720 g/kg of condensed tannins and a crude extract of chestnut (Farmatan®; Tanin Sevnica, Slovenia) described as having 750 g/kg of tannins of which 637.5 g/kg were hydrolysable and 112 g/kg were condensed tannins. The other two extracts were purified in Sephadex LH 20 (Amersham Pharmacia Biotech, Portugal) in our laboratory according to Dentinho, Belo and Bessa (2014) and included a grape seed extract (AHD international LLC, Atlanta, GA, USA) and a *C. ladanifer* extract containing 950 g/kg of condensed tannins.

2.2.3. *In vitro* procedures

Before the morning meal, about 1 L of ruminal content was collected from each fistulated sheep and immediately transferred to the laboratory in a thermostatic box at 39 °C. It was then immediately filtered through four layers of cheesecloth and mixed with McDougall buffer solution, under CO_2 flux and warming at 39 °C, in a proportion of 1:2 (ruminal fluid:buffer solution, vol/vol). The rumen buffered solution was distributed into Hungate tubes (15 cm×2.5 cm) containing 60 mg of feed substrate, with no added tannins (Control) or with 6 mg of chestnut (CH), quebracho (QB), grape seed (GS) or *C.ladanifer* (CL) tannin extract. The substrate used was a ground pellet feed containing dehydrated alfalfa (700 g/kg), wheat grain (105 g/kg), soybean meal (110 g/kg) and sunflower oil (60 g/kg), as well as minerals and premix (25 g/kg). The chemical composition of the substrate was 902 g/kg DM, 175 g/kg DM of crude protein, 113 g/kg DM of starch, 81 g/kg DM of ether extract and 213 g/kg DM of crude fibre. The final concentration of tannin extract was 100 g/kg DM, considering that the substrate presented 894 g/kg DM. The Hungate tubes were filled with CO_2, closed with a butyl rubber stopper and screw cap, then incubations were conducted on a water bath (Unitronic, J.P. Selecta, Barcelona, Spain) at 39 °C with gentle agitation for 6 h. In each run, duplicate tubes from each treatment and incubation time (0 and 6h) were obtained, with one tube being used for pH measurement and VFA analysis and the other for long chain FA analysis. Both 0 and 6 h tubes were directly frozen and stored at -20 °C until further analysis. The tubes for FA analysis were freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmark), weighted and stored at -20 °C until analysis. The incubation procedure was replicated six times in six consecutive weeks.

2.2.4. Analytical procedures

Feed chemical composition was determined using routine and widespread methods described previously (Francisco *et al*., 2015). Volatile fatty acids were directly analysed by gas chromatography with flame ionization detection (GC-FID) in a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a Nukol capillary silica column (30 m; 0.25 mm i.d.; 0.25 μm film thickness, Supelco Inc., Bellefonte, PA, USA) as described in the literature (Oliveira, Alves, Santos-Silva & Bessa, 2016). Freeze-dried rumen samples were transesterified into FA methyl esters by using a combined basic followed by acidic catalysis (Alves *et al*., 2013b). The internal standard was 19:0 (1 mg/mL). Fatty acid methyl esters were separated by GC-FID using a Shimadzu GC-2010 Plus chromatograph equipped with a TR-CN100 silica capillary column (100 m; 0.25 mm i.d.; 0.20 μm film thickness; Tecknokroma, Barcelona, Spain) according to procedures described in the literature.(Oliveira *et al*., 2016) Identification of FA methyl esters (FAME) and DMA was achieved by comparison of retention times with those of authentic standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA, and a Bacterial FAME mix from Matreya LLC, Pleasant Gap, PA, USA) and by confirmation with gas chromatography-mass spectrometry (GC-MS) in a Shimadzu GC-MS QP 2010 Plus chromatograph (Kyoto, Japan) (Alves *et al*., 2013b).

2.2.5. Calculations and statistical analysis

Balances of the VFA and FA (except C18 FA) during the incubation period were calculated directly from their concentrations at 6 h minus 0 h incubation times. In each treatment and incubation run, the direct balance between the amounts present in the pairs of tubes from 0 h and 6 h was occasionally inconsistent owing to the random variation of the total amount of FA in each independent tube. Thus, we averaged the content of C18 FA (in μg/g DM of tube contents) present in the pair of tubes (0 h and 6 h) of each treatment in each incubation run, assuming that no C18 FA undergo carbon chain elongation or shortening and that the *de novo* synthesis of C18 FA would be negligible owing to the abundance of C18 FA in substrate (Demeyer, Henderson & Prins, 1978). The differences between 6 h and 0 h were then computed using the mean C18 FA content in each pair of tubes and the relative distribution of C18 FA (in % of total C18 FA) present in the tube from 0 h and 6 h incubation times.

The calculations for the balance of 18:0 are given here as an example:

1) $[\sum C18_m] = ([\sum C18_{0h}] + [\sum C18_{6h}])/2$

2) $[18:0m_{0h}] = ([\sum C18_m] \times P(18:0_{0h})/100)$ and $[18:0m_{6h}] = ([\sum C18_m] \times P(18:0_{6h})/100)$

3) $[18:0B] = [18:0m_{6h}] - [18:0m_{0h}]$

Where

$[\sum C18_{0h}]$ and $[\sum C18_{6h}]$, content (µg/g DM) of total C18 FA in 0 h and 6 h incubation tubes, respectively.

$[\sum C18_m]$, averaged content (µg/g DM) of total C18 FA in both 0 h and 6 h incubation tubes

$P(18:0_{0h})$ and $P(18:0_{6h})$, % of 18:0 in total C18 FA in 0 h and 6 h tubes, respectively.

$[18:0_{m,0h}]$ and $[18:0_{m,6h}]$, content of 18:0 (µg/g DM) expressed on $[\sum C18_m]$ basis

$[18:0_B]$, balance (µg/g DM) of 18:0 during incubation period (6 h – 0 h).

The proportional disappearance of dietary unsaturated C18 FA was then calculated as:

4) Disappearance of FA (%) = $(([FA_{m,0h}] - [FA_{m,6h}])/ [FA_{m,0h}]) \times 100$

Where FA_m can be any dietary unsaturated FA.

All C18 FA that displayed a positive balance during the 6 h of incubation were considered here as BH products. The relative yields of the main classes of BH products (18:0, 18:1 isomers; 18:2 isomers and oxo-FA) were computed from the C18 FA balance data and expressed in % of total BH products, as exemplified for 18:0:

5) (18:0Y) = $([18:0_B] \times 100)/ [BHP_B]$

Where,

(18:0Y), relative yield of 18:0 expressed as percentage of total BH products;

$[BHP_B]$, sum of BH products.

Volatile FA and FA differences between 6 and 0 h were computed and analysed as a randomized complete block design using the MIXED procedure of SAS (SAS Inst. Inc., 2002, Cary, NC), where each run was treated as a random block and inclusion of tannin extracts as a fixed factor. The variance homogeneity was checked and, when justified, the variance heterogeneity structure was accommodated in the model. When significant effects of treatments were detected, the least square means were compared using the pairwise Tukey comparison test.

2.3. Results

2.3.1. Fermentation pattern

The total VFA production averaging 8.1 mmol/L was not affected by inclusion of tannin extracts (Table 2). Consistently, no individual VFA differed among treatments and the acetic acid (2:0) predominated in all treatments. Minor but significant differences in the pH variation were observed between CL and Control, with a slight pH increase for CL and a decrease for Control treatments.

Table 2. Effect of tannin extracts on volatile fatty acids (VFA) balance (mmol/L) and pH change during incubation (difference between 6 and 0h).

Item	Treatments[1]					SEM[2]	*P*-value
	Control	CH	QB	GS	CL		
VFA							
2:0	4.14	3.83	4.20	3.83	4.29	0.406	0.884
3:0	2.84	2.60	2.82	2.55	2.08	0.214	0.127
iso-4:0	0.07	0.03	0.03	0.09	-0.03	0.065	0.686
4:0	1.19	1.18	1.19	1.16	1.08	0.122	0.957
iso-5:0	0.12	0.04	0.06	0.15	0.10	0.047	0.494
5:0	0.23	0.22	0.30	0.20	0.19	0.065	0.749
Total	8.59	7.85	8.61	7.93	7.67	0.590	0.699
pH	-0.07[b]	-0.04[ab]	-0.05[b]	0.04[ab]	0.08[a]	0.032	0.008

[1], Control, no added tannins; CH, chestnut; QB, quebracho; GS, grape seed; CL, *Cistus ladanifer* tannins; [2], standard error of mean. Means within a row with different letters are significantly different ($P < 0.05$).

2.3.2. C18 fatty acid balance and biohydrogenation

Table 3 shows the effect of tannin extracts on the balance of C18 FA during the 6 h of incubation. The *c*9-18:1, 18:2n-6 and 18:3 (mostly *c*9,*c*12,*c*15 isomer but also including minor non identified 18:3 isomers) presented large negative balances in all treatments, but significant differences among treatments were only observed for 18:2n-6. The disappearance of 18:2n-6 was larger ($P < 0.05$) for GS than for Control, CH and QB treatments, whereas the disappearance of 18:2n-6 with CL did not differ ($P > 0.05$) from Control and GS but was larger ($P < 0.05$) than for CH and QB treatments. The *c*11-18:1, the unresolved peak of conjugated *t,t*-18:2 and *c*9,*t*11-18:2 also presented negative balances, except for *c*9,*t*11-18:2 in the GS and CL treatments. All other C18 FA presented positive balances, with 18:0 ($\approx$1120 µg), unresolved 10-/9-oxo 18:0 ($\approx$340 µg), *c*12-18:1 ($\approx$169 µg) and *t*11-18:1 showing the largest increases. The *t*11-18:1 production was affected by the treatments, with larger ($P < 0.05$) increases with GS and CL than with Control, CH and QB treatments. The *t*10-18:1 production was significantly higher with GS compared with Control but not different from CL treatment. The *t*10-/*t*11-18:1 ratio remained low (0.34) and did not differ ($P = 0.605$) among treatments (data not shown). Grape seed treatment also presented lower production of unresolved 10-/9-oxo-18:0 when compared with CH and QB, although not differing from Control and CL treatments.

Table 3. Effect of tannin extracts on C18 fatty acids (FA) balance (μg/g DM of fermenters content) during incubation (difference between 6 and 0h).

Item	Treatments[1]					SEM[2]	*P*-value
	Control	**CH**	**QB**	**GS**	**CL**		
C18 FA loss							
c9-18:1	-789	-734	-740	-851	-907	48.5	0.060
c11-18:1	-41[b]	-31[b]	-20[ab]	-2[a]	-29[b]	5.7	0.002
18:2n-6	-897[ab]	-842[a]	-808[a]	-1212[c]	-1149[bc]	70.5	0.001
conj.*t,t*-18:2[3]	-38[b]	-31[ab]	-30[ab]	-20[a]	-25[ab]	3.5	0.024
*c9,t*11-18:2[4]	-24[b]±2.2	-10[ab]±7.4	-15[b]±2.6	--[a]	--[a]		0.001
18:3[5]	-325±18	-300±18	-288±20.1	-344±18	-333±18	18.4	0.232
C18 FA loss	-2147[ab]	-1926[a]	-1909[a]	-2387[ab]	-2467[b]	122.2	0.008
C18 FA gain							
18:0	1205±59	1143±59	1082±66	986±144	1200±59		0.480
18:1 isomers							
t6/t7/t8	33[b]	31[b]	31[b]	77[a]	72[a]	8.0	<0.001
t9	69[b]	41[c]	67[b]	93[a]	58[bc]	5.4	<0.001
t10	50[b]	37[b]	41[b]	107[a]	80[ab]	10.1	0.001
t11	135[b]	141[b]	123[b]	300[a]	244[a]	18.6	<0.001
t15	31	26	19	13	25	4.6	0.125
t16	21	15	14	13	16	2.5	0.174
∑*trans*-18:1[6]	356[b]	312[b]	299[b]	595[a]	475[ab]	46.8	0.001
c12	150	129	132	219	213	33.6	0.112
∑*cis*-18:1[7]	158	136	124	225	217	27.6	0.055
18:2 isomers							
t11,c15-18:2	3	9	8	11	6	2.1	0.083
n.c.-18:2[8]	19[ab]±4.4	8[b]±4.4	9[b]±4.9	43[ab]±10	30[a]±4.4		0.011
*c9,t*11-18:2[4]	--[b]	--[ab]	--[b]	11[a]±7.4	13[a]±7.4		0.001
oxo-18:0							
8-oxo-	20	17	18	17	14	1.6	0.246
10-/9-oxo-	340[abc]	300[c]	306[bc]	400[a]	365[ab]	16.3	0.001
12-/13-oxo-	84	70	72	74	71	5.5	0.167
C18 FA gain	2147[ab]	1926[b]	1909[b]	2387[ab]	2467[a]	122.2	0.008

[1], Control, no added tannins; CH, chestnut; QB, quebracho; GS, grape seed; CL, *Cistus ladanifer* tannins; [2], standard error of mean; [3], conjugated *trans*, *trans*-18:2 isomers; [4], the *c9,t*11-18:2 line is spited between loss and gain balance, although the statistical analysis is common; [5], *c9,c12,c15*-18:3 plus other minor 18:3 isomers; [6], also includes *t4*- and *t5*-18:1 isomers; [7], also includes the *c13*- and *c15*-18:1 isomers; [8], other non-conjugated 18:2 isomers. Means within a row with different letters are significantly different (*P* < 0.05).

Proportional disappearances of *c9*-18:1, 18:2n-6 and 18:3 are presented in Table 4. The proportional disappearance of *c9*-18:1 was quite low and did not differ (*P* > 0.05) among treatments. Proportional disappearances of 18:2n-6 and 18:3 were higher (*P* < 0.05) with GS and CL than with Control and the other tannin extracts. The proportional distribution of the BH products is also presented in Table 3. The 18:0 comprises about 58% of BH products for Control, Chestnut and Quebracho. Grape tannins reduced (*P* < 0.05) the proportion of 18:0 formed to 42% and *C. ladanifer* presented an intermediate value (52%), not differing from any other treatment. Consistently, the 18:1 BH products were lower for Control, CH and QB treatments (≈22%) and higher for GS (36%) with CL presenting an intermediate value (28%). The 18:2 BH products only had a positive, but small, contribution to the BH products in GS

and CL treatments, presenting negative values for the other treatments. The oxo-18:0 FA represented a large proportion of BH products (≈21%) with CL treatment presenting a slightly but significantly lower contribution than Control.

Table 4. Effect of tannin extracts on biohydrogenation (%) and relative distribution of biohydrogenation products (%) between 0 and 6h of incubation.

Item	Treatment[1]					SEM[2]	P-value
	Control	CH	QB	GS	CL		
Disappearance (%)							
c9-18:1[3]	18.5	17.8	18.0	20.8	22.2	1.15	0.037
18:2n-6	31.6[b]	27.8[b]	28.9[b]	41.9[a]	45.3[a]	1.58	<0.001
18:3[4]	41.0[b]	41.2[b]	41.1[b]	51.1[a]	48.9[a]	1.76	0.001
Products (%)							
18:0	56.6[a]	59.1[a]	57.6[a]	41.7[b]	52.5[ab]	2.62	0.001
18:1							
t11	6.5[b]	7.1[b]	6.6[b]	13.4[a]	9.6[b]	0.79	<0.001
t10	2.4[b]	1.9[b]	2.2[b]	4.6[a]	3.3[ab]	0.37	<0.001
Other trans	7.4[ab]	6.2[b]	7.0[ab]	8.5[a]	7.1[ab]	0.47	0.033
Total cis	7.8	6.8	6.9	9.8	8.9	1.01	0.198
Total	23.0[b]	21.2[b]	22.3[b]	35.7[a]	27.7[ab]	2.40	0.003
18:2	-2.2[c]	-1.1[bc]	-1.6[bc]	2.1[a]	0.7[ab]	0.60	<0.001
c9,t11	-1.1[b]	-0.5[ab]	-0.7[b]	0.6[a]	0.5[a]	0.22	<0.001
Total	-2.2[c]	-1.1[bc]	-1.6[bc]	2.1[a]	0.7[ab]	0.60	<0.001
oxo-18:0	22.6[a]	20.8[ab]	21.8[ab]	20.5[ab]	19.1[b]	0.99	0.030

[1], Control, no added tannins; CH, chestnut; QB, quebracho; GS, grape seed; CL, *Cistus ladanifer* tannins; [2], standard error of mean; [3], no significant differences among means were detected in the *pos hoc* multiple comparison; [4], c9,c12,c15-18:3 plus other minor 18:3 isomers. Means within a row with different letters are significantly different (P < 0.05).

2.3.3. Microbial structural fatty acids and dimethylacetals

The balance between 0 and 6h of incubation of other non-C18 FA, mostly derived from microbial *de novo* synthesis, and DMA are presented in Table 5. Most of these FA and DMA presented a positive balance and only four FA (iso-13:0, anteiso-15:0, 15:0 and 17:0) and two DMA (anteiso-15:0 and iso-16:0) presented differences among treatments. The CH and QB treatments showed consistently similar balance values to Control for all FA and DMA except iso-13:0 FA, which presented lower (P < 0.05) values than Control. Most of the treatment differences observed was associated with GS and CL treatments. Grape seed presented lower (P < 0.05) balances of 15:0, 17:0 and iso-16:0 DMA than Control and lower (P < 0.05) anteiso-15:0 than CH treatment. The CL treatment presented lower balances of anteiso-15:0 and 17:0 than the Control treatment. Curiously, the pattern observed regarding the influence of the treatments on 15:0 and iso-16:0 DMA was symmetric to that described for the proportional contribution of *trans*-18:1 isomers to the total BH products.

Table 5. Effect of tannin extracts on other fatty acids (FA) and dimethylacetals (DMA) balance (µg/g DM of fermenters content) during incubation (difference between 6 and 0h).

Item	Treatment[1]					SEM[2]	*P*-value
	Control	CH	QB	GS	CL		
FA							
14:0	15.7	16.7	14.1	16.1	16.1	3.03	0.956
16:0	318.9	372.7	414.2	448.8	383.9	76.27	0.794
cyclo-17:0	7.5	0.9	6.6	5.3	9.4	2.85	0.360
20:0	23.9	25.9	32.0	32.6	31.4	6.57	0.825
22:0	11.7	16.0	13.3	18.8	16.8	5.16	0.874
23:0	-4.0[b]	-2.9[b]	-6.8[ab]	19.5[a]	-13.7[b]	4.77	0.011
24:0	7.6	23.9	23.7	16.0	6.4	9.51	0.468
26:0	24.2	23.0	13.9	62.6	43.1	14.93	0.194
OBCFA[3]							
i-13:0	4.2[a]	0.6[b]	0.4[b]	2.9[ab]	1.9[ab]	0.81	0.018
13:0	3.9	3.9	2.8	3.9	3.3	1.67	0.987
i-14:0	6.3	6.0	4.8	5.1	4.9	1.41	0.925
i-15:0	9.9	3.4	13.7	7.9	6.7	3.48	0.372
a-15:0	55.4[a]	42.3[ab]	52.6[ab]	37.2[ab]	35.3[b]	4.80	0.025
15:0	36.3[a]	30.6[a]	31.1[a]	12.1[b]	23.2[ab]	4.46	0.009
i-16:0	15.5	11.4	16.1	10.3	10.9	2.10	0.205
i-17:0[4]	13.7	8.6	21.6	8.2	10.0	3.91	0.170
a-17:0	26.9	21.8	25.9	16.9	18.7	3.67	0.310
17:0	34.9[a]	27.8[abc]	33.4[ab]	20.5[bc]	17.5[c]	3.41	0.006
∑OBCFA	171.4[a]	130.4[ab]	159.6[ab]	101.4[b]	106.6[ab]	16.26	0.021
DMA							
i-15:0	4.1	1.7	-2.8	-1.1	-1.5	1.96	0.159
a-15:0	2.6[ab]	6.7[a]	1.5[ab]	-5.4[b]	-1.0[ab]	2.65	0.011
15:0	5.3	3.6	1.3	2.5	4.0	1.70	0.571
i-16:0	37.0[a]	45.8[a]	44.7[a]	9.6[b]	28.7[ab]	5.49	0.001
16:1[5]	4.2	11.5	13.3	3.9	4.1	2.28	0.018
17:0	0.4	-6.7	-3.4	-2.2	-4.5	1.79	0.078
18:1	-0.7	3.4	4.7	-1.8	1.2	3.31	0.641
Total DMA[6]	49.1[a]	51.5[a]	42.4[ab]	-4.7[b]	23.0[ab]	12.07	0.021

[1], Control, no added tannins; CH, chestnut; QB, quebracho; GS, grape seed; CL, *Cistus ladanifer* tannins; [2], standard error of mean; [3], odd- and branched-chain FA; [4], also includes *c*9-16:1; [5], no significant differences among means were detected in the *pos hoc* multiple comparison; [6], also includes 12:0, 13:0, i-14:0, 14:0 DMA. Means within a row with different letters are significantly different (P < 0.05).

2.4. Discussion

In the small-scale and short-term (6h) *in vitro* system used, the high dose of tannin extracts (100 g/kg DM) incorporated in the substrate did not reduce the fermentative activity, as revealed by the absence of effects on VFA production compared with Control.

Quebracho and chestnut commercial-grade tannins have been reported to modulate ruminal BH (Buccioni *et al*., 2011; Carreño *et al*., 2015). Thus, the use of these tannin sources was expected to provide some comparability with the literature, allowing the evaluation of the relative efficacy of the less studied grape seed and particularly *C. ladanifer* tannins. However, our results clearly indicate that, in our experimental conditions, GS and CL were effective in modulating ruminal BH, whereas QB and CH were not. It is not clear why both QB and CH did not modify the BH in the present experiment. The purity of GS and CL extracts used in this trial was greater than that of CH and QB extracts, since GS and CL were purified in Sephadex whereas CH and QB extracts were from a commercial grade, which resulted in a higher dose of tannins in both GS and CL than in CH or QB. This suggests that the difference observed between commercial-grade and purified tannin extract might be due to the dose of tannins effectively applied in the incubation tubes, although the effect of distinct procedures of tannin extraction and purification might also influence their activity as BH modulators. In fact, the dose of tannins supplied by commercial-grade extracts is large enough to be expected to elicit an effect in ruminal BH (Vasta *et al*., 2009a).

Recently, it was shown that the effects of tannins on BH might be dose dependent and that there are interactions between the tannin type and the dose used (Buccioni *et al*., 2011; Carreño *et al*., 2015). Commercial tannin extract from quebracho seems to be more active on BH at lower doses and its effects disappear when included at 80 g/kg DM (Carreño *et al*., 2015), which might explain the absence of effect at 100 g/kg DM reported by us and by other authors (Ishlak, Gunal & AbuGhazaleh, 2015). Also, the effects of GS and CH on BH were described to be tendentiously attenuated at higher doses, although not disappearing completely at 80 g/kg DM (Carreño *et al*., 2015). In our experiment, the CH was ineffective at 100 g/kg DM but GS was highly effective at even a higher dose, considering that we used purified grape seed extract. There is no available information about the effects of CL on BH at lower doses but at 100 g/kg DM it was only slightly less effective than GS in modulating ruminal BH. It is not clear to us how higher doses of tannins might induce milder effects on BH than low doses. Nevertheless, it is striking that in our experiment the purified extracts, that supplied higher tannin dose than the commercial tannin extracts, elicited a stronger response on BH.

The reported effects of tannins on *in vitro* ruminal BH generally involve an inhibition of the last step of BH with reduced formation of 18:0 and accumulation of *trans*-18:1 isomers and

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sometimes a reduction of the disappearance of 18:2n-6 and 18:3n-3 (Khiaosa-Ard *et al*., 2009; Buccioni *et al*., 2011; Carreño *et al*., 2015). In our study, the effects of GS and CL on ruminal BH point to a higher disappearance of 18:2n-6 and 18:3 and an increased accumulation of *t*11-18:1 at 6h of incubation. The formation of *t*10-18:1 and other minor *trans*-18:1 isomers also increased with GS, but the relation between *t*10-/*t*11-18:1 remained fairly constant, which indicates a general accumulation of *trans*-18:1 isomers without modification of the common BH pathways.

Despite the increase in *trans*-18:1 observed in GS and CL, the formation of 18:0 was not reduced. The relative contribution of 18:0 for the total BH products formed was reduced for GS treatment, which can be explained by a stimulation of the initial steps of BH and not by an inhibition of 18:0 formation. Similarly, increased production of *t*11-18:1 induced by CL was mostly due to the increased BH of 18:2n-6 and clearly not by the inhibition of 18:0 production, which confirms our previous data (Guerreiro *et al*., 2016a). It is not clear if the tannin stimulation of initial steps of BH reported here was a dose dependent effect or a transient effect explained by the short incubation time. In fact, the effect of quebracho and chestnut tannins in reducing 18:0 accumulation have been reported to increase with the length of *in vitro* incubation, being small at 6h but becoming clearer after 12 and 18h of incubation (Buccioni *et al*., 2011). Moreover, the FA composition of abomasal digesta from lambs fed *C. ladanifer* plant suggests that an inhibition of the last step of BH was present, with a clear reduction of 18:0 concentration (Jerónimo *et al*., 2010). In order to clarify these alternatives, the effects of *C. ladanifer* on BH must be studied at lower dosages and at several incubation times.

In all treatments, the production of oxo-FA represented a high proportion of the BH products. In fact, the amount of oxo-18:0 produced was larger than those reported in other *in vitro* batch rumen incubations (Carreño *et al*., 2015) but consistent to those reported by us using the same *in vitro* system (Guerreiro *et al*., 2016a). The occurrence of oxo-C18 FA formation in the rumen linked to BH metabolism is well established (Hudson *et al*., 1995; Jenkins, AbuGhazaleh, Freeman & Thies, 2006; Alves *et al*., 2013b), despite the fact that these FA are not usually reported in the literature.

Odd- and branched-chain FA present in the rumen are mostly derived from bacterial *de novo* synthesis and are incorporated into their cell membranes (Kaneda, 1991). Rumen bacteria also present significant amounts of plasmalogen phospholipids (Miyagawa, 1982) that can be detected by the presence of DMA, which are formed from the vinyl ether chain of plasmalogens released under acid catalysis. The DMA from microbial plasmalogens also contain odd- and branched-chains that might participate in the maintenance of the optimal membrane fluidity and stability (Alves *et al*., 2013b). Thus, the production of both odd branched chain FA and DMA should reflect microbial anabolism and eventually changes in

rumen microbiota (Saluzzi *et al*., 1995; Vlaeminck *et al*., 2006). Increased BH activity and consequent accumulation of *t*11-18:1 observed with GS and CL treatments seemed to be associated with a decreased *de novo* synthesis of some OBCFA by rumen bacteria. For GS, these effects were also extended to a decrease in the production of iso-16:0-DMA and anteiso-15:0-DMA. It is not clear if the changes in microbial structural lipids indicate a modification of microbial species abundance in the rumen or simply a remodelling of bacterial membranes structure promoted by an increased availability of *t*11-18:1.

The exact mechanism explaining the effects of tannins on BH is unknown and might involve: i) a target toxic effect to bacteria catalysing *trans*-18:1 isomers' reduction (Durmic *et al*., 2008); ii) a shift on rumen microbiota composition that disfavours a specific bacterial community or iii) a more general adaptive response involving the increase of *trans*-18:1 availability to be used in an extensively remodelling of bacterial membranes. In the present study, the increase of *trans*-18:1 under the influence of CT from grape seed and slightly from *C. ladanifer* corroborates with the third hypothesis. In fact, the higher production of *trans*-18:1 with grape seed extract was mainly due to an increase of *t*11-18:1 and *t*10-18:1, which might indicate a general adaptive response of rumen microbiota instead of a shift that would lead to a different BH pathway (Alves & Bessa, 2014). Moreover, some studies suggest that tannins may have an impact on bacterial membranes and, consequently, stimulate *trans*-18:1 production probably as an adaptive response of bacteria to stress stimuli. In fact, the UFA and polyphenols were described as possible causes of membrane damages and, curiously, both may promote the accumulation of *trans*-18:1 in the rumen (Smith, Zoetendal & Mackie, 2005; Maia *et al*., 2007). The generation of *trans*-18:1 in response to stress stimuli in the rumen ecosystem was hypothesised as one of the roles of BH in the rumen (Bessa *et al*., 2000). Also, adaptive changes of non-rumen bacteria to stress stimuli with *trans*-FA formation and its incorporation into cell membranes have been reported (Keweloh & Heipieper, 1996; Endo, Kamisada, Fujimoto & Saito, 2006). Although the third hypothesis seems to be the most probable, the three hypotheses are possible as we cannot prove either of them since more studies on rumen microbiome are needed (Keweloh & Heipieper, 1996; Bessa *et al*., 2000; Smith *et al*., 2005; Endo *et al*., 2006; Maia *et al*., 2007).

2.5. Conclusions

The purified condensed tannins from grape seed and, to lesser extent, from *C. ladanifer* induced an increased BH of 18:2n-6 and 18:3, coupled with an accumulation of *t*11-18:1, and no reduction in 18:0 production. The commercial grade quebracho and chestnut tannin extracts did not display any effects on ruminal BH. Grape seed and *C. ladanifer* tannins also induced slight modifications in microbial structural lipids. It can be concluded that, in these experimental conditions, *C. ladanifer* tannins have only slightly lower potential to modulate rumen BH than grape seed extract.

CHAPTER 3 – Mimosa condensed tannins induce higher variability of ruminal biohydrogenation than chestnut hydrolysable tannins

Mimosa condensed tannins induce higher variability of ruminal biohydrogenation than chestnut hydrolysable tannins

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Contribution of Mónica Costa to this article:

Mónica Costa participated in the animal experiment, collected the rumen content and took care of the sheep. Also, she determined the FA content, quantified the 16S rRNA gene copy numbers of bacteria and performed the data processing and statistical analysis. Furthermore, Mónica Costa participated in the interpretation and discussion of the results, as well as in the writing of the manuscript.

Abstract

It was hypothesized that condensed tannins would have a higher inhibitory effect on ruminal BH than hydrolysable tannins and that would be reflected in an accumulation of *trans*-18:1 isomers in rumen contents and bacteria. Condensed tannin extract from mimosa (M, *Acacia mearnsii*) and hydrolysable tannin extract from chestnut (C, *Castanea sativa*) or their mixture (MC) (100 g/kg dry matter (DM)) were incorporated in a diet with an oil blend (40 g/kg DM). The diet was fed to rumen fistulated sheep, following a change-over design with 3 diets, 4 sheep and 4 periods. The fatty acid (FA) and dimethylacetal (DMA) composition of rumen contents, bacterial biomass fractions and blood plasma were analysed by gas-chromatography. Selected rumen bacteria were also analysed by quantitative real time PCR. Mimosa led to lower rumen fermentative activity, DMA and 16S rRNA copy number of *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio proteoclasticus* and higher *Selenomonas ruminantium* copy number, as well as lower total *trans*-18:1 and "*trans*-/*cis*-18:1" ratio in bacterial biomass than chestnut. Also, in plasma and rumen, total oxo-18:0 was higher with M comparing with C. An almost complete inhibition of BH was detected in few samples from MC and M but never with C. The M and MC treatments resulted in higher variability of ruminal BH, suggesting that condensed tannins had a more inhibitory effect on BH than hydrolysable tannins. However, the highest accumulation of *trans*-18:1 in bacterial biomass with C suggested that *trans*-18:1 might participate in an adaptive stress response in rumen bacteria.

Keywords: biohydrogenation; fatty acids; rumen; hydrolysable tannins; condensed tannins

3.1. Introduction

The inclusion of tannins in ruminant diets has been reported to modulate ruminal BH (Vasta & Bessa, 2012). In general, the effects of tannins on rumen microbiota have been attributed to the ability of these phenolic compounds to form complexes with polymers of protein and carbohydrates and to directly interact with bacterial cell membranes (Smith *et al.*, 2005). However, the effects of tannins are dependent on several factors including their molecular structure. Concerning the molecular structure, tannins can be classified into HT and CT. Hydrolysable tannins (mainly gallotannins and ellagitannins) have a polyol as a central core, which is esterified with a phenolic group, while condensed tannins (proanthocyanidins) are composed of flavan-3-ol (epi)catechin and (epi)gallocatechin units that are linked by interflavonoid linkages (Patra & Saxena, 2011). Hydrolysable tannins are readily susceptible

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to depolymerisation in the rumen whereas the depolymerisation of condensed tannins by rumen bacteria has not been clearly demonstrated (McSweeney *et al*., 2001b). Depolymerisation can convert oligomeric hydrolysable tannins into low MW monomeric subunits that exhibit a lower affinity for extracellular or intracellular proteins, such as enzymes, or for cellular lipoproteins within the membrane lipid bilayers of bacteria (Field & Lettinga, 1992). Conversely, CT might continue to inhibit the activity and growth of microorganisms causing modifications of bacterial membrane fluidity, such as a decrease of membrane permeability due to an interaction with lipoproteins, or even a disruption of membranes when added at high concentrations (Ikigai, Nakae, Hara & Shimamura, 1993; Hashimoto, Kumazawa, Nanjo, Hara & Nakayama, 1999; Smith *et al*., 2005). However, it is possible that monomeric subunits resulting from the hydrolysis of hydrolysable tannins continue to exert some toxicity towards bacteria (Field & Lettinga, 1987; Scalbert, 1991; Field & Lettinga, 1992), causing instability in membrane fluidity but allowing bacteria to build a stress response. In the presence of toxic stimuli that affect membrane integrity, some bacteria are able to reduce membrane fluidity enriching their membrane lipids with *trans*-FA (Keweloh & Heipieper, 1996; Endo *et al*., 2006). The increased rumen availability of *trans*-FA and its incorporation into microbial cell membranes were suggested to be the main purposes of ruminal BH (Bessa *et al*., 2000). In the present study, we hypothesized that CT will exert a stronger inhibitory action on rumen microbial ecosystem than hydrolysable tannins, but the low MW products of hydrolysable tannin hydrolysis will lead to a more evident stress response of bacteria with modifications of cell membranes. Altogether, this will lead to different effects of the two types of tannins on ruminal BH, FA composition of rumen bacteria and population of selected bacterial species. Thus, a condensed tannin extract from mimosa (*Acacia mearnsii*) and a hydrolysable ellagitannin extract from chestnut (*Castanea sativa*) or their mixture were incorporated in an oil-supplemented basal diet and fed to rumen fistulated sheep, in order to compare their effects on FA composition of rumen digesta and bacteria, as well on the population of selected rumen bacteria.

3.2. Material and methods

3.2.1. Animal handling and management

Animal handling followed EU Council Directive 2010/63/EU (EC, 2010) concerning animal care and rumen fistulated animals were used after approval of the ethical committee of the Faculty of Veterinary Medicine, University of Lisbon. Four rumen fistulated approximately 2-year old sheep (52±3.03 kg of live weight) were used in a change-over design with 3

treatments and 4 experimental periods. The animals were kept in individual crates and bedded with wood shavings. The dietary treatments were defined by the inclusion of tannin extract in common basal feed ingredients: 1) Mimosa (M) (100 g/kg DM of mimosa tannin extract), 2) Chestnut (C) (100 g/kg DM of chestnut tannin extract), 3) Mimosa plus chestnut (MC) (50 g/kg DM of each mimosa and chestnut tannin extracts). The tannin extracts were commercial extracts containing 683 to 723 g/kg of CT from *A. mearnsii* (Mimosa extract ME powder®; Mimosa Extract Company (Pty) Ltd, Pietermaritzburg, South Africa) and ≥ 650 g/kg of hydrolysable ellagitannins from *C. sativa* (Gallo Tanin B®; Lamothe – Abiet, Canejan, France). The compound feeds containing the tannin extracts were pelleted and fed *ad libitum*, and were provided twice daily (09h00 and 17h00), along with grass hay at 100 g/kg of DM intake. Grass hay contained 35 g/kg DM of crude protein and 764 g/kg DM of neutral detergent fiber. The feed offered was adjusted according to the amount of feed refused. The ingredients and chemical and FA composition of the pelleted experimental compound feed are presented in Table 6. The four experimental periods had consisted of 2 weeks of adaptation to the diets and 1 week of sample collection.

Table 6. Proximal, chemical (g/kg DM) and fatty acids (FA) composition (% of total fatty acids) of pelleted experimental diets.

Item	Treatments[1]		
	M	C	MC
Ingredients			
Barley	270	270	270
Mimosa	100	-	50
Chestnut	-	100	50
Soybean meal	120	120	120
Dehydrated alfalfa	468	468	468
Linseed oil	20	20	20
Sunflower oil	20	20	20
Minerals and vitamins	2	2	2
Chemical composition			
Dry matter	913	911	912
Crude protein	156	164	143
Ether extract	44	59	45
NDF	338	319	325
Ash	83	77	80
Total FA (mg/g DM)	39.40	41.69	39.00
16:0	12.73	12.22	12.65
18:0	4.88	4.38	4.69
*c*9-18:1	24.95	24.34	24.95
18:2n-6	38.57	40.05	38.75
18:3n-3	18.87	19.00	18.97

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts.

3.2.2. Sample collection

Rumen samples were collected on days 18 and 21 of each experimental period. On the first day, total rumen contents were directly collected through rumen cannula before the morning meal and used for isolation of bacterial biomass fractions and FA analysis. On the second day, total rumen contents were collected as described for the first day and used for the evaluation of fermentative activity (pH and volatile FA analysis) and also for FA analysis. On both sampling days, sub-samples of total rumen contents were preserved for DNA extraction. Jugular venous blood samples were also collected from each animal into heparinized syringe tubes (S-Monovette®; Nümbrecht, Germany) on day 21 of each experimental period, before and 3h after the morning meal. Blood samples were immediately transported to the laboratory and centrifuged at 1650 xg for 15 min at 4 °C using a Universal 32 R, Hettich Lab Technology (Tuttlingen, Germany) centrifuge, to separate blood plasma for FA analysis.

Liquid (LAB) and solid (SAB) associated bacteria were obtained by fractionation and differential centrifugation of rumen contents using a Beckman Coulter, Avanti J-26 XPI^{TM} (Indianapolis, IN, USA) centrifuge, according to the procedure reported by Bessa *et al*. (2009) with slight modifications. These modifications included the use of four layers of surgical gauze to filter the rumen contents, as well as the re-suspension and homogenization of rumen washed particles in saline solution with carboximetilcellulose at 0.1%, which were then incubated at 39ºC for 15 min. At the end, LAB and SAB pellets were washed, at least three times, with saline solution until the washing solution became clear.

The aliquots of total rumen contents used for FA analysis and DNA extraction, as well as the bacterial pellets, were immediately stored at -20ºC, freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmark) and preserved at -20ºC until analyzed. The aliquots of rumen contents for evaluation of fermentation activity were immediately filtered through four layers of cheesecloth and then used either for pH measurement (Digital pH meter "pH-2005"; JP Selecta S.A, Barcelona, Spain) or stored at -20ºC for further volatile fatty acids (VFA) analysis. Moreover, feed chemical composition was determined using the methods described by Francisco *et al*. (2015)

3.2.3. Fatty acid analysis

Volatile fatty acids were analysed by GC-FID in a Shimadzu GC-2010 Plus chromatograph (Shimadzu, Kyoto, Japan) equipped with a Nukol capillary silica column (30 m; 0.25 mm i.d.; 0.25 µm film thickness, Supelco Inc., Bellefonte, PA, USA), as previously described (Oliveira *et al*., 2016). For long chain FA analysis, 250 mg of each sample was weighted and transesterified into FA methyl esters using a combination of basic followed by acidic catalysis (Alves *et al*., 2013b). The internal standard was 19:0 (1 mg/mL). Fatty acid methyl esters

were separated by GC-FID using a Shimadzu GC-2010 Plus chromatograph equipped with a SP-2560 capillary column (100 m, 0.25 mm i.d., 0.20 µm film thickness; Supelco Inc., Bellefont, PA, USA). Identification of FA methyl esters and DMA was achieved by comparison of retention times with those of authentic standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA, and a Bacterial FAME mix from Matreya LLC, Pleasant Gap, PA, USA) and by confirmation with GC-MS using a Shimadzu GC-MS QP 2010 Plus chromatograph (Kyoto, Japan) equipped with a SP-2560 column (Alves, Raundrup, Cabo, Bessa & Almeida, 2015).

3.2.4. Quantification of microbial 16S rRNA marker genes by qPCR

Total DNA was extracted from freeze-dried ruminal contents (30 mg) using a Qiagen QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA, USA), according to manufacturer's instructions and with small modifications to enhance lysis of Gram-positive bacteria (Oss *et al*., 2016). However, other alterations included the addition of 400 µl of the supernatant to 30 µl of proteinase K (Qiagen Inc., Valencia, CA, USA), with the resulting lysates being added to 400 µl of buffer AL (Lysis buffer; Qiagen Inc., Valencia, CA, USA) and 400 µl of ethanol. The eluted DNA was confirmed with agarose gel (0.8%) electrophoresis and quantified using the Quant-iT^{TM} PicoGreen® dsDNA Assay Kit (Invitrogen Canada Inc., Burlington, ON, Canada) with a NanoDrop 3300 fluorometer (ThermoScientific, Wilmington, DE, USA).

The extracted DNA was subjected to real-time quantitative polymerase chain reaction (qPCR), using a StepOnePlus thermocycler PCR (Applied Biosystems, Foster City, CA, USA), for quantification of copies of 16S rRNA sequences specific for *Fibrobacter succinogenes* subsp. S85, *Prevotella bryantii* B14, *Ruminococcus albus* 7, *Ruminococcus flavefaciens* C94, *Selenomonas ruminantium* subsp. *Lactilytica* (ATCC19205), *Ruminobacter amylophilus* (ATCC 29744), *Butyrivibrio fibrisolvens* JW 11(Wallace & Brammall, 1985) and *Butyrivibrio proteoclasticus* PI 18 (Wallace *et al*., 2006). The PCR standards for *Butyrivibrio* spp. were created with genomic DNA from reference bacterial strains of the Rowett Institute of Nutrition and Health, University of Aberdeen (UK) and cultured in Abel Salazar Biomedical Science Institute (ICBAS) (Portugal), while the standards for the other bacteria were done with plasmid DNA containing DNA amplicons derived from strains that came from Lethbridge Research Centre culture collection. The genomic DNA was extracted from 50 mg of freeze-dried bacterial pellets of pure cultures (ICBAS, Culture Collection) using a Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA), according to manufacturer's instructions. Detailed information about plasmid standards have already been reported by Wang *et al*. (2009), as well as the primers and cycling conditions for *F. succinogenes*, *P. bryantii*, *R. flavefaciens*, *S. ruminantium*, *R. amylophilus* (Tajima *et al*., 2001) and *R. albus*

(Wang, Cao & Cerniglia, 1997). For plasmid standards, the amplicons were used at concentrations from $2.0×10^8$ to $2.0×10^6$ and from $1.0×10^6$ to $1.0×10^2$ copies per reaction. For genomic DNA standards, the number of copies considered was $3.72×10^7$ to $3.72×10^1$ for *B. fibrisolvens* and $1.90×10^7$ to $1.90×10^1$ for *B. proteoclasticus*. All concentrations of the standards were obtained by serial 10-fold dilutions. For *Butyrivibrio* group, the following conditions were applied: 95 ºC for 10 min; 40 cycles of 95 ºC for 15 s, annealing temperature of 58 ºC for 1 min (*B. fibrisolvens*) or 55 ºC for 30 s with a final extension step at 72 ºC for 30 s (*B. proteoclasticus*). For these two bacteria, the real-time PCR reaction mixtures (15 µl) contained 1 × Taqman universal PCR master mix (Applied Biosystems®, Foster City, CA, USA), 10 µM of each primer to a final concentration of 100 and 300 nM of forward and reverse primers for *B. fibrisolvens* and *B. proteoclasticus*, respectively, 250 nM of both Taqman probes and 100 × purified BSA (BioLabs® Ipswich, MA, USA). For the other bacteria, each qPCR reaction (25 µl) contained 1 × iQ SYBR Green Supermix (Bio-Rad Laboratories®, Hercules, CA, USA), 100 × purified BSA (BioLabs®, Ipswich, MA, USA) and 10 µM of each primer to a final concentration of 300 nM. For all bacteria, BSA was used to overcome the presence of tannins as PCR inhibitors. The primers and probes for *Butyrivibrio* spp. were designed using Primer Express 2.0 software (Applied Biosystems®, Foster City, CA, USA) and the homology of their sequences was searched against genetic sequence database GenBank (GenBank®, Bethesda, MD, USA), showing that they were specific for sequences of 16S rRNA of the corresponding bacterial species. The sequences of the primers and probes were as follows: 5´-CCGCGTCAGATTAGCCAGTT-3´ (forward), 5´-GTAGGAGTTTGGGCCGTGTCT-3´ (reverse) and 5´-FAM-CCAAAGCAACGATCTGTA GCCGGACTG-TAMRA-3´ (probe) for *B. proteoclasticus*; 5´-ACACACCGCCCGTCACAC-3´ (forward), 5´-TCCTTACGGTTGGGTCACAGA-3´ (reverse) and 5´-JOE-CATGGGAG TTGGGAATGCCCGA-TAMRA-3´ (probe) for *B. fibrisolvens*. Primers were synthesised by NZYTech (Lisbon, Portugal) and probes were synthesised by Stabvida (Almada, Portugal). For all qPCR reactions, 2 µl of extracted DNA template was added to a final amount of 20 ng or 10 ng per reaction and was amplified in duplicate.

3.2.5. Statistical analysis and calculations

Data were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA) considering a change-over design with 3 diets, 4 sheep and 4 periods. The animal and period were included in the model as random blocks. Measurements repeated within the same animal and period were treated as subsampling considering either the unstructured or compound symmetry covariance matrix depending on the best model fit. The variance homogeneity was tested and, when significant, the variance heterogeneity structure was accommodated in the model. The effects of treatments were evaluated using 2 orthogonal

contrasts: 1) "M vs. C" contrasting the least square means of M against C and 2) MC vs. (M + C)/2, contrasting the least square means of MC against the average of M and C.

Data of rumen bacterial 16S rRNA copy numbers were averaged for each animal (period) and analysed with Proc GLIMMIX using a negative binomial distribution and log as link function and considering the effect of treatment, animal and period as fixed effects.

The ruminal BH (% of disappearance) of dietary c9-18:1, 18:2n-6 and 18:3n-3 and the BH completeness (18:0 concentration relative to potential 18:0 concentration assuming a complete BH of substrates) were calculated as previously reported (Alves et al., 2017). The concentrations of microbial structural FA in rumen bacterial fractions were used as microbial biomass markers to estimate the crude bacterial biomass in the rumen as follows:

$$Biom = \frac{M_{rumen}}{(0.5 \times M_{SAB} + 0.5 \times M_{LAB})}$$

Where, Biom is the estimated bacterial biomass (mg/g DM), M_{rumen}, M_{SAB} and M_{LAB} are the concentrations (mg/g DM) of the selected FA marker in the rumen, SAB and LAB fractions, respectively.

3.3. Results

3.3.1. Feed intake and rumen fermentation

The DMI was about 200 g/d lower (P = 0.025) with M than with C treatment and intermediate with MC treatment (Table 7). Rumen pH measured before the morning meal averaged 6.80 and did not differ among treatments (Table 7). Consistently with DMI, the VFA concentration (mmol/L) was greater (P < 0.001) with C than with M and was greater (P = 0.014) with MC than the average of M and C treatments (Table 7). The molar proportions of linear chain VFA (2:0, 3:0, 4:0 and 5:0) did not differ (P > 0.10) between M and C treatments, but the branched chain VFA (iso-4:0 and iso-5:0) presented higher (P < 0.04) proportions with C than with M. The MC treatment presented higher 3:0 (P = 0.027) and lower 2:0 (P = 0.004) and 4:0 (P = 0.049) proportions than the average of M and C treatments. The ratio between acetate and propionate was higher (P = 0.029) with C than with M and much lower (P = 0.001) with MC than the average of M and C.

Table 7. Effect of tannin extracts on DMI (dry matter intake) (g/d), rumen pH and total volatile fatty acid (VFA) concentration (mmol/L) and composition (mmol/100 mmol of total VFA).

Item	Treatments[1]			SEM[2]	Contrasts[3]	
	M	C	MC		C1	C2
DMI	743	927	849	134.1	0.025	0.737
Rumen pH	6.77	6.79	6.83	0.120	0.761	0.475
Total VFA	79.3	152.2	136.1	8.43	<0.001	0.014
2:0	44.6	46.1	42.1	1.37	0.121	0.004
3:0	26.4	22.3	31.4	3.41	0.118	0.027
iso-4:0	1.38	2.72	1.52	0.309	0.014	0.172
4:0	23.6	23.6	20.2	2.97	0.997	0.049
iso-5:0	1.89	3.54	1.80	0.509	0.036	0.138
5:0	2.06	1.78	2.93	0.527	0.711	0.154
2:0/3:0 ratio	1.79	2.11	1.41	0.210	0.029	0.001

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], standard error of means; [3], C1, mimosa versus chestnut; C2, (mimosa + chestnut)/2 versus mimosa plus chestnut treatments. Contrasts were significantly different for *P* < 0.05.

3.3.2. Abundance of selected rumen bacteria and the microbial biomass estimate

The abundance of selected bacterial species expressed as the number of copies×10^5 of 16S rRNA per mg DM of rumen content is presented in Table 8. The abundance of specialized fibrolytic bacteria (i.e. *F. succinogenes*, *R. albus, R. flavefaciens, B. proteoclasticus*) was reduced ($P \leq 0.020$) more with M than with C treatments. Conversely, *S. ruminantium* was more ($P = 0.019$) abundant with M than with C with no differences ($P > 0.100$) between these two treatments for *B. fibrisolvens*, *P. bryantii and R. amylophilus*. The abundances of *F. succinogenes*, *R. flavefaciens* and *S. ruminantium* were higher ($P < 0.050$) with MC than the average of abundances for M and C.

The crude bacterial biomass in the rumen was estimated using the sum of DMA that showed no significant differences between bacterial fractions (iso-14:0; 14:0, anteiso-15:0, 15:0, 16:0, Table 5). The crude bacterial biomass was lower ($P = 0.019$) with M than with C (218 vs. 301 g/kg DM of rumen content).

Table 8. Effect of tannin extracts on bacterial species (nº copies×10^5/mg dry matter) of rumen contents.

Item	Treatments[1]			Contrasts[2]	
	M	**C**	**MC**	**C1**	**C2**
Rumen bacteria					
F. succinogenes	2.78±1.52	207±113	153±66	0.018	0.043
P. bryantii	36.7±25.8	2.23±1.57	15.5±8.96	0.103	0.539
R. albus	0.04±0.02	6.69±3.14	0.31±0.16	0.005	0.547
R. amylophilus	2.15±3.46	0.59±1.61	0.65±1.05	0.722	0.877
R. flavefaciens	2.96±0.98	30.9±10.2	37.8±11.5	0.020	0.037
S. ruminantium	37.6±7.9	8.80±1.86	52.3±10.5	0.019	0.023
B. fibrisolvens	3.18±0.97	5.05±1.54	4.63±1.48	0.371	0.749
B. proteoclasticus	19.1±1.02	27.7±1.48	21.0±1.12	0.016	0.266
Biomass estimate (g/kg DM)					
DMA[3]	21.8±2.43	30.1±2.43	23.1±2.43	0.019	0.256

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], C1, mimosa versus chestnut; C2, (mimosa + chestnut)/2 versus mimosa plus chestnut treatments; [3], dimethylacetals (iso-14:0; 14:0, anteiso-15:0, 15:0, 16:0). Contrasts were significantly different for *P* < 0.05.

3.3.3. Fatty acid and dimethylacetals of rumen content

The FA and DMA composition of rumen content is presented in Table 9, whereas the detailed distribution of C18 FA is shown in Table 10. Total FA concentration and C18 FA proportion in the rumen presented no significant differences among treatments. Total FA averaged 43.7 mg/g DM and total C18 FA averaged 82.7% of total FA. The sum of branched-chain FA (BCFA) did not differ among treatments, although there were higher concentrations of iso-14:0, iso-16:0 and a lower concentration of iso-13:0 with C than with M. The sum of odd-chain FA (OCFA) was higher (*P* = 0.040) with C than with M treatments, exclusively due to the contribution of 15:0. Although the sum of even-chain FA was not significantly different between C and M, a higher (P = 0.023) concentration of 16:0 was found with C than with M treatments. The differences between M and C treatments were more extensive for DMA. In fact, eight DMA (anteiso-15:0¸15:0, 16:0, 13:0, iso-14:0, 14:0, *c*9-18:1, *c*11-18:1) and the sum of DMA were present in higher (*P* < 0.022) concentration with C than with M treatments. In general, for DMA, the MC treatment did not differ from the average of M and C treatments but concentrations of 13:0 and *c*11-18:1-DMA were higher (*P* < 0.02) with MC than the average of M and C.

Table 9. Effect of tannin extracts on total fatty acids (FA) and C18 FA (mg/g DM), FA and dimethylacetals (DMA) composition (mg/100g DM) of rumen contents.

Item	Treatments[1]			SEM[2]	Contrasts[3]	
	M	C	MC		C1	C2
∑FA	40.9	48.1	42.1	4.25	0.171	0.549
∑C18 FA	33.2	38.2	34.0	3.37	0.201	0.582
BCFA[4]						
i-13:0	4.12	1.88	1.66	0.473	0.011	0.030
i-14:0	3.58	6.16	5.61	0.541	0.005	0.075
i-15:0	10.6	13.3	12.3	2.20	0.288	0.875
a-15:0	20.3	23.5	22.0	2.12	0.315	0.973
i-16:0	6.44	15.2	7.51	1.829	0.008	0.172
i-17:0	7.04	7.87	6.05	1.806	0.620	0.373
a-17:0	7.62	12.9	5.72	1.753	0.061	0.063
i-18:0	0.74	1.00	0.84	0.297	0.489	0.922
Total	60.9	82.6	61.8	8.64	0.121	0.375
OCFA[5]						
13:0	0.76	0.85	1.20	0.143	0.545	0.010
15:0	18.4	24.9	24.6	1.38	0.013	0.124
17:0	11.2	13.1	12.6	0.89	0.167	0.660
21:0	2.01	1.99	2.04	0.105	0.818	0.613
23:0	5.03	5.08	5.13	0.271	0.575	0.403
Total	37.3	45.8	45.4	2.33	0.040	0.215
ECFA[6]						
14:0	21.8	22.8	20.0	1.38	0.575	0.190
16:0	482	625	501	48.6	0.023	0.171
20:0	22.5	25.2	22.5	2.70	0.243	0.472
22:0	27.8	29.3	28.0	2.10	0.335	0.619
24:0	19.9	20.3	19.3	1.43	0.679	0.404
26:0	25.5	34.5	34.7	6.26	0.130	0.303
28:0	11.5	12.6	13.8	0.01	0.249	0.065
Total	30.9	40.3	33.6	4.68	0.114	0.629
DMA[7]						
13:0	0.61	1.16	1.02	0.165	0.021	0.401
i-14:0	3.02	8.01	4.42	0.772	0.001	0.143
14:0	6.71	9.50	5.59	0.789	0.020	0.017
a-15:0	5.13	9.45	6.35	1.069	0.019	0.492
15:0	2.14	4.47	3.28	0.412	0.005	0.953
16:0	15.1	27.7	20.4	2.64	0.008	0.754
16:1	10.6	8.83	11.5	0.85	0.180	0.131
18:0	2.21	3.40	2.14	0.461	0.090	0.244
*t*11-18:1	0.57±0.045	1.19±0.388	0.77±0.045		0.210	0.630
*c*9-18:1	3.44	5.20	4.01	0.345	0.011	0.488
*c*11-18:1	1.32	2.76	1.76	0.252	<0.001	0.018
Total	50.8	81.8	61.1	5.16	0.002	0.434
Others[8]	0.13	0.16	0.19	0.019	0.061	0.167

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], standard error of means; [3], C1, mimosa versus chestnut; C2, (mimosa + chestnut)/2 versus mimosa plus chestnut treatments; [4], branched-chain FA; [5], odd-chain FA; [6], even and linear-chain fatty acids; [7], dimethylacetals; [8], includes 16:0-3,7,11,15Me; 5, 11-cyclohexyl-11:0; total 16:1 and 20:1. Contrasts were significantly different for $P < 0.05$.

The means of all C18 FA, except the 10-oxo-18:0, did not differ significantly between M and C treatments. However, M and MC treatments consistently presented greater variances for the majority of C18 FA than C treatment, as perceived by the largest standard errors of the means (Table 10). Such higher variance found with M and MC than with C treatments derived from a suppression of BH (animal 2, period 2, sampling day 1, treatment M; animal 3, period 1, sampling day 1, treatment MC; animal 4, period 2, sampling day 1, treatment MC). In these samples, dietary unsaturated FA (i.e. c9-18:1, 18:2n-6, and 18:3n-3) remained quite high, comprising collectively more than 40 % of total C18 FA in the rumen. Consequently, the estimates of disappearance of unsaturated FA (i.e. biohydrogenation, %), as well as the BH completeness were numerical higher and much less variable with C treatment than with M or MC treatments. Despite such variance pattern, the 10-oxo-18:0 was higher (P = 0.027) with M than with C.

Table 10. Effect of tannin extracts on C18 fatty acid (FA) composition (% of total C18 FA) and biohydrogenation estimates (%) of rumen contents.

Item	Treatments[1]			Contrasts[2]	
	M	C	MC	C1	C2
18:0	56.9±6.81	62.2±5.95	52.7±6.81	0.627	0.460
trans-18:1					
*t*2	0.13±0.028	0.16±0.028	0.15±0.028	0.294	0.781
*t*4	0.17±0.025	0.21±0.003	0.21±0.025	0.187	0.421
*t*5	0.07±0.031	0.10±0.015	0.12±0.031	0.902	0.326
*t*6/ *t*7/ *t*8	0.92±0.155	0.98±0.022	0.99±0.155	0.696	0.824
*t*9	0.45±0.055	0.55±0.016	0.50±0.055	0.139	0.953
*t*10	2.64±1.62	0.78±0.72	1.95±1.62	0.485	0.911
*t*11	9.6±1.76	12.6±0.23	10.8±1.76	0.121	0.903
*t*12	1.08±0.21	1.11±0.10	1.38±0.21	0.908	0.301
*t*15	1.41±0.154	1.13±0.154	1.40±0.154	0.296	0.541
*t*16[3]	1.25±0.19	1.16±0.12	1.16±0.18	0.725	0.848
Total	17.6±2.57	18.8±0.64	18.6±2.56	0.702	0.909
cis-18:1					
*c*9	9.6±1.81	8.5±0.35	10.7±1.81	0.573	0.442
*c*11	0.39±0.132	0.32±0.017	0.64±0.132	0.639	0.081
*c*12	0.87±0.093	0.78±0.093	1.02±0.093	0.518	0.185
*c*13	0.09±0.006	0.07±0.006	0.09±0.006	0.093	0.615
*c*15	0.43±0.059	0.31±0.008	0.52±0.059	0.065	0.061
*c*16	0.21±0.037	0.17±0.037	0.22±0.037	0.453	0.648
Total	11.6±1.95	10.2±0.35	13.1±1.95	0.614	0.437
Total 18:1	29.2±2.43	29.0±0.39	31.7±2.43	0.928	0.351
18:2 isomers					
n-6	6.52±2.24	4.51±0.58	7.42±2.24	0.403	0.466
trans/trans	0.08±0.079	0.07±0.005	0.20±0.079	0.878	0.271
*c*9,*t*12	0.10±0.041	0.08±0.002	0.17±0.041	0.459	0.721
*c*9,*t*11	0.22±0.035	0.19±0.035	0.27±0.035	0.585	0.214
*t*11,*c*15[4]	1.24±0.29	0.63±0.29	2.18±1.40	0.149	0.438
*c*9,*c*15	0.05±0.039	0.05±0.039	0.12±0.039	0.406	0.001
*c*12,*c*15	0.10±0.015	0.07±0.015	0.22±0.058	0.258	0.038
Others[5]	0.09±0.025	0.09±0.025	0.11±0.025	0.794	0.460
Total	8.42±2.98	5.68±0.61	10.67±2.98	0.386	0.301
18:3 isomers					
n-3	2.67±1.08	1.40±0.28	3.23±1.08	0.277	0.342
*c*9,*t*11,*c*15	0.07±0.013	0.04±0.013	0.07±0.013	0.183	0.422
Total	2.74±1.09	1.45±0.29	3.32±1.09	0.272	0.339
oxo-18:0 isomers					
10-oxo[6]	2.10±0.37	1.13±0.11	1.16±0.37	0.027	0.304
13-oxo	0.34±0.052	0.35±0.044	0.27±0.052	0.887	0.258
15-oxo	0.08±0.012	0.09±0.009	0.06±0.017	0.457	0.275
Total	2.52±0.40	1.56±0.12	1.49±0.40	0.041	0.248
Total BI[7]	23.9±2.83	23.1±0.37	25.3±2.83	0.787	0.527
Biohydrogenation Disappearance					
*c*9-18:1	66.5±6.41	69.3±1.37	62.7±6.41	0.670	0.476
18:2n-6	85.2±5.06	90.1±1.46	83.3±5.06	0.357	0.446
18:3n-3	87.6±5.19	93.5±1.64	85.1±5.19	0.276	0.350
Completeness	67.8±6.17	72.6±1.39	65.4±6.17	0.439	0.492

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], C1, mimosa.

3.3.4. Fatty acid and dimethylacetals of rumen bacterial fractions

The FA and DMA composition of SAB and LAB is presented in Table 11, whereas the detailed C18 FA composition of bacterial fractions is presented in Table 12. The total of FA and C18 FA did not differ among treatments and not even between SAB and LAB. The FA and C18 FA content of bacterial fractions averaged 106 and 85 mg/g DM, respectively. The majority of FA corresponded to C18 FA followed by 16:0, which also did not differ among treatments or bacterial fractions. Considering the FA composition in more detail, the sum of BCFA was higher (P = 0.019) in LAB than in SAB, and this was due to the higher concentrations of iso-13:0 and mostly of anteiso-15:0 in LAB. The treatments did not affect the sum of BCFA but iso-14:0 and iso-16:0 were lower (P < 0.01) and iso-13:0 was higher (P = 0.009) with M than with C.

Odd- and linear-chain FA (OCFA) were higher (P = 0.022) in LAB than in SAB, mostly due to 15:0 and 17:0. The only OCFA that differed between M and C treatments was the 23:0, which was higher (P = 0.003) with M compared to C. However, the bacterial 15:0 and 17:0 contents were higher (P < 0.05) with MC than the average of contents of M and C.
Rumen bacteria presented a considerable amount of ECFA with carbon chain length ranging from 20 to 28 carbons. The concentrations of 22:0 and 24:0 were higher (P < 0.020) with M than with C and those of 26:0 and 28:0 were greater (P < 0.010) with MC than the average of M and C.
The total DMA content of bacteria averaged 240 mg/100 g DM and did not differ between LAB and SAB, although the concentrations of c11-18:1, c9-18:1 and 16:1-DMA (P < 0.040) were higher in LAB than in SAB and that of 18:0-DMA was greater (P = 0.023) in SAB than in LAB. Nevertheless, the sum of DMA and most of individual DMA (i.e. 13:0-, iso-14:0-, 15:0-, 16:0-, 18:0- and c11-18:1- DMA) were higher (P < 0.030) with C than with M treatments with only an opposite response for 16:1-DMA (P < 0.001). Also, the 16:1-DMA was higher (P < 0.001) with MC than the average of M and C treatments.

Table 11. Effect of tannin extracts on total fatty acids (FA) and C18 FA (mg/g DM), FA and dimethylacetals (DMA) composition (mg/100g DM) of solid (SAB) and liquid (LAB) associated bacterial fractions.

Item	Treatments[1]			P-value		Bacterial fractions		P-value
	M	C	MC	C1[2]	C2	SAB	LAB	
∑FA	109±8.9	100±8.9	107±8.9	0.475	0.817	105±7.2	106±7.2	0.890
∑C18 FA	88±8.5	73±8.5	93±8.5	0.240	0.234	82±7.0	87±7.0	0.612
BCFA[3]								
i-13:0	15±2.3	6.6±0.24	5.9±0.24	0.009	0.004	8.4±0.79	10±0.79	0.002
i-14:0	14±1.0	26±1.4	21±4.6	<0.001	0.931	18±1.9	22±1.9	0.075
i-15:0	45±9.7	54±1.8	42±1.8	0.397	0.204	46±3.6	47±3.6	0.613
a-15:0	87±9.6	107±9.6	80±9.6	0.155	0.176	73±7.8	110±7.8	0.005
i-16:0	23±5.1	48±5.1	26±5.1	0.003	0.153	34±4.2	31±4.2	0.550
i-17:0	22±4.6	23±0.7	20±0.7	0.958	0.260	22±1.7	21±1.7	0.951
a-17:0	25±7.8	37±4.2	20±4.2	0.139	0.019	30±4.7	25±4.7	0.070
i-18:0	3.8±0.37	3.9±0.37	4.0±0.37	0.833	0.815	3.5±0.30	4.3±0.30	0.091
Total	235±51	305±11	218±11	0.219	0.096	229±19	276±19	0.019
OCFA[4]								
13:0	4.4±0.62	4.4±0.62	6.6±1.39	0.992	0.165	4.5±0.71	5.8±0.71	0.173
15:0	71±8.6	82±8.6	96±8.6	0.174	0.013	76±8.0	90±8.0	0.037
17:0	37±3.2	35±3.2	46±3.2	0.798	0.023	35±2.6	44±2.6	0.023
21:0	4.6±0.32	3.6±0.32	4.6±0.32	0.053	0.231	4.3±0.26	4.1±0.26	0.594
23:0	14±0.85	9.8±0.85	13±0.85	0.003	0.473	13±0.69	12±0.69	0.535
Total	131±12	136±12	166±12	0.708	0.006	132±11	157±11	0.022
ECFA[5]								
14:0	87±8.1	77±8.1	84±8.1	0.393	0.838	72±6.7	93±6.7	0.033
16:0	1509±205	1467±85	1434±85	0.857	0.707	1497±99	1443±99	0.664
20:0	61±4.8	52±4.8	60±4.8	0.197	0.533	56±3.9	59±3.9	0.628
22:0	70±4.7	51±4.7	66±4.7	0.014	0.341	62±3.9	63±3.9	0.966
24:0	51±3.1	37±3.1	47±3.1	0.007	0.358	45±2.5	45±2.5	0.874
26:0	53±11	52±11	72±11	0.891	0.005	55±11	62±11	0.248
28:0	25±1.8	20±1.8	29±1.8	0.098	0.008	24±1.5	26±1.5	0.346
Total	1887±157	1792±157	1845±157	0.676	0.979	1920±129	1763±129	0.401
DMA[6]								
13:0	1.5±0.19	2.5±0.19	3.5±1.3	0.007	0.293	2.5±0.46	2.5±0.46	0.910
i-14:0	14±1.5	26±1.4	18±4.6	<0.001	0.666	17±1.9	21±1.9	0.169
14:0	28±2.4	29±1.4	21±3.9	0.684	0.125	25±2.1	27±2.1	0.596
a-15:0	26±1.1	33±5.2	30±5.2	0.196	0.997	31±2.7	28±3.7	0.207
15:0	9.4±0.60	15±0.60	13±1.7	<0.001	0.632	12±0.77	13±0.77	0.361
16:0	70±8.5	95±8.5	90±1.5	0.029	0.617	84±8.9	87±8.9	0.773
16:1	33±2.7	22±2.0	34±2.7	<0.001	<0.001	21±2.2	32±2.2	<0.001
18:0	8.1±0.51	12±0.51	9.4±3.1	0.001	0.889	11±1.1	8.7±1.1	0.023
*t*11-18:1	3.3±0.71	5.1±0.71	4.2±0.71	0.081	0.959	3.4±0.57	5.1±0.57	0.057
*c*9-18:1	15±2.8	14±0.62	17±2.8	0.921	0.448	12±1.5	19±1.5	0.008
*c*11-18:1	6.0±0.63	10±0.63	12±2.8	0.001	0.248	8.2±1.1	11±1.1	0.033
Total	213±14	264±14	242±30	0.006	0.922	230±16	250±16	0.176
Others[7]	52.0±1.10	59.6±8.38	64.9±8.38	0.396	0.363	44.8±6.24	72.9±6.24	<0.001

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], C1, mimosa versus chestnut; C2, (mimosa + chestnut)/2 versus mimosa plus chestnut treatments; [3], branched-chain FA; [4], odd-chain FA [5], even and linear-chain fatty acids; [6], dimethylacetals; [7], includes 16:0-3,7,11,15Me; 5, 11-cyclohexyl-11:0; total 16:1 and 20:1; standard error of means (±). Contrasts were significantly different for *P* < 0.05.

Attending to C18 FA composition, 18:0 was the main C18 FA and tended to be higher (P = 0.074) in LAB than in SAB but it was unaffected by treatments (Table 12). The C18 FA composition of LAB was fairly similar to that of SAB, although SAB presented higher (P < 0.020) proportions of some BH-derived FA than LAB (i.e. *t*11-18:1, *c*9,*t*11-18:2, *c*9,*t*11,*c*15-18:3 and 10-oxo-18:0). The sum of *trans*-18:1, with significant contributions of the proportions of *t*6-/*t*7-/*t*8-18:1, *t*9-18:1, *t*11-18:1, was higher (P = 0.003) with C than with M, whereas the opposite was found for *t*15-18:1, *t*16-18:1, *c*13-18:1, *t,t*-18:2, and *c*9,*c*15-18:2 (P < 0.050) with a tendency for *c*15-18:1 (P = 0.058). Overall, the sum of BH intermediates tended to be higher (P = 0.055) with C than with M. Moreover, the "*trans*-/*cis*-18:1" ratio was greater (P = 0.002) with C comparing with M. The C18 FA composition with MC was mostly similar to the average of M and C treatments, although with a lower (P = 0.039) total of oxo-18:0 and higher (P < 0.040) *c*9,*c*15-18:2 and *c*12,*c*15-18:2.

Table 12. Effect of tannin extracts on C18 FA composition (% total C18 FA) of solid (SAB) and liquid (LAB) associated bacterial fractions.

Item	Treatments[1]			P-value		Bacterial fractions		P-value
	M	C	MC	C1[2]	C2	SAB	LAB	
18:0	62.6±5.95	65.7±1.11	59.1±5.95	0.612	0.463	59.5±3.05	65.3±3.05	0.074
trans-18:1								
*t*2	0.15±0.01	0.16±0.003	0.15±0.01	0.361	0.914	0.15±0.006	0.15±0.006	0.523
*t*4	0.26±0.04	0.26±0.01	0.29±0.04	0.935	0.503	0.26±0.02	0.29±0.02	0.207
*t*5	0.14±0.06	0.09±0.001	0.22±0.06	0.479	0.172	0.15±0.03	0.15±0.02	0.324
*t*6/ *t*7/ *t*8	0.56±0.10	0.97±0.01	0.75±0.10	0.002	0.888	0.75±0.05	0.77±0.05	0.592
*t*9	0.29±0.08	0.55±0.05	0.38±.08	0.001	0.556	0.43±0.06	0.38±0.06	0.186
*t*10	0.99±0.16	0.97±0.05	1.18±0.16	0.907	0.281	1.04±0.09	1.06±0.09	0.803
*t*11	4.57±1.57	10.9±0.38	6.63±0.38	0.007	0.257	8.30±0.62	6.41±0.62	0.011
*t*12	1.33±0.36	1.16±0.02	2.09±0.36	0.650	0.063	1.50±0.17	1.55±0.117	0.288
*t*15	1.70±0.07	1.22±0.02	1.32±0.07	<0.001	0.121	1.36±0.04	1.47±0.04	0.059
*t*16[3]	1.40±0.06	1.20±0.06	1.15±0.06	0.037	0.057	1.20±0.05	1.29±0.05	0.213
Total	11.4±1.35	17.4±0.35	14.±0.35	0.003	0.756	15.1±0.54	13.6±0.54	0.017
cis-18:1								
*c*9	11.7±2.44	7.6±0.40	10.4±2.44	0.122	0.789	11.1±1.23	8.7±1.23	0.056
*c*11	0.56±0.53	0.61±0.09	1.59±0.53	0.921	0.124	0.72±0.27	1.12±0.27	0.112
*c*12	1.26±0.27	0.88±0.02	0.97±0.02	0.219	0.487	0.99±0.09	1.08±0.09	0.017
*c*13	0.11±0.018	0.06±0.002	0.08±0.018	0.027	0.945	0.08±0.009	0.08±0.008	0.902
*c*15	0.64±0.15	0.31±0.01	0.78±0.15	0.058	0.102	0.56±0.07	0.58±0.07	0.408
*c*16	0.23±0.015	0.17±0.015	0.22±0.015	0.023	0.289	0.20±0.01	0.22±0.01	0.259
Total	14.5±3.18	9.6±0.44	14.1±3.18	0.156	0.589	13.7±1.57	11.8±1.57	0.114
Total 18:1	25.9±2.86	27.1±0.72	28.2±2.86	0.698	0.602	28.9±1.55	25.3±1.55	0.076
trans/*cis* ratio	1.01±0.15	1.84±0.15	1.40±0.55	0.002	0.896	1.33±0.12	1.50±0.12	0.333

Table 12. Effect of tannin extracts on C18 FA composition (% total C18 FA) of solid (SAB) and liquid (LAB) associated bacterial fractions (continuation).

Item	Treatments[1]			P-value		Bacterial fractions		P-value
	M	C	MC	C1[2]	C2	SAB	LAB	
18:2								
n-6	3.78±0.59	2.84±0.18	3.83±0.59	0.157	0.451	3.79±0.34	3.18±0.34	0.193
trans/trans	0.08±0.004	0.02±0.022	0.33±0.19	0.047	0.212	0.14±0.066	0.14±0.066	0.763
*c*9,*t*12	0.13±0.03	0.07±0.003	0.24±0.11	0.147	0.263	0.16±0.04	0.14±0.04	0.064
*c*9,*t*11	0.27±0.08	0.61±0.19	0.30±0.08	0.129	0.286	0.54±0.09	0.24±0.09	0.015
*t*11,*c*15[4]	1.45±0.43	0.55±0.03	4.63±2.52	0.093	0.201	2.25±0.85	2.17±0.85	0.228
*c*9,*c*15	0.08±0.004	0.05±0.004	0.11±0.016	0.001	0.036	0.09±0.006	0.08±0.006	0.355
*c*12,*c*15	0.09±0.006	0.07±0.006	0.14±0.006	0.106	<0.001	0.10±0.005	0.11±0.005	0.207
Others[5]	0.08±0.01	0.06±0.01	0.23±0.11	0.086	0.215	0.13±0.04	0.12±0.04	0.254
Total	5.96±0.854	4.37±0.54	9.82±3.66	0.060	0.249	7.42±1.30	5.95±1.30	0.092
18:3								
n-3	1.18±0.27	0.78±0.05	1.34±0.27	0.173	0.261	1.19±0.14	1.01±0.14	0.151
*c*9,*t*11,*c*15	0.05±0.02	0.10±0.02	0.08±0.02	0.263	0.771	0.12±0.02	0.04±0.02	0.019
Total	1.23±0.28	0.87±0.07	1.42±0.28	0.241	0.271	1.33±0.16	1.02±0.16	0.114
oxo-18:0								
10-oxo-[6]	3.82±1.35	1.38±0.04	1.09±0.04	0.114	0.059	2.18±0.45	2.01±0.45	0.013
13-oxo-	0.39±0.005	0.39±0.019	0.26±0.068	0.965	0.107	0.35±0.024	0.35±0.024	0.699
15-oxo-	0.09±0.003	0.09±0.003	0.05±0.013	0.933	0.017	0.08±0.005	0.08±0.005	0.901
Total	4.35±1.35	2.07±0.04	1.49±0.04	0.137	0.039	2.75±0.45	2.53±0.45	0.011
Total BI[7]	20.0±0.73	22.3±0.73	23.6±4.02	0.055	0.573	22.9±1.48	21.0±1.48	0.108

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], contrasts: C1, mimosa versus chestnut; C2, (mimosa + chestnut)/2 versus mimosa plus chestnut treatments; [3], includes *c*14-18:1; [4], includes *t*10,*c*15-18:2; [5], conjugated *trans,cis/cis,trans*-18:2 isomers; [6], includes oxo-12 as minor component; [7], biohydrogenation intermediates, sum of total 18:1, 18:2, 18:3 and 18:0-oxo except *c*9-18:1, *c*11-18:1, 18:2n-6 and 18:3n-3; standard error of means (±). Contrasts were significantly different for $P < 0.05$.

3.3.5. Fatty acid and dimethylacetal composition of blood plasma

The FA and DMA composition of blood plasma is presented in Table 13. The total FA and total C18 FA concentrations averaged 1.91 and 1.53 mg/mL, respectively, and did not differ among treatments. The sums of 18:2 and 18:3 were slightly but not significantly higher with M than with C, although the concentration of t11,c15-18:2 was higher (P = 0.039) and that of c9,c15-18:2 tended (P = 0.084) to be higher with M than with C treatments. The sums of *trans*-18:1 and *cis*-18:1 did not differ among treatments, but c16-18:1 was higher (P = 0.037) and c15-18:1 tended (P = 0.082) to be higher with MC than the average of M and C. The oxo-18:0 was higher (P = 0.015) with M than with C. Attending to other FA, the only differences found were for the sum of FA with more than 18 carbons (LC-FA) and for the sum of DMA, which tended (P < 0.060) to be higher with M than with C treatments.

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Table 13. Effect of tannin extracts on total C18 fatty acids (FA), total FA (mg/mL) and other C18 FA and FA (µg/mL) of blood plasma.

Item	Treatments[1]			SEM[2]	Contrasts[3]	
	M	C	MC		C1	C2
Total C18 FA	1.61	1.51	1.48	0.092	0.480	0.486
18:0	337	308	324	49.4	0.585	0.969
trans-18:1						
*t*6/ *t*7/ *t*8	5.74	4.93	6.16	1.168	0.455	0.390
*t*9	5.02	4.02	4.63	0.557	0.177	0.854
*t*10	6.77	0.65	4.90	2.877	0.145	0.719
*t*11	34.3	33.7	40.5	8.46	0.930	0.325
*t*12	8.82	6.75	8.28	1.843	0.310	0.762
*t*15	4.74	2.88	3.90	1.168	0.172	0.932
*t*16	5.87	4.08	5.32	1.276	0.226	0.763
Total	73.4	59.4	78.6	15.18	0.323	0.321
cis-18:1						
*c*9	264	225	209	30.9	0.351	0.332
*c*11	7.15	6.17	7.07	0.783	0.255	0.567
*c*12	19.2	14.1	20.3	5.06	0.302	0.390
*c*13	0.88	0.59	0.88	0.306	0.409	0.624
*c*15	0.80	0.88	1.15	0.180	0.626	0.082
*c*16	0.95	0.95	1.64	0.454	0.989	0.037
Total	294	248	240	32.8	0.329	0.447
Total 18:1	397	338	339	37.9	0.301	0.555
18:2						
18:2n-6	609	552	621	62.1	0.447	0.534
*c*9,*t*11	3.89	4.10	4.40	0.442	0.674	0.387
*t*11,*c*15[4]	3.54	1.84	3.57	0.758	0.039	0.166
*c*9,*c*15	0.77	0.38	0.69	0.139	0.084	0.513
*c*12,*c*15	1.38	0.77	1.38	0.375	0.277	0.506
Other 18:2[5]	12.8	11.2	11.5	1.69	0.388	0.734
Total	539	465	551	30.7	0.189	0.282
18:3	46.6	43.2	53.5	10.93	0.794	0.467
18:3n-3	36.1	32.2	42.7	9.09	0.662	0.313
18:3n-6	4.78	4.57	5.42	1.176	0.817	0.390
*c*9,*t*11,c15[6]	1.50	1.58	1.64	0.307	0.850	0.799
Total						
Oxo-18:0	1.92	0.53	1.03	0.395	0.015	0.463
Total BI[7]	128	105	131	24.2	0.391	0.528
Total FA	2.02	1.87	1.83	0.125	0.416	0.474
Total ECFA[8]	572	506	528	60.1	0.307	0.837
Total OCFA[9]	21.2	19.4	21.1	2.03	0.502	0.721
Total BCFA[10]	10.2	11.5	9.95	1.231	0.475	0.568
Total DMA[11]	10.4	7.70	8.47	1.752	0.055	0.585
Total LC-FA[12]	97.3	75.0	86.2	8.05	0.053	0.995
Others[13]	20.2	19.3	20.5	2.37	0.722	0.726

[1], M, mimosa; C, chestnut; MC, mimosa plus chestnut tannin extracts; [2], standard error of means; [3], C1, mimosa versus chestnut; C2, (mimosa + chestnut)/2 versus mimosa plus chestnut treatments; [4], includes *t*10,*c*15-18:2; [5], conjugated *trans,cis-/cis,trans/trans,trans*-18:2 isomers and 5, 11-cyclohexyl-11:0; [6], includes 20:3n-9; [7], biohydrogenation intermediates, sum of total 18:1, 18:2, 18:3 and 18:0-oxo except *c*9-18:1; *c*11-18:1; 18:2n-6; 18:3n-3; and 18:3n-6; [8], even and linear-chain FA; [9], odd-chain FA; [10], branched-chain FA;[11], dimethylacetals; [12], long chain FA with higher than 18 carbons; [13], 16:0-3,7,11,15Me and total 16:1. Contrasts were significantly different for *P* < 0.05.

3.4. Discussion

The dietary incorporation of mimosa condensed tannins led to lower DMI, VFA concentration in the rumen, abundance of rumen specialized fibrolytic bacteria and DMA-based estimates of bacterial biomass than that of chestnut hydrolysable ellagitannins. Thus, condensed tannins led to a general depression of rumen activity when compared with hydrolysable tannins, confirming that condensed tannins exert a more inhibitory action on rumen microorganisms than hydrolysable tannins (O'Donovan & Brooker, 2001; Krause, Smith, Brooker & McSweeney, 2005; Bhatta *et al*., 2009; Buccioni *et al*., 2015).

The differential effect of tannin extracts on DMI was partially due to the lowest palatability of M pelleted diet as compared to C and MC diets. In fact, throughout the experiment, it was clear that the appetence for M pellets of all sheep was lower than that for C and even MC pellets. Additionally, the eventual greater ability of condensed tannins to bind to organic matter and thus to inhibit its digestibility may have also contributed to the lowest DMI of sheep fed M diet, as other authors have also observed post-ingestive adverse effects in ruminants fed condensed tannins (Silanikove, Perevolotsky & Provenza, 2001).

The stronger affinity of condensed tannins for proteins and, consequently, the more markedly reduced rumen degradability of dietary protein with condensed tannins as compared to hydrolysable tannins was clearly evident by the quantitative and proportional reductions of iso-4:0 and iso-5:0, as previously reported (Bhatta *et al*., 2009; Buccioni *et al*., 2015). In fact, iso-VFA derived from the fermentation of branched-chain amino acids (Mackie & White, 1990). Other reports have also described a more marked decrease of VFA, mostly of acetate, with condensed in relation with hydrolysable tannins (Makkar, Blummel & Becker, 1995; Getachew *et al*., 2008; Buccioni *et al*., 2015; Jayanegara, Goel, Makkar & Becker, 2015). It seems that the highest dose of mimosa tannins (100 g/kg DM) was the main factor for the inhibition of ruminal fermentation, considering that the MC treatment presented a higher total VFA production than that of the mean of M and C treatments.

The reduced growth of specialized fibrolytic bacteria with condensed comparing with hydrolysable tannins was shown by lower abundance of *F. succinogenes*, *R. albus*, *R. flavefaciens* and *B. proteoclasticus* with M than with C treatments. These results contrast with those obtained *in vitro* using tannin sources extracted and purified in laboratorial conditions, as Jayanegara *et al*. (2015) reported that the abundance of *R. flavefaciens* was more reduced with chestnut hydrolysable tannins than mimosa condensed tannins and that of *F. succinogenes* was similarly reduced by both mimosa and chestnut tannins. Factors like the type of extract (commercial vs. purified extracts), dose (100 vs. 73 g/kg of DM) and experimental model (*in vivo* vs. *in vitro*) make it difficult to make comparisons across studies,

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even though the effects of tannins on rumen metabolism are largely inconsistent in the literature (Makkar, 2003).

Beside the specialized fibrolytic bacteria, *S. ruminantium* was more abundant with M than with C treatments. Moreover, *S. ruminantium* seems to be consistently favoured by the presence of mimosa tannins, since its abundance was also higher with MC treatment. Conversely, the *F. succinogenes* was consistently favoured by the presence of chestnut extract, being more abundant with C and MC treatments. The explanation for differential responses of rumen bacteria to condensed and hydrolysable tannins is unclear, but it has been described that some species of rumen bacteria are more susceptible than others to the effects of tannins. In fact, Gram-negative bacteria, *S. ruminantium* in the present study, were shown to be less affected by condensed tannins than Gram-positive bacteria (Smith & Mackie, 2004). This response likely reflects the greater affinity of tannins for bacteria with Gram-positive as compared to Gram-negative cell walls (Smith *et al.*, 2005). Although *F. succinogenes* is also a Gram-negative bacterium, its susceptibility to condensed tannins has been documented (Bae, Mcallister, Yanke, Cheng & Muir, 1993; McSweeney, Palmer, Bunch & Krause, 2001a). This observation may explain why the 16S rRNA copy numbers of this bacterium were higher in the presence of hydrolysable as compared to condensed tannins.

In the present study, the crude microbial biomass in rumen contents was estimated using DMA as an internal microbial marker. Previously, Saluzzi *et al.* (1995) evaluated the DMA profile of bacterial fractions and, more recently, Alves *et al.* (2013b) suggested the utility of DMA as a microbial marker. The DMA are not exclusively of bacterial origin as their presence has also been reported in ciliate protozoa (Harfoot & Hazlewood, 1997). Thus, microbial biomass estimates based only on DMA concentration of mixed rumen bacterial fractions can be influenced by both bacterial and protozoal populations. Nevertheless, the biomass was estimated to account for 22 to 30% of rumen DM content shortly before feeding, which is consistent with previous estimates obtained using other methods (Craig, Brown, Broderick & Ricker, 1987; Legay-Carmier & Bauchart, 1989; Bessa *et al.*, 2009). The rumen biomass estimates also pointed to a general microbial depression with mimosa than with chestnut tannin extracts.

Overall, these results probably reflect a higher effectiveness of condensed tannins to bind to polymers, mainly proteins and carbohydrates, than hydrolysable tannins, reducing rumen microbial activity and population. These differential tannin effects were probably due to differences in the molecular structures of mimosa condensed as compared to chestnut hydrolysable tannins. Indeed, commercial polyflavonoid tannins of mimosa were described to be oligomers, mainly trimers and tetramers of 809, 906 and 1179 Da, respectively, with prorobinetinidin (288 Da) as the predominant repeat unit (Pasch, Pizzi & Rode, 2001). In contrast, commercial hydrolysable tannins from chestnut were reported to be composed

mostly of ellagitannins, with castalagin and vescalagin of molecular weights of 935 Da being the main constituents (Pasch & Pizzi, 2002). Hydrolysable tannins have been shown to be extensively hydrolysed in the rumen into simpler units, while mimosa oligomers linked by carbon-carbon bonds are less susceptible to hydrolysis. Thus, the high MW and large number of phenolic hydroxyl groups of mimosa tannins are expected to form strong complexes via hydrogen bonds with carboxyl groups of aliphatic and aromatic side chains of proteins and other macromolecules, including lipoproteins from cell membranes and bacterial enzymes (Field & Lettinga, 1992; McAllister *et al.*, 2005; Patra & Saxena, 2011).

Despite the general depressive effects on rumen fermentation of mimosa condensed tannins when compared with chestnut hydrolysable tannins, it was not possible to detect clear effects of these compounds on ruminal BH or impacts on blood plasma FA profiles. The estimated BH extent (disappearance of substrates) and completeness were fairly high and comparable to what was expected (Fievez, Vlaeminck, Jenkins, Enjalbert & Doreau, 2007). Ruminal BH can be considered a response to stress stimuli towards rumen microbiota induced by high concentration of UFA in the rumen milieu (Bessa *et al.*, 2000) and, eventually, also by additional stress factors like high tannin concentrations. Several studies reported a modulation of BH by tannins usually with increased BH intermediates, particularly *trans*-18:1 isomers, in the rumen (Vasta *et al.*, 2009a; Buccioni *et al.*, 2011; Carreño *et al.*, 2015). Our experimental model intended to induce such cumulative environmental stress in the rumen (i.e. high PUFA and high tannins) but, due to low statistical power of the experiment, the only clear response detected was the increased variability in BH with M and MC treatments. This increased variability was mostly caused by an occasional disruption of BH pathways observed over a few of the sampling days. This response was not clearly associated with the individual sheep, period or even with the DMI from the previous day. Such high variability was not reflected in the amount of bacterial species quantified by real time PCR, including those of *Butyrivibrio* spp., which have been considered to be the major bacteria responsible for BH in the rumen (Jenkins *et al.*, 2008; Lourenço *et al.*, 2010). Nevertheless, the abundance of *B. proteoclasticus*, a 18:0-producer, was higher with C compared to M and MC treatments and that might explain the highest numerical and less variable BH completeness (i.e. recover as 18:0 of biohydrogenated UFA) with C treatment.

Interestingly, a higher abundance of *S. ruminantium* with M than with C treatments might have contributed to a greater accumulation of oxo-18:0 FA in the rumen and blood plasma with condensed than with hydrolysable tannins. Indeed, this bacterium has the ability to hydrate *c*9-18:1 to 10-OH-18:0 (Hudson *et al.*, 1995), which is a precursor of 10-oxo-18:0 (Jenkins *et al.*, 2006). However, a higher abundance of *S. ruminantium* in the rumen together with a lower sum of oxo-18:0, mainly 15-oxo-18:0 and slightly 10-oxo-18:0, in bacterial

fractions with MC than the average of M and C might reflect the involvement of other rumen bacteria in the production of oxo-18:0.

It has been proposed that the accumulation of *trans*-18:1 and its incorporation into microbial cell membranes or a direct *cis* to *trans* isomerisation in the membranes favours the adaptation of bacteria to compounds, like PUFA and tannins, that can alter cell membrane integrity (Keweloh & Heipieper, 1996; Heipieper, Fischer & Meinhardt, 2010). Consistently with our hypothesis, the suppression of BH observed with condensed as compared to hydrolysable tannins may reflect their ability to more readily form complexes with lipoproteins from bacterial cell membranes and with enzymes (Field & Lettinga, 1992). However, hydrolysable tannins are easily hydrolysed into membrane-disturbing compounds (Field & Lettinga, 1987; Scalbert, 1991) that could potentially stimulate the incorporation of *trans*-18:1 into bacterial cell membranes, which might explain the higher "*trans*-/*cis*-18:1" ratio with hydrolysable than with condensed tannins in bacterial biomass. The increased bacterial incorporation of *trans*-18:1 with hydrolysable tannins was mainly due to a higher production of *t*11-18:1 during ruminal BH comparing with condensed tannins rather than *cis* to *trans* isomerisation.

3.5. Conclusions

In the present study, mimosa tannin extract led to lower rumen fermentative activity and abundance of specialized fibrolytic bacteria compared with chestnut tannin extract, when both were incorporated at high doses (100 g/kg DM) in sheep's diet. These results were possibly caused by the stronger ability of condensed tannins to bind to dietary and microbial polymers, causing a general inhibition of rumen microbial activity. Moreover, the instability of bacterial cell membrane caused by tannins was more evident with hydrolysable than with condensed tannins with a greater production of *trans*-18:1 by rumen bacteria and possible incorporation into their cell membranes. Further studies are needed to evaluate the influence of the two types of tannins, from the same or from different sources, at less than 100 g/kg DM in the diet, on ruminal BH.

Acknowledgements

CHAPTER 4 – The induction of *trans*-10 shift in sheep fed with a concentrate-based diet supplemented with sunflower oil

The induction of *trans*-10 shift in sheep fed with a concentrate-based diet supplemented with sunflower oil

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Article in preparation

Contribution of Mónica Costa to this article:

Mónica Costa participated in the animal experiment and management and sample collection. Also, she determined the FA content and performed the data processing and statistical analysis. Furthermore, Mónica Costa participated in the interpretation and discussion of the results, as well as in the writing of the manuscript.

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ABSTRACT

The hypothesis of gradual occurrence and maintenance of the shift from *t*11 to *t*10 ruminal BH pathways (*t*10-shift) associated with rumen pH reduction and an impact on FA composition of blood plasma was tested. Two rumen fistulated rams were housed in metabolic cages and adapted to a diet composed of 723 g/kg dry matter (DM) of ground wheat grain with sunflower oil (41 g/kg DM) (*t*10-shift inducing concentrate), 191 g/kg DM of commercial compound feed and 191 g/kg DM of forage, for 29 days of experimental period. Rumen contents were collected before and 3h after the morning meal. Once a week, rumen contents were obtained every 1h30 from 9h30 to 20h00 and blood plasma was collected at 9h30 and 12h30. The *t*10-shift appeared between days 8 and 11 and on day 15 in ram 1 and from days 15 to 17 in ram 2. The shift was associated with an increase of feed intake following its decrease and, only in ram 1, to an increase of PUFA in rumen contents. Also, there was an increment of the total of *trans*-18:1 and a decrease of 18:0 during the *t*10-shift and an increase of oxo-18:0 before the shift. No clear associations between *t*10-shift´s induction and rumen pH or blood plasma FA profiles were found, but ram 2 presented a higher post-prandial *t*10-/*t*11-18:1 ratio together with a lower pH in the rumen. These differences between animals are probably due to variability of rumen microbiota. During a selected period of the trial, there was an inferior rumen bacterial diversity with lower abundance of *Bacteroidetes* and *Firmicutes* and higher abundance of *Actinobacteria* and, to a lesser extent, of *Spirochaetae* phyla in ram 1 compared to ram 2.

Keywords: *t*10-shift´s induction, fatty acids, ruminal biohydrogenation, starch, lipid supplementation

4.1. Introduction

The occurrence of a shift from *t*11 to *t*10 ruminal BH pathways (*t*10-shift) has been reported when high-starch diets with or without supplementation with oils rich in PUFA are fed to ruminants (Loor *et al*., 2004; Bessa *et al*., 2005; Rosa *et al*., 2014). The major consequences of the *t*10-shift are a higher accumulation of *t*10-18:1 at the expense of *t*11-18:1 and the diminishment of *c*9,*t*11-18:2 to the detriment of *t*10,*c*12-18:2 in the rumen and tissues (Aldai *et al*., 2013; Bessa *et al*., 2015). A better understanding of shift's establishment is essential to prevent its occurrence, considering the potential beneficial effects of *c*9,*t*11-18:2 and *t*11-18:1 and detrimental effects of *t*10,*c*12-18:2 and *t*10-18:1 in human health (Aldai *et al*., 2013). Also, the *t*10-shift might lead to milk fat depression in dairy ruminants (Griinari *et al*.,

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1998; Bauman & Griinari, 2003) and is possibly associated with rumen acidosis (Bessa *et al*., 2015). The importance of *t*10-shift in human and animal health leads to the necessity of predicting its presence even before the analysis of rumen content, which might be accomplished by evaluating the "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the plasma, as reported in a recent study (Alves *et al*., 2017). The "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio might be a better indicator of the presence of *t*10-shift than "*t*10-/*t*11-18:1 ratio, since it considers the post absorptive desaturation of *t*11-18:1 into *c*9,*t*11-18:2 (Shingfield & Wallace, 2014). Moreover, studies about the development of *t*10-shift are scarce and its relation with rumen pH has been difficult to establish. In fact, the time required for the occurrence of the shift was reported to be about 8 to 18 days and then it remains constant (Roy *et al*., 2006; Zened *et al*., 2013b), oppositely to fast changes in rumen pH (Zened *et al*., 2013b; Bessa *et al*., 2015). The progressive establishment of the shift, reflected by the strong time dependent modifications in the "*t*10-/*t*11-18:1" ratio, suggests an adaptation of rumen microbiota (Roy *et al*., 2006; Zened *et al*., 2013a; Bessa *et al*., 2015). In the present study, we hypothesized that the *t*10-shift will appear gradually within the first two weeks of feeding rams with a high-starch diet supplemented with sunflower oil and will remain constant afterwards due to changes in the microbiota, accompanied by higher *t*10-18:1 in the plasma and rumen pH reduction. Also, dietary effects on rumen bacterial community and its individual variability were evaluated using FLX amplicon pyrosequencing, in order to relate modifications of bacterial taxonomic groups with the development of *t*10-shift.

4.2. Material and methods

4.2.1. Animal handling and management

Animal handling followed EU Council Directive 2010/63/EU (EC, 2010) concerning animal care and rumen fistulated animals were used after approval of the ethical committee of the Faculty of Veterinary Medicine, University of Lisbon. Two rumen fistulated approximately 2-year old rams (50 kg of live weight) were housed in individual metabolic cages. Before the experimental period, animals were fed with 500 g of commercial compound (CC) feed and 800 g of grass hay, both divided into two equal meals per day (9h30 and 17h00). The ingredients and chemical composition of grass hay and CC feed were previously described by Costa *et al*. (2016). The experimental diet was composed of 723 g/kg DM of ground wheat grain supplemented with 41 g/kg DM of sunflower oil (*t*10-shift inducing concentrate; T10C), 191 g/kg DM of CC feed and 191 g/kg DM of grass hay. The diet was provided twice daily (9h30 and 17h00) and grass hay was also fed at 12h30. The amount of feed refused was

weighed at every meal for all the experimental period, but the feed offered was only accordingly adjusted on the first week. During the rest of the experimental period, the feed offered per meal was constant and corresponded to 378.5 g of ground wheat grain supplemented with 21.5 g of sunflower oil (T10C) together with 100 g of CC feed. The grass hay was provided as 50 g at 9h30, 50 g at 17h00 and 100 g at 12h30. The grass hay and CC feed contained 899 and 851 g/kg DM, respectively. The ground wheat grain presented 870 g/kg DM. The experimental period had consisted of 29 days, after one week of adaptation of rams to the cages. On the first four days of the experimental period, rams were gradually transitioned from the initial diet to the experimental wheat-based diet, with the last week corresponding to the dietary transition from the experimental diet to the initial diet (CC feed and grass hay).

4.2.2. Sample collection

For each day of the experimental period, total rumen contents were collected directly though rumen cannula before the morning meal and 3h after feeding and were immediately transferred to the laboratory. On the third day of every week, total rumen contents were collected every 1h30, from 9h30 (before feeding) to 20h00. Rumen samples were stored at -20 ºC, freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmark) and preserved at -20 ºC for long chain FA analysis and, the samples from days 8 and 10, also for 16S rRNA gene amplicon pyrosequencing analysis. Sub-samples of total rumen contents from days 12 to 29 were filtered through four layers of cheesecloth for pH measurement (Digital pH meter "pH-2005"; JP Selecta S.A, Barcelona, Spain). On the third day of every week, blood samples were collected from each ram into heparinized syringe tubes (S-Monovette®; Nümbrecht, Germany), before the morning meal and 3h after feeding, and centrifuged at 1650 xg and 4 ºC for 15 min using a Universal 32 R, Hettich Lab Technology (Tuttlingen, Germany) centrifuge. The blood plasma was separated and preserved at -20 ºC for long chain FA analysis. Feed chemical composition was determined using the methods described by Francisco *et al*. (2015).

4.2.3. Fatty acid analysis

For long chain FA analysis, 250 mg of each sample was weighted and transesterified into FA methyl esters using a basic followed by acidic catalysis (Alves *et al*., 2013b) with 19:0 (1 mg/mL) as internal standard. Fatty acid methyl esters were separated by GC-FID using a Shimadzu GC-2010 Plus chromatograph equipped with a 100% cyanopropyl polysiloxane capillary column (SP-2560, 100 m, 0.25 mm i.d., 0.20 µm film thickness; Supelco Inc., Bellefont, PA, USA). Identification of FA methyl esters was achieved by comparison of the

retention times with those of authentic standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA, and a Bacterial FAME mix from Matreya LLC, Pleasant Gap, PA, USA) and by confirmation with GC-MS in a Shimadzu GC-MS QP 2010 Plus chromatograph (Kyoto, Japan) equipped with a SP-2560 column (Alves *et al.*, 2013b; Alves *et al.*, 2015). Also, for long chain FA analysis, 200 µL of blood plasma from each sample was used for direct *in situ* transesterification as descried by Glaser, Demmelmair and Koletzko (2010).

4.2.4. Pyrosequencing for 16S rRNA gene amplicon and analysis of data

The following procedures were done in collaboration with the authors Troegeler-Meynadier, A. and Enjalbert, F., as described by Zened *et al.* (2013a). Briefly, total DNA was extracted from 200 mg of freeze-dried rumen contents and quantified using NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). Amplicons from V3 to V4 regions of 16S rRNA genes (460 bp on *Echerichia coli*, GenBank number J01695) were amplified by conventional PCR with universal bacterial forward 343F (TACGGRAGGCAGCAG) (Liu, Lozupone, Hamady, Bushman & Knight, 2007) and reverse 784R (TACCAGGGTATCTAATCCT) (Andersson *et al.*, 2008) primers. After purification of PCR products, DNA was quantified using Quant-iT PicoGreen dsDNA Assay kit (Invitogen, Saint Aubin, France) on a ABI Prism 7900 HT sequence detection system (Life Technologies, Invitrogen Applied Biosystems, Villebon-sur-Yvette, France) and, for each amplicon library, 1000 ng/L of final DNA concentration was obtained. The pooled amplicons were pyrosequenced using a 454 FLX Titanium (454 Life Sciences – a Roche Company, Branford, CT) sequencer at the GeT (Genomic and Transcriptomic) platform of National Institute for Agricultural Research (INRA), Toulouse, France. For taxonomical classification of bacterial groups, filtered gene sequences were aligned using SILVA alignment database (MOTHUR software Inc., Ann Arbor, MI, USA) and clustered into Operational Taxonomic Units after calculating a pairwise distance between sequences.

4.2.5. Statistical analysis and calculations

Data corresponding to pre- and post-prandial effects on pH and "*t*10-/*t*11-18:1" ratio in the rumen and "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the plasma were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). The single fixed factor was the hour and the experimental unit was the animal. Each sample was treated as subsampling within the day using a compound symmetry covariance matrix as the best model generated. The effect of the treatment was considered as significant for *P*<0.05. All graphical representations were done with GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Shannon bacterial diversity and numerical species richness indices were calculated

using the Instat-3.37 Software (Statistical Services Centre, University of Reading, UK, 2010), as desbrided by Magurran (2004).

4.3. Results

4.3.1. Development of *t*10-shift - influence on feed intake, rumen C18 FA and pH

Figure 3 shows a graphical representation of the establishment and development of *t*10-shift and its relation with the concentrate intake and some major C18 FA concentrations in the rumen for ram 1. The shift was present between days 8 (14h) and 11 and during all day 15. In the first occurrence, the "*t*10-/*t*11-18:1" ratio averaged 1.86, reaching a maximum of 2.50 on day 8 (20h), whereas, in the second one, the ratio averaged 1.32 and its maximum was 1.52 on day 15 (11h). As expected, the concentrations of *t*10-18:1 and *t*11-18:1 followed a similar pattern of their ratio and, when ratio≈2.50, the amount of *t*10-18:1 was 7.66 mg/g DM and that of *t*11-18:1 was 3.06 mg/g DM, while the ratio value of 1.52 was associated with 3.31 mg/g DM of *t*10-18:1 and 2.18 mg/g DM of *t*11-18:1. The appearance of *t*10-shift on day 8 was preceded by a decrease of intake of dietary concentrate from days 5 (17h) to 6 (9h30) (480 to 4 g/meal), even though a temporary increase from 103 g on day 7 (17h) to 469 g on day 9 (9h30) was found. Before the second occurrence of the shift, a slight reduction of intake was verified, although, during all day 15, the intake was at a high level (average of 472 g/meal). The "*t*10,*c*12-/*c*9,*t*11-18:2" ratio considerably increased at the same time that "*t*10-/*t*11-18:1" ratio, but it was only above 1 from days 8 (20h) to 9 (12h30) (averaging 1.26) and on day 15 (11h) (ratio ≈1.13) with a higher variance of its values comparing with "*t*10-/*t*11-18:1" ratio. There was a post-prandial increase of the sum of PUFA (18:2n-6 and 18:3n-3) followed by its decrease. The relation between PUFA and the shift was similar to that reported for intake, considering an abrupt decrease of these FA just before the occurrence of the shift followed by their enhancement during the shift. In fact, the amount of PUFA was 1.39 mg/g DM on day 8 (9h30) and averaged 10.16 mg/g DM between days 8 (11h) and 10 (12h30). Also, the average of PUFA was 3.23 mg/g DM from days 13 (12h30) to 15 (9h30) but increased until 8.05 mg/g DM on day 15 (17h). Nevertheless, the maximum amount of PUFA (19.29 mg/g DM) was obtained on day 4 (12h30) of the adaptation period to wheat-based diet. The 18:0 decreased and the total of *trans*-18:1 increased during the shift, mainly from days 8 (20h) to 11 (12h30) (average of 10.20 mg/g DM for 18:0 and 19.16 mg/g DM for *trans*-18:1). However, at the second establishment of the shift, this modification was only more evident on day 15 (11h), although 18:0 was still approximately 1.66-fold higher than *trans*-18:1. Before the first occurrence of the shift, a considerable increase of oxo-18:0 was

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found, between days 5 and 7 comparing with the previous days, from an average of 0.58 to 2.88 mg/g DM. The maximum concentration of these FA (4.50 mg/g DM) was present with the lowest intake of concentrate (day 6, 9h30). Also, a slighter increment of oxo-18:0 was verified from days 10 (12h30) to 11 (9h30) (0.50 to 2.56 mg/g DM).

Figure 3. Effect of the experimental wheat-based diet in the development of *t*10-shift and its influence on major C18 fatty acids in the rumen of ram 1.
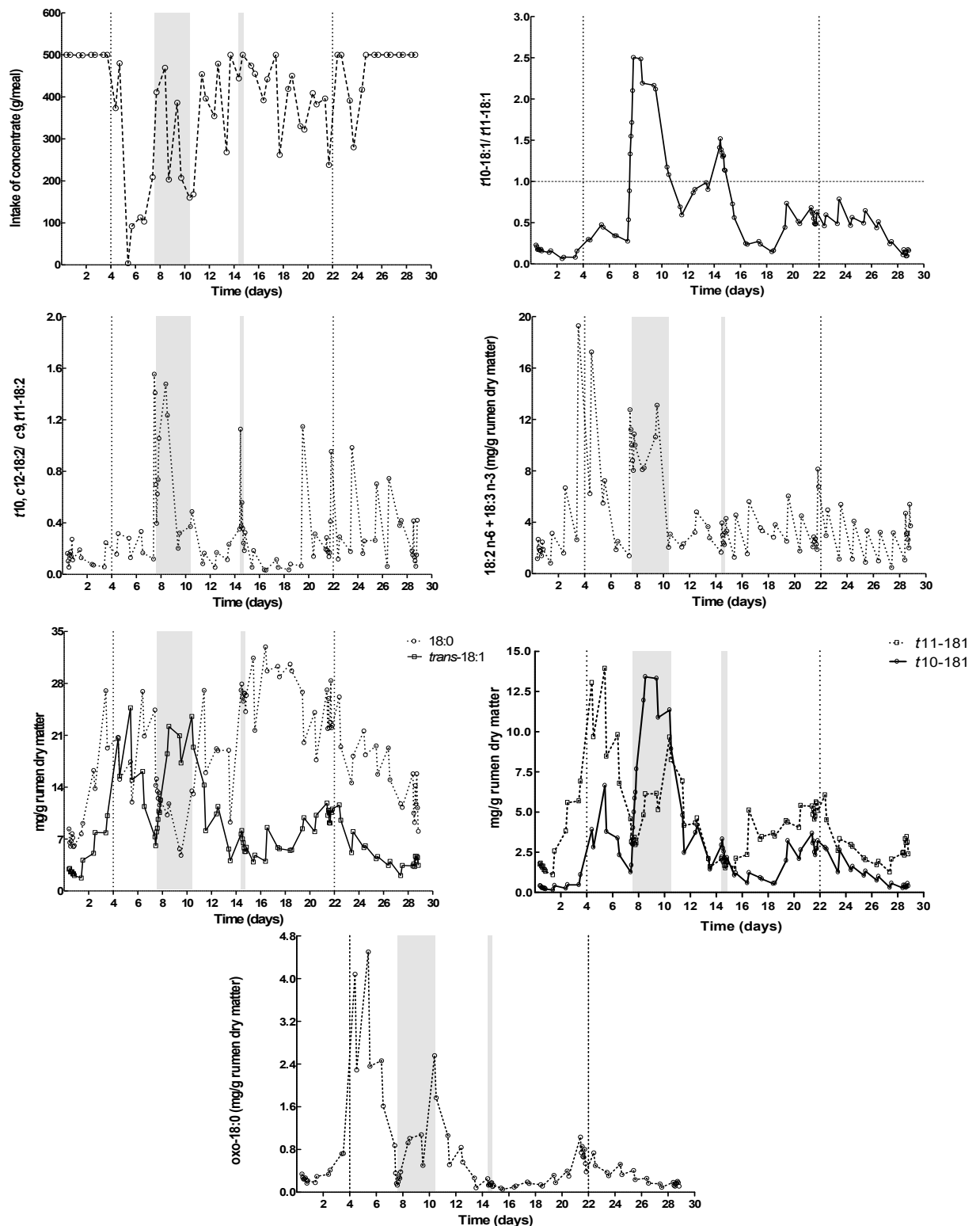
The period of feeding with the experimental wheat-based diet corresponds to the time between interrupted lines. The transition from the initial diet (commercial compound feed and forage) to the experimental diet occurred over the first 4 days and the transition back to the initial diet occurred from days 22 to 29. The threshold for the occurrence of *t*10-shift is represented by a horizontal line and the shaded area indicates the period of time in which the *t*10-shift was established.

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Figure 4 represents the same as figure 3 but for ram 2. The t10-shift occurred between days 15 and 17 with "t10-/t11-18:1" ratio averaging 1.68 and reaching a maximum of 1.96 on day 15 (12h30), although the ratio was already close to 1 on day 17 (average of 1.01). The concentrations of t10-18:1 and t11-18:1 varied according to a similar pattern of their ratio and, when ratio ≈1.96, the amount of t10-18:1 was 3.70 mg/g DM and that of t11-18:1 was 1.89 mg/g DM. The appearance of t10-shift was preceded by a decrease of the intake of dietary concentrate from days 14 (17h) to 15 (9h30) (500 to 281 g/meal) and, even though the intake returned to its maximum in the beginning of day 15, it suddenly decreased from days 15 (17h) to 16 (9h30) and it was maintained at a low level until day 18 (9h30) (averaging 236 g/meal). Similar to "t10-/t11-18:1" ratio but with a higher variance of its values, the "t10,c12-/c9,t11-18:2" ratio increased during the shift, reaching a maximum of 0.51 on day 15 (20h). However, the greatest ratio was present before and after the shift and it was never above 1.

There was a post-prandial increase of the sum of PUFA (18:2n-6 and 18:3n-3) followed by its decrease. Identically to the intake of concentrate, these FA decreased from 7.53 to 4.22 mg/g DM just before the occurrence of the shift, although the transitory increase of the intake at the time of the shift did not lead to an enhancement of the sum of 18:2n-6 and 18:3n-3. In fact, the concentration of PUFA remained low during the shift (averaging 3.37 mg/g DM). Moreover, the maximum total of PUFA (25.54 mg/g DM) was obtained on day 4 (12h30) of the adaptation period to wheat-based diet. The 18:0 was considerable higher than the total of $trans$-18:1, during the shift (average of 33.16 mg/g DM for 18:0 and 4.83 mg/g DM for $trans$-18:1). Before the establishment of the shift, there were increases alternated with decreases of total oxo-18:0 between days 9 (12h30) and 14 (averaging 0.76 mg/g DM), reaching 1.44 mg/g DM on day 11 (9h30). Afterwards, the amount of oxo-18:0 remained low with an average of 0.32 mg/g DM at the time of the shift. Nevertheless, the maximum of these FA (2.13 mg/g DM) was verified on day 4 (9h30). The progression of the shift and its relation with the concentrate intake and some C18 FA in the rumen between days 5 and 7 could not be evaluated, since rumen contents were not collected on these days.

Figure 4. Effect of the experimental wheat-based diet in the development of *t*10-shift and its influence on major fatty acids and the pH in the rumen of ram 2.
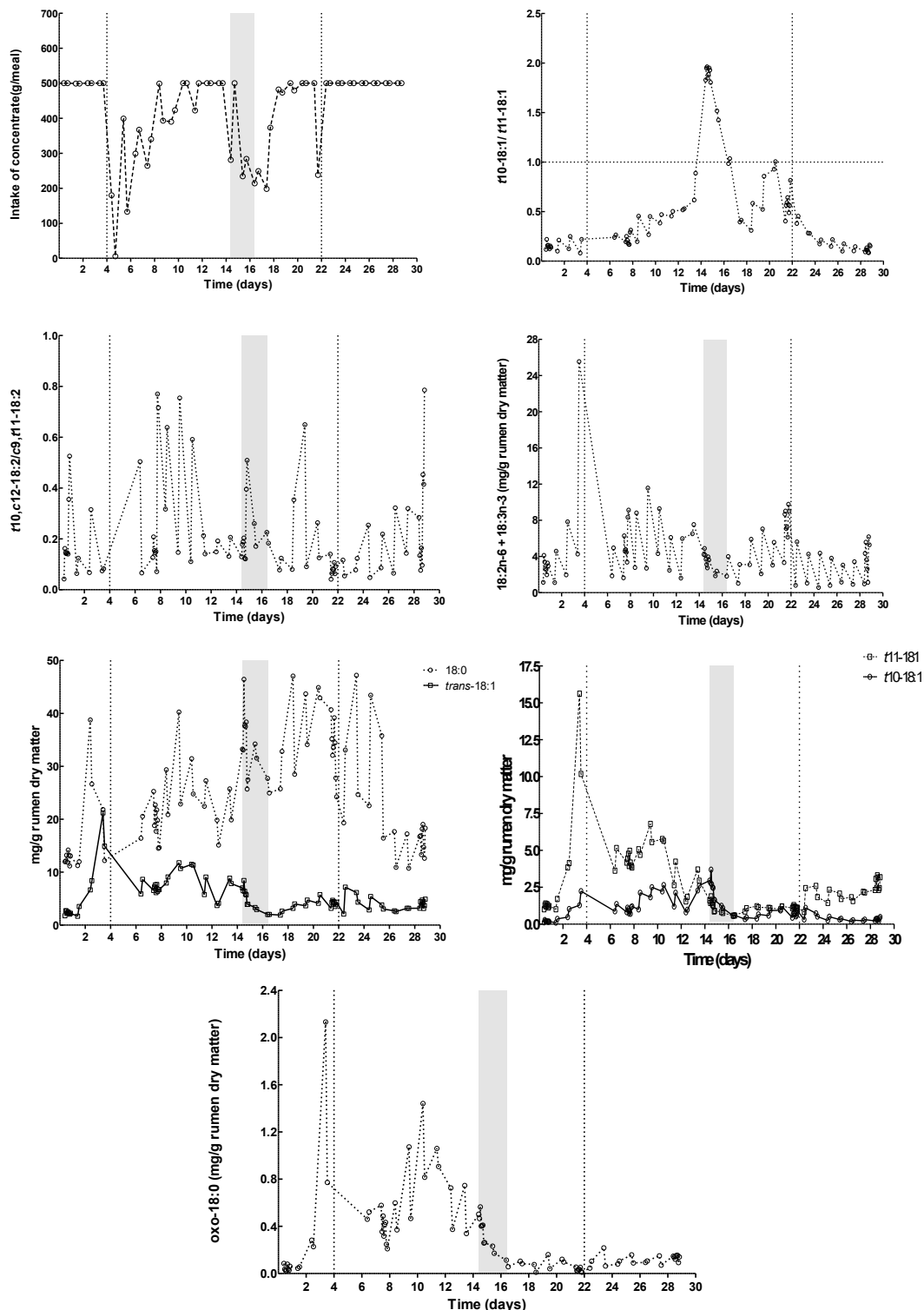
The period of feeding with the experimental wheat-based diet corresponds to the time between interrupted lines. The transition from the initial diet (commercial compound feed and forage) to the experimental diet occurred over the first 4 days and the transition back to the initial diet occurred from days 22 to 29. The threshold for the occurrence of *t*10-shift is represented by a horizontal line and the shaded area indicates the period of time in which the *t*10-shift was established, although "*t*10-/*t*11-18:1" ratio≈0.98 between 16 day and 17 day.

4.3.3. Relation between the development of *t*10-shift and rumen pH - Pre- and post-prandial effects

Figure 5 shows the progression of pH during the trial, as well as pre- and post-prandial effects on pH and "*t*10-/*t*11-18:1" ratio in the rumen. For both rams, there was no clear association between the presence of *t*10-shift on day 15 and rumen pH, which averaged 5.97 for ram 1 and 6.35 for ram 2. The post-prandial pH was significantly lower than the pre-prandial pH, for both animals ($P < 0.001$). In fact, before the mourning meal, the average of pH values was 6.82±0.062 for ram 1 and 7.10±0.077 for ram 2 and, 3 h after the meal, it averaged 5.68±0.062 for ram 1 and 5.86±0.077 for ram 2. Considering the "*t*10-/*t*11-18:1" ratio, it significantly increased ($P < 0.001$) for ram 2 after the mourning meal (0.43 to 0.52±0.083), but only a slight and non-significant ($P \approx 0.342$) increment of the ratio was verified for ram 1 (0.60 to 0.63±0.103).

Figure 5. Relation between the development of *t*10-shift and rumen pH. Pre- and post-prandial effects on *t*10-shift and pH.
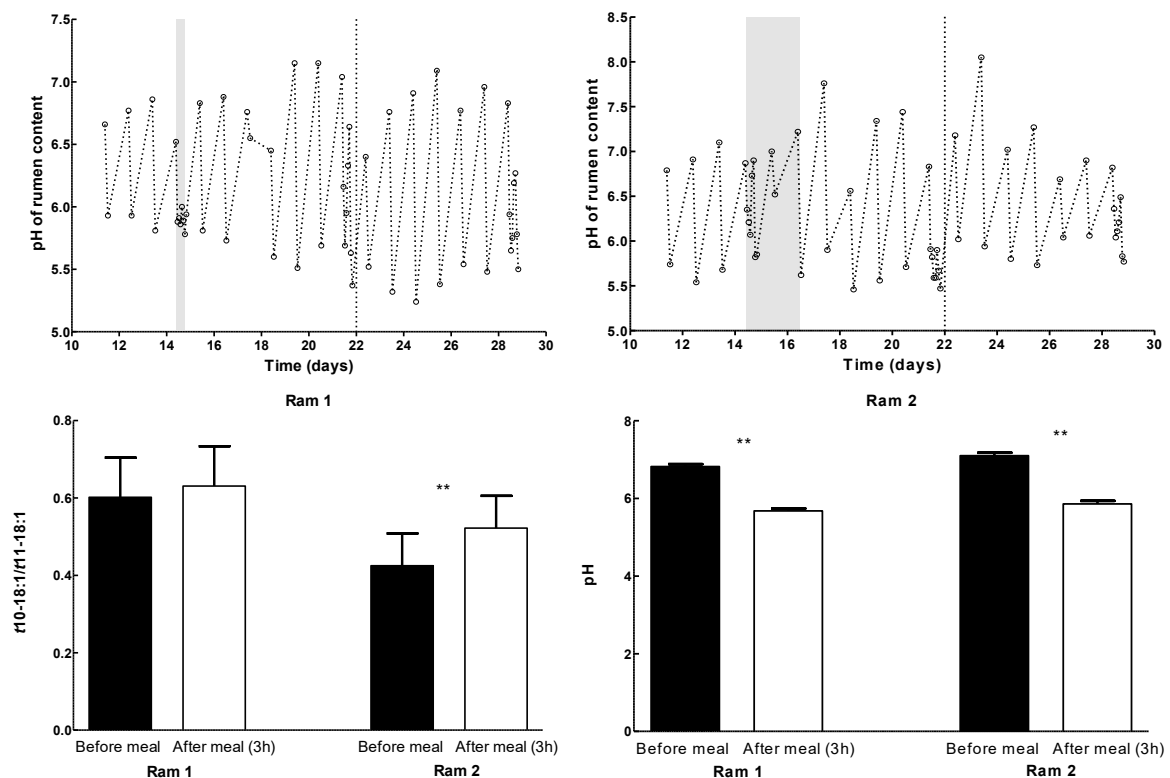
The period of feeding with the experimental wheat-based diet corresponds to the time until the interrupted vertical line and, afterwards, the transition to the initial diet (commercial compound feed and forage) took place. The shaded area indicates the period of time in which the t10-shift was established. ** *P*<0.001.

4.3.3. Relation between the development of *t*10-shift in the rumen and its expression in the blood plasma

Figure 6 represents weekly, pre- and post-prandial effects on "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the blood plasma, as well as the relation between that ratio and *t*10-/*t*11-18:1 ratio in the rumen. In ram 2, an evidence of *t*10-shift´s occurrence was verified in the plasma, after the meal at the third week (day 22) of trial, but the "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio was only slightly higher than 1 (1.09). A plasmatic evidence of shift was not so clear for ram 1, although the ratio reached its maximum value at the same time as for ram 2 and was close to 1. Moreover, no significant differences were found between pre- and post-prandial "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratios, but a tendency (P = 0.073) for a higher ratio after (0.47±0.139) than before (0.43±0.139) the meal was observed for ram 1. Also, there was no association between "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the plasma and "*t*10-/*t*11-18:1" ratio in the rumen.

Figure 6. Weekly, pre- and post-prandial effects on "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the blood plasma and relation between that ratio and *t*10-/*t*11-18:1 ratio in the rumen.
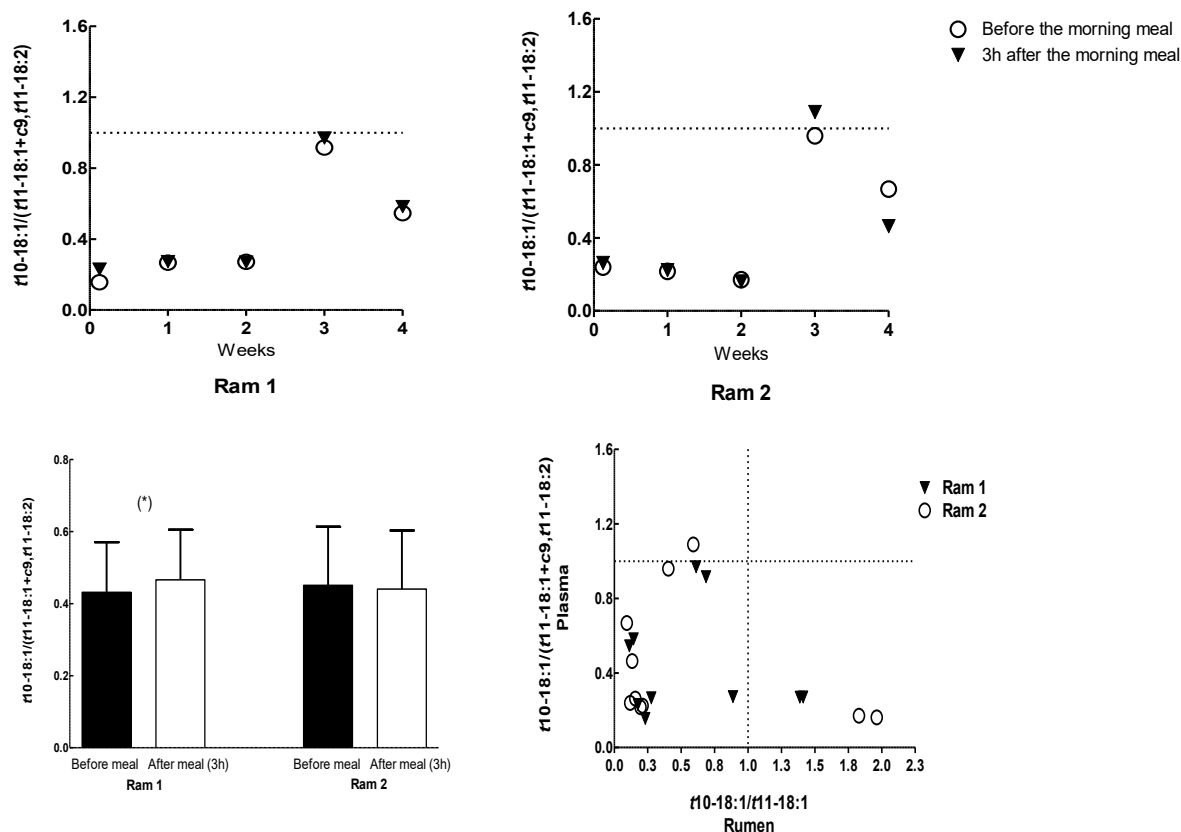
The threshold for the reflection in the plasma of *t*10-shift´s occurrence in the rumen is represented by a horizontal line, while the vertical line is for the presence of *t*10-shift in the rumen. (*) *P*<0.1.

4.3.4. Relative abundance and diversity of bacterial groups

The taxonomic evaluation of rumen bacteria according to phylum, class and order is presented in Figure 7. At the phylum level, there was a high abundance of bacteria belonging to *Actinobacteria* and *Bacteroidetes* phyla for ram 2, which corresponded to an average of 18.3% and 42.1%, respectively. Generally, *Actinobacteria* was the most predominant phylum for ram 1, reaching a maximum of 86.8%, but, in two samples (days 8 and 10 at 12h30), *Spirochaetae* was the major phylum and averaged 56.6%. The presence of bacteria belonging to *Bacteroidetes* was also proeminent for ram 1, being the second most abundant phylum in three samples (day 8; 14h00, 18h30, 20h00) with an average of 17.4%. The *Firmicutes* phylum was the fourth most abundant phylum for both rams, contributing to an average of 3.04% for ram 1 and 5.54% for ram 2. There was a highly variable increase of the abundance of *Actinobacteria* with an increment of "*t*10-/*t*11-18:1" ratio, ranging from 28.7% (ratio ≈ 2.12) to 86.8% (ratio ≈ 2.10) in ram 1. The contribution of *Firmicutes* was lower for

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ram 1 than for ram 2, although this was not found for one sample of ram 1 (ratio ≈ 1.33; day 8, 14h00) that corresponded to 12.0%, conversely to an average of 1.76% for the other samples of ram 1. Also, a lower abundance of *Bacteroidetes* was found for ram 1 compared to ram 2.

Concerning the bacterial class, the abundance of *Actinobacteria* presented a similar pattern to that observed for *Actinobacteria* phylum, ranging from 67.0 (ratio ≈ 2.10) to 15.7% (ratio ≈ 2.12) in ram 1. The *Clostridia* class was generally lower for ram 1, reaching a proportion as low as 0.76%, than for ram 2 (minimum of 3.99%), except in one sample of ram 1 (ratio ≈ 1.33) with a contribution of *Clostridia* (11.7%) almost twice the maximum percentage (6.22%) found for ram 2. Moreover, there was a lower abundance of *Bacteroidia* for ram 1 (average of 17.7%) than for ram 2 (average of 41.8%). Although the abundance of *Spirochaetes* was variable for both rams, a higher variation was found for ram 1 with proportions ranging from 0.06 (day 8, 14h00) to 62.1% (day 8, 12h30). Another proemient class was *Coriobacteriia* that ranged between 16.9 (day 10, 9h00) and 24.3% (day 8, 9h00) for ram 2 and between 8.28 (day 8, 12h00) and 20.9% (day 8, 15h30) for ram 1.

Considering the bacterial order, the main responsible for the variable increase of *Actinobacteria* phylum and class associated with an increment of "*t*10-/*t*11-18:1" ratio was *Actinomycetales*, since its abundance ranged between 4.76 and 32.5% in ram 1 and only reached a maximum of 1.00% in ram 2. The major contribution of *Actinobacteria* was also determined by slightly differential abundance of *Bifidobacteriales*. In fact, although *Bifidobacteriales* contributed to up to about 20.0% of total sequences for both rams, its greatest proportion (23.5%) was found with the highest ratio (ratio ≈ 2.50) that corresponded to one sample from ram 1 (day 8, 20h00). Moreover, the abundance of *Bifidobacteriales* was variable, ranging from 4.27 to 23.5% for ram 1 and 4.52 to 20.2% for ram 2. Also, the contribution of *Corynebacteriales* was variable and generally more abundant for ram 1 than for ram 2, being as high as 28.9% for ram 1 but always below 1.00% for ram 2 except in one sample (day 10, 9h30) (11.3%).

Figure 7. Contribution of 16S rRNA sequences evaluated at bacterial phylum (a), class (b) and order (c) levels to the total number of sequences related to "*t*10-/*t*11-18:1" ratio in the rumen content.
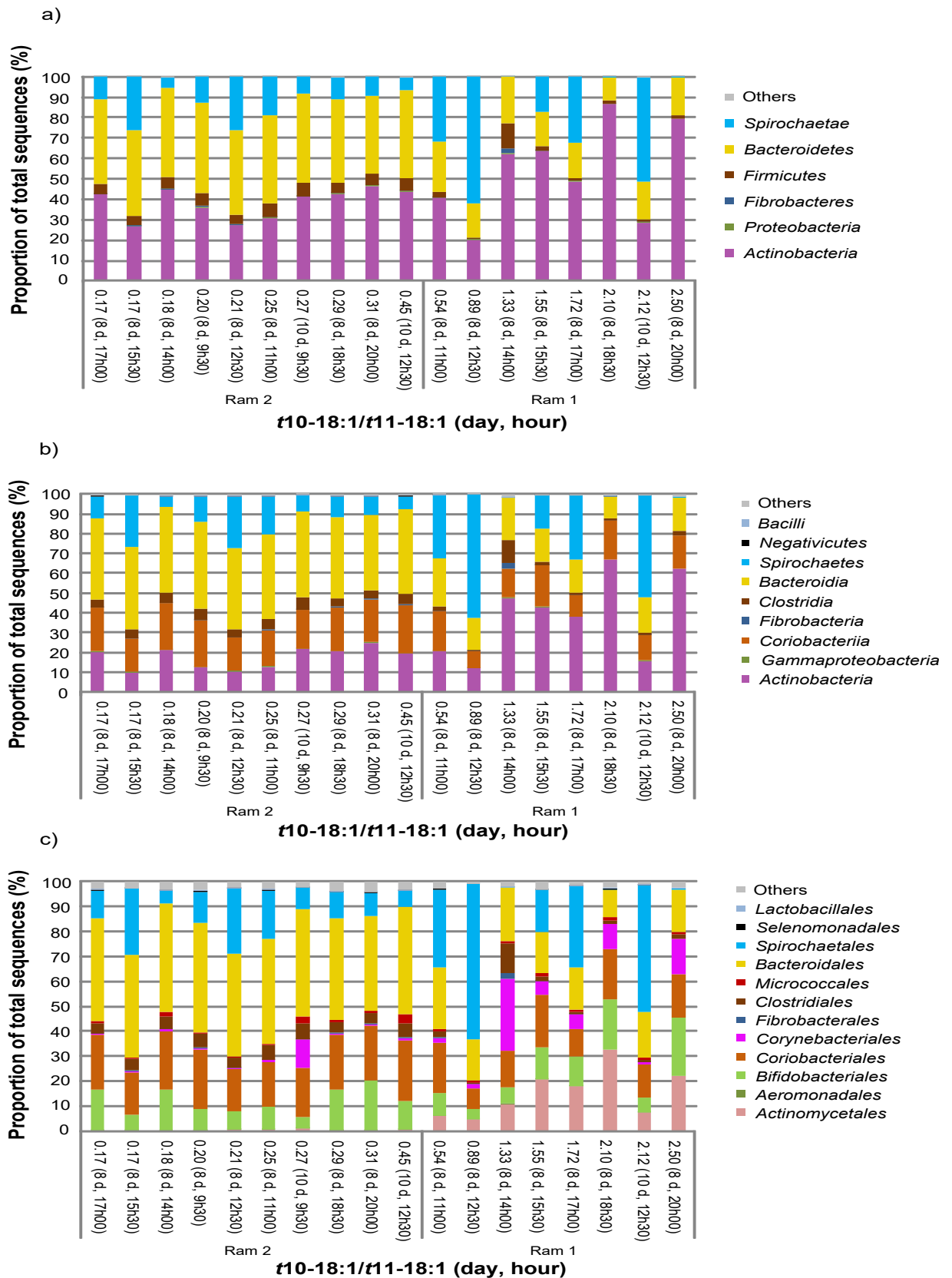
Figure 8 shows the taxonomic evaluation of rumen bacteria according to family and genus. Concerning the bacterial family, there was a lower abundance of *Porphyromonadaceae* for ram 1 than for ram 2, corresponding to 0.35 and 1.20%, respectively. The contribution of *Prevotellaceae* for the total sequences was inferior in ram 1 (7.55%) compared with ram 2 (15.5%). The abundance of *Rikenellaceae* was variable in both rams, although it was generally lower for ram 1 than for ram 2, ranging from 0.23 to 7.16% and from 3.51 to 10.4%, respectively. Also, an inferior abundance of *Rikenellaceae* was found in the three samples presenting the lowest "*t*10-/*t*11-18:1" ratio, with an average of 0.68%. The abundance of unclassified *Bacteroidales* for ram 2 was more than twice that for ram 1 and averaged 17.5 and 5.82%, respectively. The abundance of *Bacteroidaceae* family was highly variable for both rams and generally lower for ram 1 (< 1.00% in most of the samples) than for ram 2 (up to 2.44%), although its highest proportion (3.74%) was found in one sample from ram 1 (day 10, 12h30). *Spirochaetaceae*, *Corynebacteriaceae*, *Bifidobacteriaceae* and *Actinomycetales incertae sedis* families followed the same pattern as their orders.

Considering the bacterial genus, the abundance of *Prevotella* was variably lower for ram 1 than for ram 2 and ranged between 1.86 and 10.7% and between 6.84 and 13.3%, respectively. The uncultured *Prevotellaceae* genus followed a similar pattern to *Prevotella* but with a generally lower contribution, ranging from 0.37 to 4.54% for ram 1 and from 3.63 to 8.74% for ram 2. The abundance of uncultured *Coriobacteriaceae* was higher for ram 1 (0.63 to 4.83%) than for ram 2 (< 0.3%). The contribution of this genus was variably increased with an increment of "*t*10-/*t*11-18:1" ratio, as it averaged 4.34% for ratio ≈ 2.10 (day 8, 18h30) and 2.15 (day 8, 20h) but considerably decreased to 1.24% when ratio ≈ 2.12. The *Enterorhabdus* genus was mostly abundant in ram 1, mainly in three samples (day 8, 18h30; day 8, 20h00 and day 10, 12h30) with the highest ratio, reaching a maximum of 7.48% when ratio ≈ 2.12. The contribution of *Denitrobacterium* genus was variably higher for ram 1 than for ram 2, ranging from 0.17 to 2.17% and from 0.25 to 0.65%, respectively. The *Atopobium* genus showed a similar pattern to *Denitrobacterium*, even though with a higher variable abundance for ram 1 (0.20 to 3.44%) compared to ram 2 (0.22 to 0.67%). The abundance of *Olsenella* was generally lower for ram 1 than for ram 2, reaching a maximum of 7.52% and 13.5%, respectively. Although there was no clear relation between modifications of *Olsenella* proportion and of "*t*10-/*t*11-18:1" ratio, the lowest contribution of this genus was found in two samples of ram 1 with the highest ratio and averaged 1.23%. *Treponema*, *Corynebacterium*, *Bifidobacterium*, *Bacteroides* and uncultured *Actinomycetales* genera followed the same pattern as their orders and families, since they were the only genera belonging to them. Moreover, other bacterial genera, which included uncultured and unidentified *Rikenellaceae*, mostly *Rikenellaceae*_RC9 genus, and *Porphyromonadaceae*, were lower and more variable for ram 1 than for ram 2, ranging from 6.18 to 17.1% and from 23.9 to 27.1% respectively.

The unkown genera had a considerable contribution for the total number of sequences, corresponding to up to 11.8% for ram 1 and 24.1% for ram 2.

Figure 8. Contribution of 16S rRNA sequences evaluated at bacterial family (a) and genus (b) levels to the total number of sequences related to "t10-/t11-18:1" ratio in the rumen content.

a)

b)

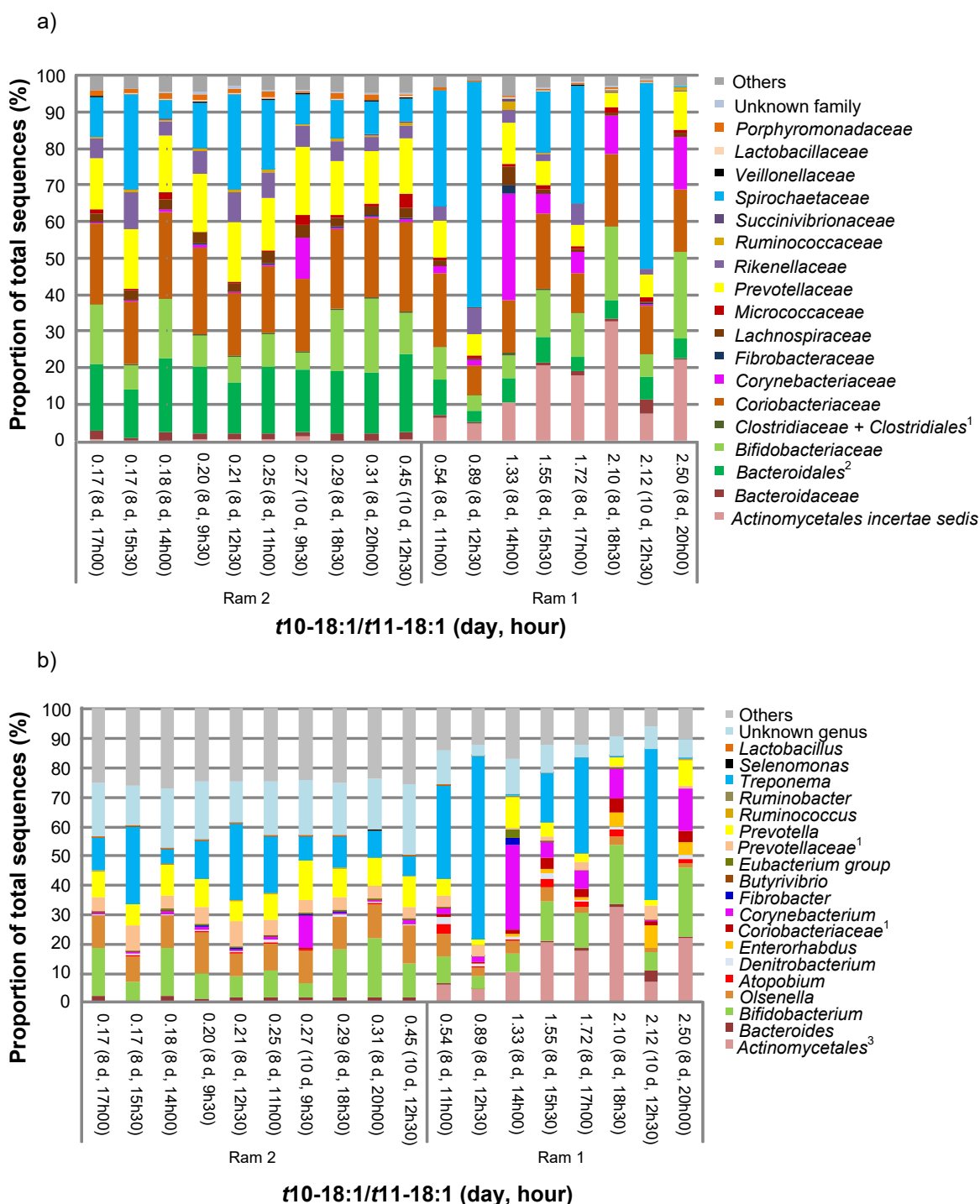

[1], Uncultured; [2], uncultured and *incertae sedis*; [3], uncultured bacterium proposed to belong to the genus *Ancilulla* ("*Candidatus* Ancilulla").

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The relation between bacterial diversity and "$t10$-/$t11$-18:1" ratio is presented in Figure 9. The numerical species richness index was variable for both rams, especially for ram 1, and was lower for ram 1 than for ram 2, ranging from 98 to 558 and from 319 to 635 respectively. An inferior and less constant Shannon diversity index was also found for ram 1 (2.66 to 4.17) than for ram 2 (average of 4.54). There was a tendency for a lower (P = 0.100) numerical species richness index, as well as a significantly lower Shannon diversity index (P = 0.016), with a higher "$t10$-/$t11$-18:1" ratio, in ram 1. However, the species richness index (P = 0.593) and Shannon diversity index (P = 0.357) were not related to the ratio, in ram 2.

Figure 9. Relation between estimators of bacterial diversity and $t10$-/$t11$-18:1 ratio in the rumen.
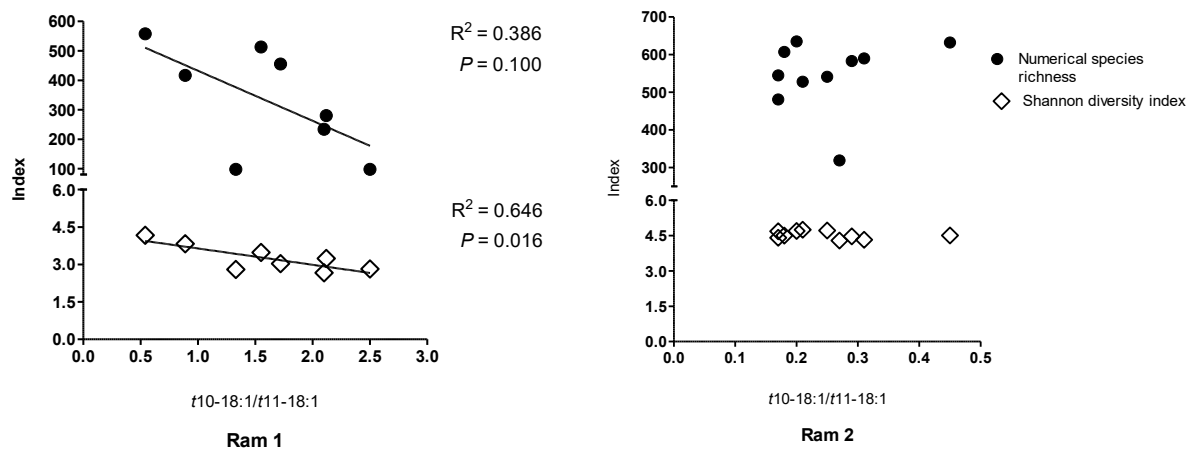
4.4. Discussion

To our knowledge, this is the first study describing a more detailed induction of the $t10$-shift in sheep, since there are only few studies about this issue in dairy cows (Roy *et al.*, 2006; Zened *et al.*, 2013b). As we hypothesized, the $t10$-shift was present within the first two weeks of feeding rams with a high-starch plus sunflower oil diet, but, oppositely to what was expected, it was only maintained during a short period of time. This discrepancy between our results and the ones reported by Roy *et al.* (2006) and Zened *et al.* (2013b) might be due to different species' metabolism and levels of feed intake. The lowest feed intake observed in the present study may be caused by an increase of starch content in the diet (Colman *et al.*, 2012) and, as suggested by Zened *et al.* (2013b), it can be responsible for a lack of $t10$-shift. Indeed, in our study, the $t10$-shift only occurred during an increase of intake that followed its decrease. This fact was particularly evident for the first appearance of the shift in ram 1 and

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was accompanied by an enhancement of *trans*-18:1 together with a decrease of 18:0 in the rumen. A lower efficiency of *trans*-18:1 reduction was previously found to be associated with a saturation of the conversion of *trans*-18:1 into 18:0 in the rumen (Troegeler-Meynadier, Bret-Bennis & Enjalbert, 2006). Also, a diminished activity of bacteria responsible for this reduction might be present (Zened *et al.*, 2013a) but it is possible that those bacteria had become more active and adapted to wheat-based diet with the progression of the trial, since, after day 6 for ram 2 and day 15 for ram 1, the ruminal BH was more complete with a much higher concentration of 18:0 than *trans*-18:1. The fact that the abundance of *Butyrivibrio* genus was considerably low (0 to 0.25%) on days 8 and 10 for both rams with even *B. proteoclasticus*, which is the main bacterium described as responsible for the production of 18:0 (Wallace *et al.*, 2006; Paillard *et al.*, 2007), not being identified, evidences that other bacteria are probably involved in the formation of 18:0 (Huws *et al.*, 2011). Additionally, the highest production of *trans*-18:1 may have been a protective mechanism against stress stimuli in the rumen ecosystem caused by a higher feed intake associated with acidotic environmental conditions. In fact, the generation of *trans*-18:1 and its incorporation into bacterial cell membranes in response to stress stimuli were hypothesized as one of the roles of ruminal BH. The increase of PUFA, when the intake was re-established during the *t*10-shift in ram 1, may have also exacerbated the *trans*-18:1 production as a protection from lipid overload (Bessa *et al.*, 2000; Vasta & Bessa, 2012). Nevertheless, for both rams, the highest total of 18:3n-3 and 18:2n-6 in the rumen was verified during the adaptation to wheat-based diet, which can be explained by the concomitant introduction of oil rich in PUFA.

Furthermore and as hypothesized, the *t*10-shift appeared progressively with an increase of *t*11-18:1 previously to that of *t*10-18:1, as described by Zened *et al.* (2013b), which probably indicates a gradual adaptive change on the structure or activity of rumen microbiota towards the production of *t*10-FA (Zened *et al.*, 2011; Zened *et al.*, 2013a). This fact was evident in ram 1, considering an increment of *t*11-18:1 between days 2 and 5 followed by its decrease until day 8, whereas *t*10-18:1 increased from days 8 to 11. Not only was this progression clearer for ram 1 than for ram 2, but also the "*t*10-/*t*11-18:1" ratio reached a higher value in ram 1 comparing with ram 2 (2.50 versus 1.86). The differences between the two rams in the patterns of *t*10-shift are in accordance to an individual variation in susceptibility to shift´s induction already described by Rosa *et al.* (2014) and Santos-Silva *et al.* (2016), which can be related to a variability of rumen microbiota among animals (Bessa *et al.*, 2015). This variability was more evident with the analysis of diversity of bacterial groups identified by short-length pyrosequencing. In fact, the lower numerical species richness and Shannon diversity indices for ram 1 compared to ram 2 on days 8 and 10 indicate an inferior rumen bacterial diversity in ram 1. The differential bacterial diversity was accompanied by different proportions of bacterial taxonomic groups between animals, which might not only be caused

by an individual variability of rumen microbiota (Chen, Penner, Li, Oba & Guan, 2011) but also by dietary effects (Mao, Zhang, Wang & Zhu, 2013; Petri *et al.*, 2013; Zened *et al.*, 2013a). In fact, although both animals were fed with a high-starch diet, the more variable feed intake in ram 1 might have contributed to greater stress stimuli in the rumen induced by the presence of starch and, consequently, to a higher modification of rumen microbiota in this animal. So, it is possible that, in ram 2, rumen bacteria were more adapted to the new environmental conditions than in ram 1. Generally, there was a predominance of *Actinobacteria*, *Bacteroidetes*, *Spirochaetae* and *Firmicutes* phyla. However, higher abundances of *Actinobacteria* and, in some samples, *Spirochaetae*, as well as lower abundance of *Bacteroidetes* and a slight reduction of *Firmicutes* were found in ram 1 compared to ram 2. Similarly, in some previous studies, an increase (Mao *et al.*, 2013; Petri *et al.*, 2013) or a tendency for an increase (Zened *et al.*, 2013a) of *Actinobacteria* and a decrease of *Bacteroidetes* (Mao *et al.*, 2013) were also reported when high-starch diets were fed to ruminants.

In the present study, the main bacterial order that contributed to a high abundance of *Actinobacteria* phylum in ram 1 (up to 62.0%) was *Actinomycetales* that included only one family (*Actinomycetales incertae sedis*) and one proposed genus (*Actinomycetales* uncultured). The participation of bacteria from *Actinomycetales* order on feed digestion occurring in the rumen has never been explored, although Tan, Deng & Cao (2009) reported that some of them have amylolytic activity, which could explain their high abundance with starch feedstuff. However, it is not possible to conclude about an involvement of *Actinomycetales* on t10-shifted BH pathways due to an absence of knowledge about the role of these bacteria on ruminal BH, even with its highest abundance being associated with an increase of "t10-/t11-18:1" ratio. Other families that contributed to a greater abundance of *Actinobacteria* in ram 1 than in ram 2 were, in a variable extent, *Coriobacteriaceae*, mainly *Atopobium*, *Enterorhabdus*, *Denitrobacterium* and uncultured genera, *Bifidobacteriaceae* and *Corynebacteriaceae*. Although the role of some bacteria belonging to *Coriobacteriaceae* family, such as *Enterorhabdus*, is unknown, others, including *Atopobium*, were shown to ferment carbohydrates to lactic acid (Harmsen *et al.*, 2000; Kraatz, Wallace & Svensson, 2011). The *Atopobium* genus was also found to be increased in the rumen with high-grain diets (Mao *et al.*, 2013; Petri *et al.*, 2013). The *Denitrobacterium* genus and, specifically, its only identified species (*D. detoxificans*) can enhance detoxication in the rumen by metabolizing nitrocompounds, although that was not demonstrated for concentrate feed (Anderson, Rasmussen, Jensen & Allison, 2000). Interestingly, there was a lower contribution of *Olsenella* genus for ram 1 than for ram 2, despite the fact that this genus was described as having similar acitivity in the rumen to *Atopobium* (Kraatz, *et al.*, 2011). However, these results might be due to an individual variation of rumen microbiota or some

differences between *Olsenella* and *Atopobium* in respect to rumen metabolism under acidotic conditions. The *Bifidobacteriaceae* family that included only the *Bifidobacterium* genus had a predominant contribution (4.27 to 23.5%) to the total of bacteria for both animals, which can be explained by the ability of *Bifidobacterium* spp. to metabolize carbohydrates into acetic and lactic acids (Nagaraja & Titgemeyer, 2007). Also, Mao *et al*. (2013) and Zened *et al*. (2013a) reported an increase of *Bifidobacterium* spp. in the rumen with starch feedstuffs. The *Bifidobacterium* spp. may still participate on ruminal BH, since this genus was described as a producer of CLA and CLNA (Gorissen *et al*., 2010; Park *et al*., 2011), although it was never reported as being associated with *t*10-shift. The high contribution of *Corynebacteriaceae* family, including only *Corynebacterium* genus, mostly in ram 1 (up to 28.9%), may be due to the ability of these bacteria to produce organic acids, such as lactic and succinic acids, from glucose (Okino, Inui & Yukawa, 2005; Fukui *et al*., 2011). Considering *Spirochaetae* phylum and mostly *Spirochaetaceae* family and *Treponema* genus, the variable high abundance (0.06 to 62.1%) of bacteria belonging to these groups might be due to the variability of concentrate feed intake between days 8 and 10 in ram 1, since *Treponema* genus can be involved in an adaptation to high-grain diets. In fact, these bacteria were previously described as being increased with 72% and 89% DM of dietary grain (Chen *et al*., 2011) and decreased with a lower proportion of grain (45% DM) (Mao *et al*., 2013).

The bacterial families that mostly contributed to the lower abundance of *Bacteroidetes* phylum, Bacteroidia class and Bacteroidales order in ram 1 were uncultured *Bacteroidales*, *Bacteroidaceae*, *Porphyromonadaceae*, *Rikenellaceae* (uncultured *Rikenellaceae_RC9* genus) and *Prevotellaceae* (uncultured *Prevotellaceae* and *Prevotella* genera). Considering that the majority of these bacteria are Gram negative, the predominance of Gram positive comparing with Gram negative bacteria with a high influence of starch on rumen microbiota (Nagaraja & Titgemeyer, 2007) might help to explain the inferior contribution of *Bacteroidetes* phylum in ram 1. Moreover, a decrease of *Rikenellaceae*_RC9 genus in the rumen was already described with starch feedstuffs (Zened *et al*., 2013a). Although inconsistent results have been reported for the contribution of *Prevotella* genus to total rumen bacteria (Tajima *et al*., 2001; Bekele, Koike & Kobayashi, 2010), some studies revealed a lower diversity (Bekele *et al*., 2010; Pitta *et al*., 2010) and abundance (Mao *et al*., 2013) of these bacteria with concentrate- than with forage-based diets. However, none of these bacterial groups has been previously associated with *t*10-shift, despite the fact that, in the present study, a higher abundance of *Rikenellaceae*_RC9 genus was observed with an increment of "*t*10-/*t*11-18:1" ratio. Still, uncultured *Bacteroidales* and *Prevotella* genera were recently related to the formation of *c*9,*t*11-18:2 and *t*11-18:1 in the rumen (Huws *et al*, 2011).

Attending to *Firmicutes* phylum, bacterial groups with a slightly lower abundance in ram 1 were *Clostridia* class, *Clostridiales* order and particularly *Lachnospiraceae* family and

uncultured *Lachnospiraceae* genera. Consistently, Zened *et al*. (2013a) reported a negative effect of high starch plus oil addition in the diet on the proportion of uncultured *Lachnospiraceae* in the rumen. Interestingly, in the present study, *Selenomonas* genus presented a low abundance in both animals (up to 0.26%) and especially in ram 1 (0 to 0.02%) on days 8 and 10. These results may be associated with the decrease of oxo-18:0 production observed during this period, considering that *S. ruminantium* was reported to participate in the hydration of *c*9-18:1 into 10-OH-18:0 (Hudson *et al*., 1995) that is a precursor of 10-oxo-18:0 (Shingfield *et al*., 2010a). Also, *P. acnes*, another bacterial genus described as capable of producing 10-OH-18:0 (McKain *et al*., 2010), was not even detected in both rams, although it belongs to a predominant phylum (*Actinobacteria*). However, the involvement of other bacteria in the formation of oxo-18:0 cannot be excluded. Additionally, bacteria involved in *t*10-shifted BH pathways might be somehow associated with the ones responsible for the production of oxo-18:0, since there was a consistent increase of oxo-18:0 before the establishment of *t*10-shift. Overall, more studies are needed for a better comprehension about the bacterial diversity in the rumen, as well as the role of bacteria on ruminal BH.

Moreover, conversely to what was hypothesized, no clear association between rumen pH and *t*10-shift was found, although there was a post-prandial increase of "*t*10-/*t*11-18:1" ratio in ram 2 that was related to a reduction of pH. These results might have been caused by the absence of a continuous measurement of pH values that is essential to evaluate the variation of pH all along the day. Indeed, not only a low but also a fluctuating pH was described as being necessary for the development of the shift (Colman *et al*., 2012). Additionally, the presence of *t*10-shift in the rumen could not be predicted by the evaluation of the "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the plasma, since there was only an evidence of the shift in the plasma after its establishment in the rumen. Conversely, Aldai, Dugan, Rolland and Aalhus (2012), Mapiye *et al*. (2013) and Alves *et al*. (2017) found that the blood proportions of *t*10-18:1 and *t*11-18:1 were predictors of *t*10-shift, although Aldai *et al*. (2012) and Mapiye *et al*. (2013) evaluated correlations of *t*10-18:1 and *t*11-18:1 between erythrocytes and adipose tissue with only Alves *et al*. (2017) reporting an association between "*t*10-18:1/(*t*11-18:1 + *c*9,*t*11-18:2)" ratio in the plasma and "*t*10-/*t*11-18:1" ratio in the rumen. The transient behaviour of *t*10-shift and the individual variation on its appearance might explain the discrepancies between the present results and the ones observed in the previous studies.

4.5. Conclusions

In the present study, the t10-shift appeared progressively within the first two weeks of feeding rams with a wheat-based diet supplemented with oil and was accompanied by an enhancement of t10-18:1 after that of t11-18:1 in the rumen. These results, together with an increase of oxo-18:0 before the t10-shift, probably indicate an adaptation of rumen microbiota to starch feedstuffs. In fact, there was a lower rumen bacterial diversity during the shift, which can be due to individual variability of microbiota or dietary effects. Moreover, the t10-shift´s induction was also variable between animals and the shift was only maintained during a short period of time, which might have contributed to the difficulty in predicting the occurence of t10-shift in the rumen by evaluating the t10-18:1/(t11-18:1 + c9,t11-18:2)" ratio in the blood plasma. Also, no clear association between rumen pH and t10-shift was found, but one ram showed a post-prandial (lower pH) increase of "t10-/t11-18:1" ratio. Further studies are needed for a more detailed evaluation of the biology behind the development of t10-shift.

CHAPTER 5 – The reduction of starch in finishing diets supplemented with oil does not prevent the accumulation of *trans*-10 18:1 in lamb meat

The reduction of starch in finishing diets supplemented with oil does not prevent the accumulation of *trans*-10 18:1 in lamb meat

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Contribution of Mónica Costa to this article:

Mónica Costa participated in the animal experiment and management and sample collection. Also, she determined the FA content and performed the data processing and statistical analysis. Furthermore, Mónica Costa participated in the interpretation and discussion of the results, as well as in the writing of the manuscript.

ABSTRACT

The experiment was conducted to test the hypothesis that the replacement of cereal with low-starch feed ingredients in lambs' finishing diets supplemented with oils could prevent the accumulation of t10-18:1 in meat. Forty lambs were fed with one of 4 diets supplemented with soybean oil (59 g/kg dry matter, DM) and fish oil (10 g/kg DM) during 6 weeks before slaughter. Control diet contained barley at 42% DM (cereal, C) and, in the other diets, barley was completely replaced for dehydrated citrus pulp (DCP), dehydrated beet pulp (DBP) or soybean hulls (SH). Growth performance, feed intake, carcass and meat quality traits were analyzed. At slaughter, *Longissimus* muscle (LM) samples were collected for gene expression evaluation and, 3 days after slaughter, LM and subcutaneous fat samples were obtained and analyzed for fatty acid (FA) composition. None of the diets affected meat quality, but DCP diet reduced ADG ($P < 0.05$) and DCP and SH diets decreased feed efficiency ($P < 0.01$). The DCP diet increased ($P < 0.05$) the risk of occurrence of parakeratosis and the severity of the lesions. Moreover, DBP led to larger a* (redness) and b* (yellowness) color parameters values of subcutaneous fat than C treatment ($P < 0.05$). Lipid content of LM did not differ ($P > 0.05$) with treatments and averaged 34.4 g/kg of meat. Diets had no effect ($P > 0.05$) on SFA, PUFA and *cis*-MUFA sums and on "n-6/n-3" ratio in both LM and subcutaneous fat. A lower expression of FASN gene was found with DCP than with the other treatments ($P < 0.001$). All treatments showed a high accumulation of t10-18:1, averaging 90.5 mg/g FA in LM and 147.3 mg/g FA in subcutaneous fat. The concentration of t11-18:1 in the tissues was considerably lower than that of t10-18:1, and thus the "t10-/t11-18:1" ratio was above 3 with all treatments. Despite that, SH treatment clearly promoted a larger deposition of t11-18:1 and c9,t11-18:2 in the tissues compared to the other treatments. Stearoyl-CoA desaturase gene expression and SCD activity index in LM were reduced with SH diet compared with C and DCP diets. Overall, these results clearly showed, for the first time, that the establishment of t10-shifted biohydrogenation pathways in the rumen, evaluated by the deposition of biohydrogenation intermediates in meat and fat, is not exclusively dependent of starch level in the diet.

Keywords: t10-shift, fatty acids, biohydrogenation, meat, starch, lipid supplementation

Doctoral Thesis in Veterinary Science – Mónica Mendes da Costa

5.1. Introduction

Public health implications of *trans*-FA from ruminant products are controversial as *trans*-11 FA, i.e. vaccenic (*t*11-18:1) and rumenic acids (*c*9,*t*11-18:2), are known to be beneficial to human health (Gebauer *et al*., 2011; Lim *et al*., 2014), whereas *t*10-18:1 are thought to increase the risk of cardiovascular diseases (Hodgson *et al*., 1996).

Ruminant edible fats are the richest natural dietary sources of *trans*-18:1 fatty acids (FA) and of CLA isomers (mostly *c*9,*t*11-18:2) as consequence of ruminal BH of dietary unsaturated FA (Shingfield & Wallace, 2014). Strategies to enrich ruminant meat with *t*11-18:1 and *c*9,*t*11-18:2 have been extensively researched, and supplementation of high- forage diets with C18 unsaturated FA has been defined as the most effective approach (Bessa *et al*., 2005). Simultaneous dietary inclusion of marine oils also reduces the completeness of BH resulting in large rumen outflow of *t*11-18:1 (Shingfield *et al*., 2011). However, high-starch finishing diets used in intensive ruminant production are known to alter ruminal BH pathways through a shift towards the formation of *t*10-18:1 at the expense of t11-18:1 as the major BI (hereafter *t*10-shift). When the *t*10-shift is established in the rumen, the supplementation with C18 PUFA fails to increase the *t*11-18:1 and *c*9,*t*11-18:2 in meat, and a large accumulation of the undesirable *t*10-18:1 isomer is observed in tissues (Bessa *et al*., 2015). Very little is known about microbiological and metabolic mechanisms that determine the occurrence of *t*10-shift, but a high-starch/low-fibre diet seems to be mandatory (Bessa *et al*., 2005; Rosa *et al*., 2014). Thus, we hypothesize that the reduction of starch content of oil supplemented finishing diets for lambs, via replacing cereals with low-starch feed ingredients, will prevent the establishment of *t*10-shift in the rumen, resulting in higher proportions of *c*9,*t*11-18:2 and *t*11-18:1 in the meat and subcutaneous fat.

5.2. Materials and methods

5.2.1. Animals, diets and management

Animal were raised and handled in compliance with the EU Council Directive 2010/63/EU (EC, 2010) concerning animal care. The experiment was conducted at the facilities of National Institute of Agronomic and Veterinary Research (INIAV), Portugal. Forty Merino Branco ram lambs approximately 90 days of age were randomly assigned to 20 pens, with 2 lambs per pen and 5 pens per treatment. The treatments consisted of 4 ground complete diets composed of forage (dehydrated alfalfa) and a concentrate meal containing either barley (cereal, C), dehydrated citrus pulp (DCP), dehydrated sugar beet pulp (DBP) or soybean hulls (SH) as the main energy source. All diets included 59 g/kg DM of soybean oil and 10 g/kg DM of fish oil. The ingredients and chemical composition of the diets are presented in Table 14. The trial started after 7 d of adaptation to the experimental conditions and lasted for 6 weeks. The BW of lambs at the beginning of the trial was 26.6 ± 1.05 kg. Feed was offered *ad libitum*, and feed intake was daily controlled by weighing the amounts of feed offered and refused, considering 10% orts. The animals were weighed weekly just before feeding. Two composite samples of each diet were obtained from a daily collection of feed samples and were analyzed for DM, CP, NDF, ADF, FA, ether extract, ash, starch and sugar composition as described by Santos-Silva *et al*. (2016).

Table 14. Ingredients, chemical composition and fatty acid (FA) profile of the experimental diets.

Item	Diets[1]			
	C	DCP	DBP	SH
Ingredients, g/kg DM[2]				
Barley	416	-	-	-
Citrus pulp	-	426	-	-
Beet pulp	-	-	436	-
Soybean hulls	-	-	-	446
Wheat bran	114	59	94	89
Soybean meal	178	223	178	173
Dehydrated alfalfa	198	198	198	198
Soybean oil	59	59	59	59
Fish oil	10	10	10	10
Calcium carbonate	13	13	13	13
Sodium bicarbonate	5	5	5	5
Salt	4	4	4	4
Premix	3	3	3	3
Chemical composition, g/kg DM				
DM[3]	893	886	901	896
CP[4]	178	181	181	174
Ether extract	105	99	92	100
FA[5]	70.8	77.0	74.3	73.2
Starch	318	50	56	54
Sugar	68	229	106	74
NDF[6]	260	236	369	463
ADF[7]	124	159	188	261
Ash	74	93	86	85
FA profile, g/kg FA				
16:0	183	150	157	154
18:0	50.7	41.8	42.8	49.4
c9-18:1	235	233	236	239
18:2n-6	453	482	475	475
18:3n-3	49.7	60.0	55.2	59.0
20:3n-6	6.1	5.7	5.6	5.7
20:4n-6	1.1	1.5	1.6	0.9
20:5n-3	8.0	9.8	9.5	6.1
22:5n-3	1.0	1.3	1.1	0.7
22:6n-3	12.3	15.9	15.4	10.4

[1] C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], dry matter; [3], g/kg feed, [4], crude protein; [5], fatty acids; [6], neutral detergent fibre; [7], acid detergent fibre.

5.2.2. Slaughter, carcass evaluation and sample collection

At the end of experimental period, lambs were weighed and transported to the experimental abattoir of INIAV, located in the same facilities, to be stunned and slaughtered by exsanguination. After slaughter, the rumen content was strained through four layers of cheesecloth and the rumen pH was measured (pH meter - Metrohm, 744, Herisau, Switzerland). Also, rumen mucosa parakeratosis lesions were evaluated using a 4 point visual scale (with 0 being normal rumen papillae and 3 representing strong parakeratosis lesions), according to Tamate, Nagatani, Yoneya, Sakata and Miura (1973). Samples of *Longissimus* muscle (LM) were excised from the right side of carcasses (at the level of 12th

vertebra) within 10 min post-slaughter, snap-frozen in liquid nitrogen and stored at -80ºC until mRNA expression analysis. The hot carcass weight was recorded and the carcasses were kept at 10 ± 1°C for 24 h, to prevent cold-induced shortening. Thereafter, cold carcass weights were recorded, and the carcasses were graded for conformation and fat cover according to EUROP classification systems for lamb carcasses (EC, 2011) and chilled at 2±1ºC for 48 h. On the third day after slaughter, kidney knob channel fats and kidneys were removed; the carcasses were split along the spine and their left sides were separated into eight joints (Santos-Silva *et al*., 2002b). The weights of the individual joints were recorded and the proportion of the higher priced joints (leg + chump + loin + ribs) was determined. The chump and shoulders were totally dissected to determine the proportions of muscle, subcutaneous and intermuscular fats and bone.

In the left halves of carcasses, the loin joints containing LM were vacuum-packed and frozen at -20°C until shear force analysis. In the rib joint, at the level of 12th vertebra, a sample of LM with 1.5 cm thickness was collected to evaluate the color after 1 h of blooming. The remaining portion of LM was isolated and, after the removal of the epimysium, was minced in a food processor (3 × 5 s), vacuum-packed, freeze-dried, and stored at -20°C until lipid analysis.

In the right halves, the subcutaneous fat of rib joints was detached and used for color evaluation in the inner face. Afterwards, samples of subcutaneous fat were stored at -20°C until lipid analysis. The right loin joints containing LM were vacuum packed and stored at -20°C to be used for sensory analysis.

5.2.3. Color, pH and shear force determinations

Muscle color was measured using a Chroma Meter CR-400 (Konica Minolta, Inc., Japan), calibrated with white plate (Y = 84.9; x = 0.3199; y = 0.3359) using illuminant D65, 1 cm diameter observed area, 2°-viewing angle. Three measurements per sample were recorded according to CIE L*, a*, b* system, where L* is lightness, a* redness and b* yellowness. For muscle pH determination, 5 g of LM samples were homogenized in 50 mL of 0.1 M potassium chloride solution (ISO-2917, 1999). The pH of suspended samples was determined using a pH meter (Metrohm, 744, Herisau, Switzerland) equipped with a combined glass electrode. For shear force determinations, the frozen loin joints were thawed for 24 h at 2±1°C. Thereafter, the LM was isolated from vertebra bones, weighed, and roasted in an electric oven at 170 °C until the internal meat temperature reached 70 °C, which was individually monitored with a type T thermocouple (Thermometer, Eomega RDXL4SD, Manchester, USA). After cooling for 24 h at 2±1°C, each meat sample was cut longitudinally in the direction of fibers into subsamples with 1 cm^2 of section for shear force determination using a Warner-Bratzler shear device mounted in a Texture Analyser (TA-tx2i

Texture Analyser, Stable Micro Systems, Surrey, UK), according to procedures described by Francisco *et al*. (2015). The measurement of cores from each loin was recorded as the average of a minimum of 15 repeats.

5.2.4. Sensory analysis

Meat sensory characteristics were evaluated in 7 sessions by a trained sensory panel composed of 9 members of INIAV. For each session, 5 or 6 LM samples were randomly selected and were allowed to thaw for 24h at 2°C. The LM was cooked in the same way as for shear force determinations and samples were prepared according to procedures previously described by Francisco *et al*. (2015). The meat attributes evaluated were odor, tenderness, juiciness, flavor and overall acceptability. The scale applied in the sensory analysis was structured into eight points, where: 1 – extremely soft (odor and flavor), extremely tough (tenderness), extremely dry (juiciness) or extremely inacceptable (overall acceptability); 8 – extremely intense (odor and flavor), extremely tender (tenderness), extremely juicy (juiciness) or extremely acceptable (overall acceptability).

5.2.5. Fatty acid analysis

Fatty acid methyl esters of feed lipids were prepared by one-step extraction using 10% HCl in methanol and 19:0 as internal standard (Sukhija & Palmquist, 1988). Muscle and subcutaneous fat lipids were extracted with dichoromethane:methanol (2:1, vol/vol) from freeze-dried tissue samples and transesterified into FA methyl esters using a combined basic and acidic catalysis as described by Oliveira *et al*. (2016). Fatty acid methyl esters were analyzed by GC-FID (Shimadzu GC-2010 Plus, Kyoto, Japan) using a 100% cyanopropyl polysiloxane capillary column (SP-2560, 100 m, 0.25 mm i.d., 0.20 µm film thickness; Supelco Inc., Bellefont, PA, USA). Identification of FA methyl esters was achieved by comparison of retention times with those of authentic standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA, and a Bacterial FAME mix from Matreya LLC, Pleasant Gap, PA, USA) and, particularly in the case of non-terminal mono-methyl BCFA, with published chromatograms (Alves *et al*., 2013a). In addition, identifications were confirmed by GC-MS in a GC-MS QP 2010 Plus chromatograph (Shimadzu, Kyoto, Japan) with a SP-2560 column. The GC-MS and GC-FID conditions were as described previously (Alves *et al*., 2015; Oliveira *et al*., 2016). For quantification of individual CLA isomers present in the muscle, a combination of gas chromatography and silver high performace liquid chromatography (3 Ag+ - HPLC) was used as reported by Bessa *et al*. (2007).

5.2.6. Gene expression

Total RNA was isolated from LM samples using QIAzol lysis reagent and further purified with RNeasy mini columns with on-column DNase digestion (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 0.5 µg total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems CA, USA) according to manufacturer´s instructions. Real-time PCR was performed as described previously (Francisco *et al*., 2016) using a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences used for amplification of SCD and ACACA genes were reported by Madeira *et al*. (2013) and Francisco *et al*. (2016). Gene-specific intron-spanning primers for FASN were designed as described by Madeira *et al*. (2013) and Francisco *et al*. (2016) and their sequences (from 5' end to 3' end) were CCAAGTACAATGGCACCCTGA and TCTCCTCGGTGAGCTGCG for forward and reverse primer, respectively. For each gene, a standard curve was established using a series of 5-fold dilutions of pooled cDNA samples. The standard curve was used to determine the relative gene expression variation after normalization with the geometric mean of two housekeeping genes (ACTB and RPLP0). Subsequently the specificity of the amplification was verified and relative expression levels of mRNAs were calculated as a variation of the Livak method (Livak & Schmittgen, 2001).

5.2.7. Statistical analysis

Data were analyzed as completely randomized design using the MIXED procedure of SAS (SAS Institute Inc., Cary NC). A single fixed factor was the type of diet and a pen was the experimental unit. Lambs within pens were treated as sub-sampling using either a compound symmetry or an unstructured covariance matrix depending on the best model generated. The variance homogeneity was tested for a level of $P = 0.01$ and, when significant, the variance heterogeneity was accommodated in the model. When significant effects of treatments were detected, least square means were compared using the pairwise Tukey comparison test. Weekly BW data were analyzed by random intercept regression model considering a pen as the experimental unit and lambs within pens as subsampling in order to estimate ADG (i.e. slopes). Daily DMI data, collected in each pen, was the observational unit and was averaged per week and divided by 2, to obtain mean intake values expressed as g/d per lamb. A repeated measurements model was adjusted in order to estimate the average DMI per lamb during the experiment. Slaughter BW, hot and cold carcass weights were adjusted to initial BW, and FA composition of LM was adjusted to lipid content of LM. Dressing, carcass cuts and chump and shoulder composition were adjusted to hot carcass weight. Data from meat sensory evaluation were analyzed considering observations from each panelist as the repeated measurement and assuming a first-order autoregressive covariance matrix. The

probability of occurrence of different grades of rumen parakeratosis was analyzed using the PROC GLIMMIX (SAS Institute Inc., Cary NC), considering the multinomial distribution and the cumulative logit as link function.

5.3. Results

5.3.1. Intake and productive performance

As planned, the experimental diets allowed large differences in starch, sugar and NDF intake (Table 15). The dietary replacement of barley with citrus pulp, beet pulp or soybean hulls reduced ($P < 0.05$) the starch intake from an average of 377 to 68 g/d. Moreover, the DCP diet resulted in the largest sugar intake ($P < 0.05$) and the SH diet led to the largest ($P < 0.05$) NDF and ADF intake. The DBP diet allowed an intermediate intake of sugar and NDF between DCP and SH. The intake of starch + sugar decreased ($P < 0.05$) gradually from C to DCP to DBP and to SH. Despite those differences, DMI did not differ ($P = 0.14$) among diets. Nevertheless, ADG of lambs was lower ($P < 0.05$) with DCP diet than with C and DBP diets (Table 15). Lambs fed SH diet presented an intermediate ADG that did not differ ($P > 0.05$) from those observed with the other diets. Thus, feed efficiency (kg gain/kg DMI) observed with DCP was lower ($P < 0.05$) than those attained with C and DBP, but similar ($P > 0.05$) to SH. Slaughter BW did not differ among treatments, averaging 40.7±0.73 kg.

Table 15. Effect of replacing cereal in the diet with low starch feed ingredients on nutrient intake and growth performance of lambs.

Item	Diets[1]				SEM[2]	P-value
	C	DCP	DBP	SH		
Intake, g/d						
Dry matter	1177	1231	1306	1276	39.7	0.144
Crude protein	209	222	235	222	7.1	0.107
Ether extract	123	121	121	128	4.0	0.613
Starch	377^a	62^b	74^b	68^b	8.0	<0.001
Sugar	80^d	280^a	138^b	95^c	4.4	<0.001
Starch + sugar	452^a	343^b	213^c	165^d	10.8	<0.001
NDF[3]	306^c	292^c	480^b	587^a	14.4	<0.001
ADF[4]	146^d	196^c	245^b	332^a	7.9	<0.001
Initial BW, kg	25.7	26.5	27.5	26.6	1.05	0.697
Slaughter BW[5], kg	41.1	38.9	42.0	40.6	0.73	0.064
BW gain, kg	14.6	12.4	15.3	14.1	0.74	0.073
ADG[6], g/d	349^a	288^b	354^a	324^{ab}	17.7	0.035
Feed efficiency[7]	0.29^a	0.23^c	0.27^{ab}	0.25^{bc}	0.008	0.002
Rumen pH	5.97^{ab}	6.09^a	5.55^b	5.84^{ab}	0.121	0.035

[a-c], Means within a row with different superscripts differ ($P < 0.05$); [1], C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], standard error of means; [3], neutral detergent fibre; [4], acid detergent fibre; [5], BW, body weight; [6], average daily gain; [7], kg gain/kg dry matter intake.

The occurrence and severity of rumen parakeratosis lesions are presented in Figure 10. The probability of developing rumen parakeratosis was influenced by diets and it was higher ($P < 0.05$) for DCP (0.94±0.083) than for the other diets (0.44±0.083). Also, more severe lesions (grades 2 and 3) were found for DCP (average of 24.2%) comparing with the other treatments (averaging 2.2%). Rumen pH measured post-mortem was below 6.1 for all the treatments, being lower for DBP (5.55) than for DCP (6.09) and intermediate for C and SH.

Figure 10. Effect of dietary energy source on the probability of occurrence of rumen mucosa parakeratosis evaluated by analysis of the severity of lesions.
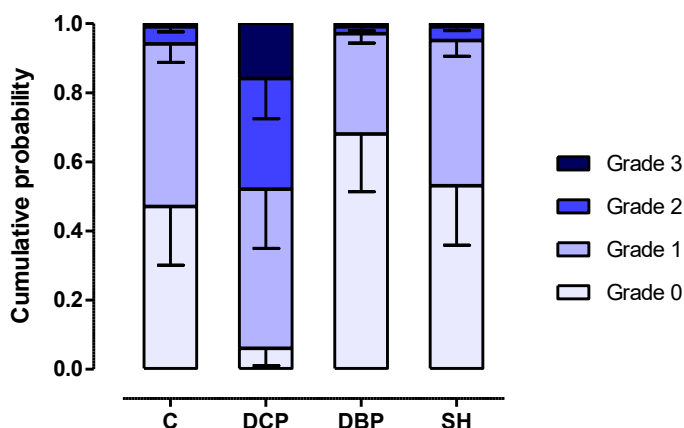
Treatments: C, cereal; DCP, citrus pulp; DBP, beet pulp; SH, soybean hulls. The patterns observed with C, DBP, and SH diets were similar (P > 0.05). The pattern observed with DCP diet differed (P < 0.03) from that of other diets.

5.3.2. Carcass traits and meat quality traits

There was no effect of treatments on carcass traits (Table 16). Hot carcass weight, cold carcass weight, dressing and higher priced cuts percentages averaged 20.2±0.44 kg, 19.7±0.41 kg, 49.7±0.44% and 52.9±0.46%, respectively. Dissection of chump and shoulder cuts showed that treatments had no effect on the percentages of muscle (P = 0.526), bone (P = 0.423) and intermuscular (P = 0. 493) and subcutaneous (P = 0.484) fat. For carcass conformation, 88.9% of the carcasses were graded as class R (good) and 11.1% as O (regular). For fat cover, 56.7% was graded as 5 (very high) and 43.3% as 4 (high).

Subcutaneous fat color was influenced by diet. In fact, the redness parameter (a*) was higher (P < 0.001) for DBP than for the other treatments. Moreover, the yellowness parameter (b*) was higher for DBP (P < 0.05) than for SH, presenting intermediate values for C and DCP.

Treatments did not affect LM quality traits (Table 16). Meat pH average was 5.65±0.009 and did not differ (P = 0.71) among treatments. Shear force average was 3.93±0.428 kgF/cm2 and did not differ (P = 0.79) among treatments with LM color not being affected (P > 0.25) by treatments, averaging in 41.3±0.73 for L*, 17.3±0.42 for a* and 8.6±0.37 for b*. Meat sensorial evaluation was not affected (*P* > 0.40) by the treatments either, and the sensory panel considered the meat as tender (6.51±0.116), with moderate juiciness (5.81±0.119), soft odor (3.42±0.114), soft flavor (3.96±0.15) and good overall acceptability (6.28±0.109).

Table 16. Effect of replacing cereal in the diet with low starch feed ingredients on carcass composition and meat quality of lambs.

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
Carcass traits						
Hot carcass weight, kg	20.7	19.3	20.9	20.0	0.44	0.063
Cold carcass weight, kg	20.0	18.8	20.3	19.5	0.41	0.101
Dressing, %	50.2	49.8	49.3	49.3	0.44	0.334
KKCF[3], %	4.2	3.5	3.6	4.1	0.29	0.333
Higher priced cuts[4], %	52.8	53.1	53.3	52.4	0.46	0.574
Chump and shoulder tissues[5], %						
Muscle	51.0	51.6	52.9	50.6	1.11	0.526
Bone	18.7	18.6	18.4	18.0	0.33	0.423
Intermuscular fat	16.2	15.8	15.0	17.1	0.94	0.493
Subcutaneous fat	17.0	16.6	15.9	17.9	0.87	0.484
Meat quality traits						
Subcutaneous fat color						
L*	74.4	74.7	74.8	77.6	1.31	0.324
a*	3.4^b	2.9^b	4.8^a	2.9^b	0.29	0.001
b*	7.4^{ab}	8.2^{ab}	8.7^a	6.8^b	0.38	0.018
Longissimus muscle						
Color						
L*	39.9	42.0	41.7	41.6	0.73	0.252
a*	17.6	17.4	17.4	16.8	0.42	0.530
b*	8.3	8.7	8.7	8.6	0.37	0.851
pH	5.65	5.65	5.64	5.65	0.009	0.714
Shear force, kgF/cm^2	3.85	3.95	3.63	4.27	0.428	0.794
Sensorial evaluation						
Tenderness	6.51	6.46	6.50	6.57	0.116	0.912
Juiciness	5.76	5.80	5.76	5.91	0.119	0.756
Odor intensity	3.48	3.22	3.48	3.49	0.114	0.409
Flavor intensity	4.04	4.06	3.80	3.95	0.145	0.606
Flavor acceptability	6.37	6.35	6.50	6.39	0.105	0.795
Overall acceptability	6.29	6.30	6.32	6.19	0.109	0.800

[a-b], Means within a row with different superscripts differ (*P* < 0.05); [1] C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], standard error of means; [3], kidney knob channel fat; [4], leg + chump + loin + ribs; [5], data from the dissection of rump and shoulder cuts.

5.3.3. Fatty acid composition of muscle

The lipid content and general FA composition of LM are presented in Table 17. Lipid and FA content of LM averaged 34.3 and 27.4 g/kg in fresh meat respectively, and did not differ (P > 0.05) among treatments, although the variability with DBP and SH was larger (P < 0.01) than in C and DCP. The linear chain SFA averaged 402 mg/g FA in LM for all the treatments. The major linear SFA present was 16:0 and its content was higher (P < 0.05) in case of DCP treatment (259 mg/g FA) and lower (P < 0.05) with SH treatment (233 mg/g FA). This was followed by 18:0 (ranging from 122 to 136 mg/g FA) that was lower (P < 0.05) with DBP (122 mg/g FA) than with C treatment (136 mg/g FA). Several terminal and non-terminal BCFA have been identified in LM lipids and, although all of them were present in residual amounts, collectively they averaged 11 mg/g FA for C, DBP and SH treatments and only in 8.8 mg/g

FA for DCP (P < 0.05). In general, terminal BCFA (i.e. iso and anteiso) were higher for SH, lower for DCP (P < 0.05) and intermediate for the other treatments. Otherwise, NT-BCFA were higher for C, lower for SH (P < 0.05) and intermediate for the other treatments.

The major FA present in LM lipids was c9-18:1 with an average value of 301 mg/g FA across treatments. However, c9-18:1 least square means did not differ ($P > 0.05$) among treatments and the variance was much larger ($P < 0.001$) for DCP treatment than for the other treatments. This larger variance found with DCP treatment was due to 3 lambs that presented consistently high c9-18:1 content and low BI in both, LM and subcutaneous fat. It was not possible to relate that variability with pen, ADG, intramuscular lipid content or rumen parakeratosis lesions of lambs. Some other minor cis-MUFA (i.e. c9-14:1, c9-16:1 and c11-20:1) presented small but significant differences among treatments.

The total PUFA content of LM was lower ($P < 0.05$) with DCP treatment (84 mg/g FA) and higher ($P < 0.05$) with DBP treatment (111 mg/g FA), being the other 2 treatments intermediate. This pattern arose from differences observed in the 18:2n-6, the major PUFA present in LM. Most of other PUFA did not present significant differences among treatments (including all n-3 PUFA), or present only small, although significant, differences.

The sum of $trans$-FA (excluding the t11-18:1 and CLA isomers) present in LM was fairly high, averaging in 122 mg/g FA among treatments. $Trans$-FA did not differ ($P > 0.05$) among treatments but the variance was much higher ($P < 0.001$) for DCP treatment than for the other treatments.

Table 17. Effect of replacing cereal in the diet with low starch feed ingredients on lipids and fatty acid (FA) profile (mg/g of total fatty acids) of *Longissimus* muscle of lambs.

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
Lipids, g/kg meat	33.5±1.27	31.0±1.27	35.1±3.03	37.8±2.75		0.196
FA, g/kg meat	24.5±1.13	24.0±1.13	27.4±3.12	30.0±3.12		0.326
FA profile						
LC-SFA[3]						
10:0	1.12[ab]	1.28[a]	1.02[ab]	0.92[b]	0.037	0.087
12:0	0.62	0.67	0.53	0.52	0.046	0.115
14:0	19.6[ab]	21.9[a]	18.0[ab]	16.8[b]	1.12	0.033
15:0	1.93	1.90	2.15	2.01	0.084	0.234
16:0	236[ab]	259[a]	238[ab]	233[b]	6.1	0.037
17:0	6.76	6.72	7.05	6.76	0.229	0.753
18:0	136[a]	127[ab]	122[b]	134[ab]	3.1	0.028
20:0	0.79[b]	0.75[b]	0.85[ab]	0.98[a]	0.033	0.002
Sum	404	420	391	394	7.3	0.055
T-BCFA[4]						
iso-14:0	0.09[b]	0.08[b]	0.21[a]	0.19[a]	0.016	<0.001
iso-15:0	0.34[b]	0.35[b]	0.43[b]	0.73[a]	0.050	<0.001
anteiso-15:0	0.59[b]	0.45[b]	0.83[a]	0.83[a]	0.043	<0.001
iso-16:0	0.71[b]	0.54[c]	0.96[a]	0.79[ab]	0.040	<0.001
iso-17:0	1.98[b]	1.65[b]	1.83[b]	2.66[a]	0.131	<0.001
anteiso-17:0	3.01[a]	2.25[b]	3.29[a]	3.18[a]	0.091	<0.001
iso-18:0	0.94[ab]	0.79[b]	1.15[a]	0.94[ab]	0.059	0.007
Sum	7.64[b]	6.12[c]	8.67[ab]	9.32[a]	0.332	<0.001
NT-BCFA[5]						
6 Me-14:0	0.14[a]	0.13[ab]	0.12[ab]	0.07[b]	0.015	0.047
8 Me-14:0	0.10	0.10	0.08	0.07	0.095	0.247
4 Me-14:0	0.23	0.23	0.18	0.13	0.031	0.152
10 Me-14:0	0.09	0.07	0.05	0.06	0.013	0.222
2 Me-16:0	0.12	0.15	0.15	0.10	0.019	0.277
6 Me-16:0	0.31[ab]	0.33[a]	0.25[ab]	0.21[b]	0.024	0.017
8 Me-16:0	0.15[ab]	0.19[a]	0.14[ab]	0.10[b]	0.017	0.023
4 Me-16:0	0.79	0.76	0.70	0.65	0.046	0.216
12 Me-16:0	1.02[a]	0.84[b]	0.92[ab]	0.80[b]	0.042	0.010
Sum	2.92[a]	2.70[ab]	2.49[ab]	2.06[b]	0.172	0.024
Total BCFA	10.55[a]	8.80[b]	11.15[a]	11.40[a]	0.372	<0.001

Table 17. Effect of replacing cereal in the diet with low starch feed ingredients on lipids and fatty acid (FA) profile (mg/g of total fatty acids) of *Longissimus* muscle of lambs (continuation).

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
cis-MUFA[6]						
*c*9-14:1	0.54[ab]	0.61[a]	0.46[ab]	0.39[b]	0.048	0.031
*c*7-16:1	1.54	1.68	1.60	1.46	0.050	0.063
*c*9-16:1	12.0[a]	13.0[a]	11.3[ab]	9.7[b]	0.54	0.005
*c*9-17:1	3.24	3.44	3.13	2.75	0.198	0.168
*c*9-18:1	304±6.0	322±17.6	286±6.4	290±6.5		0.167
*c*11-18:1	14.1	12.7	13.9	12.9	0.45	0.113
*c*11-20:1	1.35[ab]	1.22[b]	1.51[a]	1.43[ab]	0.057	0.024
Sum	323±6.4	341±18.4	304±6.7	306±6.6		0.150
n-6 PUFA[7]						
18:2n-6	69.7[ab]	55.2[b]	76.0[a]	62.8[ab]	4.18	0.021
18:3n-6	0.33[a]	0.26[b]	0.33[a]	0.29[ab]	0.015	0.001
20:2n-6	0.73	0.76	0.87	0.71	0.045	0.132
20:3n-6	1.75	1.72	1.77	1.62	0.094	0.732
20:4n-6	10.03	9.10	10.65	9.27	0.908	0.613
22:4n-6	0.60[b]	0.71[ab]	0.82[a]	0.71[ab]	0.044	0.035
22:5n-6	1.64	1.29	1.74	1.78	0.128	0.085
Sum	84.9[ab]	69.1[b]	92.6[a]	77.1[ab]	5.16	0.037
n-3 PUFA						
18:3n-3	4.81	4.41	5.12	4.84	0.177	0.085
20:4n-3	0.71	0.64	0.90	0.71	0.107	0.427
20:5n-3	3.33	2.95	3.59	2.95	0.360	0.542
22:5n-3	3.96	4.03	4.38	3.78	0.259	0.465
22:6n-3	3.84	2.67	3.55	2.85	0.284	0.189
Sum	15.6	14.7	17.6	15.2	1.08	0.285
Total PUFA[8]	100.5[ab]	83.9[b]	110.5[a]	92.2[ab]	6.14	0.048
Total *trans*-FA[9]	123±8.7	99±16.0	138±8.9	128±8.9		0.301
Total C18 FA[10]	674[ab]	650[b]	670[ab]	683[a]	6.82	0.030
Total DMA[11]	1.39	1.04	1.80	1.07	0.406	0.538

[a-c], Means within a row with different superscripts differ ($P < 0.05$); [1] C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], standard error of means; [3], linear chain saturated FA; [4], terminal branched chain FA (iso and anteiso); [5], non-terminal branched chain FA; [6], *cis*-monounsaturated FA excluding biohydrogenation intermediates, [7], polyunsaturated FA; [8], sum of all *cis*, methylene interrupted PUFA; [9], sum of *trans*-FA excluding *t*11-18:1 and conjugated linoleic acid isomers; [10], sum of all C18 FA; [11], sum of dimethylacetals.

The detailed profile of C18 BI in LM is presented in Table 18. The main BI was the *t*10-18:1 that did not differ among the treatments and averaged 91 mg/g FA. About 67% of all the *t*18:1 BI and 59% of all the BI consisted of *t*10-18:1. The *t*11-18:1 was the second most abundant BI, but differed widely among the treatments, being lower for C (9.1 mg/g FA), higher for SH (35.6 mg/g FA) and intermediate for the other treatments. Thus, *t*10-/*t*11-18:1 ratio was greater than 1 in all the treatments and was higher ($P < 0.05$) for C than for DCP and SH and intermediate for DBP (Figure 11). Only two animals, both fed SH diet, presented a "*t*10-/*t*11-18:1" ratio close to 1. The level of some of the minor octadecenoic isomers differed between the treatments with the lowest value in DCP. Nevertheless, the sum of 18:1

BI did not differ among treatments and averaged 135 mg/g FA with a larger variance in DCP treatment ($P < 0.001$) compared to the other treatments.

The major 18:2 BI were non-conjugated isomer t10,c15-18:2 (which included t11,c15-18:2), that averaged 5.8 mg/g FA across treatments; and conjugated isomer c9,t11-18:2 (rumenic acid) that ranged from 2.8 in C to 8.3 mg/g FA in SH. The content of c9,t11-18:2 was higher ($P < 0.05$) in SH than in the other treatments. Generally, 18:2 BI were higher with SH (23.1 mg/g FA), lower with C and DCP (≈16.5 mg/g FA) and intermediate with DBP (18.2 mg/g FA).

Table 18. Effect of replacing cereal in the diet with low starch feed ingredients on C18 biohydrogenation intermediates (mg/g of total fatty acids) present in *Longissimus* muscle of lambs.

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
18:1 isomers						
*t*4	0.14	0.19	0.19	0.19	0.027	0.550
*t*5	0.23	0.23	0.30	0.26	0.030	0.358
*t*6 /*t*7 /*t*8	7.70[a]	3.89[b]	5.37[ab]	6.63[a]	0.591	0.002
*t*9	6.37[a]	3.16[b]	4.00[b]	5.09[ab]	0.529	0.003
*t*10	91.1	74.1	106.1	90.5	9.24	0.165
*t*11	9.1[c]	17.7[b]	16.2[b]	35.6[a]	2.51	<0.001
*t*12	6.33[ab]	5.01[b]	6.86[a]	6.18[ab]	0.425	0.049
*t*15	0.82	0.71	0.87	1.28	0.154	0.109
*c*12	2.31	2.27	2.48	2.82	0.307	0.637
*c*13	0.88	0.88	0.85	0.89	0.037	0.875
*t*16[3]	1.31[b]	1.30[b]	1.24[b]	1.80[a]	0.109	0.010
*c*15	1.72[a]	1.38[b]	1.68[ab]	1.81[a]	0.076	0.008
*c*16	1.09	0.91	1.09	0.89	0.082	0.179
Sum	128±8.6	110±18.0	147±8.8	156±8.8		0.122
18:2 isomers						
*t*8,*c*12	0.35	0.34	0.34	0.28	0.043	0.368
*c*9,*t*13	0.25	0.27	0.32	0.26	0.021	0.226
*t*8,*c*13	0.65[ab]	0.56[b]	0.51[b]	0.77[a]	0.058	0.037
*c*9,*t*12	0.52[b]	0.53[b]	0.58[ab]	0.69[a]	0.038	0.035
*t*9,*c*12	0.72[b]	1.03[ab]	1.03[ab]	1.45[a]	0.113	0.004
*t*10,*c*15[4]	4.49	5.65	6.17	6.08	0.565	0.192
*t*12,*t*14	0.028[c]	0.048[ab]	0.051[a]	0.033[bc]	0.0036	<0.001
*t*11,*t*13	0.049[b]	0.064[ab]	0.063[ab]	0.085[a]	0.0071	0.031
*t*10,*t*12	0.099	0.116	0.116	0.132	0.0105	0.261
*t*9,*t*11	0.103[b]	0.135[ab]	0.134[ab]	0.187[a]	0.0152	0.015
*t*8,*t*10	0.040	0.044	0.048	0.057	0.0048	0.179
*t*7,*t*9	0.076	0.060	0.071	0.081	0.0096	0.481
*t*6,*t*8	0.054	0.059	0.064	0.067	0.0034	0.107
c/*t*12,14	0.019	0.023	0.018	0.020	0.0028	0.648
*t*11,*c*13	0.039[b]	0.076[a]	0.062[ab]	0.090[a]	0.0080	0.003
*c*11,*t*13	0.011	0.017	0.015	0.018	0.0029	0.431
*t*10,*c*12	0.384	0.563	0.568	0.488	0.0714	0.262
*c*9,*t*11	2.77[b]	4.98[b]	4.33[b]	8.28[a]	0.632	<0.001
*t*9,*c*11	0.83[ab]	0.65[b]	0.90[a]	0.69[ab]	0.052	0.012
*t*7,*c*9	1.87[a]	0.86[c]	1.21[bc]	1.64[a]	0.152	0.001
Sum	15.1[b]	17.8[b]	18.2[ab]	23.1[a]	1.19	0.004
18:3 isomer						
*c*9,*t*11,*c*15[5]	0.99	1.16	1.05	1.02	0.089	0.613
Total BI[6]	144±9.3	129±19.7	166±9.6	180±9.5		0.079
*t*10/*t*11ratio[7]	10.7[a]	6.4[b]	7.3[ab]	3.6[b]	0.88	<0.001

[a-c] Means within a row with different superscripts differ (*P* < 0.05); [1] C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], standard error of means; [3], includes small amounts of *c*14-18:1; [4], includes *t*11,*c*15-18:2 isomer; [5], includes 20:3n-9; [6], Sum of biohydrogenation intermediates (i.e. all FA listed in the table); [7], ratio between *t*10-18:1 and *t*11-18:1.

Figure 11. Effect of replacing cereal in the diet with low starch feed ingredients on t10-/t11-18:1 ratio in *Longissimus* muscle (LM) and subcutaneous fat.
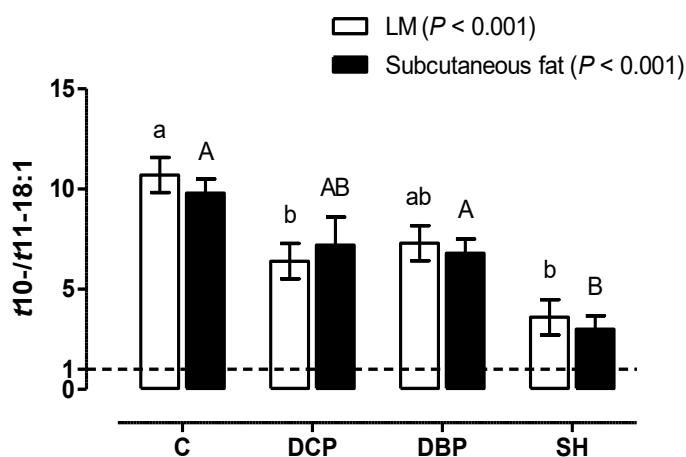
Lowercase superscripts indicate differences among means of treatments in LM (P < 0.05). Uppercase superscripts indicate differences among means of treatments in subcutaneous fat (P < 0.05). Treatments: C, cereal; DCP, citrus pulp; DBP, beet pulp; SH, soybean hulls.

5.3.4. Fatty acid composition of subcutaneous fat

The lipid content and general FA composition of subcutaneous fat are presented in Table 19. The lipid content of subcutaneous fat samples did not differ among treatments and averaged 843 mg/g tissue DM, whereas the FA content was larger (P < 0.05) for C than for DBP (697 vs. 660 mg/g tissue DM) and it had an intermediate value for DCP and SH treatments. The sum of LC-SFA did not differ among treatments and averaged 380 mg/g FA. The major LC-SFA were 16:0 (averaging in 208 mg/g FA across treatments) and 18:0 that ranged from 128 to 162 mg/g FA; it was higher (P < 0.05) for C compared to DCP and DBP and had intermediate values for SH treatment. The content of both, terminal and non-terminal BCFA was high across treatments with an average value of 31 mg/g FA. There were no significant differences between the treatments in the content of NT-BCFA and the average value was 16.7 mg/g FA. Some differences were observed for terminal BCFA, with the DCP treatment resulting in consistently lower proportion of these FA compared to DBP (i.e. iso-16:0), SH (iso-15:0 and iso-17:0) or both (i.e. iso-14:0) treatments. However, the sum of terminal BCFA did not differ among the treatments and averaged 14.5 mg/g FA. The major FA present in subcutaneous fat was c9-18:1, averaging 236 mg/g FA across treatments, and it made up the majority of *cis*-MUFA. Although the least square means of c9-18:1 did not differ (P > 0.05) among treatments, the variance was much larger (P < 0.001) for DCP treatment than for the other treatments. There were small but significant differences between the treatments in the content of some other minor *cis*-MUFA (i.e. c7-16:1 and c11-20:1).

The total PUFA content of subcutaneous fat did not differ among treatments and averaged 57 mg/g FA. The major PUFA present across treatments was 18:2n-6 with an average value of 45 mg/g FA. In the case of DBP and C, 18:2n-6 values tended (P = 0.054) to be larger (50.4 and 48 mg/g FA, respectively) compared to the other treatments (41.2 mg/g FA). The 18:3n-3 was the major n-3 PUFA and did not differ among the treatments, averaging in 5.2 mg/g FA. Despite of very low content of n-3 LC-PUFA, some differences (P > 0.05) among the treatments were observed. The largest values of 20:5n-3 and 22:5n-3 were observed for DBP and the lowest values were found for SH, with intermediate values for the other treatments. The 22:6n-6 was also higher for DBP but lower for C, with intermediate values for the other treatments.

The sum of *trans*-FA (excluding *t*11-18:1 and CLA isomers) in subcutaneous fat was fairly high, ranging from 167 to 222 mg/g FA, and had a tendency to be lower for DCP than for DBP, with no differences for the other treatments.

Table 19. Effect of replacing cereal in the diet with low starch feed ingredients on subcutaneous fat lipids and fatty acid (FA) composition (mg/g of total fatty acids) of subcutaneous fat.

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
Lipids, mg/g DM	839	842	843	848	6.6	0.800
FA, mg/g DM	697[a]	679[ab]	660[b]	668[ab]	8.1	0.029
FA profile, mg/g FA						
LC-SFA[3]						
10:0	1.13	1.36	1.09	1.22	0.093	0.194
12:0	0.67	0.70	0.60	0.65	0.057	0.617
14:0	21.1	24.2	21.8	22.9	1.646	0.555
15:0	5.10	6.00	5.94	5.42	0.639	0.723
16:0	202	218	203	208	7.083	0.393
17:0	15.1	19.2	15.9	14.6	1.87	0.317
18:0	162[a]	132[b]	128[b]	141[ab]	5.2	0.002
20:0	1.19[ab]	1.07[b]	1.24[ab]	1.35[a]	0.056	0.017
Sum	391	384	363	380	11.2	0.413
T-BCFA[4]						
iso-14:0	0.22[b]	0.18[b]	0.45[a]	0.39[a]	0.027	<0.001
iso-15:0	0.60[b]	0.58[b]	0.77[b]	1.35[a]	0.087	<0.001
anteiso-15:0	1.64	1.63	2.38	2.09	0.193	0.042
iso-16:0	1.42[ab]	1.26[b]	1.90[a]	1.46[ab]	0.143	0.037
iso-17:0	2.68[ab]	2.12[b]	2.34[b]	3.06[a]	0.163	0.004
anteiso-17:0	5.77	5.85	6.63	5.80	0.602	0.719
iso-18:0	1.25	1.18	1.55	1.04	0.127	0.073
Sum	13.5	12.8	16.3	15.2	1.18	0.184

Table 19. Effect of replacing cereal in the diet with low starch feed ingredients on subcutaneous fat lipids and fatty acid composition (mg/g of total fatty acids) of subcutaneous fat (continuation).

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
NT-BCFA[5]						
6 Me-14:0	1.10	1.77	1.26	1.05	0.273	0.248
8 Me-14:0	0.74	1.18	0.90	0.73	0.197	0.334
4 Me-14:0	1.65	2.77	1.98	1.66	0.419	0.216
10 Me-14:0	0.56	0.91	0.75	0.55	0.158	0.334
2 Me-16:0	1.07	1.93	1.25	1.04	0.264	0.090
6 Me-16:0	1.56	3.27	2.20	1.60	0.509	0.096
8 Me-16:0	1.08	2.07	1.37	1.04	0.293	0.080
4 Me-16:0	2.37	3.95	2.77	2.34	0.519	0.126
12 Me-16:0	3.25	4.67	3.75	3.12	0.547	0.203
Sum	13.7	23.0	16.5	13.4	3.27	0.143
Total BCFA	27.2	35.8	32.7	28.6	4.35	0.473
cis-MUFA[6]						
*c*9-14:1	0.33	0.46	0.36	0.42	0.035	0.086
*c*7-16:1	2.26[b]	2.77[a]	2.45[ab]	2.10[b]	0.118	0.018
*c*9-16:1	7.47	7.76	7.58	7.24	0.251	0.512
*c*9-17:1	3.80	4.89	4.38	4.33	0.743	0.758
*c*9-18:1	232±5.4	261±18.6	226±5.4	226±5.4		0.369
*c*11-18:1	11.6	11.0	11.2	10.5	0.29	0.102
*c*11-20:1	2.21[a]	1.89[ab]	2.31[a]	1.95[b]	0.074	0.004
Sum	260±5.9	293±20.3	255±5.9	253±5.9		0.351
n-6 PUFA[7]						
18:2n-6	48.0	41.6	50.4	40.8	2.67	0.056
18:3n-6	0.16	0.17	0.17	0.15	0.021	0.921
20:2n-6	0.50[ab]	0.51[ab]	0.63[a]	0.45[b]	0.029	0.004
20:3n-6	0.65	0.67	0.75	0.68	0.035	0.245
20:4n-6	0.85	0.86	1.00	0.90	0.069	0.459
22:4n-6	0.15	0.21	0.19	0.12	0.027	0.058
22:5n-6	0.74	0.68	0.88	0.85	0.108	0.531
Sum	51.0	44.7	53.9	43.9	2.79	0.054
n-3 PUFA						
18:3n-3	5.26	5.11	5.55	4.74	0.297	0.309
20:5n-3	0.62[ab]	0.65[ab]	0.86[a]	0.57[b]	0.065	0.039
22:5n-3	1.43[ab]	1.47[ab]	1.78[a]	1.31[b]	0.111	0.064
22:6n-3	0.84[b]	0.97[ab]	1.44[a]	0.99[ab]	0.127	0.032
Sum	8.15	8.25	9.59	7.65	0.509	0.084
Total PUFA[8]	59.1	52.9	63.5	51.6	3.20	0.057
Total *trans*-FA[9,10]	214±8.3	167±20.3	222±8.3	190±8.3		0.047
Total C18 FA[11]	705[a]	663[b]	687[ab]	694[ab]	8.1	0.011

[a-c], Means within a row with different superscripts differ (*P* < 0.05); [1] C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], standard error of means; [3], linear chain saturated FA; [4], terminal branched chain FA (iso and anteiso); [5], non-terminal branched chain FA; [6], *cis*-monounsaturated FA excluding biohydrogenation intermediates, [7], polyunsaturated FA; [8], sum of all *cis,* methylene interrupted PUFA; [9], sum of *trans*-FA excluding *t*11-18:1 and conjugated linoleic acid isomers; [10]; no differences among means were present after Tukey adjustment for multiple comparisons;[11], sum of all C18 FA.

The detailed profile of C18 BI in subcutaneous fat is presented in Table 20. Taken together, FA derived from ruminal BH did not differ among the treatments (P = 0.097) and averaged 246 mg/g FA across treatments. The main BI was t10-18:1 ranging from 124 to 172 mg/g FA. The amount of t10-18:1 was higher (P < 0.05) for DBP than for SH, and the amount of this isomer for the other treatments did not differ from both DBP and SH. The variance of t10-18:1 found for DCP was much larger (P < 0.001) than for the other treatments. The t11-18:1 was the second major BI and was higher for SH treatment (60±5.6 mg/g FA) and lower for C treatment (17±1.4 mg/g FA). Moreover, the variance of t11-18:1 was higher (P < 0.001) for DCP and SH, intermediate for DBP and lower for C treatment. The "t10-/t11-18:1" ratio ranged from 3 to 9.8, being lower (P < 0.05) for SH than for C and DBP and there was no difference between DCP and the other treatments due to the largest variability found with DCP treatment (Figure 11). Significant differences were observed for some minor 18:1 BI between treatments with DCP treatment generally resulting in lower values than SH (i.e. t6-/t7-t8-18:1, t9-18:1, t15-18:1, t16-18:1, c15-18:1) and C (i.e. t6-/t7-t8-18:1, t9-18:1, c15-18:1) treatments.

The concentration of 18:2 BI present in subcutaneous fat ranged from 20 to 28 mg/g FA, being the lowest (P < 0.05) for C, the highest for SH with intermediate values for the other treatments. The major 18:2 BI were the coeluted peaks containing the non-conjugated t10,c15- and t11,c15-18:2 isomers and the conjugated c9,t11- and t7,c9-18:2 isomers. The c9,t11-/t7,c9-18:2 peak was higher for SH (11.3 mg/g FA) than for the other treatments (≈5.9 mg/g FA).

Table 20. Effect of replacing cereal in the diet with low starch feed ingredients on C18 biohydrogenation intermediates (mg/g of total fatty acids) present in subcutaneous fat of lambs.

Item	Diets[1]				SEM[2]	*P*-value
	C	DCP	DBP	SH		
18:1 isomers						
*t*4	0.47	0.72	0.58	0.56	0.072	0.137
*t*5	0.42	0.47	0.42	0.43	0.045	0.839
*t*6 /*t*7 /*t*8	14.8[a]	7.1[c]	9.8[bc]	11.6[ab]	0.90	<0.001
*t*9	9.44[a]	4.71[c]	6.13[bc]	8.40[ab]	0.684	0.001
*t*10	158[ab]±7.6	124[ab]±17.7	172[a]±7.6	135[b]±7.1		0.024
*t*11	16.8[b]±1.42	31.0[b]±6.54	27.6[b]±2.47	59.7[a]±4.38	4.08	<0.001
*t*12	10.2	8.6	10.0	9.6	0.47	0.124
*t*15	1.79[ab]	1.44[b]	1.44[b]	2.35[a]	0.155	0.002
*c*12	3.26	3.20	3.54	3.77	0.212	0.236
*c*13	0.81	0.90	0.89	0.92	0.041	0.353
*t*16[2]	2.03[b]	1.59[b]	1.67[b]	2.66[a]	0.155	0.001
*c*15	2.55[a]	2.04[b]	2.53a	2.70[a]	0.104	0.002
*c*16	1.47	1.17	1.26	1.05	0.121	0.138
Sum	223±6.8	186±23.9	238±6.8	239±6.8		0.158
18:2 isomers						
*t*8,*c*12	1.80	1.67	1.82	2.16	0.115	0.161
*c*9,*t*13	0.70	0.82	0.70	0.58	0.096	0.383
*t*8,*c*13	0.66[ab]	0.58[ab]	0.54[b]	0.81[a]	0.060	0.029
*c*9,*t*12	0.62	0.64	0.68	0.81	0.065	0.201
*t*9,*c*12	0.96[b]	1.22[ab]	1.16[ab]	1.62[a]	0.580	0.034
*t*10,*c*15[4]	7.58	9.70	9.90	8.88	0.839	0.247
*c*9,*t*11[5]	5.13[b]	6.12[b]	6.55[b]	11.32[a]	0.624	<0.001
*t*9,*c*11	0.63	0.62	0.77	0.63	0.058	0.211
*t*10,*c*12	0.64	1.11	0.93	0.70	0.108	0.050
Sum	20.0[b]	23.0[ab]	24.1[ab]	28.1[a]	1.43	0.009
18:3 isomers						
*c*9,*t*11,*c*15	0.18	0.17	0.18	0.14	0.023	0.619
Total BI[6]	244±7.6	211±25.8	263±7.6	268±7.0		0.097
*t*10/*t*11 ratio[7]	9.8[a]±0.71	7.2[ab]±1.41	6.8[a]±0.71	3.0[b]±0.67		<0.001

[a-c], Means within a row with different superscripts differ (*P* < 0.05); [1] C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls; [2], standard error of means; [3], includes small amounts of *c*14-18:1; [4], includes *t*11,*c*15-18:2 isomer; [5], includes *t*7,*c*9-18:2 isomer; [6], sum of biohydrogenation intermediates (i.e. all FA listed in the table); [7], ratio between *t*10-18:1 and *t*11-18:1.

5.3.5. Gene expression and SCD activity indices

The relative mRNA expression levels of SCD, ACACA and FASN in the LM are presented in Figure 12. The expression of ACACA did not differ between the treatments. The expression of SCD was twice higher in C and DCP when compared to DBP and SH treatments (*P* < 0.05). The expression of FASN was twice lower in DCP than in the other treatments (*P* < 0.05). The SCD activity index calculated using *c*9-17:1 FA and 17:0 product and substrate pair (SCDi-17) in LM is given in Figure 12. The SCDi-17 was lower (*P* < 0.05) for SH than for C and DCP treatments. The DBP treatment had a lower SCDi-17 compared to DCP but a SCDi-17 similar to that in SH and C treatments.

Figure 12. Effect of replacing cereal in the diet with low starch feed ingredients on relative mRNA expression level (arbitrary units) of codifying genes and on SCD activity index in *Longissimus* muscle.
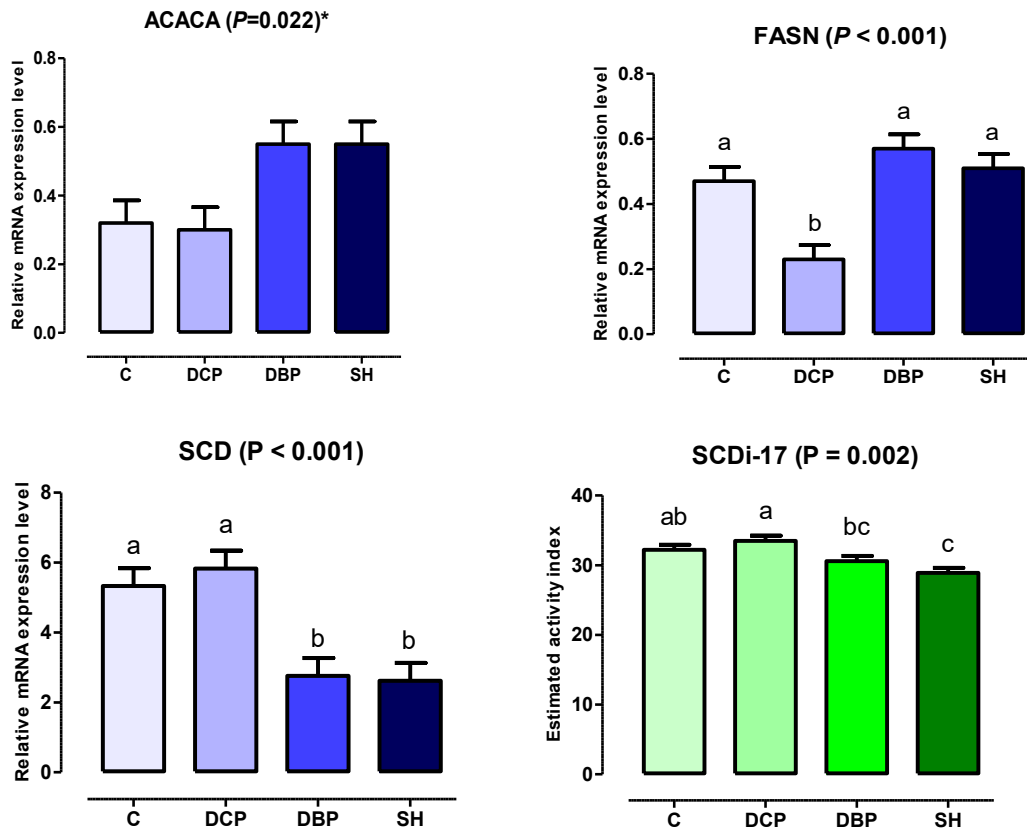
Means with different superscripts differ among treatments ($P < 0.05$); *, no differences among means were present after Tukey adjustment for multiple comparisons. Treatments: C, cereal; DCP, dehydrated citrus pulp; DBP, dehydrated beet pulp; SH, soybean hulls. ACACA, acetyl-CoA carboxylase; FASN, fatty acid synthase; SCD, stearoyl-CoA desaturase or Δ^9-desaturase; SCDi17, SCD activity index, c9-17:1/(c9-17:1 + 17:0))×100.

5.4. Discussion

The use of dehydrated citrus pulp, sugar beet pulp and soybean hulls in ruminant diets is well known as an alternative to cereals (Ludden *et al.*, 1995; Bampidis & Robinson, 2006; Vasta *et al.*, 2008). In the present study, DBP resulted in a productive performance similar to that achieved with the C diet. However, ADG was reduced for the DCP diet and the feed efficiency ratio was lower under both DCP and SH diets compared to C diet. The reduced productive performance for DCP may be related to the highest probability of developing rumen parakeratosis lesions, which might have led to a decreased capacity for VFA absorption (Hinders & Owen, 1965). In fact, it was reported that the incorporation of large

proportions of dehydrated citrus pulp into low-forage diets increases the occurrence of rumen parakeratosis lesions (Loggins, Ammerman, Moore & Simpson, 1968; Martinez-Pascual & Fernandez-Carmona, 1980) and our results are in agreement with these studies. The diminished feed efficiency observed with SH diet compared to C diet is consistent with a lower digestible energy value of soybean hulls compared to cereals (Ludden *et al*., 1995; Ferreira *et al*., 2011b).

In the present study, it was not found any impact of the diets investigated on carcass quality parameters. This is in agreement with several reports that concluded that the replacement of cereals with DCP (Prado *et al*., 2000; Lanza *et al*., 2001; Bampidis & Robinson, 2006), DBP (Normand *et al*., 2001; Cuvelier *et al*., 2006) or SH (Lage *et al*., 2014) in the diets of growing ruminants has a small or null impact on carcass quality. Moreover, meat quality traits like color, shear force and sensorial evaluation were not affected by the diets in our study. Our results on meat color parameters are consistent with reports on meat color for Portuguese lambs (Santos-Silva, Mendes, Portugal & Bessa, 2004; Teixeira *et al*., 2015). The redness of subcutaneous fat (a*) in the present study was more intense for DBP than for SH. DBP also resulted in a higher value of b* compared to SH, indicating a higher level of fat pigmentation probably due to deposition of beet pigments as vulgaxantines and betanins (Gasztonyi, Daood, Hajos & Biacs, 2001). However, the differences in effects of the diets on the color of subcutaneous fat were not detectable by visual examination and therefore unlikely to have impact on meat appreciation by consumers. Meat shear force averaged 3.93 kgF/cm^2, which is consistent with our previous data (Francisco *et al*., 2015), and sensorial evaluation indicated that all the meats had a good overall acceptability.

To the best of own knowledge, this is the first study on effect of total replacement of dietary cereals with dried beet pulp or soybean hulls on accumulation of BI in lamb tissues. However, it should be noted that there have been recent communications on effects of replacing cereals with dehydrated citrus pulp on accumulation of BI in lamb tissues (Lanza *et al*., 2015) and ewe milk (Santos-Silva *et al*., 2016). Our main objective was to test the hypothesis that the occurrence of *t*10-shift in lambs fed complete finishing diets supplemented with oil can be prevented by replacing cereals (i.e. barley) with low-starch feed ingredients. The *t*10-shift was evaluated by calculating the tissue *t*10-/*t*11-18:1 ratio which reflects BH pathways established in the rumen during the feeding period. In order to enhance accumulation of 18:1 BI in tissues, our diets were supplemented with a blend of soybean and fish oils. It is known that, inclusion of marine oils in ruminant diets consistently disrupts the the completion of BH of C18 UFA resulting in a large increase of 18:1 BI, mainly *trans*-18:1, leaving the rumen (Shingfield and Wallace, 2014). Thus, the very high levels of tissue 18:1 BI observed in the present study (135 mg/g FA in LM and 222 mg/g FA in subcutaneous fat) is consistent with the level and type of lipid supplementation used. Moreover, our diets were

designed to have two extreme points; high-starch/low-NDF content (C, barley, 318 g of starch and 260 g of NDF per kg DM) and low-starch/high-NDF content (SH, 54 g of starch and 463 g of NDF per kg DM); and intermediate points where starch was replaced by pectin rich by-products with various sugar and NDF contents (DCP and DBP). The extreme diets, C and SH, were expected to induce t10-18:1 and t11-18:1 accumulation, respectively, in tissues and we anticipated intermediate responses in case of DCP and DBP diets. These assumptions were supported by the widely accepted concept that high starch diets favor t10-18:1 production and high fiber diets favor t11-18:1 production in the rumen (Griinari *et al*., 1998; Sackmann *et al*., 2003). However, the presented results were unexpected and contradict our hypothesis as all the treatments induced a clear t10-shifted pattern, with "t10-/t11-18:1" ratio considerably above 1 in LM and subcutaneous fat. To the best of our knowledge, this is the first report of t10-shift´s induction by a low-starch/low-sugar/high-NDF diet (i.e. SH). There is a general lack of knowledge regarding both, bacteria and metabolic pathways involved in t10-shift and until now the only consistent inducing factor of t10-shift was the high-starch, low-fibre diets coupled with PUFA supplementation as an amplifying factor (Bessa *et al*., 2015). Low rumen pH might also induce t10-shift. However, it is known that an increasing content of dietary starch results in decrease of rumen pH, and therefore the two factors are generally confounded *in vivo*. A few publications reported results of *in vitro* experiments which attempted to discriminate the effects of starch availability and low rumen pH on the establishment of t10-shifted BH pathways (Fuentes, Calsamiglia, Cardozo & Vlaeminck, 2009; Maia, Bessa & Wallace, 2009; Zened, Enjalbert, Nicot & Troegeler-Meynadier, 2012). In the present study, all the treatments resulted in a low rumen pH. In fact, the rumen pH only did not drop below 6.0 under DCP. However, taking into account the high incidence of rumen parakeratosis in case of DCP and, as previously reported (Kleen, Hooijer, Rehage & Noordhuizen, 2003), the close association between rumen mucosa lesions and rumen acidosis, the rumen pH in DCP group can be viewed as fairly low throughout the experiment.

Our results suggest that the low rumen pH may have a more significant role in the production of t10-18:1 when compared to the dietary starch content. However, although none of the diets studied were able to prevent t10-shift (evaluated by the t10-/t11-18:1 ratio in tissues), the SH diet allowed for a much greater t11-18:1 deposition in the tissues than the other treatments. Moreover, the reduction of the t10-/t11-18:1 ratio under SH diet was mainly due to an increase t11-18:1 level rather than reduced content of t10-18:1 in the tissues. Some fibrolytic bacteria, such as *B. fibrisolvens* and *B. proteoclasticus* have been known as the major contributors to t11-shifted BH pathways (Shingfield & Wallace, 2014), and thus the stimulatory effect of dietary NDF on t11-18:1 production might be due to the sustenance of those fibrolytic biohydrogenation bacteria, despite the low rumen pH. It still remains unknown

what are the main bacteria responsible for conducting *t*10-shifted BH pathways. It has been reported that few strains of *M. elsdenii* (Kim *et al*., 2002) and *P. acnes* (Shingfield & Wallace, 2014) are able to produce *t*10,*c*12-18:2 in pure cultures. However, the role of the above bacteria in the rumen is questionable. The rumen microbiome studies on animals expressing *t*10-shifted BH pathways suggest that unculturable rumen microbes might play the major role in this process (Zened *et al*., 2012).

Taken together, results of our study suggest that the high dietary starch (or low NDF) inhibits *t*11-18:1 production in the rumen, but high dietary NDF (or low starch) does not an inhibit *t*10-18:1 production in the rumen. The small feed particle size from ground diets probably did not properly stimulate the rumination and salivation in lambs. This lack of physically effective NDF may be the explanation for the failure of the high-NDF diet in preventing the *t*10-shift. However, when ground dehydrated alfalfa was used as a basal diet, very low *t*10-/*t*11-18:1 ratios were consistently reported in the rumen, abomasum and tissues of lambs (Bessa *et al*., 2007; Jerónimo *et al*., 2010; Alves *et al*., 2013b). Thus, factors other than fibre particle size (e.g. the rate of NDF fermentation in the rumen) might be playing an important role in this process and need to be further investigated.

One of the consequences of *t*10-shift in ruminants is the low concentration of *c*9,*t*11-18:2 in tissues (Bravo-Lamas, Barron, Kramer, Etaio & Aldai, 2016; Oliveira *et al*., 2016; Oliveira, Alves, Santos-Silva & Bessa, 2017). Consistently, we observed a relatively low concentration of *c*9,*t*11-18:2 in LM and subcutaneous fat for all treatments. As expected, the *c*9,*t*11-18:2 concentration in tissues followed the concentration pattern of *t*11-18:1 (Palmquist *et al*., 2004), and thus the concentration of *c*9,*t*11-18:2 was lower under C diet (0.30% of total FA in LM) and higher under SH diet (0.85% of total FA in LM).

A detailed analysis of CLA isomers demonstrated that in addition to *c*9,*t*11-18:2, there also were *t*7,*c*9-18:2, *t*9,*c*11-18:2 and *t*10,*c*12-18:2 CLA isomers present in LM. This confirms association between high-starch diets and production of these specific CLA isomers (Shingfield *et al*., 2005; Alfaia *et al*., 2009). Moreover, significant amounts of nonconjugated 18:2 isomers were identified in LM and subcutaneous fat for all treatments, and some of them, such as *c*9,*t*12-18:2 and *c*9,*t*13-18:2, were recently found to be products of SCD activity (Vahmani *et al*., 2016).

Lambs fed barley-based diets have been reported to have abnormally soft subcutaneous adipose tissue due to a considerable concentration of NT-BCFA (Duncan, Lough, Garton & Brooks, 1974; Berthelot, Normand, Bas & Kristensen, 2001). Therefore, in the present study, we anticipated that replacing barley with non-starchy feed ingredients would decrease the proportion of NT-BCFA. However, the proportion of these FA in subcutaneous fat remained low and was similar across treatments. One possible explanation of these results might be

that accumulation of NT-BCFA in tissues varies between ovine breeds (Alves *et al*., 2013a), and Merino Branco breed used in the present study does not largely express that feature. High-starch finishing diets are positively associated with intramuscular fat deposition and up-regulation of SCD (Daniel *et al*., 2004; Pethick, Harper & Oddy, 2004; Costa *et al*., 2013). Both, low intramuscular fat deposition and down-regulation of SCD can be limiting factors for the enrichment of ruminant meat with *t*11-18:1 and *c*9,*t*11-18:2 (Bessa *et al*., 2015). The relative expression of SCD gene and the SCD activity index evaluated by the proportions of *c*9-17:1 and 17:0 as described by Bessa *et al*. (2015) were lower in LM for SH diet compared to DCP and C diets, which is consistent with the lowest content of insulinemia promoting nutrients (i.e. starch and sugar) in SH diet. The possible downregulation of SCD is supported by a lower level of SCD products *c*9-14:1, *c*9-16:1 and *c*9-17:1, but not *c*9-18:1, when feeding the SH diet compared with C and DCP diets. The tissue *c*9,*t*11-18:2 mostly derives from the reaction catalyzed by SCD with *t*11-18:1 as the substrate. However, our results suggest that the main reason for a higher *c*9,*t*11-18:2 content observed in the case of SH diet is the largest substrate availability rather than increased SCD activity (Daniel *et al*., 2004). Interestingly, despite of the common insulin-related regulatory mechanisms for SCD and ACACA expression, the expression of ACACA followed the pattern opposite to that for SCD e.g. with ACACA expression being lower in case of diets providing a higher level of insulinemia promoting nutrients (C and DCP). It is not clear why the above changes in the gene expression patterns occurred especially in the absence of differences in dietary effects on intramuscular fat and 16:0 content. It should be noted that, in the present experiment, *de novo* FA synthesis was expected to be reduced as all the diets were supplemented with 6.9% of oil blend in DM. However, there was a lower expression of FASN in the muscle of DCP-fed lambs, which might be related to inferior feed efficiency and ADG. Overall, these data suggest that a lower metabolizable energy (ME) provided by DCP diet as a result of diminished rumen absorption caused by parakeratosis lesions leads to reduced growth performance of lambs and affects the expression of FASN.

5.5. Conclusions

Our results indicate that the reduction of starch content in lambs' diet by replacing barley with low-starch feed ingredients does not prevent the establishment of *t*10-shifted BH pathways in the rumen, as reflected by the large accumulation of *t*10-18:1 in tissues. SH was more effective in promoting *t*11 BH pathways compared to the other diets studied, as evidenced by an increased deposition of *t*11-18:1 and *c*9,*t*11-18:2 in muscle and subcutaneous fat. These results demonstrate that low-starch/high-NDF diets on their own are not sufficient to prevent the occurrence of *t*10-shifted BH pathways evaluated by deposition of BI in lamb meat and fat.

CHAPTER 6 – General discussion

General discussion

In the present chapter, the effect of the two methodological approaches (four experiments) described in the thesis, which included the incorporation of tannin extracts (Chapters 2 and 3) in the diet and the modification of the dietary starch level (Chapters 4 and 5), on ruminal BH will be discussed.

The first main objective of the present experimental work was to acquire more knowledge about the effects of tannins on ruminal BH pathways. In both experiments with tannin extracts, the effects of condensed and hydrolysable tannins on BH were compared, although, *in vitro*, the two molecular types of tannins were mainly evaluated in relation to a control treatment (without tannins). Overall, condensed tannins showed a greater influence on BH than hydrolysable tannins. In fact, in the *in vitro* experiment, condensed tannin extracts from grape seed and *C. ladanifer* caused a higher proportional disappearance of 18:3 and 18:2n-6 than hydrolysable tannin extract from chestnut, while, in the *in vivo* experiment, condensed tannin extract from mimosa led to a higher variability of the disappearance of *c*9-18:1, 18:2n-6 and 18:3n-3 and BH completeness comparing with hydrolysable tannin extract from chestnut. The effects of tannins were reported to be influenced by their molecular nature, dose and duration of utilization (Toral *et al*., 2011; Toral *et al*., 2013; Carreño *et al*., 2015) and those factors might also explain the differences among hydrolysable and condensed tannins observed in both experiments.

Considering the molecular nature, the ability of tannins to bind to polymers has been described to be influenced by their MW (Poncet-Legrand, Gautier, Cheynier & Imberty, 2007; Patra & Saxena, 2011) and chemical structural conformations (Poncet-Legrand *et al*. 2006). In *in vitro* and *in vivo* experiments, the average MW of hydrolysable tannins from chestnut extract was probably lower than that presented by condensed tannins from grape seed, *C. ladanifer* and mimosa, since olygomeric units from chestnut tannins are prone to be hydrolysed into monomeric subunits with less than 500 Da (Pasch & Pizzi, 2002) that corresponds to the minimum MW for tannins to be able to bind to polymers (Field & Lettinga, 1992), while the hydrolysis of condensed tannins has not been clearly demonstrated (McSweeney *et al*., 2001b). Moreover, the formation of tannin oligomers, due to polymerization reactions, with up to 2000 Da from chestnut extract depends of internal rearrangements of monomers formed by tannin hydrolysis (Pasch & Pizzi, 2002), whereas mimosa and grape seed tannins are mainly composed by oligomers with mimosa tannins showing an average degree of polymerization from 4.9 (Thompson & Pizzi, 1995) to 5.4 and a maximum of 2333 Da (Pasch *et al*., 2001) and those of grape seed presenting an average degree of polymerization from 3.3 (Vivas *et al*., 2004) to 3.8 (Poncet-Legrand *et al*., 2007). Attending to the chemical structure, tannins from mimosa and grape seed have

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conformational flexibility and stability provided by the presence of prorobinetinidins polyflavonoids composed of considerable proportions of favanyl units with angular configuration in mimosa tannins (Steenkamp, Ferreira, Roux & Hull, 1983; Pasch *et al.*, 2001) and the high level of galloylation of grape seed tannins (Vivas *et al.*, 2004; Poncet-Legrand *et al.*, 2006). These fexible structural conformations and the higher MW of condensed tannins might confer them a more effective binding to substract (Poncet-Legrand *et al.*, 2006; Poncet-Legrand *et al.*, 2007; Hagerman, 2012), such as lipoproteins from cell membranes and enzymes of bacteria and dietary compounds, than hydrolysable tannins (Field & Lettinga, 1992), although that phenomenon has been more clearly shown for grape seed tannins (Ikigai *et al.*, 1993; Hashimoto *et al.*, 1999; Poncet-Legrand *et al.*, 2007). The results from the *in vitro* experiment evidences a similar ability of condensed tannins from *C. ladanifer* and from grape seed to bind to polymers and, consequently, modify ruminal BH, but further studies are needed for a better knowledge about the chemical structure of *C. ladanifer* tannins.

Attending to the different abitilies of the two molecular types of tannins to form complexes with proteins and carbohydrates, the stronger binding properties of condensed tannins might lead to an inactivation of bacteria through unavailability of substracts and inhibition of enzymes necessary for bacterial growth and activity comparing with hydrolysable tannins. Also, the ligation of tannins with lipoproteins from bacterial cell membranes may cause a decrease of membrane permeability and a disruption of essential cell processes (Ikigai *et al.*, 1993; Hashimoto *et al.*, 1999; Smith *et al.*, 2005). Moreover, when condensed tannins are present at high doses, they may lead to an increase of membrane permeability due to membrane disruption, exerting not only a bacteriostatic but also a bactericidal effect (Ikigai *et al.*, 1993; Smith *et al.*, 2005; Trentin *et al.*, 2013). Nevertheless, the low MW monomeric subunits resulting from the hydrolysis of hydrolysable tannins might present a stronger toxicity towards bacteria (Field & Lettinga, 1987; Scalbert, 1991), since they possibly penetrate more easily through cell membrane (Field & Lettinga, 1992), causing a higher instability of membrane fluidity and stimulation of homeoviscous adaptation of bacteria with the formation of *trans*-FA during ruminal BH to be incorporated into bacterial cell membranes (Keweloh & Heipieper, 1996; Endo *et al.*, 2006), as compared to condensed tannins.

Indeed, in the *in vivo* study, the greater total *trans*-18:1, mainly *t*11-18:1, and a consequent higher "*trans*-/*cis*-18:1" ratio in bacterial biomass fractions was found with chestnut hydrolysable tannins in relation with mimosa condensed tannins. Conversely, an inibition of microbial rumen ecosystem was obtained with mimosa compared with chestnut tannin extracts, being associated with a reduction of intake, fermentative activity in the rumen, abundance of specialized fibrolytic bacteria (*F. succinogenes*, *R. albus*, *R. flavefaciens* and *B. proteoclasticus*) and bacterial biomass estimates using DMA. However, these differential

effects between condensed and hydrolysable tannins cannot explain the greatest *in vitro* formation of *t*11-18:1 in the rumen with grape seed and *C. ladanifer* comparing with chestnut tannin extracts. Nevertheless, this ocurrence might have been related to a short duration of utilization of condensed tannins in the *in vitro* experiment, as it is possible that the ruminal BH occurring in the incubation tubes was still in the step of accumulation of BI. Also, the high dose (100 g/kg DM) of tannins used might have stimulated the BH of PUFA, although that is not well understood.

Considering the rumen microbial biomass, the lower bacterial biomass estimate using DMA with mimosa might indicate an inferior general abundance of rumen bacteria with condensed tannins from mimosa than with hydrolysable tannins from chestnut. Additionally, in the *in vitro* study, there was a lower total of DMA in the rumen with grape seed tannins and a slightly inferior total of DMA with *C. ladanifer* tannins comparing with chestnut tannins. Overall, these results may indicate a lower bacterial biomass in the rumen with condensed tannins comparing with hydrolysable tannins, even without a determination of biomass estimates in the in *vitro* experiment. Moreover, the importance of DMA as internal microbial markers and the fact that DMA might be a better marker than OCFA, as previously suggested by Alves *et al*. (2013b), was evident in both experiments, since there was a more pronounced effect of tannins on DMA than OCFA and, in the *in vivo* experiment, only the total of all DMA did not differ between bacterial fractions.

Conversely to what was found for fibrolytic bacteria, the mimosa tannin extract promoted a higher abundance of the amylolytic *S. ruminantium* than chestnut extract, which was probably associated with a higher proportion of 10-oxo-18:0 in the rumen and of oxo-18:0 in the blood plasma with condensed than with hydrolysable tannins, since this bacterial species can hydrate *c*9-18:1 into a precursor of 10-oxo-18:0 (i.e. 10-OH-18:0) (Hudson *et al*., 1995). Similarly to the *in vivo* experiment, the production of 10-/9-oxo-18:0 was higher with grape seed and *C. ladanifer* than with chestnut tannin extracts and that might also indicate a higher abundance of bacteria that produce oxo-18:0 with condensed comparing with hydrolysable tannins in the *in vitro* experiment. Considering these results, other studies are needed for a better comprehension of the influence of tannins on the production of oxo-18:0 FA in the rumen.

Moreover, the effect of condensed tannins on rumen ecosystem indicates a general inactivation of bacteria, although without the presence of *t*10-shift. In fact, the "*t*10-/*t*11-18:1" ratio, which is an indicator of the occurrence of *t*10-shift (Bessa *et al*., 2015), was inferior to 1 in both experiments. In the *in vitro* experiment, that ratio value was probably explained by the fact that the rumen inoculum was derived from rumen contents that had already a predominance of *t*11 BH pathways, since the sheep were fed with a high-forage and low-concentrate diet. Indeed, a preponderance of *t*11 BH pathways has been described with

forage-based diets, while concentrate feed might stimulate *t*10 BH pathways with or without the addition of oil (Bessa *et al*., 2005; Rosa *et al*., 2014). The inclusion of increasing levels of *C. ladanifer* and oil in a 1:1 forage-to-concentrate diet was described as leading to a higher *t*10-18:1 than *t*11-18:1 accumulation in the rumen (Alves *et al*., 2017) and in the *Longissimus* muscle of lambs (Francisco *et al*., 2016), whereas Jerónimo *et al*. (2010) reported a much greater increase of *t*11-18:1 comparing with *t*10-18:1 in the abomasal digesta and muscle of lambs fed with a similar diet but with a higher proportion of lucerne. Nevertheless, in the present *in vivo* experiment, the predominance of *t*11 BH pathways, even with a basal diet presenting a balanced proportion of forage and concentrate (1:1), was probably explained by the fact that the animals used in the experiment were adult sheep fed with a diet for mantainance and with a low DMI, while the results reported by Alves *et al*. (2017) and Francisco *et al*. (2016) were obtained with lambs fed with a finishing diet and with a high DMI.

Moreover, once a better comprehension of the effects of tanins on ruminal BH is acquired, it is important to notice that, if applied in pratical ruminant nutrition, the high dose (100 g/kg DM) of tannin extracts used in both experiments would lead to an impairment of animal productive performance, as suggested by a lower DMI with mimosa comparing with chestnut tannin extracts. However, the use of such high dose had the purpose of exarcebate the influence of tannins on ruminal BH, as an attempt to understand the differential mechanism of action of these compounds. So, lower doses should be considered, regarding that approximately 21 g/kg DM of condensed tannins from *C. ladanifer* (Jerónimo *et al*., 2010; Vasta *et al*. 2010b) and, in general, condensed tannins at doses up to 60 g/kg DM (Aerts, Barry & McNabb, 1999) were not associated with a diminishment of growth performance. Nevertheless, attending to the great variability and inconsistency of tannin effects on ruminal BH found in literature (Kronberg *et al*., 2007; Benchaar & Chouinard, 2009; Cabiddu *et al*., 2009; Jayanegara *et al*., 2011) and also reported in this thesis, they cannot be recommended to practical feeding conditions in ruminants. However, the tannins are able to induce stress in the rumen ecosystem and potentially modulate BH and, consequently, will continue to be very useful in experimental models to study ruminal BH. In order to disclose the mechanism of action of tannins, more detail about the structure of several tannins from different plants or even from the same plant with different origins, as well as a deeper knowledge about the structural modifications that might occur when tannins are exposed to different extraction methods and processes of preparation of diets that include the granulation of feeds, are needed. In fact, the high temperature used during the pelleting of diets might inactivate the tannins, as the formation of complexes between tannins and proteins is sensitive to temperature (Hagerman, 2012), and that could contribute to the mild influence of tannins on ruminal BH observed in the *in vivo* experiment.

The second main objective of the present experimental work was to obtain more knowledge about the factors that lead to the establishment of t10-shift and the dynamics of t10-shift´s induction. In fact, very little is known about the biology behind the t10-shift with no general consensus about what are the t10-shifted BH pathways that might occur in the rumen and the microorganisms that have a determinant role in the occurrence of t10-shift (Bessa *et al.*, 2015). The determinant factors for the establishment of t10-shift are also not well understood, although the high starch level of the diet has been described, in previous studies (Bessa *et al.*, 2005; Rosa *et al.*, 2014), as fundamental for the predominance of t10 BH pathways. Moreover, only Zened *et al.* (2011) evaluated the progression of t10-shift in the rumen and there are no reports about possible circadian oscillations of t10-shift.

In the third experiment, the t10-shift was induced in 2 rumen fistulated rams and, as expected, it was present within the first 2 weeks of feeding rams with a starch-rich diet supplemented with oil but, conversely to what was already described, it was transient (maximum of 4 days) (Roy *et al.*, 2006; Zened *et al.*, 2013b). Furthermore, there was an individual variability in the pattern of shift's induction, as reported by Rosa *et al.* (2014) and Santos-Silva *et al.* (2016), with ram 1 being more susceptible to the occurrence of t10-shift than ram 2, and this can be related to differences in rumen microbiota between animals (Bessa *et al.*, 2015). Moreover, there was no clear relation between pH and t10-shift, even though ram 2 showed a higher "t10-/t11-18:1" ratio at 3h after the mourning meal accompanied by a reduction of rumen pH comparing with that before the mourning meal. Also, conversely to what was reported by Alves *et al.* (2017), the presence of t10-shift was not evidently reflected in blood plasma, since no association between "t10-/t11-18:1" ratio in the rumen and "t10-18:1/(t11-18:1 + c9,t11-18:2)" ratio in the plasma was found for both rams.

In experiment 4 and identically to experiment 3, an individual variation on the establishment of t10-shift was found, although that was only concluded by the analysis of its reflection in meat FA composition. Also, there was a high averaged proportion of *trans*-18:1 in LM (144±10.9 mg/g FA) and subcutaneous fat (213.7±10.9 mg/g FA) and a "t10-/t11-18:1" ratio considerably higher than 1 in both tissues from lambs with barley and with alternative energy sources to barley (dehydrated citrus pulp, dehydrated beet pulp or soybean hulls) in a basal diet supplemented with an oil blend. Moreover, soybean hulls with high NDF level led to a higher deposition of c9,t11-18:2 and t11-18:1 in LM and subcutaneous fat, probably due to a stimulation of t11 BH pathways, than that obtained with other energy sources, although its low starch content could not inhibit the accumulation of t10-18:1 in the tissues. Overall, these results are striking showing that, conversely to what was expected (Bessa *et al.*, 2015), a high dietary starch level is not indispensable for the establishment of t10-shift.

For a better evaluation of meat FA composition, it is important to consider how it can be influenced by gene expression of SCD, ACACA and FASN in the muscle. The decreased SCD gene expression and SCD activity index in the muscle with soybean hulls compared to cereal was possibly due to a lower content of insulinemia promoting nutrients, mainly starch (Bessa *et al*., 2015), in the alternative source. So, the increased *c*9,*t*11-18:2 with soybean hulls was mostly a consequence of the highest availability of substrate for its production (*t*11-18:1) (Daniel *et al*., 2004). Moreover, the lowest FASN expression with citrus pulp diet might be associated with the highest probability of lambs fed with the dietary treatment to develop more severe rumen parakeratosis lesions, as previously reported by Loggins *et al*. (1968), leading to a decreased absorption of nutrients and, consequently, a reduced ME provided by the diet.

All together, the results of experiments 3 and 4 resulted in relevant conclusions about the induction and development of *t*10-shift, but still further studies are needed. Once more knowledge about the biology behind the *t*10-shift is acquired; pratical considerations for ruminants´ nutrition can be done. In this way, it is important to consider the impact of the main dietary energy sources used in experiment 4 on animal productive performance, such as the presence of a lower ADG and feed efficiency with citrus pulp treatment comparing with cereal. Therefore, the citrus pulp diet was a not so good alternative to cereal attending to animal productive performance, although also soybean hulls led to a lower feed efficiency related to cereal. Even so, the ADG with citrus pulp treatment was only slightly lower (288±17.7 g/d) than that reported by Santos-Silva *et al*. (2002b) with lighter BW lambs (322.6±8.34 g/d).

Overall, the fact that condensed tannins either stimulated *in vitro* BH of 18:2n-6 and 18:3 or caused a higher variability of *in vivo* BH of *c*9-18:1, 18:2n-6 and 18:3n-3 and BH completeness demonstrates the high diversity of effects of different tannin sources on ruminal BH and evidences how little is known about the influence of tannins on BH. Moreover, the presence of *t*10-shift when lambs are fed with low-starch diets indicates that, conversely to what was expected, a high dietary starch level is not indispensable for the occurrence of *t*10-shift. So, it is necessary to acquire a deeper knowledge about the biology behind this modification of BH pathways.

CHAPTER 7 – Conclusions, implications and future perspectives

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Conclusions, implications and future perspectives

Modulation of ruminal biohydrogenation in the presence of tannins or t10-shift

Considering the results of the four experiments presented in the thesis, it can be concluded that the experimental models used to study ruminal BH were important for a better comprehension of the dynamics of ruminal BH pathways and how they can be influenced by dietary tannins and energy sources. In fact, it seems that tannins may induce a bacterial stress response with an increase of *trans*-FA production in the rumen, similarly to the response of bacteria in the presence of PUFA from lipid supplementation of diets. Moreover, highly fermentable energy sources, and not only starchy feedstuff, induced *t*10-shifted BH pathways probably as an adaptation of rumen ecosystem to acidotic environments.

However, the incorporation of tannins in a diet or dietary substrate was shown to modulate BH in different ways, either stimulating or inhibiting the BH of PUFA. So, it is difficult to evaluate the influence of tannins on ruminal BH pathways due to the inconsistency of results obtained. The combination of the dose, time of utilization and molecular nature of tannins, as well as the basal diet, seemed to be the most important determinants of tannin effects on BH. Concerning the molecular nature of tannins, more studies should be done in order to evaluate their average MW and MW distribution to allow for a better comprehension of the ligation between tannins from different sources and proteins. The application of technologies for the structural analysis of polyphenols, such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and for the analysis of the stoichiometry of ligation, including mainly MALDI-TOF and isothermal titration microcalorimetry (ITC), can provide new insights about how the chemical structure of tannins relate to their biological effects including the modulation of BH. The use of ITC could also allow for the determination of how the conformational structure of tannins and proteins influences the nature of tannins-proteins binding. Particularly, it would be interesting to test the ligation of tannins to lipoproteins of bacterial cell membrane or enzymes that are important for the general metabolism of rumen bacteria and might be determinant for ruminal BH. Moreover, the analysis of alterations of rumen microbiota with the addition of tannins to a dietary substrate or basal diet by "omics" technologies would be stimulating, but that could be even more interesting when applied to *t*10-shift´s induction studies.

In fact, although some bacteria were reported to have a role in *t*10-shifted BH pathways, little is known about the biology behind the induction of *t*10-shift and how rumen microbiota is involved in this phenomenon. The variability among animals in the establishment of the *t*10-shift shown in the present thesis highlights the importance of rumen microbiota in shift´s ocurrence. The high-throughput pirosequencing of 16S rRNA genes using Illumina platform would be a good option for a better understanding of the phylogenetic identities and

abundance of rumen bacteria in the absence and presence of *t*10-shift. However, the sequencing of 16S rRNA genes (microbiomics) only allows for the evaluation of a small region of bacterial genome without providing information about functional genes contents of bacteria. For a phylogenetic and functional study, the microbiomic technologies should be associated with metagenomic sequencing of total bacterial genomic DNA. In fact, comparative metagenomic studies could even provide insights into inter-individual differences in respect of rumen microbiota. Moreover, a deeper knowledge about which genes actively express enzymes that are fundamental for the different steps of ruminal BH pathways, such as isomerases and reductases, is needed. For this purpose, not only microbiomic and metagenomic technologies should be applied but also metatranscriptomics, for the obtention of gene expression profile, and metaproteomics, for the analysis of protein products including enzymes, even though these technologies are more recent and technically demanding. Aditionally, the analysis of metabolic products (metabolomics) could be done as a final proof that metabolic processes have occurred in the rumen.

Exploration of possible nutritional strategies for ruminants

Further *in vivo* studies are needed, for the acquisition of a deeper knowledge about how ruminal BH would proceed and the repercussions on animal productive performance when dietary tannin extracts at distinct doses in forage- or concentrate-based diets are fed to ruminants. Only after a higher understanding of the mechanism of action of tannins on BH, effective and reproducible nutritional strategies to increase health beneficial FA in ruminants´ meat could be designed. Ideally, the dose of tannins for modulation of BH should be as low as possible. The usual doses of condensed tannins that do not compromise the productive performance of ruminants range from, approximately, 21 to 60 g/kg DM. The effect of tannins on meat FA composition should also be evaluated, in order to verify if it reflects the impact of these compounds on ruminal BH.

Additionally, more studies are needed for the evaluation of induction and development of *t*10-shift in a higher number of animals than the ones used in the *t*10-shift´s induction experiment, when alternative energy sources to cereals providing distinct levels of ME intake and with different NDF fementation rates in the rumen are incorporated in oil-supplemented diets. For those studies, the diets could present a higher "forage/concentrate" ratio or a distinct composition of forage comparing with the basal diet from the experimental model described in Chapter 5. Additionally, a better evaluation of the relation between rumen pH and *t*10-shift with a continuous measurement of pH values could be done.

The importance of the use of agro-industrial by-products instead of cereals consists in a reduction of waste disposal and, generally, animal feeding costs and a diminishment of the dependence of ruminant diets on cereal grains that can be consumed by humans.

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