"There are no such things as applied sciences, only applications of science"

(Louis Pasteur)

"Observation is a passive science, experimentation an active science"

(Claude Bernard)

"In science, read, by preference, the newest works; in literature, the oldest"

(Edward George Bulwer-Lytton)

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Influence of forages on the fatty acid composition of rumen digesta and ruminant milk and meat

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Dutch translation of the title:

Invloed van ruwvoeders op de vetzuursamenstelling van pensinhoud en van melk en vlees van herkauwers

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LIST OF ABBREVIATIONS

ADF - Acid detergent fibre
BCFA – Branched chain fatty acids
BCVFA – Branched chain volatile fatty acids
BD – Botanically diverse
BDS – Botanically diverse silage
C – Concentrate
C/M – Chloroform/methanol mixture
CLA – Conjugated linoleic acid
CV – Cardiovascular
CVB – Central Bureau for Livestock Feeding
C1600 – Cinnamaldehyde treatment
CWO – "Comissie voor Wetenschapelijk Onderzoek" (Scientific Research Committee)
DGGE – Denaturating gradient gel electrophoresis
DHA – Docosahexaenoic acid
DM – Dry matter
DMI – Dry matter intake
DNA – Deoxyribonucleic acid
DVE – True protein digested in the small intestine
E – Ethanol
EDTA – Ethylenediamine tetraacetic acid
EPA – Eicosapentaenoic acid
E800 – Eugenol treatment
F – Forage
FA – Fatty acids
FAME – Fatty acid methylesters
FD – Freeze-dried
GC – Gas chromatopgraphy
HDL – High density lipoprotein
I/C – *Iso*propanol/chloroform mixture
IM – Intramuscular
IMF – Intramuscular fat
IMS – Intensively managed silage
IR – Intensive ryegrass

IRS – Intensive ryegrass silage

IWT – Institute for the promotion of innovation by Sciente and Technology in Flanders

L – Leguminosa rich

LA – Linoleic acid

LCFA – Long chain fatty acids

LDL – Low density lipoprotein

LNA – Linolenic acid

m.a.s.l. – meters above see level

MON – monensin

MSL – Maite silage and crushed linseed

MUFA – Monounsaturated fatty acids

N – Nitrogen

n.d. – not detected

NDF – Neutral detergent fibre

NE – Net energy

NH_3-N – Ammonia nitrogen

n.r. – not reported

n.s. – not significant

OBCFA – Odd and branched chain fatty acids

OLCFA – Odd and linear chain fatty acids

PCA – Principal component analysis

PCR – Polymerase chain reaction

PHO – Partially hydrogenated oils

PL – Polar lipids

PPO – Polyphenol oxidase

PUFA – Polyunsaturated fatty acids

Q – Quercetin

QB – Quillaja bark

RC – Red clover

RCS – Red clover silage

rDNA – Recombinant DNA

rRNA – Recombinant ribonucleic acid

SC – Subcutaneous

s.d. – Standard deviation

SE – Standard error

SEM – Standard error of mean

SFA – Saturated fatty acids

Sign – Significance

SPP – Species poor

SPR – Species rich

TAG - Triacylglycerol

TVA – *Trans*-vaccenic acid

UEFA – Unesterified fatty acids

UFA – Unsaturated fatty acids

VEM – Net energy lactation in the Dutch system (1 VEM = 6.9 kJ)

VEVI – Feed unit beef cattle intensive in the Dutch system (1 VEVI = 6.9 kJ)

VFA – Volatile fatty acids

WC – White clover

WCS – White clover silage

WHO – World health organization

INTRODUCTION

Introduction

Consumption of fatty acids and nutritional recommendations

Long chain n-3 fatty acids (FA) are important in the prevention of 'modern' diseases such as cardiovascular (CV), autoimmune and inflammatory diseases, obesity and cancer (Ruxton et al., 2004). Nowadays, human diets in industrialized countries are generally characterized by high levels of saturated fat, n-6 FA and *trans*-FA, and low levels of n-3 FA (Simopoulos, 2004). Table I.1. shows the estimated intake and recommended intake in Northern Europe for the major FA groups. It is clear that consumers from Northern Europe are still far from the nutritional recommendations from the World Health Organization (WHO, Table I.1.).

Table I.1. – Estimated human daily intake and recommended human daily intake of the major groups of FA in Northern Europe, relative to the total daily energy (E) intake.

	SFA	MUFA	PUFA	n-3 PUFA	n-6 PUFA	*Trans*-FA	CLA
Intake (% total E intake)	13[a]	12[a]	6[a]	1[a]	5[a]	1.2[a]	2-3[a]
Recommendation (% total E intake)	<10[b]	<10[b]	6-10[b]	1-2[b]	5-8[b]	<1[b]	-

[a] – Lunn and Theobald, 2006; [b] – WHO, 2003;
SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; *Trans*-FA – Sum of all *trans*-fatty acid isomers, included in MUFA; CLA – Sum of conjugated linoleic acid isomers, not included in n-6 PUFA nor in n-3 PUFA.

There is evidence that medium chain saturated FA (SFA), particularly C12:0, C14:0 and C16:0 increase plasma cholesterol levels and the risk of CV diseases, whereas short chain SFA (C4:0 and C6:0) and C18:0 are considered neutral in this regard (Givens, 2005; Lee et al., 2006a). Substitution of SFA by monounsaturated FA (MUFA) or polyunsaturated FA (PUFA), results in reduced plasma total cholesterol and low density lipoprotein (LDL)-cholesterol (Givens, 2005). However, it seems that the cholesterol-lowering capacity of PUFA is greater than that of MUFA (Givens, 2005), as PUFA lowered both LDL and high density lipoprotein (HDL)-cholesterol, whereas MUFA only reduced the levels of LDL- and not of HDL-cholesterol.

The ratio of n-6 to n-3 PUFA, the two groups of PUFA, is also important in

human health. Northern European diets are generally characterised by an n-6/n-3 ratioabove the recommended ratio 2-5/1 (Simopoulos, 2004) and to lower this ratio the intake of n-3 PUFA should increase. Both groups of PUFA are essential to human health yet n-6 and n-3 PUFA show distinctive functions in human health, with n-6 PUFA changing in general the health status to pro-thrombotic, pro-constrictive and pro-inflammatory and increasing HDL-cholesterol (Calder, 2004; Simopoulos, 2004; Lunn and Theobald, 2006). Further, within n-3 PUFA, C18:3 n-3, C20:5 n-3 (EPA) and C22:6 n-3 (DHA) show distinctive functions in terms of human health. Health benefits of increased C18:3 n-3 intake have been suggested to be primarily associated with an increased synthesis of EPA (Givens et al., 2006). Hence, nutritional research has focused on the health benefits of EPA and DHA. Both EPA and DHA are synthesized endogenously in the human body, but conversion rates are low and are considered inadequate to meet the requirements of long chain n-3 PUFA. Hence research also focused on the direct supply from different sources to increase the human intake of EPA and DHA.

Another important group of FA are the *trans*-FA. Particularly the *trans*-FA from industrially (partially) hydrogenated oils (PHO) are described to increase LDL-cholesterol levels (Kühlsen et al., 2005; Pfeuffer and Schrezenmeir, 2006). More recently, Pfeuffer and Schrezenmeir (2006) compared the intake of *trans*-FA from PHO and ruminant products in terms of their effects on human health and found no evidence that *trans*-FA from ruminant origin (mainly C18:1 t11) exerts adverse effects on human health. Nevertheless, the dietary recommendation from the WHO (2003) is to drastically reduce or even avoid the overall consumption of *trans*-FA (Table I.1.).

Almost 15 years ago, conjugated linoleic acids (CLA), another important group of FA, were claimed to have positive effects on human health (Ip et al., 1994). Positive effects of CLA were found in research done in laboratory animals and more recently in humans, such as reducing levels of HDL- and LDL-cholesterol and triacylglycerols in plasma (Yaqoob et al., 2006 in humans); atherosclerosis inhibition (Lee et al., 1994 in rabbits), carcinogenesis inhibition (Ha et al., 1990 and Ip et al., 1999 in mice) and decreasing body fat deposition (Park et al., 1997 and Pariza et al., 1999 in mice). Recommendations for the intake of CLA are not established yet, but increasing the intake of CLA would benefit human health. However, different CLA isomers might be responsible for the various physiological effects mentioned before, and more research is needed to assess the *in vivo* effects in humans.

Dietary sources of fatty acids with emphasis on ruminant products

Ruminant products are criticised for their high SFA and low PUFA proportion, compared to pork or chicken meat (monogastric species) and fish (Table I.2.). In particular, milk and dairy products are richer in SFA and poorer in MUFA and PUFA compared to beef and lamb meat (Table I.2.). On the other hand, microbial conversions prior to the small intestine result in the production and accumulation of intermediates, among which CLA that are relatively unique to ruminant products (Table I.2.). Fish is a source rich in EPA and DHA (up to 25 g/100 g total FA), whereas milk has no EPA or DHA and meat has typically less than 1 g/100 g total FA (Table I.2.).

Table I.2. – Typical fatty acid composition (g/100 g total fatty acids) of different foods of human diet

	SFA	MUFA	PUFA		Specific FA groups			
			n-3	n-6	*Trans*-FA	EPA	DHA	CLA
Milk & Dairy products	73[1]	23[1]	4[1]		4.24[2]	0[3]	0[3]	0.72[4]
Meat								
Beef	46[5]	48[5]	1.1[5]	4.3[5]	2.84[6]	0.25[7]	0.05[7]	0.53[8]
Lamb	53[5]	41[5]	1.9[5]	4.0[5]	3.17[6]	0.45[7]	0.25[7]	1.4[8]
Pork	38[5]	43[5]	2.0[5]	17[5]	n.r.	0.23[3]	0.23[3]	0.14[8]
Fish								
Lean fish (Trout)	27[9]	31[9]	32[9]	7.0[9]	n.r.	6.0[9]	19[9]	0.07[9]
Fatty fish (Salmon)	24[10]	50[10]	19[10]	3.9[10]	n.r.	3.4[10]	10[10]	0[10]

[1] – Givens and Shingfield, 2004; [2] – Jensen, 2002; [3] – Lunn and Theobald, 2006; [4] – Dhiman et al., 2005; [5] – Williamson et al., 2005; [6] – Givens et al., 2006; [7] – Raes et al., 2004a [8] – Schmid et al., 2006; [9] – Valente et al., 2007; [10] – Berge et al., 2005; n.r. – not reported;
SFA – Saturated fatty acids; MUFA – Monounsaturated fatty acids; PUFA – Polyunsaturated fatty acids; *Trans*-FA – Sum of all *trans*-fatty acid isomers, included in MUFA; EPA – Eicosapentaenoic acid, included in n-3 PUFA; DHA – Docosahexaenoic acid, included in n-3 PUFA; CLA – Sum of isomers t10c12 and c9t11, not included in PUFA

Modifying fatty acid composition of animal products and their impact on dietary fatty acid intake

To meet the nutritional recommendations of the WHO (Table I.1.) and the consumer demands, there is a large interest in the animal industry to improve the composition and health value of products of animal origin. From their simulation study, De Henauw et al (2007) concluded that enriching pork meat with n-3 PUFA by increasing the animal dietary supply of C18:3 n-3 would increase the human intake of

C18:3 n-3 and of the long chain n-3 PUFA by 19% and 6.7% respectively, compared to a standard scenario wherein animal products were not enriched in n-3 PUFA. Another projected scenario in the latter study was enriching all fat from the animals (pork, beef, poultry, eggs and dairy products) with n-3 PUFA by increasing the animal dietary supply of C18:3 n-3. In the latter scenario, the human intake of C18:3 n-3 was increased by 39% and that of long chain n-3 PUFA by 37%. These two projected scenarios would meet the nutritional recommendations of the WHO (2003) for C18:2 n-6 (>2 % total Energy), n-6 PUFA and n-3 PUFA human intake. However, only in the scenario wherein all animal fat was enriched with n-3 PUFA the nutritional recommendations for human C18:3 n-3 intake would be met (>1% total Energy). Although nutritional recommendations for long chain n-3 PUFA were not met with any of these two projected scenarios, the human intake of long chain n-3 PUFA was shifted in favourable direction in the scenario wherein all animal fat was enriched (De Henauw et al., 2007). These results clearly show the positive impact of changing the FA profile of animal products. To achieve this goal, two distinctive approaches are possible: 1) modification of the FA profile during meat or milk processing or 2) modification via changes in the animal diet. However, no simulation study has been performed yet to assess the impact of modified ruminant dairy products only. Nevertheless, regarding the major contribution of ruminant dairy products to SFA intake, some non-negligible impact might be expected, as suggested from the simulation study of De Henauw et al. (2007), where human SFA intake decreases upon consuming a n-3 PUFA enriched diet.

Origin of fatty acids in ruminant products

When aiming at modifying ruminant product FA composition, some background on the metabolic origin of these FA is required. Thus, in the following sub-sections, the metabolic origin of FA in ruminants is mentioned.

Saturated FA

Several factors determine the FA composition of ruminant products, both animal and diet related. In their review, Chilliard and Ferlay (2004) reported that 60% of the FA secreted in milk are taken up from plasma while 40% are *de novo* synthesized (FA up to C16) in the mammary gland. Dietary unsaturated FA, mainly of 18 carbon length,

are saturated in the rumen resulting in C18:0 (stearic acid) as an end product next to *cis* and *trans* isomers, which are available for direct incorporation in the mammary gland (Jensen, 2002). Nevertheless, the high SFA content of milk is mainly due to *de novo* synthesis of short and medium chain FA (C4:0 to C16:0), from acetate and β-hydroxybutyrate (Jensen, 2002; Chilliard and Ferlay, 2004). The latter are the end products resulting from the microbial fermentation in the rumen (Jensen, 2002). *De novo* synthesis of SFA is also dependent on the stage of lactation. Cows in early stages of lactation produce milk with lower levels of short and medium chain SFA (C4:0 to C14:0) compared to cows in mid and late lactation stages (Palmquist et al., 1993; Jensen, 2002; Chilliard and Ferlay, 2004). This is related to the physiological state of cows in early lactation, with increasing importance of mobilized FA when cows are in negative energy balance (Jensen, 2002). This higher uptake of long chain PUFA by the mammary gland might inhibit *de novo* FA synthesis and dilute *de novo* synthesized FA in milk fat (Palmquist et al., 1993; Chilliard and Ferlay, 2004; Walker et al., 2004). Another important animal factor is the breed of the animals. Recently, Soyeurt et al. (2006) have shown variation in the SFA composition of milk due to genetic/breed differences, with breeds showing lower Δ^9-desaturase activity in the mammary gland, producing saturated milk fat (Soyeurt et al., 2006). Other factors affecting the levels of SFA in the milk are of dietary origin. However, short chain SFA (C4:0 to C8:0) are hardly affected by the diet or lipid body mobilization, as these FA are synthesized by pathways not dependent of acetyl CoA carboxylase (Chilliard and Ferlay, 2004).

Unsaturated FA

In general, the levels of milk unsaturated FA, mainly MUFA, are closely related to the activity of the enzyme Δ^9-desaturase. This enzyme predominantly desaturates SFA arriving in the mammary gland, contributing to the unsaturated FA levels in the milk (Chilliard and Ferlay, 2004). The higher or lower activity of this enzyme will determine the higher or lower levels of milk unsaturated FA, in particular of MUFA and CLA, as these FA are end products of the desaturation of SFA and C18:1 t11, respectively (Jensen, 2002; Chilliard and Ferlay, 2004). It has been shown that Δ^9-desaturase activity differs between breeds of dairy cattle (Soyeurt et al., 2006). As for milk SFA, milk MUFA can also be affected by the lactation stage. As mentioned before, during early lactation, the body lipid mobilization is high and the major MUFA mobilized is C18:1 c9 (oleic acid), which is directly incorporated in the milk,

contributing to the increased levels of MUFA in early stages of lactation (Chilliard and Ferlay, 2004; Walker et al., 2004). Other factors influencing the unsaturated FA content of milk are of dietary origin. The mammary gland is not able to synthesize n-6 or n-3 PUFA and their levels are closely related to the intestinal absorption of PUFA that is, the amounts of PUFA leaving the rumen (Chilliard and Ferlay, 2004). Hence, dietary strategies to increase the milk PUFA content should provide dietary PUFA precursors (increased PUFA dietary intake) and preferentially modify the microbial biohydrogenation in order to limit breakdown of dietary PUFA (Chilliard and Ferlay, 2004; Walker et al., 2004; Collomb et al., 2006). More recently, some limited evidence for potential production of EPA by FA elongation of C18:3 n-3 in the mammary gland was given, but not always revealed to be true and regarding the negligible amounts of milk EPA and DHA (Table I.2.), it is of minor physiological importance (Scollan et al., 2005).

Meat fatty acid composition is also affected by several factors such as animal and dietary factors. Fatness is a major animal factor influencing the FA composition of muscle (Nürnberg et al., 1998; De Smet et al., 2004), and several factors will affect the level of fatness, such as age, sex and breed of the animals. Deposition of fat in the muscle occurs mainly in the neutral lipid fraction (triacylglycerols), with mainly SFA and CLA being deposited in this fraction (Nürnberg et al., 1998; De Smet et al., 2004; De la Torre et al., 2006; Schmid et al., 2006), whereas PUFA are mainly present in the cell membrane of the muscle cells, the phospholipid fraction (Nürnberg et al., 1998; De Smet et al., 2004). With increasing age/weight, animals have a tendency to deposit higher amounts of fat, leading to an increase of SFA and a decrease of PUFA proportions through a dilution effect of the phospholipid fraction (Nürnberg et al., 1998; Malau-Aduli et al., 2000; De Smet et al., 2004). Sex and hormones are also related to fatness level, with male animals being leaner and having higher intramuscular (IM) PUFA and lower SFA proportions than females (Nürnberg et al., 1998; Malau-Aduli et al., 2000). The breed of the animals is related with fat deposition, but also with the enzyme activity and gene expression (Nürnberg et al., 1998; De Smet et al., 2004; Scollan et al., 2006b). The expression level and activity of the Δ^9-desaturase enzyme is associated with the MUFA levels in the muscle of ruminants (Scollan et al., 2006b), as in milk. Other enzymes (elongase, Δ^5 and Δ^6-desaturase) are also present in the muscle and are responsible for the desaturation and elongation of C18:2 n-6 and C18:3 n-3, resulting in the production of the long chain PUFA, such as C20:4 n-6, EPA and DHA.

Both enzyme activity as well as precursor supply will determine the extent of these processes and the proportions of the long chain PUFA in the IM fat (De Smet et al., 2004; Scollan et al., 2006b). However, differences in the IM FA composition originating of genetic and animal factors are smaller than those induced by dietary factors (Raes et al., 2004a; Scollan et al., 2006b). As with milk, the transfer of dietary PUFA to intramuscular and other fats of ruminants will depend on the level of PUFA in the diet and on the extent of rumen biohydrogenation (Scollan et al., 2005).

Conjugated linoleic acid

The levels of muscle CLA are also dependent of animal factors, such as breed, age and sex, all linked with the animal fatness level (Dhiman et al., 2005; De la Torre et al., 2006; Scollan et al., 2006b), and of dietary factors, such as the basal diet and the extent of rumen biohydrogenation (Mir et al., 2003; Dhiman et al., 2005; Schmid et al., 2006). Breed of animals that tend to deposit more fat will have higher CLA, as CLA deposits in the neutral lipid fraction (triacylglycerols) of the muscle (Scollan et al., 2006b; De la Torre et al., 2006; Schmid et al., 2006). Milk CLA is also influenced by animal factors such as breed, age and lactation stage (Dhiman et al., 2005). Further, the level of expression of Δ^9-desaturase enzyme also affects the levels of CLA in the milk and IM fat, as it converts the C18:1 t11 absorbed in the intestines to CLA c9t11, the major CLA isomer (~90%) in ruminant fat (Dhiman et al., 2005). However, the major factor determining milk and meat CLA is the accumulation in the rumen and the outflow rate from the rumen of C18:1 t11 (Fievez et al., 2003b).

Dietary strategies to modify fatty acid composition of ruminant products

Despite rumen microbial conversion of dietary PUFA, increasing dietary PUFA intake enhances the PUFA content of milk and IM fat (Mir et al., 2003; Dewhurst et al., 2006; Elgersma et al., 2006; Scollan et al., 2006b). Different nutritional strategies have been used to modify the FA profile of ruminant milk and muscle, such as forage feeding and supply of oilseeds or marine products. Examples of responses to these dietary strategies are given in Tables I.3. and I.4. for milk and muscle, respectively. Moreover, some strategies additionally aim at modifying the rumen FA metabolism, with protected fat sources aiming at by-passing the rumen metabolism and rumen microbial modifiers

aiming at disturbing the rumen environment and causing shifts in the hydrogenating microbial population. The latter approach has been mainly investigated in dairy cattle, with positive effects on the levels of C18:3 n-3, CLA c9t11 and total CLA (Table I.3.).

The use of different vegetable seeds or oils has been the main focus of dietary manipulation in ruminant nutrition through lipid supplementation. However, it is clear that feeding seeds or oils results in different responses on the milk FA profile. E.g. feeding whole linseed only increased the levels of C18:3 n-3 of dairy cows compared to feeding linseed oil, which increased the C18:1 t11 and CLA c9t11 milk proportions (Table I.3.). This is most probably due to the fact that in the oil the FA are more easily accessible for lipolysis and hydrogenation by the microbial lipases than in the seeds, where the coating of the seed might physically protect the C18:3 n-3 from the microbial attack (Chilliard and Ferlay, 2004).

Table I.3. – Examples of responses to diet manipulation of dairy cows on important milk fatty acids (g/100 g total FA), with the difference between the modified and the control diet.

	Amount[‡]	SFA	Vaccenic	Oleic	LA	LNA	CLA c9t11
F *vs*. C*	-	-19.0[1]	+3.85[1]	+1.70[1]	-0.29[1]	+0.48[1]	+1.17[1]
Unprotected fat							
Linseed	1.6[2]	-3.21[2]	-0.37[2]	n.r.	-0.72[2]	+0.75[2]	-0.24[2]
Linseed oil	3.0[3§]	-12.1[3]	+3.10[3]	+4.00[3]	-0.10[3]	+0.02[3]	+1.20[3]
Sunflower oil	3.0[3§]	-13.0[3]	+4.00[3]	+5.20[3]	+0.40[3]	-0.10[3]	+1.60[3]
Rapeseed oil	n.r.[4]	+0.13[4]	+1.20[4†]	+0.53[4]	-0.07[5]	+0.36[4]	n.r.
Fish oil	0.25[6]	-6.40[6]	+8.70[6†]	-3.60[7]	+0.50[6]	+0.02[6]	n.r.
Algae	0.91[8]	-3.04[8]	+6.30[8]	-10.2[8]	-0.10[8]	-0.07[8]	+2.25[8¤]

[1] – Couvreur et al., 2006; [2] – Ward et al., 2002; [3] – Chilliard and Ferlay, 2004; [4] – Givens and Shingfield, 2004; [5] – Bell et al., 2006; [6] – Dewhurst et al., 2006; [7] – Rego et al., 2005; [8] – Franklin et al., 1999;
* – Effect of forage (F) feeding compared to concentrate (C) feeding; [‡] - Amount (kg/day) of fat source supplemented in the diet; n.r. – not reported; [§] - Expressed as % in the total diet; [†] – Represents the sum of all C18:1 *trans* isomers; [¤] – Represents the total CLA;
SFA – Saturated fatty acids (C4:0 up to C18:0); *Trans*-vaccenic acid – C18:1 t11; Oleic acid – C18:1 c9; LA – C18:2 n-6; LNA – C18:3 n-3

Different responses are also observed when vegetable oils from different sources are fed to the animals. These differences are linked to the higher or lower content of C18:2 n-6 and C18:3 n-3 of the seeds. E.g. feeding sunflower oil (C18:2 n-6 rich oil) to dairy cows resulted in a higher response in C18:1 t11 and CLA c9t11 proportions in milk than feeding linseed oil (C18:3 n-3 rich oil; Table I.3.). Feeding unprotected vegetable seeds or oils only slightly increased the milk levels of C18:2 n-6 or C18:3 n-

3, whereas higher responses are observed on biohydrogenation intermediates and SFA (Table I.3.). Yet, duodenal infusion of PUFA has been shown to increase milk PUFA (Litherland et al., 2005). Hence, protecting the lipid supplement against microbial metabolism might be a good strategy to increase milk PUFA (Schroeder et al., 2004; Sinclair et al., 2005; Gulati et al., 2005). Several strategies (e.g. calcium salts (Brzóska, 2006), formaldehyde (Gulati et al., 2005; Petit, 2006)) have been tried to achieve a good ruminal protection of the lipid source, and recently the inclusion of soybean oil in a whey protein gel matrix proved to be effective in protecting C18:2 n-6 and C18:3 n-3 from rumen microbial hydrogenation (Carroll et al., 2006). As vegetable seeds or oils (protected or not) are no dietary source of EPA and DHA, effects on the EPA and DHA levels of milk were negligible. To increase the milk EPA and DHA levels, dairy cow diets should be supplemented with fish oil or algae (Givens and Shingfield, 2004; Givens and Gibbs, 2006), although responses are often marginal (+0.02 and +0.04 g/100g total FA for EPA and DHA, respectively; Dewhurst et al., 2006). Yet, feeding algae was reported to increase the levels of DHA to a larger extent (+0.5 g/100g total FA; Singh et al., 2004).

Similar nutritional strategies have been used to modify the ruminant muscle FA composition (Table I.4.).

Table I.4. – Examples of responses to diet manipulation of beef cattle on important muscle fatty acids (g/100 g total FA), with the difference between the modified and the control diet.

	Amount‡	SFA	TVA	OA	LA	LNA	EPA	DHA	CLA c9t11
F *vs*. C*	-	+1.9[1]	+1.5[1†]	-5.2[1]	+0.21[1]	+1.3[1]	+0.44[1]	+0.06[1]	+0.09[1]
Unprotected fat									
Linseed	2.1[2]	-3.8[2]	+1.7[2†]	+0.32[2]	-0.41[2]	+0.47[2]	+0.12[2]	+0.01[2]	+0.20[3]
C. linseed[Δ]	6.8[4¤]	+0.80[4]	+0.07[4†]	+1.4[4]	-2.0[4]	+0.68[4]	+0.13[4]	-0.01[4]	+0.03[4]
Raw soybean	2.0[5]	-0.90[5]	-0.06[5†]	n.r.	+0.14[5]	+0.04[5]	n.r.	n.r.	-0.01[5]
Ext soybean[§]	1.8[6]	+1.2[6]	+0.4[6]	-1.5[6]	+0.30[6]	+0.07[6]	n.r.	n.r.	+0.40[6]
Linseed oil	0.24[7]	-2.4[7]	+3.3[7]	-2.9[7]	+0.24[7]	-0.02[7]	-0.02[7]	-0.04[7]	+0.53[7]
Sunflower oil	0.24[7]	-4.3[7]	+5.5[7]	-2.4[7]	+0.82[7]	-0.50[7]	-0.04[7]	-0.02[7]	+1.1[7]
Fish oil	0.52[2]	+0.97[2]	+2.4[2†]	-5.5[2]	-0.89[2]	+0.03[2]	+0.25[2]	+0.47[2]	n.r.

[1] – Nuernberg et al., 2005; [2] – Scollan et al., 2001; [3] – Schmid et al., 2006; [4] – Raes et al., 2004b [5] – McNiven et al., 2004; [6] – Madron et al., 2002; [7] – Noci et al., 2007;

* – Effect of forage (F) feeding compared to concentrate (C) feeding; ‡ - Amount (kg/day) of fat source supplemented in the diet; [Δ] - Crushed linseed; ¤ - Expressed as % DM in the total diet; † – Represents the sum of all C18:1 *trans* isomers; § – Extruded full fat soybeans; n.r. – not reported;

SFA – Saturated fatty acids (C12:0 up to C18:0); TVA – C18:1 t11; OA – C18:1 c9; LA – C18:2 n-6; LNA – C18:3 n-3; EPA – C20:5 n-3; DHA – C22:6 n-3

The effects of feeding different lipid sources is related to the processing of the seeds (e.g. crushing, extrusion), seed coating protection (difference between seed and oil), type of seed or oil used (in terms of content in C18:2 n-6 and C18:3 n-3) and the protection or not of the lipid supplement against rumen microbial metabolism. E.g. feeding extruded soybeans resulted in a higher positive response on the levels of *trans*-C18:1 FA compared to feeding raw soybeans, due to the easier access of microbial lipases and hydrogenases to C18:2 n-6 and C18:3 n-3 when feeding extruded soybeans (Table I.4.). Another example of diet manipulation is feeding oils rich in C18:2 n-6 (e.g. sunflower oil) that resulted in a higher increase of the levels of CLA c9t11 in the IM fat of ruminants than feeding oils rich in C18:3 n-3 (e.g. linseed oil; Table I.4.). Feeding supplemented diets rich in C18:3 n-3 will increase the levels of EPA and DHA in the IM fat of ruminants. However, higher increases in the levels of these FA were reported when fish oil was added to the diet of beef cattle (Scollan et al., 2001). Further, protected fish oil (formaldehyde treatment) can lead to an increase of EPA and DHA by 1 g/100 g total FA and to a significant increase of C18:2 n-6 and C18:3 n-3 (Gulati et al., 2005), whereas protection (formaldehyde treatment) of vegetable oils from the rumen microbial metabolism resulted in the highest increase of C18:2 n-6 and C18:3 n-3 levels and in minor increases of EPA and DHA levels in the IM fat of beef cattle (Scollan et al., 2003).

Besides supplementation of ruminant diets with lipid sources, forage feeding has been shown to be a good strategy to increase n-3 PUFA, C18:1 t11 and CLA c9t11 proportions in ruminant milk and meat, as forages are a major source of C18:3 n-3 (Dewhurst et al., 2006; Elgersma et al., 2006; Scollan et al., 2006b). The response to forage feeding will depend on several factors such as the forage:concentrate ratio in the diet, conservation of the forage (fresh *vs.* conserved), grazing period, maturity of the forages and type of forage used (Dewhurst et al., 2006; Elgersma et al., 2006). Thus, within forage based strategies, several systems have been reported to modify the milk FA composition. For e.g., organic faming systems, based on high dietary forage proportions have been described to result in higher PUFA proportions, compared to conventional farming systems, based on lower dietary forage proportions and higher concentrate/grain dietary proportions (Ellis et al., 2006). Moreover, higher levels of C18:3 n-3 in milk and beef have been reported upon partial replacement of grass by legume (clover) forages (Dewhurst et al., 2006; Elgersma et al., 2006). Other systems reported to increase n-3 PUFA and CLA c9t11 proportions are alpine/mountain

systems. The latter systems are characterized by high forage proportions and botanical diversity compared to the other systems, mainly based on monocultures (Dewhurst et al., 2006). However, there is still a lack of knowledge on the "origin" of the differences in the milk and meat FA profile, when feeding botanically diverse forages to ruminants.

The main objective of this PhD thesis was to assess the "origin" of the observed differences when feeding botanically diverse forages to the animals. However, studying forage feeding requires appropriate handling and analysis of the basal material (the forages). Thus, the first part of this PhD thesis (Chapters 1 and 2) is dedicated to methodologies aiming at optimising forage FA analysis with regards to handling and storage procedures as well as extraction solvents. In the second part of this PhD thesis the effects of the botanical composition of forages (fresh or conserved) on the milk (Chapter 3) and lamb meat (Chapters 4 and 5) FA pattern are examined *in vivo*. The overall hypothesis that these changes might be related to modification of the rumen FA metabolism was assessed and discussed throughout Chapters 3, 4 and 5. Further, Chapter 6 offers a general discussion of both experimental data obtained during this PhD research as well as literature data in an attempt to link changes in the rumen FA metabolism to explain the observed changes in milk and meat of ruminants fed clover and botanically diverse forages. Possible factors contributing to differences in rumen FA metabolism are also proposed in this Chapter 6. This includes e.g. the polyphenol oxidase enzyme found in red clover and plant secondary metabolites present in botanically diverse forages. Finally, in the last part of this PhD research, the effect of some plant secondary metabolites on rumen biohydrogenation of forage C18:3 n-3 was studied using continuous *in vitro* cultures of which results are presented in Chapter 7.

PART I

Methods for fatty acid extraction of fresh grass and sample storage conditions

Chapter 1

Extraction solvents and sample storage conditions affect content, pattern and esterification of fatty acids in fresh grass

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Chapter 1

Extraction solvents and sample storage conditions affect content, pattern and esterification of fatty acids in fresh grass

Fatty acids were extracted from grass samples either directly after harvest, after 3h of storage in liquid N_2 or after 24h frozen storage at -20ºC, -80ºC and -20ºC in the extraction solvent. Further, the effectiveness of *iso*propanol to deactivate plant enzyme activity was considered. Results show that the total amount of fatty acids in extracts of grass stored for 3h in liquid N_2 (32.5 mg/g DM) were similar to those in grass extracts obtained directly after harvest (32.3 mg/g DM) and higher than in extracts of grass which were stored frozen for 24h (26.3 mg/g DM). The method generally recommended in literature for lipid extraction from plant tissues, a pre-extraction with *iso*propanol, resulted in a reduced recovery of the fatty acids compared to a method based on chloroform/methanol (2/1, vol/vol) (34.5 mg/g DM with chloroform/methanol *vs*. 24.2 mg/g DM for *iso*propanol/chloroform). Results from a second experiment revealed fatty acids could not be recovered quantitatively from freeze-dried grass samples and C18:3 n-3 proportions were significantly reduced. In conclusion, fatty acid analysis should be performed as soon as possible after harvesting and if not possible, conservation of samples for short period in liquid N_2 is the best option. Chloroform/methanol (2/1, vol/vol) seems more powerful than *iso*propanol/chloroform (1/1, vol/vol) in extracting lipids from plant tissues.

Key words: Fatty Acid Extraction, Fresh Grass Storage, *Lolium multiform*, *Lolium perenne*.

INTRODUCTION

A growing number of studies focus on forage fatty acid (FA) composition in relation to milk and meat quality. However, enzyme activity in grass samples (Christie, 1993) might provoke deterioration and transformation of FA during sample storage, particularly of the unsaturated grass lipid constituents, being most susceptible to oxidation (Christie, 1992). Our objectives were to study the total content and profile of FA extracted from grass samples stored under different conditions prior to FA extraction, using different extraction solvents. Storage conditions and extraction solvents were chosen according to common practice, reported in recent literature.

MATERIALS AND METHODS

Sampling and experimental design

Fresh grass samples were obtained at 08:00 in March and May 2004, from two pastures of the Ghent University (Melle, Belgium, 50º59 N/03º49 E, 11 m.a.s.1.). The first was sown with pure diploid *Lolium multiflorum* (Italian ryegrass) in September 2003, the second with *Lolium perenne* (perennial ryegrass) in April 2002. The plots were fertilized according to common practice. Grass samples (100 g) were cut 5 cm above the soil, taken to the lab and immediately prepared for FA extraction (5.0 g, n=3), while the rest was immersed in the field under liquid N_2 and stored for 3h. Prior to solvent addition, grass samples of *Lolium multiflorum* were cut into 1 cm strips and 5.0 g per treatment and per repetition were weighed for FA extraction or storage during 24h at -20ºC, -80ºC or in the extraction solvent at -20ºC. Alternatively, grass (5.0 g per treatment) was stored for 24h without prior cutting and cut into 1 cm strips after frozen storage. Half of all samples were extracted with chloroform/methanol (C/M; 2/1, vol/vol), according to a method adopted from Folch et al. (1957), while for the others *iso*propanol/chloroform (I/C; 1/1, vol/vol) was used, according to Lee et al. (2004) (Figure 1.1.).

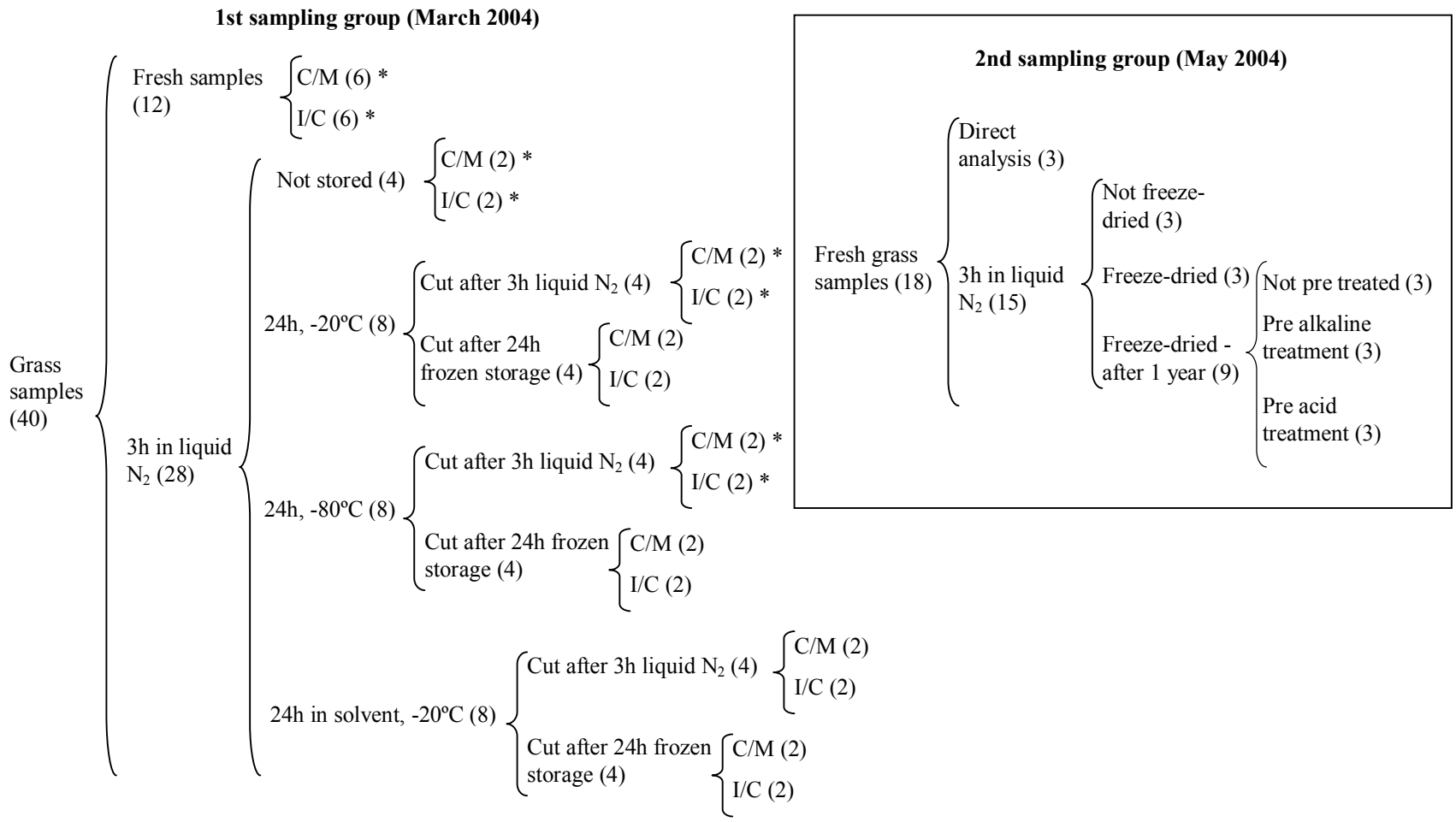

Figure 1.1. – Scheme of sampling and sample handling concerning the two groups of samples. Numbers between brackets correspond to number of samples per group. * - Samples taken for thin layer chromatography (n=1).

Fatty acid analysis

- *Extraction*

Handling time of the samples prior to FA extraction was always less than 5 min. *Lolium perenne* samples were extracted, using C/M (2/1; vol/vol), immediately after harvest, after 3h of storage in liquid N_2 and after freeze drying (immediately and after 1 year; Figure 1.1.). Before overnight extraction (at room temperature for C/M and at 4ºC for I/C), all samples were homogenized with an ultra-turrax for 1 min (Ultra-Turrax T25, IKA-Labortechnik, Belgium), and their endogenous water content was determined (105ºC for 4h) in order to adjust ratios of chloroform/methanol/water and *iso*propanol/chloroform/water to 8/4/3 and 2/2/1 (vol/vol/vol), respectively. Nonadecanoic acid (C19:0, 10 mg; Sigma, Bornem, Belgium) was used as internal standard. C/M extracts were prepared by consecutive extractions with 40, 30 and 20 ml of C/M (2/1, vol/vol). I/C extracts (Lee et al., 2004) were obtained by extraction with 40 and 5 ml of I/C (1/1, vol/vol). Freeze-dried samples (1.0 g) were extracted with C/M (2/1, vol/vol) (30, 20 and 20 ml) with the addition of 20 ml of distilled water before overnight extraction. Additionally, the effect of prior acid and alkaline hydrolysis on FA extraction was tested for freeze-dried samples (n=3), of which 1.0 g was boiled for 1h with 100 ml of HCl 3M, filtrated with distilled water until the filtrate reached a pH of 7.0 and the filtrate was extracted with C/M (2/1, vol/vol) as described before for freeze-dried samples. Alkaline hydrolysis was as follows: 3 ml of NaOH (2M) were added to the samples (1.0 g, n=3), which were vortexed and the walls of the tubes rinsed with another 4 mL of NaOH (2M). Samples were then put in the oven at 85ºC for 45 min. The extraction procedure was as described before for freeze dried samples, except for the third extraction step in which C/M has been replaced by ethanol/HCl (6M)/C (1/1/2, vol/vol/vol).

- *Thin Layer Chromatography*

Separation in lipid classes of *Lolium multiflorum* extracts (fresh, stored 3h in liquid N_2 and stored frozen for 24h at -20ºC and -80ºC or not; Figure 1.1.) was performed by thin layer chromatography (Dohme et al., 2003). Before methylation, 1 mg of C19:0 (Sigma, Bornem, Belgium) was added to the triacylglycerol (TAG) and polar lipid (PL) fractions.

- *Methylation*

For methylation, 10 ml of extract was used. Fatty acids were methylated at 50ºC

with NaOH in methanol (0.5M) followed by HCl/Methanol (1/1, vol/vol) according to Raes et al. (2001).

- *Gas Chromatography*

Fatty acid methyl esters (FAME) were analyzed on a Hewlet-Packard 6890 gas chromatograph (Hewlett-Packard Co., Belgium) with a CP-Sil88 column for FAME (100m x 0.25mm x 0.2 μm; Chrompack Inc., The Netherlands). For more detailed information about the GC analysis of the samples we refer to Raes et al. (2004b).

Statistics

A general linear ANOVA-model was used to evaluate the effect of cutting, extraction solvent and sample storage on grass FA content and composition, for the *Lolium multiflorum* samples, according to: $Y_{ijk}= \mu + C_i + S_j + T_k + \varepsilon_{ijk}$, where μ is the overall mean, C_i the effect of cutting (cut after harvest *vs.* after liquid N_2 storage *vs.* after 24h frozen storage), S_j the solvent effect (C/M *vs.* I/C), T_k the effect of storage (extracted immediately after harvest *vs.* after 3h in liquid N_2 *vs.* after 24h frozen storage) and ε_{ijk} the residual error. An additional statistical analysis, using the same model but only with 24h frozen storage results, has been performed to compare storage at -20ºC *vs.* -80ºC *vs.* -20ºC in solvent. Comparison of means was done using Duncan as post-hoc test.

A one-way ANOVA was used to evaluate the effect of freeze-drying on *Lolium perenne* FA content and pattern, according to: $Y_i= \mu + P_i + \varepsilon_i$, where μ is the overall mean, P_i the effect of preservation, and ε_i the residual error, using three orthogonal contrasts (direct analysis *vs*. 3h in liquid N_2; 3h in liquid N_2 *vs*. freeze-drying and immediately *vs*. 1 year after freeze-drying). Comparison of means was done using Duncan as post-hoc test.

All statistical analyses were performed using SPSS 11.0 (SPSS software for Windows, Release 11.0, SPSS Inc., USA).

RESULTS AND DISCUSSION

Chloroform/methanol (2/1, vol/vol) is more powerful than I/C (1/1, vol/vol) to extract FA from plant tissues, particularly C18:3 n-3 (Table 1.1.), which might be related to the

reduced polarity of I/C resulting in reduced unsaturated to saturated FA ratios (UFA/SFA) in I/C compared to C/M extracts (Table 1.1.). Further, smaller amounts of FA are extracted after 24h storage (Table 1.1.).

Table 1.1. – Total fatty acid content (mg/g DM) and individual fatty acid proportions (g/100 g of FAME) extracted from grass samples of *Lolium multiflorum* (fresh or stored 3h in liquid N_2, 24h at -20ºC, at -80ºC or in solvent at – 20ºC) when extracted with C/M (2/1, vol/vol) or with I/C (1/1, vol/vol) and when cut after harvest, after 3h in liquid N_2 or after 24h storage

Fatty acids	Extraction method	Fresh	Stored 3h in liquid N_2							Statistics			
			Cut after liquid N_2 storage				Cut after 24h frozen storage						
		Cut after harvest	Not stored	Frozen storage 24h			Frozen storage 24h			St.[1]	Ct[2]	Sl.[3]	Fr.[4]
				-20ºC	-80ºC	Solvent	-20ºC	-80ºC	Solvent				
n		6	2	2	2	2	2	2	2				
Total	C/M	35.0	39.5	33.1	34.5	28.8	29.1	32.8	32.4	*	n.s.	***	n.s.
	I/C	29.6	25.5	20.4	21.9	21.0	20.2	20.1	21.6				
C12:0	C/M	0.847	0.934	0.965	0.944	0.855	0.846	0.938	0.825	n.s.	n.s.	*	n.s.
	I/C	0.874	0.979	1.08	1.11	1.05	0.926	1.03	1.06				
C14:0	C/M	1.61	1.66	1.69	1.69	1.75	1.64	1.81	1.68	n.s.	n.s.	n.s.	n.s.
	I/C	1.59	1.78	1.86	1.75	1.77	1.57	1.79	1.82				
C16:0	C/M	11.3	10.8	11.3	11.2	10.9	11.4	11.8	11.2	n.s.	n.s.	†	†
	I/C	11.5	11.3	11.8	12.5	11.2	13.0	12.3	11.5				
C16:1 c9	C/M	1.64	1.73	1.77	1.77	1.66	1.64	1.83	1.71	n.s.	n.s.	n.s.	n.s.
	I/C	1.72	1.75	1.90	1.75	1.69	1.63	1.89	1.79				
C18:0	C/M	2.12	1.78	1.23	1.13	1.14	1.12	1.22	1.09	**	n.s.	*	n.s.
	I/C	2.18	2.21	1.42	1.12	1.56	1.82	1.65	1.36				
C18:1 c9	C/M	0.947	0.849	0.721	0.725	0.707	0.938	0.730	0.876	n.s.	†	**	n.s.
	I/C	1.89	1.24	1.06	0.891	1.51	3.32	2.27	1.49				
C18:2 n-6	C/M	8.22	7.63	8.38	8.51	8.33	9.19	9.06	8.96	n.s.	n.s.	n.s.	n.s.
	I/C	8.10	7.78	8.37	8.41	7.69	8.49	8.96	8.29				
C18:3 n-3	C/M	64.5	67.4	66.8	66.8	67.9	66.2	65.2	66.6	n.s.	n.s.	*	n.s.
	I/C	63.2	64.3	64.2	58.8	64.9	56.1	62.3	64.6				
UFA/SFA ratio[5]	C/M	4.78	5.16	5.14	5.22	5.39	5.27	4.99	5.27	n.s.	n.s.	†	n.s.
	I/C	4.67	4.67	4.67	4.35	4.86	4.15	4.51	4.88				

[1] Significance of comparison between storage conditions (fresh *vs*. 3h in liquid N_2 without further storage *vs*. 3h in liquid N_2 and 24h frozen storage);
[2] Significance of comparison between cut after harvest, after 3h in liquid N_2 and after 24h storage;
[3] Significance of comparison between C/M and I/C;
[4] Significance of comparison between frozen storage for 24h at -20ºC, -80ºC and in solvent at -20ºC;
[5] UFA = unsaturated fatty acids; SFA = saturated fatty acids;
n.s. – not significantly different ($p>0.1$); † - $0.1>p>0.05$; * - $0.05>p>0.01$; ** - $0.01>p>0.001$; *** - $p<0.001$

The loss might be due to ongoing lipolysis and subsequent oxidation during or after freezing (Frankel, 1998), to thawing losses (Christie, 1993; Park et al., 2002) or to a reduced ability to extract FA from frozen samples.

Increased proportions of unesterified fatty acids (UEFA, expressed as a proportion of TAG + UEFA + PL; Table 1.2.) are indicative of ongoing lipolysis during or after sample storage (Dierick and Decuypere, 2002). However, as polyunsaturated FA (PUFA) proportions were not reduced (Table 1.1.), there is no indication of oxidative FA loss. Important thawing losses are also unlikely as sample handling has been performed within some minutes. Hence, a reduction in the amount of FA extracted after frozen storage is the most logical explanation for the losses. This might be due to the reduced ability for appropriate pre-treatment of frozen samples prior to extraction using an ultra-turrax. Indeed, ultra-turraxing of grass samples is crucial to ensure complete FA extraction as observed in former work at our lab (unpublished results). In the latter study, significantly more FA were recovered when using an ultra-turrax (14.0 mg/g DM *vs*. 4.99 mg/g DM, SEM=0.397, P<0.001).

Table 1.2. – Fatty acids (mg/g DM) extracted from the major lipid classes of grass samples (*Lolium multiflorum*) (fresh or stored 3h in liquid N_2 or 24h at -20ºC or -80ºC), extracted with C/M or I/C (n=1)

Lipid fraction	Fresh		Stored 3h in liquid N_2						Statistics		
			Cut after liquid N_2 storage								
	Cut after harvest		Not stored		Frozen storage for 24h				St.[1]	Sl.[2]	Fr.[3]
					-20ºC		-80ºC				
Solvent	C/M	I/C	C/M	I/C	C/M	I/C	C/M	I/C			
UEFA	0.533	0.953	1.35	1.55	2.53	1.87	2.22	1.93	†	n.s.	n.s.
PL	30.1	20.5	21.9	13.2	17.6	10.0	20.4	9.84	**	**	n.s.
TAG	4.32	3.49	3.58	3.74	2.86	1.92	2.50	1.71	*	n.s.	n.s.

[1] Significance of comparison between storage conditions (fresh *vs*. 3h in liquid N_2 without further storage *vs*. 3h in liquid N_2 and 24h frozen storage);
[2] Significance of comparison between C/M and I/C;
[3] Significance of comparison between frozen storage for 24h at -20ºC and -80ºC;
UEFA – unesterified fatty acids; PL – polar lipids; TAG – triacylglycerols;
n.s. – not significantly different (p>0.1); † - 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001

As the first experiment indicated that grass samples ideally should be extracted as soon as possible after harvest, using C/M (2/1, vol/vol) as the extraction solvent, a

second experiment (Table 1.3.), with more repetitions (n=3), has been conducted to compare analysis directly after harvest and after short term storage in liquid N_2, e.g. to allow transport to the lab.

Table 1.3. - Total fatty acid content (mg/g DM) and major fatty acids proportions (g/100 g of FAME) of grass (*Lolium perenne*) samples (fresh, stored 3h in liquid N_2 or freeze-dried), extracted with C/M (n=3)

Fatty acids	Direct analysis	3h liq N_2	3h liq N_2 and FD	3h liq N_2 and 1 year FD	SEM	Sign.[1]	Sign.[2]	Sign.[3]
Total	38.7	31.6	19.2	14.6	1.45	**	***	†
C12:0	0.566	0.588	0.739	0.357	0.026	n.s.	**	***
C14:0	1.96	1.92	2.39	2.89	0.037	n.s.	***	***
C16:0	11.5	12.3	11.9	20.0	0.152	**	n.s.	***
C16:1c9	1.96	2.06	2.03	3.19	0.036	n.s.	n.s.	***
C18:0	1.39	1.32	1.37	2.31	0.115	n.s.	n.s.	***
C18:1c9	1.55	1.25	1.47	2.27	0.193	n.s.	n.s.	*
C18:2 n-6	10.5	11.2	11.7	9.52	0.282	n.s.	n.s.	**
C18:3 n-3	65.4	63.3	60.5	50.6	0.501	*	**	***
SFA[4]	15.5	16.2	16.4	26.1	o.238	†	n.s.	***
MUFA[4]	5.85	5.69	6.52	6.01	0.202	n.s.	*	n.s.
PUFA[4]	76.3	75.1	72.9	60.4	0.566	n.s.	*	***
UFA/SFA[4]	5.29	4.95	4.82	2.54	0.088	*	n.s.	***

[1] Orthogonal contrast direct analysis *vs*. 3h liq. N_2;
[2] Orthogonal contrast 3h liq. N_2 *vs*. 3h liq. N_2 and FD;
[3] Orthogonal contrast extraction of FD samples, directly after FD *vs*. after 1 year of FD storage;
[4] SFA, MUFA, PUFA, UFA – saturated, mono-unsaturated, poly-unsaturated and unsaturated fatty acids;
n.s. – not significantly different ($p>0.1$); † - $0.1>p>0.05$; * - $0.05>p>0.01$; ** - $0.01>p>0.001$; *** - $p<0.001$

Moreover, freeze-drying has been evaluated as a possible alternative for storage over a longer period. Our results (Table 1.3.) confirm previously reported difficulties (Christie, 1993) to obtain quantitative recovery of lipids from freeze-dried samples, even after alkaline or acid hydrolysis (14.6^a mg/g DM *vs*. 14.9^a mg/g DM *vs*. 13.0^b mg/g DM, SEM=0.339, $P<0.05$, for FD samples without prior hydrolysis and with alkaline or acid hydrolysis, respectively). Furthermore, freeze-dried samples kept at ambient

temperature, for an extended period, undergo oxidation, as indicated by the increased proportion of saturated and mono-unsaturated FA at the expense of PUFA.

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Chapter 2

Comparison of two different procedures to analyze the fatty acid composition by gas-liquid chromatography after extraction of photosynthetic tissues and methylation of extracted fatty acids

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Chapter 2

Comparison of two different procedures to analyze the fatty acid composition by gas-liquid chromatography after extraction of photosynthetic tissues and methylation of extracted fatty acids

Two methods, used as standard methods for fatty acid (FA) analysis of plant material at two different European Universities were compared. The procedures tested were a mixture of chloroform/methanol (2/1, vol/vol) for FA extraction followed by an acid-alkaline methylation (C/M), and absolute ethanol for FA extraction followed by an alkaline methylation (E).

Higher total amounts of FA extracted from plant tissue were obtained with the C/M method. Significant differences in the proportions of individual FA were observed between the two methods, with the E method resulting in higher C18:3 n-3 proportions. The observed differences are suggested to be associated with different lipid classes being extracted by the different solvents tested (for fresh and silage grass samples) and to the different methylation procedures used in the different extraction methods (for silage grass samples). Further and more detailed investigations are required to confirm these hypotheses.

Key words: Fatty Acid Analysis, Fresh Grass Samples

INTRODUCTION

Fatty acid (FA) analysis is normally comprised of three steps: extraction, derivatization and identification/quantification by means of separation techniques, from which the most common is gas chromatography. Different solvents and methods have been described both for the extraction step as well as for the derivatization step, but not all are suitable for FA analysis of plant material. The FA composition of photosynthetic tissue differs from that in other tissues. In plant leaves, the lipid fraction constitutes less than 10% of the dry weight and is mostly located in the chloroplasts (Hawke, 1973). These complex lipids are polar constituents of membranes and highly unsaturated. Hence, an appropriate extraction solvent should not only be suitable to readily dissolve polar lipids but should also overcome the interactions between the lipids and cellular constituents, such as proteins and polysaccharides (Christie, 1993).

It is well known that the extractability of tissue FA is variable and depends both on the nature of the tissue and of the lipids to be extracted (Christie, 2003). Simple lipids such as triacylglycerols are extracted with relative ease, whereas complex lipids, such as galactolipids and phospholipids, usually constituents of cellular membranes, are more difficult to extract as they are in close association with proteins and polysaccharides (Christie, 1993, 2003). Thus, lipids lacking polar groups, such as triacylglycerols or cholesterol esters, will be very soluble in non-polar solvents, such as hexane, toluene or cyclohexane, and in moderately polar solvents, such as diethyl ether or chloroform. In contrast, lipids with polar groups, such as phospholipids and galactolipids, will dissolve readily in more polar solvents such as methanol, ethanol and in moderately polar solvents, such as chloroform. Hence, no single pure solvent appears to be suitable as a general-purpose lipid extractant. Most analysts accepted a mixture of chloroform/methanol (2/1, vol/vol) to extract more fully both polar and non-polar lipids from a variety of tissues (Christie, 2003). Nonetheless, both chloroform and methanol are toxic to the analysts and to the environment, and are not completely stable (Christie, 1993). Other solvents with lower toxicity have been suggested such as ethanol (Elgersma et al., 2003) or *iso*propanol (Hawke, 1973; Lee et al., 2004) for FA extraction of plant tissues.

The objectives of this study were to compare two standard procedures for FA analysis of plant tissue: the classical chloroform/methanol (2/1, vol/vol) mixture

followed by an acid-alkaline methylation *vs.* absolute ethanol followed by an alkaline methylation. Each procedure was run in one laboratory on the same material at the same time, and no distinctive evaluation of the extraction and transesterification procedure and GC conditions was done. As a consequence, the individual steps can not explain differences in the values between the two procedures tested, hence differences are considered to be due to the overall procedure.

MATERIALS AND METHODS

Fresh plant material was collected from an experimental field of Wageningen University, The Netherlands (51º58'N/5º40'E, 7 m.a.s.l.). Fresh herbage (± 200g) was sampled and immediately, collected in plastic bags immerged in dry ice (-80ºC) and transported to the lab. Three *Lolium perenne* (perennial ryegrass) samples with two different leaf blade proportions were chosen. Additionally, a grass silage sample was taken at the same experimental farm of Wageningen University. Dry matter content of fresh grass and grass silage samples was determined at Wageningen University, by oven-drying fresh grass and grass silage sub-samples at 70ºC for 3 days. A paper cutter was used to cut the samples for FA analysis into 5-10 mm pieces, and samples were representatively divided into four plastic tubes, as individual replicates for each method. Tubes were transported frozen (-80°C) and analyzed on the same day (11 days after harvest) in each lab at either Ghent University or Wageningen University, using their standard method, as described in Chapter 1 of this PhD thesis and by Elgersma et al., (2003), respectively. At Ghent University and before extraction, grass samples were transferred into glass centrifugation tubes and the plastic tubes rinsed 3 times with 5 ml of chloroform/methanol (2/1, vol/vol). Three extraction steps were performed with the chloroform/methanol procedure, whereas two extraction steps were performed with the ethanol procedure. Extracts obtained with chloroform/methanol procedure were further methylated at 50ºC with NaOH in methanol (0.5 M) followed by HCl/methanol (1/1, vol/vol) as described by Raes et al. (2001). Extracts obtained with the ethanol procedure were further methylated with sodium methanolate in methanol (2 M) according to Badings and De Jong (1983). Fatty acid methylesters (FAME) obtained with the chloroform/methanol procedure were analyzed by gas chromatography (GC), on a

Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Belgium) with a CP-Sil-88 column for FAME (100 m x 0.25 mm x 0.2 μm; Chrompack Inc., The Netherlands) and according to the following temperature program: 150ºC for 2 min, followed by an increase of 1ºC per min up to 158ºC, hold at 158ºC for 28 min, followed by linear increase of 1ºC per min up to 200ºC. FAME obtained with the ethanol procedure were also analyzed by GC, on an Interscience GC 8000 top CE instruments gas chromatograph (Interscience, The Netherlands), with a ZB-Wax column (30 m x 0.53 mm x 0.5 μm; Zebron, Phenomenex) and according to the following temperature program: starting at 80ºC, followed by an increase of 20ºC per min up to 160ºC, and afterwards with an increase of 5ºC per min up to 260ºC. Nonadecanoic (C19:0, Sigma, Bornem, Belgium) and heptadecanoic (C17:0, Sigma, The Netherlands) acids were used as internal standards for the method used at Ghent University and Wageningen University, respectively. The method used at Ghent University will be referred to as C/M and the method of Wageningen University as E.

A one-way ANOVA was used to evaluate the effect of the two procedures on fresh grass FA, according to $Y_{ij} = \mu + M_i + GO_j + M_i*GO_j + \varepsilon_{ij}$, where M_i is the effect of the FA analysis procedure, GO_j refers to the grass origin (N level of fertilization and days of regrowth), M_i*GO_j refers to the interaction between the FA analysis procedure and the grass origin, and ε_{ij} the residual error. Comparison of means was done using Duncan as post-hoc test.

A one-way ANOVA was used to evaluate the effect of the two procedures on grass silage FA, according to $Y_i = \mu + M_i + \varepsilon_i$, where M_i is the effect of the FA analysis procedure and ε_i the residual error. Comparison of means was done using Duncan as post-hoc test.

All statistical analysis were performed using SPSS 12.0 (SPSS software for Windows, Release 12.0, SPSS Inc., USA).

RESULTS

The assessment of the effect of the extraction solvent and the methylation procedure was based on the absolute amount (mg/g DM) of FA extracted from the grass products, as well as on the proportions (g/100 g of FAME) of the major grass FA:

C14:0, C16:0; C16:1 c9, C18:0; C18:1 c9; C18:2 n-6 and C18:3 n-3 in the total fat. For the C/M method, 94 g/100 g of FAME are presented and 95 g/100 g of FAME for the E method. For fresh grass samples, the total amount of FA and the proportions of individual FA differed significantly between the C/M and E methods (Table 2.1.).

Table 2.1. - Influence of the two analytical procedures on the total amount of FA extracted (mg/g DM) and proportions of individual FA (g/100 g FAME) of fresh grass samples (n=12)

Fatty acids	C/M	E	SEM	Sign.[1]
Total FA	41.1	34.3	1.31	**
C14:0	1.95	0.224	0.048	***
C16:0	12.8	12.0	0.455	n.s.
C16:1 c9	1.12	0.184	0.061	***
C18:0	2.08	0.931	0.252	**
C18:1 c9	3.43	1.87	0.492	*
C18:2 n-6	10.7	11.7	0.301	*
C18:3 n-3	61.0	68.0	1.44	**
SFA/UFA	0.232	0.161	0.012	**

C/M – Chloroform/methanol (2/1, vol/vol) extraction (as described in Chapter 1); E – Ethanol extraction (according to Elgersma et al., 2003); SFA/UFA – Ratio between saturated fatty acids (C14:0, C16:0 and C18:0) and unsaturated fatty acids (C16:1 c9, C18:1 c9, C18:2 n-6 and C18:3 n-3); SEM – Standard error of mean; [1] – Significance of the FA analysis method; n.s. – not significantly different ($p>0.05$); * - $0.05>p>0.01$; ** - $0.01>p>0.001$; *** - $p<0.001$;

The C/M method allowed the extraction of a higher amount of FA than the E method. The proportions of C14:0, C16:1 c9, C18:0 and C18:1 c9 were highest for the C/M method, whereas proportions of C18:2 n-6 and C18:3 n-3 were highest for the E method. Proportions of C16:0 did not differ between both methods. However, the absolute amount of all individual FA was always lower for the E method (data not shown) compared to the C/M method. In addition, no interaction was observed between the analytical procedure and the grass origin (including both the N level of fertilization and days of regrowth). No differences were observed in the total amount of FA extracted for days of regrowth (36.7 *vs.* 39.8 mg/g DM for 38 and 30 days of regrowth, respectively) nor for the N level of fertilization (39.5 *vs.* 36.8 mg/g DM for 100 and 45 kg N/ha). However, proportions of C18:2 n-6 were highest for *Lolium perenne* with 38 days regrowth (11.6 g/100g of FAME) compared with *Lolium perenne* with 30 days of regrowth (10.5 g/100g of FAME), but were not affected by the level of N fertilization. On the other hand, proportions of C18:3 n-3 were affected by both days of regrowth (62.4 vs. 68.7 g/100 g of FAME for 38 and 30 days of regrowth, respectively) and the N

level of fertilization (60.7 vs. 66.3 g/100g of FAME for 100 and 45 kg N/ha). Nevertheless, these data will not be discussed further as it do not fall within the main objective of this study.

The same FA as for fresh grass samples were used to assess the effectiveness of FA extraction from grass silage samples. Results regarding the total amount of FA (mg/g DM) and the proportions of individual FA (g/100g of FAME) analyzed by both methods are presented in Table 2.2. For the C/M method 92 g/100 g of FAME are presented and 95 g/100 g of FAME for the E method. Again, a higher amount of FA was extracted with the C/M method compared to the E method. Proportions of C14:0, C16:0, C16:1 c9 and C18:0 were highest for the C/M method, whereas proportions of C18:2 n-6 and C18:3 n-3 were highest for the E method. Proportions of C18:1 c9 did not differ between treatments.

Table 2.2. - Influence of the two analytical procedures on the total amount of FA extracted (mg/g DM) and proportions of individual FA (g/100 g FAME) of silage grass samples (n=4)

Fatty acids	C/M	E	SEM	Sign.
Total FA	19.1	8.54	0.429	***
C12:0	0.434	0.304	0.017	**
C14:0	2.32	0.767	0.059	***
C16:0	17.0	14.4	0.131	***
C16:1 c9	1.34	0.228	0.030	***
C18:0	1.57	1.28	0.033	**
C18:1 c9	2.90	2.41	0.190	n.s.
C18:2 n-6	17.1	20.5	0.211	***
C18:3 n-3	48.7	55.1	0.354	***

C/M – Chloroform/methanol (2/1, vol/vol) extraction (as described in Chapter 1); E – Ethanol extraction (according to Elgersma et al., 2003); SEM – Standard error of mean; n.s. – not significantly different; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001;

DISCUSSION

This discussion will be limited to the first two steps of the procedure: extraction and methylation, as the GC conditions applied and equipment used for the FAME analysis were rather similar for the two methods studied.

Polar ethanol has been used for the extraction of lipids from the liver tissue (Lucas and Ridout, 1970) and *Cyanobacterium* (Mendes et al., 2005, 2006). In our

study, extraction of lipids from fresh grass samples with ethanol yielded lower total amounts and individual FA proportions than the chloroform/methanol mixture, in agreement with the results of Mendes et al. (2005, 2006). Further, the different proportions of individual FA and the different ratio of saturated to unsaturated FA (SFA/UFA; Table 2.1.) obtained for the two methods suggest a certain level of selectivity in extracting different lipid classes from the tissue matrix. Indeed, plant C18:3 n-3 is mainly present as a galactolipid in the membrane of chloroplasts (Hawke, 1973; Christie, 2003) and its proportions were highest for the E method, suggesting ethanol to be a good solvent for the extraction of the more polar membrane-associated lipids, in particular galactolipids. Lower C18:3 n-3 proportions obtained with the C/M method are the result of a dilution effect, as the C/M method allowed the extraction of higher amounts of total FA than the E method. Indeed, the absolute amount of C18:3 n-3 extracted was approximately similar in both methods (25.1 *vs.* 23.3 mg/g DM for the C/M and E methods, respectively). Nevertheless, more detailed studies with separation of lipid classes by thin layer chromatography are necessary to assess this hypothesis. From the results obtained in this study, chloroform/methanol (2/1, vol/vol) appears to be a more general solvent mixture for lipid extraction than ethanol in terms of lipid classes extracted.

For grass silage samples, it is rather unlikely that the former hypothesis would completely explain the differences between the 2 methods tested, as it is known that FA in grass silage are mainly present as free FA. Thus, differences in the total amount of FA and in the proportions of individual FA between the two methods could be explained by the different methylation procedures used. Samples extracted with chloroform/methanol were further transesterified into methylesters with an acid-alkaline based methylation, whereas samples extracted with ethanol were further transesterified with an alkaline based methylation. Methylation in alkalic environment guarantees the complete transesterification of esterified FA only, whereas with an acid based methylation, both esterified and unesterified FA (free FA) are converted into methylesters (Carrapiso and García, 2000; Christie, 2003; Palmquist and Jenkins, 2003). Hence, it might be possible that the total amount of FA and proportions of individual FA presented in Table 2.2. for the C/M method, reflect both the free FA as well as the esterified FA extracted from grass silage samples, whereas the total amount of FA and individual proportions of FA presented for the E method would mainly refer to the esterified FA. Thus, the observed differences in the total amount of FA analyzed in

grass silage samples with the C/M and E methods might be explained by the different solvents used but also by the methylation procedure used following the different extraction methods.

CONCLUSIONS

The two analytical methods compared in this study resulted in significantly different amounts of FA and proportions of individual FA, both for fresh grass and grass silage samples. Higher amounts of FA were reported when applying the C/M method compared to the E method, both in fresh grass as well as in grass silage samples. In fresh grass samples, these differences might be related to different lipid classes being selectively extracted by the different solvents used. In grass silage samples the observed differences could be associated to the different extraction solvents used as well as to the different methylation procedures used (acid-alkaline in the chloroform/methanol method *vs.* alkaline in the ethanol method).

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PART II

Botanical composition of forages and effects on fatty acid metabolism of dairy cattle and lamb

Introduction

Introduction

Feeding forages to ruminants has been shown to increase the n-3 polyunsaturated fatty acid (PUFA) content in milk and meat (Dewhurst et al., 2006; Scollan et al., 2006b, respectively) as forages are naturally rich sources of C18:3 n-3. Nevertheless, differences within forage based systems could not always be related to changes in dietary precursor supply (C18:2 n-6 + C18:3 n-3). In the Introduction of this PhD thesis, it was indicated that the gastro-intestinal and endogenous fatty acid (FA) metabolism also might contribute to changes in ruminant product FA profile. As outlined in the Introduction of this PhD thesis, FA might undergo major modifications in the rumen, mammary gland and subcutaneous and intramuscular tissue. Hence, these tissues and organs are emphasized in the following experiments of this PhD research.

Indeed, Dewhurst et al. (2003a) suggested a higher rumen outflow rate to be responsible for the lower rumen lipolysis of C18:2 n-6 and C18:3 n-3 and for the higher C18:3 n-3 proportions observed in the milk of white clover fed dairy cows. Further, Lee et al. (2003, 2004, 2007) hypothesized a lipase inhibitory compound, polyphenol oxidase, present in red clover to cause lower ruminal lipolysis of C18:2 n-6 and C18:3 n-3 associated with a lower rumen biohydrogenation of dietary PUFA. Nevertheless, no specific mechanisms have yet been suggested to explain the observed differences when feeding botanically diverse forages *vs.* grass based diets. In some (e.g. Leiber et al., 2005) but not all cases (e.g. Collomb et al., 2002), this could be related to an increased dietary precursor (C18:2 n-6 + C18:3 n-3) supply. However, some specific herbs of botanically diverse forages have been described to have some antimicrobial activity (Chao et al., 2000; Wallace, 2004). Moreover, the antimicrobial activity of these herbs has been reported to be comparable to the antimicrobial activity of ionophores (Greathead, 2003). Within the latter, monensin is described to lower complete rumen biohydrogenation to stearic acid, leading to accumulation of hydrogenation intermediates (Van Nevel and Demeyer, 1995; Jenkins et al., 2003).

The first objective of this part of the present PhD thesis was to report the effect of botanically diverse forages on the FA composition of milk of dairy cows (Chapter 3) and on the FA pattern of lamb intramuscular and subcutaneous fat (Chapters 4 and 5).

Intensive ryegrass forages were chosen as a representation of the common practice in forage feeding to ruminants, and were used as a control. Leguminous forages, in particular clover silages, were used as these forages haven been described in literature to have the potential to manipulate rumen FA metabolism. In addition, plant secondary metabolites of the latter (saponins in white clover and polyphenol oxidase in red clover) are described to change the rumen fermentation pattern and possibly the ruminal FA metabolism.

The second objective was to link the observed differences to changes in the rumen FA metabolism as assessed through the collection and FA analysis of rumen pool samples (Chapter 3) or rumen spot samples at slaughter (Chapters 4 and 5). Further, a general discussion combining both experimental and literature data is given, reviewing effects on milk and meat FA composition as well as possible explanations for their origin (Chapter 6).

Chapter 3

Milk fatty acid composition and associated rumen lipolysis and fatty acid hydrogenation when feeding silages from intensively managed or semi-natural grasslands

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Chapter 3

Milk fatty acid composition and associated rumen lipolysis and fatty acid hydrogenation when feeding silages from intensively managed or semi-natural grasslands

To evaluate the effect of replacing intensive forage by semi-natural grassland products on rumen lipid metabolism and milk fatty acid composition, four lactating and rumen canulated Holstein cows were used in a 4x4 Latin square design. Four different diets were fed: diet 100 IMS – 100% intensively managed silage (IMS), diet 20 SPP – 80% IMS plus 20% semi-natural but species poor silage (SPP), diet 60 SPP – 40% IMS plus 60% SPP and diet 60 SPR – 40% IMS plus 60% semi-natural species rich silage (SPR). Silages showed significant differences in total fat content and in proportions of C18:2 n-6 and C18:3 n-3. Despite the reduced dietary supply of C18:3 n-3 with diets 60 SPP and 60 SPR, differences in milk C18:3 n-3 were small, suggesting higher recoveries of C18:3 n-3. Presumably, the latter are related to a higher transfer efficiency of C18:3 n-3 from the duodenum to the mammary gland, as rumen biohydrogenation, estimated from rumen pool size and first order rumen clearance kinetics, were similar among diets. CLA c9t11 in milk from cows fed diet 60 SPR were almost doubled compared to feeding one of the other diets. This has been related to partial inhibition of rumen biohydrogenation of C18:3 n-3 and/or C18:2 n-6, as suggested by the increased proportions of hydrogenation isomers and reduced stearic acid proportions in rumen pool samples. In conclusion, the results suggest that the use of semi-natural grasslands in the diet of the animals reduce to some extent complete rumen biohydrogenation, which leads to an increase in milk CLA.

Key words: CLA, Grasslands, Hydrogenation, Milk Fatty Acids, Rumen

OBJECTIVES

The objectives of this study were to evaluate the effect of replacing intensive forage by semi-natural grassland products on milk fatty acid composition, and to examine whether these differences could be linked to changes in rumen fatty acid metabolism.

MATERIALS AND METHODS

Experimental design

The experiment was carried out as described by Bruinenberg et al. (2004). Briefly four lactating and rumen canulated Holstein cows (647 ± 69 kg at the start of the trial), 20 to 25 kg milk per day (249 ± 76 days in lactation at the beginning of the experiment) were used. The experiment was carried out from May until August 2001 and was designed as a Latin square. Each experimental period lasted three weeks, comprising of an adaptation period of two weeks and the last week for sampling. Cows were fed twice daily, receiving 40% of the daily dry matter (DM) intake in the morning (6:00) and 60% in the evening (16:00).

Diets

As described by Bruinenberg et al. (2004), four different diets were used, containing forages with different combinations of three grassland silages. The latter were obtained from one intensively managed grassland (IMS) – a monoculture of *Lolium perenne* – and two semi-natural grasslands: one species poor (SPP), composed (proportions expressed on a DM basis) mainly (95.9%) of mature grasses, of which *Lolium perenne* (perennial ryegrass) represented 5.9% [other grass species present in the silage were *Holcus lanatus* (35.5%; yorkshire fog), *Poa trivialis* (13.9%; rough bluegrass), *Alopecurus geniculatus* (13.3%; marsh foxtail), *Agrostis stolonifera* (12.3%; creeping bentgrass)], 0.03% legumes and 4.0% non-legminous herbs [mainly *Ranunculus repens* (3.2%; creeping buttercup)]; and one species rich (SPR), consisting of 34 % non-leguminous herbs (4.1% *Anthiscus sylvestris* (cow parsley), 3.9%

Ranunculus acris (buttercup), 3.9% *Galium mollugo* (hedge bedstraw), 3.8% *Crepis biennis* (rough hawksbeard), 3.6% *Cirsium arvense* (canada thistle), 3.4% *Plantago lanceolata* (narrowleaf plantain), 3.3% *Achillea millefolium* (common yarrow), 3.1% *Heracleum sphondylium* (eltrot)), 11 % legumes (4.9% *Lathyrus pratensis* (meadow pea), 2.9% *Trifolium pratense* (red clover) 1.7% *Trifolium repens* (white clover)) and 55 % grasses (13.2% *Arrhenatherum elatius* (tall oatgrass), 4.1% *Lolium perenne* (perennial ryegrass), 3.8% *Alopercurus pratensis* (meadow foxtail), 3.6% *Dactylis glomerata* (orchardgrass or cocksfoot), 3.3% *Agrostis stolonifera* (creeping bentgrass), 3.1% *Festuca rubra* (red fescue), 1.8% *Poa trivialis* (rough bluegrass)). Diet one (100 IMS) was composed of IMS silage only; the second diet (20 SPP) consisted of 80% IMS silage and 20% SPP silage; diet three (60 SPP) of 40% IMS silage and 60% SPP silage and the fourth diet (60 SPR) of 40% IMS silage and 60% SPR silage. The pasture of SPP was managed to encourage nesting of birds, and was fertilized on March 10th 2000 with 20 m^3 cattle slurry/ha. In order to allow birds to complete nesting, harvesting of this grassland was not allowed before June 7th 2000, when cutting of grasses took place. The pasture of SPR was part of a nature reserve and had not been fertilized since approximately 1980.

Table 3.1. – Chemical composition (g/kg DM), net energy content (MJ/kg DM), fatty acid content (g/kg DM) and proportion of predominant fatty acids (g/100 g of FAME) of the mixed silages used in the four dietary treatments

Diet	100 IMS	20 SPP	60 SPP	60 SPR	SE[2]
Intake of silage (kg DM/d)	13.9	13.6	12.2	13.0	1.20
DM (g/kg fresh material)	601	625	674	594	91.8
Chemical composition					
Organic matter	885	889	897	897	1.7
Crude protein	191	181	159	139	1.8
Neutral detergent fibre	524	540	568	547	8.8
Net energy [1]	5.9	5.6	5.0	4.7	0.08
Fatty acids					
Total FA	15.9	14.9	11.8	13.2	0.37
C16:0	14.8	15.3	16.0	15.7	0.35
C18:1 c9	2.9	3.7	4.5	4.9	0.36
C18:2 n-6	11.0	11.9	13.9	14.6	0.58
C18:3 n-3	48.0	44.7	39.1	38.6	0.87

[1] – Net Energy calculated based on VEM system (Van Es, 1978);
[2] – Standard error

In order to maintain biological diversity, harvesting of the grasslands was not allowed before June 15th and hence the herbage was harvested on June 21st 2000. The pasture of IMS was from a sward growing on a clay soil, which received 112 kg N/ha on March 22nd 2000 and was harvested on May 5th 2000, in order to achieve a high quality of the forage. The forages were pre-wilted (< 72h) until a DM content of 600-750 g/kg and were ensiled in big bales of 400-600 kg. Table 3.1. represents some characteristics of the silages in terms of their chemical composition, net energy content and fatty acid composition. In addition to the silages (mean intake of 13.2 kg DM/day), the cows were fed 4.5 kg of concentrate (from the same production lot) per day (chemical and fatty acid composition shown in Table 3.2.).

Table 2.2. – Chemical composition (g/kg DM), net energy content (MJ/kg DM), total fatty acid content (g/kg DM) and proportion of predominant fatty acids (g/100 g of FAME) of the concentrate used in the four dietary treatments

		Predominant fatty acids	
Intake concentrate (kg DM/d)	4.5	Total fatty acids	68.8
DM (g/kg fresh material)	874	C12:0	25.9
Chemical composition		C16:0	13.7
Organic matter	907	C18:1 c9	18.0
Crude protein	247	C18:2 n-6	20.2
Neutral detergent fibre	311	C18:3 n-3	1.9
Net energy[1]	7.4		

[1] Net Energy calculated based on VEM system (Van Es, 1978).

Feed refusals were weighed daily and feed intake was measured during 48h, in the measuring week (Bruinenberg et al., 2003). Negative protein balances were avoided and negative energy balances did not occur as all cows were in an advanced stage of the lactation.

Measurements and sampling

The silage sampling procedure was as described by Bruinenberg et al. (2004). Briefly the samples of the silage mixtures were taken immediately after mixing for feeding and stored at -18ºC. Per experimental period one composite sample of silage was used for further analysis (n=4). The concentrate was sampled once per experimental

period, stored frozen (-18ºC) and one composite sample (from the four periods) was used for further analysis (n=1).

Consecutive milk samples were collected at two morning and two evening milkings, on days 2 and 3 of the measuring week. The samples were stored at -18ºC with potassium dichromate (1 tablet per 50 ml) as preservative (Merck, Darmstad, Germany).

Rumen contents were completely evacuated manually at 4:00, 10:00 and 20:00 of day 4 and at 9:00 of day 5 of the measuring week, and the animals were deprived of food between the latter 2 evacuations (Taweel et al., 2004; Van Vuuren et al., 1992). The contents were mixed thoroughly and a sample of 1 kg was taken, which was then freeze-dried and stored at ambient temperature until analysis.

Analysis

Lipids of feed samples were extracted following the method by Folch et al. (1957) with some adaptations as described by Raes et al. (2001). Briefly, from the frozen diet samples, a representative subsample of 5 g was weighed in an extraction tube and chloroform/methanol (C/M; 2/1, vol/vol) and 10 mg of internal standard (heptadecanoic acid, C17:0; Sigma Bornem, Belgium) were added. Samples were homogenized by an ultra-turrax mixer (Ultra-Turrax T25, IKA-Labortechnik, Belgium) and were extracted overnight. After extraction the samples were methylated (Raes et al., 2001). Fatty acid methyl esters (FAME) were identified by gas-liquid chromatography as described by Raes et al. (2001).

Fatty acids (FA) in rumen samples (2.5 g of freeze-dried material) were extracted overnight with 30 ml of chloroform/methanol (C/M; 2/1, vol/vol), 20 ml of distilled water and 10 mg of nonadecanoic acid (C19:0; Sigma, Bornem, Belgium) used as the internal standard. The extracts were centrifuged for 15 min at 1821 x *g*. This procedure was repeated twice by adding 20 ml C/M (2/1, vol/vol). The extracts were combined, washed once with distilled water to avoid the formation of methyl esters during further analysis of the samples and brought to a final volume of 100 ml with C/M (2/1, vol/vol). Fatty acids were methylated with NaOH in methanol (0.5 mol/l) followed by HCl in methanol (1/1 v/v), at 50ºC (Raes et al., 2001). Fatty acid methyl esters were extracted twice with 2 ml of hexane and pooled extracts were evaporated to dryness under N_2. The residue was dissolved in 1 ml hexane and analysed by gas chromatography Raes et al., (2004b).

Milk samples were extracted according to the isomethod (ISO-3889) using Rosein Gotlieb extraction tubes, in three steps (Vlaeminck et al., 2005). In the first step samples were extracted with ammonium hydroxide solution, ethanol, diethyl ether and petroleum ether. In the second step, samples were extracted with ethanol, diethyl ether and petroleum ether. In the final extraction step the solvents used were diethyl ether and petroleum ether. Extracts were combined, evaporated, methylated and analysed separately for short chain FA (C4:0-C10:0) and medium and long chain FA (C12:0-C24:0). Standard curves were used to determine the response factors for milk short chain fatty acids, taking into account tridecanoic acid (C13:0; Sigma Bornem, Belgium) as internal standard, whereas the other fatty acids were quantified with nonadecanoic acid as internal standard (C19:0; Sigma Bornem, Belgium), according to Vlaeminck et al. (2005).

Chromatography

Fatty acid methylesters were analyzed on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Brussels, Belgium) with a CP-Sil88 column for FAME (100 m x 0.25 mm x 0.2 µm; Chrompack Inc., Middelburg, The Netherlands). For more detailed information about the GC analysis of feed and rumen FA we refer to Raes et al. (2004b) and to Vlaeminck et al. (2005) for milk FA. Separation of the isomers C18:1 t10 and C18:1 t11 was possible in milk samples but not in rumen samples, due to the status of the GC column, which was new at the time of analysis of milk samples, compared to the analysis of the rumen samples, which took place 2 to 3 months later.

Calculations

1. Calculations of C18:3 n-3 biohydrogenation rates and duodenal flow of C18:3 n-3 were based on the afternoon intake (at 16:00), on the clearance rate as estimated from the intake at 16:00 - which equals the C18:3 n-3 amount at 0h - and the rumen evacuations at 4h (20:00) and 17h (9:00) after feeding and on the effective hydrogenation based on hydrogenation and passage rates: a) Clearance rate: The rumen clearance rate of a compound represents both the degradation as well as the passage rate (Taweel et al., 2004). Applied to polyunsaturated FA (PUFA), clearance rates (k_c) of PUFA can be assumed to represent both the hydrogenation as well as the passage rate: $k_c = k_h + k_p$, where k_h is the hydrogenation rate and k_p is the passage rate. The clearance

rate was estimated based on rumen pool size and first order rumen clearance kinetics as: $C = C_0 \times e^{(-kc \times t)}$, where C is the amount of C18:3 n-3 remaining in the rumen (g) at time t, C_0 is the intake of C18:3 n-3 (g), k_c is the clearance rate (%/h) and t is the time (h). Curve fitting was based on C18:3 intake at 16:00 (t = 0h) and rumen C18:3 n-3 pool size (C18:3 n-3 concentration x rumen volume) at 20:00 (t = 4h) and 9:00 (t =17h); b) Hydrogenation rate: The hydrogenation rate is calculated by subtracting the passage rate from the clearance rate, assuming the passage rate to be equal to the Acid Detergent Lignin (ADL) clearance rate (Taweel et al., 2004); c) Effective biohydrogenation: The effective biohydrogenation of C18:3 n-3 was then calculated as follows: *effective hydrogenation (g/d)* = C18:3 n-3 intake (g/d) x $[k_h/(k_h + k_p)]$ (Ørskov and McDonald, 1979); d) Relative biohydrogenation: The percentage of biohydrogenation can then be calculated as: *% Biohydrogenation* = [effective hydrogenation (g/d)/C18:3 n-3 intake (g/d)] x 100; e) daily duodenal flow of C18:3 n-3: Based on the effective biohydrogenation, the daily amount of C18:3 n-3 passing to the duodenum was calculated as: C18:3 n-3_d (g/d) = C18:3 n-3 intake (g/d) – effective hydrogenation (g/d), where C18:3 n-3_d is the amount of C18:3 n-3 that leaves the rumen into the duodenum.

2. Recovery of C18:2 n-6 and C18:3 n-3 in milk were calculated based on the amount of these FA in the milk and their intake from the silage and the concentrate, with a) milk secretion calculated as: *FA milk (g/d)* = [FA milk (g/100 g of FAME) x milk production (kg/d) x milk fat (%) x 1000]/10 000; b) intake as: *FA intake (g/d)* = [(FA silage (g/100 g of FAME) x silage intake (kg/d) x FA silage (g/kg DM))/100] + [(FA conc. (g/100 g of FAME) x conc. intake (kg/d) x FA conc. (g/kg DM)/100] and finally the c) proportion of dietary C18:2 n-6 or C18:3 n-3 recovered in the milk as: $Recovery_{feed}$ *(%)* = [FA milk (g/d) / FA intake (g/d)] x 100. The transfer efficiency of C18:3 n-3 and C18:2 n-6 from the duodenum to the milk was calculated as: $Recovery_{duodenum}$ *(%)* = [FA milk (g/d) / (FA duodenum (g/d))] x 100.

3. The ratios C14:1 c9/C14:0, C16:1 c9/C16:0 and CLA c9t11/C18:1 t11 give some measure of the activity of the Δ^9-desaturase enzyme (Fievez et al., 2003b; Lock et al., 2002). Nevertheless, recently, Palmquist et al. (2004) proposed a nutritional model which allows **calculation of the endogenous CLA c9t11 synthesis in the mammary gland**, from the amount of CLA and C18:1 t11 measured in the milk, according to the model: $CLATot_{in} = CLArum_i + \{[k_i/(1-k_i)] \times TVAis_{in}\}$, where $CLATot_{in}$ is the total

amount of CLA of the milk of the n^{th} animal on the i^{th} diet; $CLArum_i$ is the milk CLA from ruminal origin; k_i is the proportion of total C18:1 t11 that is converted to CLA c9t11 in the milk when fed the i^{th} diet, $TVAis_{in}$ is the amount of C18:1 t11 measured in milk and with amounts of all fatty acids expressed as g/100 g total fatty acids in the milk. Statistically this equation has the form of a simple regression: $CLATot_{ijn} = B^0_{ij} + B^1_{ij} TVAis_{ijn} + \varepsilon_{in}$, where, B^0_i is an intercept specific to diet, B^1_i is a slope specific to diet, and ε_{in} is an error term. In fact, B^0_i corresponds to $CLArum_i$ and k_i can then be calculated from B^1_i as follows: $k_i = B^1_i/(1+B^1_i)$.

Statistics

A split plot analysis for repeated measures (time sampling at 4:00, 10:00 and 20:00 of day 4 and 9:00 of day 5 of the measuring week) was used to evaluate the effect on rumen FA content and composition of dietary treatments, variations between animals and the interaction between diets and animals. The effect of sampling time was evaluated from the "within-subject-effect" (Wilks' lambda value is presented). The diets (100 IMS, 20 SPP, 60 SPP and 60 SPR) and the animals (1, 2, 3 and 4) were introduced as "between-subject-factors" and their effects evaluated from the "between-subject-effects", using orthogonal contrasts to evaluate dietary effects. Three orthogonal contrasts were applied: 1) diet 100 IMS *vs*. the other 3 diets (20SPP, 60SPP, 60SPR), to compare 100% intensively managed ryegrass silage with combined silages of IMS and another forage type; 2) diet 20 SPP *vs*. diets 60 SPP and 60 SPR, to evaluate the effect of proportion of semi-natural grassland in the diet; 3) diet 60 SPP *vs*. diet 60 SPR, to compare a species poor silage and a species rich silage.

A general linear ANOVA-model was used to evaluate the effect of diet, animal, sampling time and experimental period on milk FA content and composition, according to: $Y_{ijkl} = D_i + S_1 + C_j + P_k + \varepsilon_{ijkl}$, where Y_{ijkl} is the individual observation, D_i the effect of diet (fixed factor), S_1 the effect of sampling time (morning vs. evening sampling) (fixed factor), C_j the animal effect (random factor), P_k the effect of experimental period (random factor) and ε_{ijk} the residual error. Again the same three orthogonal contrasts as described before were applied.

Significances presented in Table 3.5. for k_i were accessed comparing the 95% confidence intervals for each slope of each diet. The comparisons were made in the same way as for the orthogonal contrasts, that is diet 100 IMS *vs*. the other 3 diets; diet 20 SPP *vs*. diets 60 SPP + 60 SPR and diet 60 SPP *vs*. diet 60 SPR.

All statistical analysis were performed using SPSS 11.0 (SPSS software for Windows, release 11.0, SPSS, Inc., USA).

RESULTS

Chemical composition and fatty acid characteristics of diets.

As shown in Table 3.1., silages and diets had similar organic matter contents. The crude protein was higher for diet 100 IMS and decreased as the percentage of intensive ryegrasss silage in the diet diminished (Table 3.1.). Net energy content of silage 60 SPR was 20% lower than that of 100 IMS silage. Nevertheless, no negative energy balances occurred during the experiment, as cows were in late stages of lactation (249 ± 76 days of lactation at the beginning of the experimental period). Indeed, the net energy supplied (115, 114, 105 and 110 MJ NE/d for diets 100 IMS, 20 SPP, 60SPP and 60 SPR, respectively), always covered the requirements of the animals (104, 97, 97 and 99 MJ NE/d when fed diets 100 IMS, 20 SPP, 60 SPP and 60 SPR, respectively). Calculations of net energy were made according to Van Es (1978).

It is clear that the dietary supply of C18:3 n-3 is mainly originating from the silages whereas both silages and concentrate provide C18:2 n-6 (Tables 3.1. and 3.2.). Moreover, switching from a diet typical for intensive production systems to a more diverse diet (in terms of both botanical composition and maturity of the plant species present in the forage), characteristic of extensive systems, increases C18:2 n-6 proportions whereas a decrease is observed in the concentration of C18:3 n-3.

Rumen fatty acid characteristics

Total FA content (mg/g DM) in the rumen decreased with decreasing proportions of intensive ryegrass silage in the diet (Table 3.3.). This also reflects differences in total rumen fatty acid pool size, as total rumen volume did not differ among diets.

As can be expected, rumen biohydrogenation resulted in an extensive reduction of the proportions of PUFA compared to the PUFA proportions in the diet (Tables 3.1. and 3.3.), as well as in an accumulation of biohydrogenation intermediates, with C18:1 t10 + t11 being the most abundant intermediate (2 to 4 g/100 g of total FAME, Table

2.3.). C18:1 t11; C18:1 c15; C18:2 t11c15 and C18:3 c9t11c15 have been suggested as the major hydrogenation intermediates of either C18:2 n-6 and C18:3 n-3. These intermediates represented a significantly higher proportion of total FAME when cows were fed diet 60 SPR (5.9 g/100 g of total FAME) compared to feeding intensive ryegrass silage only (100 IMS, 3.9 g/100 g of total FAME). Accumulation of these intermediates in the rumen of the cows fed diet 20 SPP and 60 SPP represented 3.9 and 4.4 g/100 g of total FAME, respectively.

Table 2.3. – Rumen volume (kg DM), total fatty acid content (mg/g DM) and fatty acid composition (g/100 g of FAME) of rumen contents (n=16).

	Sampling time				SEM[1]	Diet				SEM[1]	Statistics			
	4:00	10:00	20:00	9:00		100IMS	20SPP	60SPP	60SPR		Sign.[2]	Sign.[3]	Sign.[4]	Sign.[5]
Rumen volume	12.5	13.5	15.8	8.09	0.910	12.4	12.6	12.5	12.5	1.02	***	n.s.	n.s.	n.s.
Total fatty acids	32.9	32.9	30.6	34.6	0.706	41.1	35.4	27.8	26.8	0.572	n.s.	***	***	n.s.
C12:0	3.78	5.97	6.14	2.81	0.114	4.04	4.26	5.17	5.22	0.106	***	***	***	n.s.
C14:0	3.76	4.41	4.39	3.86	0.071	3.67	3.87	4.54	4.34	0.093	**	**	**	n.s.
C16:0	18.8	18.5	18.7	19.2	0.079	18.0	18.5	19.4	19.2	0.117	***	***	**	n.s.
C18:0	45.3	41.4	38.3	48.0	0.478	45.2	44.9	43.5	39.4	0.542	**	**	**	**
C18:1 t6 - t8	0.174	0.188	0.190	0.171	0.009	0.165	0.147	0.189	0.223	0.012	n.s.	n.s.	**	†
C18:1 t9	0.167	0.187	0.179	0.151	0.011	0.160	0.156	0.171	0.197	0.016	n.s.	n.s.	n.s.	n.s.
C18:1 t10 + t11	2.78	2.64	2.63	2.62	0.076	2.14	2.17	2.66	3.71	0.093	n.s.	**	***	***
C18:1 c9	3.80	4.50	4.77	3.48	0.086	3.77	3.87	4.06	4.85	0.097	***	**	**	**
C18:1 c11	1.19	1.06	1.32	1.27	0.032	1.15	1.14	1.15	1.40	0.043	**	n.s.	*	**
C18:1 c15	0.328	0.333	0.362	0.306	0.011	0.311	0.315	0.317	0.386	0.012	n.s.	†	*	**
C18:2 t11c15	0.587	0.631	0.746	0.389	0.025	0.527	0.498	0.582	0.745	0.032	***	†	**	*
CLA c9t11	0.085	0.067	0.109	0.067	0.013	0.068	0.080	0.103	0.077	0.012	n.s.	n.s.	n.s.	n.s.
CLA t10c12	0.071	0.070	0.071	0.046	0.006	0.045	0.035	0.054	0.124	0.007	*	*	**	***
C18:3 c9t11c15	0.074	0.091	0.110	0.089	0.012	0.075	0.086	0.099	0.103	0.017	n.s.	n.s.	n.s.	n.s.
C18:2 n-6	2.18	2.81	3.45	1.50	0.075	2.45	2.43	2.23	2.84	0.072	***	n.s.	n.s.	**
C18:3 n-3	2.04	2.55	3.60	1.01	0.131	2.96	2.34	1.59	2.30	0.157	***	**	†	*

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † - 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01 >p>0.001; *** - p<0.001;

[2] – Wilks' Lambda value for effect of sampling time;

[3] – Orthogonal contrast 100 IMS *vs.* other 3 diets;

4 – Orthogonal contrast 80 % of IMS in diet *vs.* 40 % of IMS in diet;

[5] – Orthogonal contrast 60SPP *vs.* 60SPR

Despite the significantly different dietary C18:3 n-3 proportions (Table 3.1.), cows fed diet 20 SPP and diet 60 SPR showed similar rumen C18:3 n-3 proportions (Table 3.3.). Nevertheless, percentage of biohydrogenation, as calculated from fractional rates of biohydrogenation and passage based on clearance rate of the rumen, remained similar across diets (93, 94, 93 and 94 % for diets 100IMS, 20 SPP, 60 SPP and 60 SPR, respectively).

Milk fatty acids characteristics

Total short chain FA in milk (C4:0 to C10:0) did not differ significantly when feeding different silages, varying between 9.3 g/100 g of total FAME in milk of cows fed 60 SPR to 9.7 g/100 g of total FAME for cows on diet 100 IMS (Table 3.4.). There was a higher proportion of medium chain fatty acids (C12:0 to C16:0) in milk of cows fed diet 100 IMS (48.8 g/100 g of total FAME), compared to feeding a combination of IMS silage and natural grassland products (on average 46.8 g/100 g of total FAME for diets 20 SPP, 60 SPP and 60 SPR) (Table 3.4.). Proportions of C18:0 for diets representing intensive systems (diet 100 IMS) and extensive systems (diet 60 SPR) were very similar (Table 3.4.).

As for the FA considered health promoting, like C18:1 c9 and PUFA there was a clearly lower proportion of PUFA in milk from cows on 100 IMS (19.5 g/100 g of total FAME) when compared to the other diets, particularly diet 60 SPR (21.2 g/100 g of total FAME). Despite these higher proportions of “healthy” FA when feeding the 60 SPR, omega-3 FA were proportionally lower for this diet (0.69 g/100 g of total FAME compared to 0.74 g/100 g of total FAME for diet 20 SPP and 0.75 g/100 g of total FAME for diet 100 IMS) (Table 3.4.). Consequently the n-6/n-3 ratio is slightly higher in milk of cows fed 60 SPR (1.4) compared to feeding 100 IM (1.1).

Table 3.4. – Milk production (kg/d), total fatty acid content (mg/g milk) and proportions of individual fatty acids (g/100 g of FAME) of milk (n=16).

Diet	100 IMS	20 SPP	60 SPP	60 SPR	SEM[1]	Sign.[2]	Sign.[3]	Sign.[4]
Milk production	21.9	20.2	20.5	20.5	0.917	n.s.	n.s.	n.s.
Total fat	40.9	39.9	39.4	42.3	0.161	n.s.	n.s.	n.s.
C4:0	3.99	4.13	4.27	4.29	0.143	n.s.	n.s.	n.s.
C6:0	2.10	2.12	2.08	2.00	0.065	n.s.	n.s.	n.s.
C8:0	1.19	1.18	1.11	1.03	0.037	†	*	n.s.
C10:0	2.46	2.42	2.14	1.94	0.075	**	**	†
C12:0	4.23	4.14	3.83	4.09	0.076	*	†	*
C14:0	11.8	11.5	10.6	10.8	0.197	**	**	n.s.
C14:1 c9	1.40	1.25	1.20	1.24	0.024	***	n.s.	n.s.
C16:0	30.0	29.1	28.7	29.7	0.289	*	n.s.	*
C16:1 c9	1.40	1.26	1.43	1.40	0.037	n.s.	**	n.s.
C18:0	7.63	8.47	8.20	7.83	0.157	**	*	n.s.
C18:1 t6 - t8	0.114	0.118	0.126	0.151	0.005	**	**	**
C18:1 t9	0.113	0.117	0.119	0.149	0.003	***	***	***
C18:1 t10	0.106	0.099	0.116	0.151	0.007	†	**	**
C18:1 t11	0.469	0.478	0.497	0.822	0.017	***	***	***
C18:1 c9	17.6	17.8	19.6	18.9	0.422	*	*	n.s.
C18:1 c11	0.370	0.423	0.561	0.512	0.023	***	**	n.s.
C18:1 c15	0.144	0.141	0.124	0.178	0.004	n.s.	*	***
C18:2 t11c15	0.099	0.090	0.083	0.177	0.005	**	***	***
CLA c9t11	0.259	0.240	0.264	0.438	0.012	**	***	***
CLA t10c12	0.014	0.009	0.013	0.033	0.002	*	***	***
C18:2 n-6	0.798	0.805	0.857	0.948	0.024	*	**	*
C18:3 n-3	0.612	0.604	0.549	0.590	0.013	†	*	*
C20:4 n-6	0.031	0.029	0.022	0.032	0.002	n.s.	n.s.	**
C20:5 n-3	0.095	0.105	0.075	0.067	0.007	n.s.	**	n.s.
C22:6 n-3	0.039	0.032	0.040	0.033	0.004	n.s.	n.s.	n.s.

[1] – Standard error of mean; n.s. – not significantly different ($p>0.1$); † - $0.1>p>0.05$; * - $0.05>p>0.01$; ** - $0.01>p>0.001$; *** - $p<0.001$;

[2] – Orthogonal contrast 100 IMS *vs.* other 3 diets;

[3] – Orthogonal contrast 80 % of IMS in diet *vs.* 40 % of IMS in diet;

[4] – Orthigonal contrast 60SPP *vs.* 60SPR;

Nevertheless, differences in milk C18:3 n-3 (Table 3.4.) were less than expected from the significantly reduced dietary C18:3 n-3 supply when feeding lower intensive ryegrass silage proportions (Table 3.1.), which resulted in a higher recovery of dietary C18:3 n-3 in milk when feeding 60 SPP (7.6 %) and 60 SPR (7.3%) compared to diets

with higher intensive ryegrass proportions (5.1 to 5.2 %; Table 3.5.). Rumen biohydrogenation intermediates of C18:2 n-6 and C18:3 n-3 (e.g. C18:1 t11, C18:1 c15, C18:2 t11c15) in milk of cows on diet 60 SPR represent 1.54 g/100 g of total FAME, whereas proportions in milk of cows fed diet 100 IM were slightly lower (1.05 g/100 g of total FAME; Table 3.4.).

Table 3.5. – Estimates of mammary Δ^9-desaturase activity and milk recoveries of dietary C18:2 n-6 and C18:3 n-3 (n=16).

Diet	100 IMS	20 SPP	60 SPP	60 SPR	SEM[1]	Sign.[2]	Sign.[3]	Sign.[4]
Ratio C14:1 c9/C14:0	0.121	0.109	0.114	0.114	0.002	**	n.s.	n.s.
Ratio C16:1 c9/C16:0	0.047	0.043	0.050	0.048	0.001	n.s.	**	n.s.
Ratio CLA c9t11/C18:1 t11	0.554	0.504	0.531	0.532	0.011	*	†	n.s.
k_i	0.355	0.334	0.346	0.348	0.039	n.s.	n.s.	n.s.
$Recovery_{feed}$ (%)								
C18:2 n-6	10.9	9.55	10.9	12.2	0.599	n.s.	*	n.s.
C18:3 n-3	5.11	5.16	7.63	7.31	0.394	*	**	n.s.

k_i – proportion of C18:1 t11 converted into CLA c9t11 in the mammary gland (Palmquist et al., 2004);

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † - 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001;

[2] – Orthogonal contrast 100 IMS *vs.* other 3 diets;

[3] – Orthogonal contrast 80 % of IMS in diet *vs.* 40 % of IMS in diet;

[4] – Orthogonal contrast 60SPP *vs.* 60SPR

Significant differences were found concerning the CLA isomers in milk upon replacing of the intensive ryegrass silage by SPP. Feeding a more species diverse silage significantly increased the levels of CLA isomers (0.47 g/100 g of total FAME) compared to feeding a species poor silage (0.28 g/100 g of total FAME; Table 3.4.). Estimates of Δ^9-desaturase activity in the mammary gland, based on the ratios C14:1 c9/C14:0 and CLA c9t11/C18:1 t11, revealed slightly higher activities when feeding diet 100 IMS compared to the other three diets (Table 3.5.). Nevertheless, calculations of endogenous CLA c9t11, based on variations upon animals in milk C18:1 t11 and CLA c9t11, revealed the proportion of C18:1 t11 converted to CLA c9t11 in the mammary gland to be similar for all diets (Table 3.5.).

DISCUSSION

Chemical composition of diet and fatty acid characteristics

Differences in FA composition of milk were not induced by differences in negative energy balance as net energy supply always covered net energy requirements (maintenance and production), as indicated before (Van Es, 1978).

Silage was the major dietary C18:3 n-3 source and the proportions of the latter decreased as the silage became more species diverse. Feeding lower percentages of intensive ryegrass silage reduced the daily intake of C18:3 n-3 (109 g/d, 95 g/d, 61 g/d and 70 g/d when feeding 100 IMS, 20 SPP, 60 SPP and 60 SPR respectively), but the recovery of this fatty acid in the milk was higher (7.63 and 7.31 %) in diets 60 SPP and 60 SPR.

Rumen fatty acid characteristics

Fatty acid composition of the rumen contents is in accordance to dietary FA composition for diets 100 IMS, 20 SPP and 60 SPP. However, the increased diversity of the 60 SPR diet presumably affected rumen hydrogenation, as suggested by significantly higher accumulation of hydrogenation intermediates for diet 60 SPR, indicating a less complete hydrogenation in the rumen (i.e., reduced accumulation of C18:0 in the rumen). Most probably, substances in herbs prevent complete hydrogenation of C18:3 n-3 and C18:2 n-6, with the conversion of C18:1 t11 to C18:0 being most sensitive to inhibition, as observed before, when supplementing for e.g. fish oil (Chow et al., 2004b; Loor et al., 2003a, 2004). In the current study, this is supported by the proportions of these fatty acids in the rumen, both expressed as proportion of total FAME (C18:0 – 39.4 g/100 g of total FAME; C18:1 t10+t11 – 3.71 g/100 g of total FAME for diet 60 SPR *vs.* C18:0 – 45.2 g/100 g of total FAME; C18:1 t10+t11 – 2.14 g/100 g of total FAME for diet 100 IMS) as well as proportion of the sum of C18-FA (C18:0 – 68.3 g/100 g of total C18; C18:1 t10+t11 – 8.4 g/100 g of total C18 for diet 60 SPR *vs.* C18:0 – 75.1 g/100 g of total C18; C18:1 t10+t11 – 6.3 g/100 g of total C18 for diet 100 IMS), with the latter units being most representative for evaluation of the extent of biohydrogenation (Chow et al., 2004a). Although the method used did not allow distinction between C18:1 t10 and C18:1 t11 isomers, *trans*-vaccenic acid (C18:1 t11) can be assumed to be the predominant isomer when feeding grass silage based diets

(Dewhurst et al., 2003c), in agreement with our milk FA profiles.

Milk fatty acid characteristics

The higher presence of hydrogenation intermediates in milk when fed the 60 SPR diet reinforces the data observed in the rumen contents. Increased CLA c9t11 content in milk of cows fed 60 SPR most probably is provoked by a higher supply of C18:1 t11 (Fievez et al., 2003b) in accordance to the observations in the rumen samples. Indeed, CLA is mainly produced in the mammary gland from C18:1 t11 through the activity of Δ^9-desaturase enzyme (Griinari et al., 2000). Presumably, differences in C18:1 t11 supply rather than in Δ^9-desaturase enzyme activity determine milk CLA c9t11 concentrations (Fievez et al., 2003b), as the C14:1 c9/C14:0 ratio (0.113 for diet 60 SPR and 0.121 for diet 100 IMS, Table 3.5.) suggested the latter to be lower when feeding a more diverse silage. Indeed, the ratios C14:1 c9/C14:0, C16:1 c9/C16:0 and CLA c9t11/C18:1 t11 give some measure of the activity of the Δ^9-desaturase enzyme activity. The most reliable ratio is C14:1 c9/C14:0 as all C14:0 and C14:1 c9 in milk fat is produced via *de novo* synthesis in the mammary gland. However, conclusions based on these product/substance ratios might be erroneous due to differences in substrate concentrations as well as in affinities of the desaturase for fatty acids of different chain length. The recently described approach, based on the estimations of the intercept and slope of the linear regression fitted to milk C18:1 t11 and CLA c9t11 proportions (Palmquist et al., 2004), revealed the amount of C18:1 t11 converted in CLA c9t11, in the mammary gland by *de novo* synthesis, to be similar among diets (Table 3.5.).

The similar milk C18:3 n-3 concentrations despite significant differences in dietary C18:3 n-3 supply is remarkable and provoked a higher recovery of dietary C18:3 n-3 in the milk for the 60 SPP and 60 SPR diets. This higher recovery could be due to a change in rumen metabolism of C18:3 n-3, when feeding a more species diverse silage, resulting in an increased amount of C18:3 n-3 reaching the duodenum. However, estimates of duodenal flow of C18:3 n-3 based on rumen pool size and first order rumen clearance kinetics (Harvatine and Allen, 2004) reveal the amount of C18:3 n-3 passing to the duodenum to be lower when feeding diet 60 SPR (4.68 g/d) or diet 60 SPP (4.30 g/d), compared to diets 100 IMS (7.81 g/d) and 20 SPP (5.13 g/d). Accordingly, these data suggest a higher transfer efficiency of C18:3 n-3 from the duodenum to the mammary gland for diets 60 SPR (104%) and 60 SPP (103%), than for diets 100 IMS (69.6%) and 20 SPP (94.7%). Others (Chilliard et al., 2000) also reported a curvilinear

relation between duodenal flow of C18:3 n-3 (g/d) and transfer efficiency from the duodenum to the milk. The reason for this varying transfer efficiency could be the necessity to maintain a certain level of milk fat plasticity by the animal (Chilliard et al., 2000).

It has long been recognised that medium chain FA may be detrimental to human health, opposite to oleic acid and PUFA, particularly n-3 FA. The latter are known to lower blood cholesterol concentrations and hence contribute positively to human health (Dietschy, 1998). Feeding decreasing proportions of IM silage increased oleic acid proportion in milk and reduced medium chain FA. Hence, taking together these observations, one could say dairy products from animals fed a diverse pasture might be health promoting, due to an increased content of oleic acid and CLA c9t11 and slightly reduced concentrations of medium chain FA. On the other hand, we have to be aware of the concomitant increase of C18:1 t11. Indeed, consumption of *trans* FA has been associated with an increased risk of coronary heart diseases although these associations generally do not apply to *trans* FA of animal origin (Parodi, 2004). *Trans*-vaccenic acid has been suggested to be even health promoting due to its possible conversion to CLA c9t11 in humans (Turpeinen et al., 2004).

CONCLUSIONS

Rumen and milk data of this study gave some evidence of a partial inhibition of the rumen biohydrogenation when replacing dietary intensive forage by semi-natural grassland products, resulting in a higher accumulation of biohydrogenation intermediates in the rumen and in an higher milk CLA content.

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Chapter 4

Effect of grazing pastures with different botanical composition by lambs on rumen fatty acid metabolism and fatty acid pattern of *Longissimus* muscle and subcutaneous fat

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Chapter 4

Effect of grazing pastures with different botanical composition by lambs on rumen fatty acid metabolism and rumen fatty acid pattern of *Longissimus* muscle and subcutaneous fat

In order to study the effect of grazing pastures with a different botanical composition on rumen and intramuscular fatty acid (FA) metabolism, 21 male lambs were assigned to three botanically different pastures: Botanically diverse (BD) (consisting for 65% of a variety of grass species); Leguminosa rich (L) (consisting for 61% of *Leguminosae*) and Intensive perennial ryegrass (IR) (with 69% *Lolium perenne*). Pastures were sampled weekly for 12 weeks for analysis of their FA content and composition and on nine occasions to determine the botanical composition. Ruminal and abomasal contents were sampled at slaughter and muscle and subcutaneous fat 24h after slaughter. All samples were prepared and analysed for FA composition. The L pasture showed a higher FA content (29.8 mg/g DM *vs.* 18.5 and 25.5 mg/g DM, for BD and IR pastures, respectively), but the sum of the proportions of the major polyunsaturated FA, C18:2 n-6 and C18:3 n-3, were similar for the three pastures (69.9, 69.4 and 71.1 g/100 g of FAME for BD, L and IR pastures, respectively). The BD pasture was richer in C18:2 n-6 (18.2 g/100 g of FAME), while IR pasture had a higher C18:3 n-3 content (57.2 g/100 g of FAME). Rumen data showed that animals grazing the BD pasture presented higher proportions of biohydrogenation intermediates, mainly C18:1 t11, C18:2 t11c15 and CLA c9t11, suggesting an inhibition of biohydrogenation. These changes were associated with shifts in the rumen microbial population as indicated by differences in the rumen pattern of volatile FA and of microbial odd and branched chain FA. In L pasture animals, the content of C18:2 n-6 and C18:3 n-3 in the abomasum and subcutaneous fat was higher. Finally, higher proportions of C20:4 n-6, C20:5 n-3 and C22:5 n-3 and higher indices for elongation and desaturation activity in the intramuscular fat of BD grazing animals suggest some stimulation of elongation and desaturation of long chain fatty acids, although this also might have been provoked partially by reduced fat deposition (due to a lower growth rate of the animals).

Keywords: Biohydrogenation, Fatty Acids, Grazing, Pastures

OBJECTIVES

The objectives of this experiment were to study the fatty acid (FA) content of three botanically different pastures and to examine whether grazing these pastures resulted in a modified rumen FA metabolism and FA pattern in muscle and subcutaneous fat of lambs.

MATERIALS AND METHODS

Animals

Twenty-one male lambs of similar genetic background ("Vlaams Kuddeschaap", a typical "herding" sheep breed), all born from yearling ewes and originating from an organic farm (Berendrecht, Belgium) were used for this experiment. Until the beginning of the experiment, the lambs were grazing with their mothers on the pastures of the organic farm of origin. At weaning, animals were assigned to one of three pastures, based on their age and live weight (n=7 each), i.e. a botanically diverse (BD), leguminosa rich (L) or intensive Perennial ryegrass (IR) pasture. It is obvious that in botanically diverse pastures not only the botanical composition is diverse but the maturity stages of the different plant species present differ. For better reading of this chapter we will refer to the latter pastures as to 'botanically diverse pastures' only. The average age and live weight at the onset of the experimental period was 86 (s.d. 9) days and 22.3 (s.d. 3.1) kg respectively, and did not significantly differ between groups.

Pastures

The experiment was carried out for 12 weeks (July 1st 2004 until September 22nd 2004). Animals were grazing *ad libitum* and did not receive any supplemental feed. Essential minerals were provided by a mineral block for sheep (Timac Potasco, Belgium), with the following mineral and micro element composition: Sodium (270 g/kg), Calcium (60 g/kg), Phosphor (2 g/kg) and Magnesium (1 g/kg)) and micronutrients (Zinc (18 000 mg/kg), Manganese (2 000 mg/kg), Iodine (100 mg/kg), Cobalt (40 mg/kg) and Selenium (10 mg/kg)).

The botanical composition of the pastures was determined according to De Vries (1933) on samples taken on nine occasions during the trial. A pooled sample from several sampling squares (100 cm^2) was used for the determination. The sampling squares were taken every 15 m by crossing the fields in zigzag, assuring that also the sides were sampled. Different plants inside the sampling square were identified and numerated by order of frequency. For each species the frequency was calculated and expressed as proportion of all investigated sample sites. In each sampling site, the most predominant species (ranking a) received a score of 3, the second predominant species (ranking b) 2 and the third (ranking c) a score of 1. All the other species identified got score 0. Per species Bi (that represents how many times a species received the ranking a, b and c) has been calculated as B = ((a) x 3) + ((b) x 2) + ((c) x 1). The relative importance of a species (B_{rel}i (%)) represents how many times a species received the ranking a, b and c and is calculated according to the formula: $B_{rel}i$ (%) = $Bi/\sum_{i=1}^{j} Bi$, with Bi as defined before and j the total number of species identified. This determination was made for every sampling.

Table 4.1. – Botanical composition (main species) of the three pastures (n=9)

Plant Family	BD	L	IR
Poaceae	*Agrostis sp.* bentgrass (38% ± 5.8) *Bromus hordeaceus* soft brome (18% ± 4.9) *Phleum pratense* timothy (9% ± 3.5)	*Lolium perenne* perennial ryegrass (19% ± 6.6) *Phleum pratense* timothy (14% ± 2.5)	*Lolium perenne* perennial ryegrass (69% ± 4.4) *Bromus hordeaceus* soft brome (17% ± 3.1) *Lolium multiflorum* Italian ryegrass (5% ± 2.6)
Asteraceae	*Carduus sp.* thistle (12% ± 6.6) *Taraxacum officinale* dandelion (4% ± 1.7)		
Ranunculaceae	*Rannunculus sp.* buttercup (5% ± 2.9)		
Fabaceae		*Trifolium repens* white clover (41% ± 5.8) *Medicago sativa* luzern (20% ± 5.8)	

The importance of each individual species over the entire trial period is reported which is the average of the nine calculated $B_{rel}i$ values. The botanical composition of the different pastures is presented in Table 4.1.

The BD and L pastures were situated on the farm of origin of the lambs (Berendrecht, Belgium, 51º20 N/04º28 E, 14 m.a.s.l.). These pastures were natural grasslands without any kind of fertilization. The IR pasture was situated at the experimental farm of the Ghent University (Melle, Belgium, 50º59 N/03º49E, 11 m.a.s.l.) and was fertilized on May 4th 2004 with 50 kg N/ha and 30 kg/ha of compound fertilizer (corresponding to 9 kg N/ha and 3 kg P_2O_5/ha); and on August 18th 2004 with 35 kg N/ha. Stocking density was lower than 850 kg live weight/ha in the BD pasture and lower than 1200 kg live weight/ha in the L and IR pastures. Height of the pastures was constant at 10 to 15 cm. This was achieved by mowing the fields on the first week of August 2004 or by adjusting stocking density when the growth of the pastures became excessive.

Measurements and sampling

During the experimental period, representative samples of the pastures were taken weekly for dry matter (DM), chemical composition determination and FA analysis. Sampling of the pastures was done according to an adapted technique described by Madeira de Carvalho (2002). Plant material was cut with scissors at a height of approximately 5 cm, every 10 m when crossing the fields on their width and assuring that the sides were also sampled. Thistles were however not cut, as careful observation of the lambs grazing showed animals were not consuming this species. After collection, the fresh samples of each pasture were mixed and a representative sub-sample (30 g) was stored immediately in liquid N_2 for 4h (maximum time of travel to the lab), after which the FA extraction was performed immediately.

At the end of the experimental period, the lambs were transported to a private abattoir (Ronse, Belgium) without prior fastening and slaughtered according to conventional practice. Ruminal (1 L) and abomasal (0.5 L) contents were sampled into plastic pots after thorough mixing, and kept refrigerated until arrival in the lab. To ensure correct sampling, the pH of both stomach contents was measured at three different locations in each stomach. Rumen samples were prepared for volatile fatty acid (VFA) analysis, as soon as they arrived in the lab. The sample residues were freeze-dried and kept at ambient temperature (one month) until analysis of FA. Samples

for VFA analysis were acidified with phosphoric/formic acid (10/1 vol/vol) and centrifuged for 15 min at 31 000 x *g*. The supernatant was recovered and 1 ml was transferred to vials and analysed by gas chromatography (Schimadzu GC-14A, Belgium), according to Van Nevel and Demeyer (1977).

Meat and subcutaneous fat samples were taken 24h after slaughter, from chilled carcasses (4ºC). Meat samples were taken from the *m. longissimus thoracis*, from the left side of the carcass (between T7 and T8). Meat and subcutaneous fat samples were stored vacuum packed at -20ºC until FA analysis.

Chemical composition analysis

Samples for chemical composition determination were dried at 50ºC for 48h, finely (0.5 to 1 mm) grinded (Grindomix GM 200, Retsch, Germany) and further analysed. Chemical composition analysis consisted of determination of crude protein, according to the Khjedal method (European Community, 1993), Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) using the Van Soest method (Van Soest et al., 1991), lignin according to the method described by Van Soest and Wine (1968) and crude fat with the Soxhlet method (International Organization for Standardization, 1973). Results are presented in Table 4.2.

Table 4.2. – Chemical composition of the three pastures, expressed as g/kg DM (n=3)

	BD	L	IR	SEM[1]	Sign.
DM (g/kg)	932[a]	926[b]	933[a]	0.154	*
Crude protein	98.8[b]	235[a]	148[b]	2.23	**
Crude fat	36.0	37.3	37.2 [2]	0.504	n.s.
ADF	342	293	327	2.63	n.s.
NDF	571[a]	396[b]	567[a]	3.60	*
Ash in NDF	37.6	27.6	24.8	0.332	n.s.
Lignin	54.0	70.7	43.7	0.938	n.s.
Net energy (MJ/kg DM)	1.23	3.14	1.86	-	-

[1] – Standard error of mean; [2] n=2;
n.s. – not significantly different (p>0.05); * - 0.05>p>0.01; ** - 0.01>p>0.001; [a,b,c] – Different superscripts in the same row differ significantly

Net energy (NE) of pastures was calculated as: *Net energy (MJ/kg DM)* = 0.55 x 0.82 x DE (MJ/kg DM), where DE is the digestible energy of the pastures. Digestible

energy was calculated based on NRC (2000), using the formula: *Digestible energy (MJ/kg DM)* = [4.22 – 0.115 x (ADF %) + 0.0332 (CP%) + 0.00112 (ADF%)] x 4.184 MJ.

Fatty acid analysis

- *Extraction*

Fatty acids of fresh grass samples (from the three different pastures) were extracted in triplicate with chloroform/methanol (C/M; 2/1, vol/vol), as described in Chapter 1. Briefly, 5 g of fresh material was cut into 1 cm stripes and homogenised for 1 min at 9000 rpm (Ultra-Turrax T25, IKA-Labortechnik, Belgium). The endogenous water was determined (105ºC for 4h) in order to adjust the ratio of chloroform/methanol/water to 8/4/3 (vol/vol/vol). In all samples, 40 ml of C/M (2/1, vol/vol) and 10 mg of nonadecanoic acid (C19:0; Sigma, Belgium) as internal standard were added and samples were extracted overnight. The next morning, samples were centrifuged at 1821 x *g* for 10 min and the C/M layer was recovered. In a second and third extraction step, 30 ml and 20 ml of C/M (2/1, vol/vol) respectively, were added and the samples were centrifuged at 1821 x *g* for 10 min for every extraction step. The extracts were combined and washed once with distilled water and the C/M layer was recovered. Finally, the extracts were brought to a final volume of 100 ml with C/M (2/1, vol/vol).

Rumen and abomasum samples were analysed in duplicate for FA as described for rumen samples in Chapter 3. Briefly 2.5 g of freeze-dried sample was extracted overnight with 30 ml of C/M (2/1, vol/vol), 20 ml of distilled water and 10 mg of nonadecanoic acid (C19:0; Sigma, Belgium) as internal standard. Samples were then centrifuged at 1821 x *g* for 10 min and the C/M layer was recovered. This procedure was repeated twice, adding 25 ml of C/M (2/1, vol/vol) in the second and 20 ml in the third extraction step. Finally, samples were washed with distilled water and the C/M layer was recovered. Extracts were brought to a final volume of 100 ml with C/M (2/1, vol/vol).

Meat samples were extracted in duplicate as described by Raes et al. (2001). Briefly, 5 g of meat was homogenised for 30 sec at 9000 rpm (Ultra-Turrax T25, IKA-Labortechnik, Belgium) and extracted overnight with 30 ml of C/M (2/1, vol/vol) and 3 ml of BHT in chloroform (0.1%, w/vol). Samples were then filtered (Fiorini, S.A.) and the filtrate was collected. The filter was washed twice with 10 ml of C/M (2/1, vol/vol).

The filtrate was transferred to the extraction tubes and 15 ml of distilled water was added. Samples were centrifuged at 1821 x *g* for 10 min and the C/M layer was recovered and evaporated with a rotavapor (Laborota 4000 WB, Germany) at 40ºC. The dry residue was then re-suspended in 10 ml of C/M (2/1, vol/vol).

Subcutaneous fat samples (1 g) were extracted using a similar procedure as described before for FA extraction of meat (Raes et al., 2001), however the bottom layer was recovered into volumetric flasks after washing with distilled water and was brought to a final volume of 100 ml with C/M (2/1, vol/vol).

- *Methylation*

For methylation of intramuscular and subcutaneous FA, 2 ml of extract was taken and 1 ml of nonadecanoic acid (2 mg/ml; C19:0; Sigma, Belgium) was added. For methylation of grass, rumen or abomasum FA, 10 ml of extract was used. Samples were methylated at 50ºC with NaOH in methanol (0.5M) followed by HCl/Methanol (1/1, vol/vol) according to Raes et al. (2001).

- *Gas Chromatography*

Fatty acids methylesters (FAME) were analyzed on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Belgium) with a CP-Sil88 column for FAME (100m x 0.25mm x 0.2 µm; Chrompack Inc., The Netherlands). For more detailed information about the GC analysis of rumen, abomasum, intramuscular and subcutaneous fat samples we refer to Raes et al. (2004b). For the grass FA the following temperature program was used: 150ºC for 2 min, followed by an increase at a rate of 1ºC/min until 200ºC. Temperature of the injector and detector was 250ºC and 280ºC, respectively. Separation of the isomers C18:1 t10 and C18:1 t11 was not possible due to the status of the GC column. CLA *cis-cis* (CLA cc) isomers and CLA *trans-trans* (CLA tt) isomers are reported as the sum of all CLA isomers with two *cis* and *trans* double bounds, respectively, as with the GC method used it is not possible to have a clear separation of these isomers.

Ciliates identification

- *Total DNA extraction and Polymerase Chain Reaction (PCR)*

Total DNA extractions of 0.5 g of rumen sample were performed following the method of Griffiths et al. (2000) with adaptations as described by Boon et al. (2003). Approximately 223 bp of the 18S rDNA gene were amplified using the ciliate protozoal-specific primers 316f and 539r (Sylvester et al., 2004) with a 42 bp GC-clamp

linked to the 5' terminus of the reverse primer. Amplification conditions were adapted from Sylvester et al. (2004) according to the following steps: initial denaturation at 94°C for 1 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 2 min; final extension at 72°C for 6 min. Amplicons were analysed by electrophoresis on a 1% agarose gel and visualized after staining with ethidium bromide.

- *Denaturating Gradient Gel Electrophoresis (DGGE) analysis*

DGGE was performed using the Bio-Rad D gene system (Bio-Rad, Hercules, CA) as described by Muyzer et al. (1993). PCR fragments were loaded onto a 7% (w/vol) polyacrylamide gel in 1 x TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 8.5) with denaturing gradients ranging from 45% to 60%. The electrophoresis was run for 16h at 60°C and 38V. DNA was visualized by staining with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene OR, USA). The obtained DGGE patterns were analysed with the BioNumerics software version 3.0 (Applied Maths, Kortrijk, Belgium) and clustered following Dice correlation.

- *Cloning and identification of ciliates*

The 16S rRNA gene fragments were cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Twenty four white clones were picked from LB agar plates (50 µg/ml ampicillin, 40 mg/ml X-gal in dimethylformamide) and grown in liquid LB medium overnight at 37°C. An aliquot of 800 µl was stored in 40% glycol (vol/vol) while plasmid DNA was isolated from the remaining liquid (4.2 ml) using the high pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing of the partial 16S rRNA fragments was performed by ITT Biotech (Bielefeld, Germany). Analysis of DNA sequences and sequence identity searches were completed with standard DNA sequencing programs and the BLAST server of the NCBI using the BLAST algorithm (Altschul et al. 1997) and BLASTN program for the comparison of a nucleotide query sequence against a nucleotide sequence database.

Statistics

A one way ANOVA was used to evaluate the effect of the different pastures on grass, rumen, abomasum, intramucular and subcutaneous fat FA and rumen VFA, according to $Y_i = \mu + B_i + \varepsilon_i$, where μ is the overall mean, B_i the effect of the different

pastures and ε_i the residual error. Comparison of means was done using Duncan as post-hoc test.

Principal component analysis (PCA), based on the correlation matrix, was conducted to determine components which account for most of the total variation in odd and branched chain fatty acids (OBCFA). Each object (animal x treatment, n = 21) was considered to be a data vector of 11 variables (*iso* C13:0, *anteiso* C13:0, C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0 and C17:0 all expressed as g/100 g of total OBCFA). The principal component scores are presented in a scatter plot to evaluate grouping of treatments (Figure 4.1.).

All statistical analyses were performed using SPSS 11.0 (SPSS software for Windows, release 11.0, SPSS inc., USA).

RESULTS

Live weight gain of the animals grazing pasture BD was very low and significantly lower than for animals grazing pastures L or IR. Average live weight at slaughter was 24.8 (s.d. 4.2) kg for the pasture BD group compared to 35.6 (3.8) and 36.4 (3.5) kg for the L and IR groups, respectively.

Chemical composition and fatty acid composition of the pastures

As shown in Table 4.2., protein content was lowest and NDF content was highest for BD pasture. Unexpectedly, NDF content of pasture IR was similar to the NDF content of pasture BD.

Fatty acid composition of the pastures is presented in Table 4.3. It is clear that pasture L had the highest total FA content (29.8 mg/g DM). Nevertheless, the proportions of polyunsaturated (PUFA; C18:3 n-3 + C18:2 n-6) were rather similar between groups, with the IR pasture being richer in C18:3 n-3 and the BD pasture having a higher proportion of C18:2 n-6.

Table 4.3. – Total fatty acid content (mg/g DM) and proportions of FA (g/100 g of FAME) of the grass samples taken during the 12 weeks of the experimental period, of the three different pastures (n=12)

	BD	L	IR	SEM[1]	Sign.
DM (g/100 g)	25.5[a]	17.1[b]	18.6[b]	0.014	***
Total fatty acids	18.5[c]	29.8[a]	25.5[b]	1.23	***
C12:0	0.622[a]	0.404[b]	0.599[a]	0.044	**
C14:0	1.82	2.06	2.04	0.147	n.s.
C16:0	14.5	15.1	14.4	0.403	n.s.
C16:1 c9	1.73[b]	2.14[a]	1.79[b]	0.081	**
C18:0	3.00	3.50	2.78	0.418	n.s.
C18:1 c9	3.83[a]	2.61[b]	2.88[b]	0.248	**
C18:2 n-6	18.2[a]	17.3[a]	13.9[b]	0.778	**
C18:3 n-3	51.7[b]	52.1[b]	57.2[a]	1.67	*
Total C18	76.8	76.7	75.5	0.638	n.s.

[1] – Standard error of mean; n.s. – not significantly different (p>0.05); * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001; [a,b,c] – Different superscripts in the same row differ significantly

Fatty acid composition of rumen and abomasum contents

Total rumen concentrations of VFA, proportions of individual VFA (mmol/mol total VFA) and some ratios are presented in Table 4.4. Fermentation patterns were as expected showing the highest acetate and lowest propionate proportions for the animals grazing BD pasture. Pasture L induced lower acetate and higher valerate and butyrate proportions.

Table 4.4. - Total volatile fatty acid content (mol/L) and relative proportions of individual VFA (mmol/mol total VFA) and pH in the rumen of the animals grazing three different pastures (n=7)

VFA	BD	L	IR	SEM[1]	Sign.
Total	0.108[b]	0.156[a]	0.109[b]	0.006	***
Acetate	672[a]	598[c]	636[b]	6.05	***
Propionate	174[b]	197[a]	197[a]	6.69	*
Butyrate	114[c]	146[a]	130[b]	4.08	***
Valerate	12.8[b]	18.2[a]	13.6[b]	0.484	***
Ratios					
Acetate/Propionate	3.25[b]	3.09[b]	3.91[a]	0.142	**
(Ac+But)/Prop	3.91[b]	3.84[b]	4.57[a]	0.175	*
Rumen pH	6.11	6.15	6.33	0.087	n.s.

Ac – Acetate; But – Butyrate; Prop – Propionate;
[1] – Standard error of mean; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001; [a,b,c] – Different superscripts in the same row differ significantly

Table 4.5. – Total medium and long chain fatty acid content (mg/g DM) and fatty acid composition (g/100 g FAME) of rumen contents of the animals grazing three different pastures (n=7)

Fatty acids	BD	L	IR	SEM[1]	Sign.
Total	45.2^{c}	55.4^{b}	63.6^{a}	1.56	***
C12:0	0.293^{a}	0.210^{b}	0.218^{b}	0.012	***
C14:0	0.741^{a}	0.618^{b}	0.812^{a}	0.039	**
C16:0	13.1	13.4	14.1	0.302	†
C18:0	53.1^{b}	55.4^{ab}	57.6^{a}	0.787	**
C18:1 t9	0.470^{a}	0.339^{b}	0.236^{c}	0.031	***
C18:1 t10+t11	7.29^{a}	4.72^{b}	4.47^{b}	0.365	***
C18:1 c9	3.51^{b}	4.69^{a}	3.89^{b}	0.238	**
C18:1 c15	0.185^{b}	0.456^{a}	0.406^{a}	0.043	**
C18:2 t11c15	0.867	0.803	0.625	0.069	†
CLA c9t11	0.113^{a}	0.068^{b}	0.057^{b}	0.010	**
CLA t10c12	0.172^{a}	0.077^{c}	0.125^{b}	0.012	***
CLAtt	0.084	0.094	0.084	0.009	n.s.
C18:3 c9t11c15	0.237^{b}	0.371^{a}	0.266^{b}	0.027	**
C18:2 n-6	1.53^{ab}	1.78^{a}	1.27^{b}	0.109	*
C18:3 n-3	1.79	1.77	1.87	0.098	n.s.
Total C18	72.6^{b}	75.1^{a}	74.5^{a}	0.311	***
Total OLCFA	3.07^{a}	2.65^{b}	2.39^{c}	0.072	***
Total BCFA	4.08^{a}	3.29^{b}	3.04^{b}	0.100	***
Total MUFA	16.5^{a}	16.1^{a}	14.1^{b}	0.661	*
anteiso C17:0	0.402^{a}	0.324^{b}	0.349^{b}	0.018	*
C18 fatty acids as % of total C18					
C18:0	73.2^{b}	73.8^{b}	77.3^{a}	1.03	*
C18:1 t9	0.648^{a}	0.452^{b}	0.316^{c}	0.043	***
C18:1 t10+t11	10.0^{a}	6.28^{b}	6.00^{b}	0.473	***
C18:1 c9	4.84^{b}	6.24^{a}	5.23^{b}	0.323	**
C18:1 c15	0.255^{b}	0.606^{a}	0.545^{a}	0.056	**
C18:2 t11c15	1.20^{a}	1.07^{ab}	0.839^{b}	0.096	*
CLA c9t11	0.156^{a}	0.094^{b}	0.079^{b}	0.013	**
CLA t10c12	0.238^{a}	0.106^{c}	0.172^{b}	0.017	***
CLAtt	0.115	0.130	0.128	0.013	n.s.
C18:3 c9t11c15	0.326^{b}	0.494^{a}	0.358^{b}	0.036	*
C18:2 n-6	2.11^{ab}	2.38^{a}	1.70^{b}	0.151	*
C18:3 n-3	2.47	2.49	2.37	0.136	n.s.

[1] Standard error of mean; n.s. – not significantly different (p>0.05); † - 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001; ^{a,b,c} – Different superscripts in the same row differ significantly;
Total OLCFA – Sum of odd linear chain fatty acids: C13:0, C15:0, C17:0 and C17:1 c9;
Total BCFA – Sum of branched chain fatty acids: *iso* C12:0, *iso* C13:0, *anteiso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, *iso* C16:0, *iso* C17:0 and *anteiso* C17:0;
Total MUFA – Sum of monounsaturated fatty acids: C14:1 c9, C15:1 c9, C16:1 t9, C16:1c9, C18:1 t6-t8, C18:1 t9, C18:1 t10+t11, C18:1 t12-t14, C18:1 c9, C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c14+t16 and C18:1 c15

Total long chain FA content and proportions of rumen contents are presented in Table 4.5. For rumen and abomasum contents, the proportions of C18-FA are also

expressed relative to the sum of all C18-FA, as this allows a better evaluation of rumen hydrogenation when dietary supply of C18-FA differs (Chow et al., 2004b). Rumen contents of BD pasture animals clearly had the lowest amount of total FA, reflecting the lower total FA content of the pasture samples. Proportions of C18:3 n-3 in rumen contents were very similar among treatments, whereas proportions of C18:2 n-6 were significantly higher in the rumen of animals grazing the L pasture compared to the IR pasture animals, with the BD pasture group being intermediate. Rumen contents of animals grazing the BD pasture contained higher proportions of intermediates (C18:1 t10+t11; C18:2 t11c15 and CLA c9t11) of the major rumen biohydrogenation pathways of C18:2 n-6 and C18:3 n-3 (8.27 g/100 g of FAME *vs.* 5.59 and 5.15 g/100 g of FAME for the BD *vs.* L and IR pastures, respectively). This is particularly true for the isomers C18:1 t10+t11 and for the CLA isomer c9t11, whereas other intermediates (e.g. C18:3 c9t11c15) of the major biohydrogenation pathway of C18:3 n-3 were reduced. When expressed relative to the sum of all C18-FA, the difference between the three treatments becomes even more obvious (Table 4.5.). Some intermediates of secondary biohydrogenation pathways (e.g. C18:1 t9, CLA t10c12) were also higher in the rumen contents of BD pasture animals, whereas end and intermediate products of other pathways (e.g. C18:1 c15) were significantly reduced.

Compared to the rumen, the abomasum contents were richer in total amount of FA and in saturated fat (Table 4.6.), which is not surprising considering the absorption from the rumen of fermentation end products of carbohydrates and proteins, and the biohydrogenation of unsaturated FA, respectively. IR and L pastures induced higher amounts of total FA but animals of pastures BD and L had lower amounts of C18:0, as for rumen samples. However, the proportion of biohydrogenation intermediates (C18:1 t10+t11, C18:1 c15, CLA c9t11, C18:2 t11c15 and C18:3 c9t11c15) was lower than in the rumen for all three groups (5.52, 5.20 and 5.32 g/100 g of FAME for animals of pasture BD, L and IR, respectively). Most of the differences found between the groups in the rumen samples were no longer apparent in the abomasum samples.

Similarly to what was observed in the rumen contents, proportions of C18:2 n-6 in the abomasum contents were significantly higher for the L pasture lambs than for the IR pasture lambs. Moreover - compared to both other groups - abomasal contents of L pasture lambs was significantly enriched in C18:3 n-3, although this difference has not been observed in the rumen.

Table 4.6. - Total fatty acid content (mg/g DM) and fatty acid composition (g/100 g of FAME) of abomasum contents of the animals grazing the three different pastures (n=7)

Fatty acids	BD	L	IR	SEM[1]	Sign.
Total	52.4^{b}	76.0^{a}	84.1^{a}	3.10	***
C12:0	0.244^{a}	0.138^{b}	0.100^{b}	0.015	***
C14:0	0.588^{a}	0.436^{b}	0.556^{a}	0.022	***
C16:0	11.9^{b}	11.5^{b}	12.6^{a}	0.243	*
C18.0	59.4^{b}	60.6^{b}	63.8^{a}	0.893	**
C18:1 t9	0.444	0.498	0.448	0.040	n.s.
C18:1 t10+t11	4.40	3.58	4.14	0.411	n.s.
C18:1 c9	4.76^{a}	4.89^{a}	3.47^{b}	0.313	**
C18:1 c15	0.522^{b}	0.831^{a}	0.564^{b}	0.052	**
C18:2 t11c15	0.335	0.417	0.391	0.035	n.s.
CLA c9t11	0.058	0.019	0.007	0.018	n.s.
CLA t10c12	0.105^{a}	0.049^{b}	0.091^{ab}	0.014	*
CLA tt	0.078^{b}	0.145^{a}	0.109^{ab}	0.017	*
C18:3 c9t11c15	0.200^{b}	0.350^{a}	0.219^{b}	0.028	**
C18:2 n-6	1.54^{a}	1.87^{a}	0.872^{b}	0.114	***
C18:3 n-3	1.59^{b}	2.00^{a}	1.24^{b}	0.133	**
Total C18	77.6^{c}	80.1^{a}	78.7^{b}	0.355	***
Total OLCFA	2.01^{a}	1.59^{c}	1.84^{b}	0.053	***
Total BCFA	2.56^{a}	2.23^{b}	1.89^{c}	0.062	***
Total MUFA	15.6^{a}	15.7^{a}	12.9^{b}	0.829	*
anteiso C17:0	0.310^{a}	0.189^{b}	0.220^{b}	0.018	***
C18 Fatty acids relative to the sum of all C18-Fatty acids					
C18:0	76.5^{b}	75.7^{b}	81.3^{a}	1.12	**
C18:1t9	0.573	0.622	0.568	0.048	n.s.
C18:1 t10+t11	5.66	5.19	4.46	0.503	n.s.
C18:1 c9	6.14^{a}	6.10^{a}	4.45^{b}	0.368	**
C18:1 c15	0.673^{b}	1.04^{a}	0.700^{b}	0.062	**
C18:2 t11c15	0.431	0.521	0.478	0.042	n.s.
CLA c9t11	0.075	0.024	0.071	0.043	n.s.
CLA t10c12	0.136^{a}	0.061^{b}	0.116^{a}	0.018	*
CLAtt	0.100^{b}	0.181^{a}	0.136^{ab}	0.021	*
C18:3 c9t11c15	0.259^{b}	0.437^{a}	0.291^{b}	0.035	**
C18:2 n-6	1.98^{a}	2.33^{a}	1.05^{b}	0.144	***
C18:3 n-3	2.06^{a}	2.50^{a}	1.49^{b}	0.168	**

[1] Standard error of mean; n.s. – not significantly different ($p>0.05$); * - $0.05>p>0.01$; ** - $0.01>p>0.001$; *** - $p<0.001$; ^{a,b,c} – Different superscripts in the same row differ significantly;
Total OLCFA – Sum of odd linear chain fatty acids: C13:0, C15:0, C17:0 and C17:1;
Total BCFA – Sum of branched chain fatty acids: *iso* C12:0, *iso* C13:0, *anteiso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, *iso* C16:0, *iso* C17:0 and *anteiso* C17:0;
Total MUFA – Sum of monounsaturated fatty acids: C14:1 c9, C15:1 c9, C16:1 t9, C16:1c9, C18:1 t9, C18:1 t10+t11, C18:1 t12-t14, C18:1 c9, C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c14+t16 and C18:1 c15

Rumen biohydrogenation intermediates as well as changes in rumen OBCFA can give an indication for changes in the rumen microbial population. Thus, OBCFA

were used in a biplot analysis (Figure 4.1.), to determine the components which account for most of the variation in OBCFA. The separation of the different dietary groups was mainly based on the first component, with a positive higher score for the BD pasture animals and a more negative score for the IR pasture animals. *Iso* C14:0 was more negatively correlated with the first component as well as C17:0 and *anteiso* C15:0, whereas *iso* C13:0, *iso* C15:0 and C15:0 were strongly positive correlated with the first component.

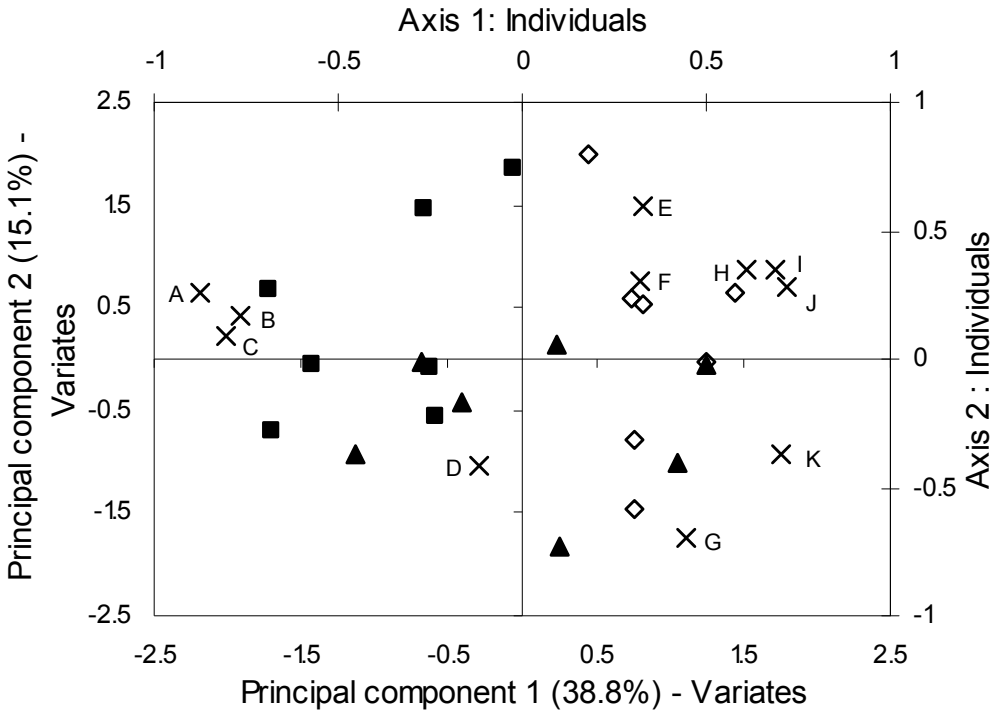

Figure 4.1. – Biplot representing both regression factor scores according to the pasture groups (Botanical diverse (◊), Intensive ryegrass (■), Leguminosa rich (▲)) and loadings (x) of the first two principal components, based on proportions (g/100 g of total OBCFA) of rumen OBCFA. The letters refer to individual OBCFA: A – *iso* C14:0; B – C17:0; C – *anteiso* C15:0; D – *anteiso* C17:0; E – *anteiso* C13:0; F – *iso* C17:0; G – C13:0; H – *iso* C16:0; I – *iso* C15:0; J – *iso* C13:0; K – C15:0.

Subcutaneous and intramuscular fatty acid composition

The FA content of subcutaneous fat was relatively low and its pattern was mainly a reflection of what was found in the abomasum (Table 4.7.), although some differences between groups were no longer significant (e.g. C18:0). Higher proportions for C18:3 n-3 and C18:2 n-6 were found in the subcutaneous fat of the animals grazing

the L pasture, which is consistent with the abomasum data. The proportion of CLA c9t11 in the subcutaneous fat was significantly higher for animals grazing BD pasture compared to the other pasture groups. Concerning other CLA isomers, BD pasture animals presented the lowest CLA cc and a trend for higher CLA t10c12 and lower CLA tt proportions.

Table 4.7. - Total fatty acid content (mg/g fat) and fatty acid composition (g/100 g of FAME) of subcutaneous fat of the animals grazing the three different pastures (n=7)

Fatty acids	BD	L	IR	SEM[1]	Sign.
Total	609	669	660	18.8	†
C12:0	0.644^{a}	0.131^{b}	0.225^{b}	0.065	***
C14:0	5.98^{a}	2.40^{b}	2.96^{b}	0.445	***
C16:0	20.5	19.8	18.2	0.668	†
C18:0	25.7	25.1	29.1	1.95	n.s.
C18:1 t9	0.667^{b}	0.802^{a}	0.441^{c}	0.040	***
C18:1 t10+t11	4.57^{a}	3.25^{b}	4.47^{a}	0.222	**
C18:1 c9	24.7	25.7	26.4	1.11	n.s.
C18:1 c15	0.232^{c}	0.468^{a}	0.309^{b}	0.023	***
CLA c9t11	1.32^{a}	0.676^{c}	1.01^{b}	0.091	**
CLA t10c12	0.100	0.084	0.101	0.006	†
CLA cc	0.013^{c}	0.027^{a}	0.019^{b}	0.001	***
CLA tt	0.044	0.047	0.058	0.004	†
C18:3 c9t11c15	0.196^{b}	0.267^{a}	0.238^{ab}	0.017	*
C18:2 n-6	1.19^{b}	2.35^{a}	0.862^{c}	0.078	***
C18:3 n-3	1.30^{b}	3.53^{a}	1.50^{b}	0.166	***
Total OLCFA	2.86	3.35	3.29	0.283	n.s.
Total BCFA	2.91^{a}	2.37^{b}	2.74^{a}	0.110	**
anteiso C17:0	0.666	0.763	0.780	0.059	n.s.
Total PUFA	4.63^{b}	7.78^{a}	4.55^{b}	0.287	***
Total MUFA	33.2	35.3	35.9	1.37	n.s.
Total SFA	55.7	50.3	53.4	1.68	n.s.
n-6/n-3 ratio	1.18^{a}	0.733^{b}	0.721^{b}	0.052	***
P/S ratio	0.046^{b}	0.125^{a}	0.048^{b}	0.005	***

[1] Standard error of mean; n.s. – not significantly different (p>0.05); † - 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001; ^{a,b,c} – Different superscripts in the same row differ significantly

Total OLCFA – Sum of odd linear chain fatty acids: C13:0, C15:0, C17:0 and C17:1;

Total BCFA – Sum of branched chain fatty acids: *iso* C12:0, *iso* C13:0, *anteiso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, *iso* C16:0, *iso* C17:0 and *anteiso* C17:0;

Total PUFA – Sum of polyunsaturated fatty acids: C18:2 t11c15, C18:2 n-6, C18:3 n-6, C18:3 n-3, CLA c9t11, CLA t10c12, CLA cc, CLA tt, C18:3 c9t11c15 and C20:2 n-6;

Total MUFA – Sum of monounsaturated fatty acids: C14:1 c9, C15:1 c9, C16:1 t9, C16:1c9, C17:1 c9, C18:1 t6-t8, C18:1 t9, C18:1 t10+t11, C18:1 t12-t14, C18:1 c9, C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c14+t16, C18:1 c15 and C20:1 c9;

Total SFA – Sum of saturated fatty acids: C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0 and C22:0;

n-6/n-3 ratio – Ratio between the sum of C18:2 n-6, C18:3 n-6 and C20:2 n-6, and C18:3 n-3;

P/S ratio – Ratio between the sum of C18:2 n-6 and C18:3 n-3, and the sum of C14:0, C16:0 and C18:0

Table 4.8. - Total fatty acid content (mg/g meat) and fatty acid composition (g/100 g of FAME) of intramuscular fat of the animals grazing the three different pastures (n=7)

Fatty acids	BD	L	IR	SEM[1]	Sign.
Total	16.0	24.4	19.6	2.58	†
C12:0	0.288	0.179	0.212	0.040	n.s.
C14:0	2.45	2.25	2.29	0.351	n.s.
C16:0	15.7^{b}	20.5^{a}	19.1^{a}	0.829	**
C18.0	16.6	17.4	19.1	0.696	†
C18:1 t	2.22	2.41	2.74	0.258	n.s.
C18:1 c9	22.8^{b}	28.9^{a}	29.3^{a}	1.13	**
C18:1 c15	0.170^{c}	0.346^{a}	0.259^{b}	0.025	***
CLA c9t11	0.897	0.738	0.903	0.097	n.s.
CLA t10c12	0.045	0.047	0.052	0.006	n.s.
CLA cc	0.042	0.064	0.052	0.006	†
CLA tt	0.061^{b}	0.123^{a}	0.093^{ab}	0.015	*
C18:3 c9t11c15	0.034	0.037	0.034	0.004	n.s.
C18:2 n-6	7.06^{a}	5.28^{b}	3.37^{c}	0.564	**
C18.3 n-3	2.64^{b}	3.99^{a}	2.59^{b}	0.235	**
C20:4 n-6	4.16^{a}	1.17^{b}	1.33^{b}	0.549	**
C20:5 n-3	2.76^{a}	1.09^{b}	1.33^{b}	0.315	**
C22:5 n-3	2.69^{a}	1.08^{b}	1.26^{b}	0.296	**
C22:6 n-3	0.427	0.293	0.340	0.037	†
Total OLCFA	2.04	2.37	2.12	0.128	n.s.
Total BCFA	5.13^{a}	2.96^{b}	3.76^{b}	0.360	**
Total PUFA	21.6^{a}	14.5^{b}	11.9^{b}	1.80	**
Total MUFA	28.9^{b}	35.0^{a}	35.6^{a}	1.31	**
Total SFA	37.7^{b}	42.6^{a}	43.0^{a}	1.32	*
n-6/n-3 ratio	1.37^{a}	1.05^{b}	0.902^{c}	0.033	***
P/S ratio	0.294^{a}	0.233^{ab}	0.148^{b}	0.029	**
Indices for elongation and desaturation activity (calculated as ratios of FA)					
C20:4 n-6/C18:2 n-6	0.543^{a}	0.393^{b}	0.219^{c}	0.043	***
C20:5 n-3/C18:3 n-3	1.02^{a}	0.518^{b}	0.273^{b}	0.103	***
C22:5 n-3/C18:3 n-3	0.993^{a}	0.487^{b}	0.271^{b}	0.094	***
C22:6 n-3/C18:3 n-3	0.161^{a}	0.076^{b}	0.131^{a}	0.012	***
C22:5 n-3/C20:5 n-3	0.991	1.00	0.944	0.036	n.s.
C22:6 n-3/C20:5 n-3	0.176^{b}	0.282^{a}	0.254^{a}	0.022	**

[1] Standard error of mean; n.s. – not significantly different (P>0.05); † - 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001; ^{a,b,c} – Different superscripts in the same row differ significantly;

Total OLCFA – Sum of odd linear chain fatty acids: C13:0, C15:0, C17:0 and C17:1 c9;

Total BCFA – Sum of branched chain fatty acids: *iso* C12:0, *iso* C13:0, *anteiso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, *iso* C16:0, *iso* C17:0 and *anteiso* C17:0;

Total PUFA – Sum of polyunsaturated fatty acids: C18:2 t11c15, C18:2 n-6, C18:3 n-6, C18:3 n-3, CLA c9t11, CLA t10c12, CLA cc, CLA tt, C18:3 c9t11c15, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6, C22:5 n-3 and C22:6 n-3;

Total MUFA – Sum of monounsaturated fatty acids: C14:1 c9, C15:1 c9, C16:1 t9, C16:1c9, C17:1 c9, C18:1 t (all C18:1 *trans* isomers) C18:1 c9, C18:1 c11, C20:1 c9 and C22:1 c9;

Total SFA – Sum of saturated fatty acids: C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0 and C22:0;

n-6/n-3 ratio – Ratio between the sum of C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6 and C22:4 n-6 and the sum of C18:3 n-3, C20:5 n-3, C22:5 n-3 and C22:6 n-3;

P/S ratio – Ratio between the sum of C18:2 n-6 and C18:3 n-3, and the sum of C14:0, C16:0 and C18:0

Total content of FA in the intramuscular fat did not differ between groups (Table 4.8.). However, significantly higher proportions of C18:3 n-3 were found in the intramuscular fat of the animals grazing the L pasture, being consistent with abomasum and subcutaneous fat data. On the other hand, proportions of C18:2 n-6 were significantly higher in the intramuscular fat of BD pasture animals. Neither intramuscular fat proportions of CLA c9t11, nor CLA t10c12 differed between the three treatments. CLA tt proportions were significantly higher and CLA cc proportions tended to be higher in intramuscular fat of L pasture animals. Proportions of C18:1 c9 were significantly higher for animals of L and IR pastures compared to the BD pasture animals. Concerning FA of longer chain length, significantly higher proportions of C20:4 n-6, C20:5 n-3 and C22:5 n-3 were present in the muscle of BD pasture animals, but no significant difference was found for C22:6 n-3. On the other hand, the content of these long chain FA (LCFA – FA with 20 or more C atoms) did not differ between groups (data not shown), except for C20:4 n-6 remaining significantly higher for the BD pasture lambs. Most indices of elongation and desaturation activity, as calculated by ratios of product to precursor FA, were significantly higher in muscle of BD grazing animals (Table 4.8.).

DISCUSSION

This study aimed to compare grazing pastures differing in botanical composition on FA metabolism in growing lambs. Stocking density was low and plant biomass was not limiting. Nevertheless, average daily weight gain of the BD pasture animals was very poor, as BD pasture could not meet the energy requirements for growing lambs (3.47 MJ NE/d, CVB, 2004) from the calculated NE presented in Table 4.1 (1.23 MJ/ kg DM), which means that lambs would have to consume 2.8 kg DM/d, which is not feasible. Moreover the low protein content might also have impaired rumen microbial growth (Hume et al., 1970; Orkie et al., 1977). Significantly higher proportions of hydrogenation intermediates, particularly C18:1 t10+t11, C18:2 t11c15 and CLA c9t11, were found in the rumen of animals grazing the BD pasture, despite the similar precursor supply for the different pastures. This suggests that other factors associated with BD pastures could provoke shifts in the rumen microbial population. Indeed, the

different pastures were associated with different rumen fermentation patterns suggesting different microbial populations, which is in accordance with changes in rumen OBCFA (Figure 4.1.). These FA have been suggested as rumen microbial markers (Vlaeminck et al., 2005). In this study, proportions of *iso* C17:0 were particularly increased in the rumen contents of BD pasture animals compared with the other groups (0.133 g/100 g of FAME *vs.* 0.088 and 0.071 g/100 g of FAME for BD *vs.* L and IR pasture animals, respectively, P=0.019). From their literature survey, Vlaeminck et al. (2006) observed a positive correlation between *iso* C17:0 and C18:1 t11, from which they suggested group B bacteria, responsible for the final hydrogenation step, to have lower *iso* C17:0 proportions. These suggested shifts in rumen microbial populations of BD pasture animals might have provoked the observed changes in rumen biohydrogenation intermediates.

Moreover, compared to rumen contents, treatment differences in accumulation of biohydrogenation intermediates disappear to a large extent in the abomasum contents and subcutaneous fat (Tables 4.6. and 4.7., respectively). This could suggest that changes in the extent of rumen biohydrogenation through feeding more botanically diverse forages are also associated with the rumen protozoa population, as the latter mainly sequestrate in the rumen or pass to the abomasum at a very low rate (Williams and Coleman, 1992). Although protozoa have been considered to play only a minor role in the rumen biohydrogenation process, Devillard et al. (2004) recently indicated higher CLA c9t11 proportions in rumen protozoa compared to bacteria. Moreover, in a detailed comparison of the FA profile of rumen protozoa and bacteria, Or-Rashid et al. (2007) found rumen protozoa to be richer in C18:1 t11, CLA c9t11 and C18:2 t11c15 (intermediates from the major biohydrogenation pathway of C18:2 n-6 and C18:3 n-3) than rumen bacteria. Hence we could assume the higher proportion of biohydrogenation intermediates in the rumen of BD pasture grazing animals to be associated with increased protozoal numbers and/or modified protozoal populations. Or-Rashid et al. (2007) have recently suggested *anteiso* C17:0 as a marker to quantify protozoal biomass, as *anteiso* C17:0 proportions were two times higher in rumen protozoa than in rumen bacteria. Indeed, *anteiso* C17:0 proportions were higher in the rumen and abomasum contents of BD pasture animals compared to the other two groups (Table 4.5. and 4.6., respectively), but differences disappeared in the SC fat (Table 4.7.). Again, we could assume an increase in protozoal numbers in the rumen contents of BD pasture animals *vs.* L and IR pasture animals. However, this is conflicting with the

lower rumen butyrate proportions (Newbold et al., 2001) and a defaunating effect that is often attributed to plant secondary metabolites and essential oils (Newbold et al., 2001), which might be present in some herbs of the BD pasture. Alternatively, shifts in the predominance of specific rumen protozoal species might have been induced by grazing the BD pasture. This was further investigated using molecular techniques. Cluster analysis of the DGGE profiles of rumen ciliates (Figure 4.2.) clearly showed the DGGE profiles of BD pasture animals to cluster, suggesting shifts in the rumen protozoal population of these animals. Cloning of the ciliates revealed the appearance of a specific protozoa species, *Diplodinium dentatum*, in the rumen content of BD pasture animals.

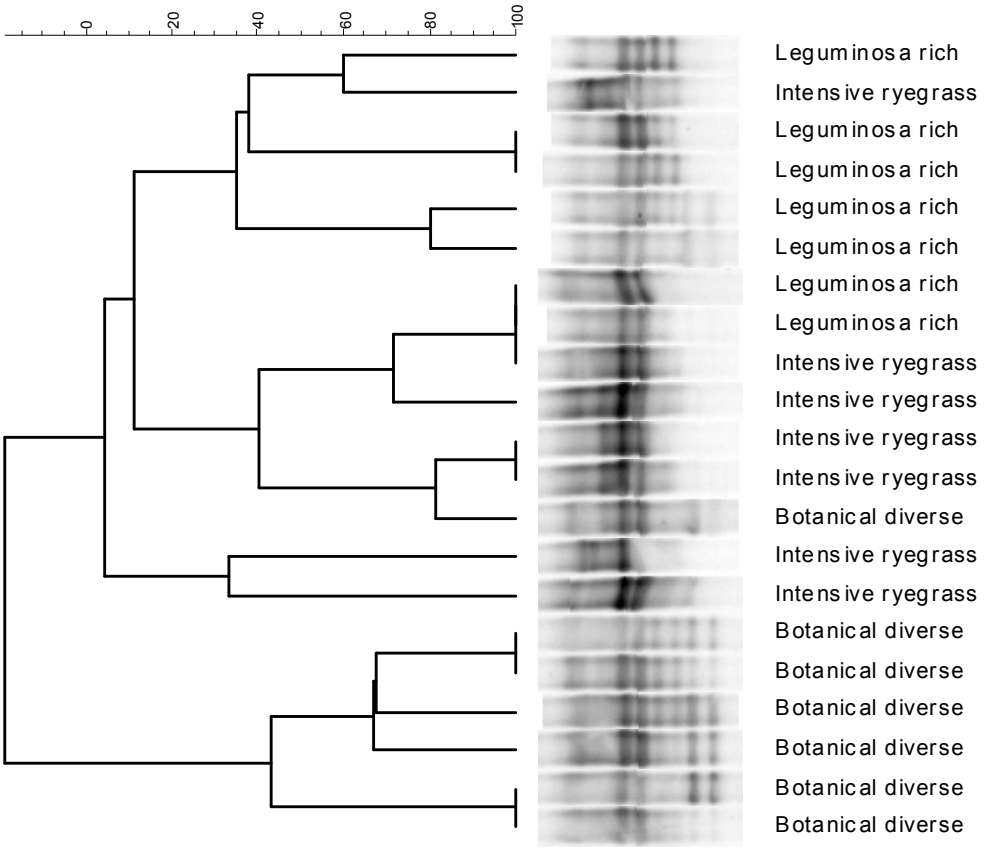

Figure 4.2. – Cluster analysis of the DGGE profiles of ciliates present in the rumen contents of animals grazing the three different pastures.

Another important finding in this study was the significantly higher proportions of C18:2 n-6 and C18:3 n-3 in the abomasum contents and subcutaneous fat but not in

the rumen contents of the L pasture animals. Differences in abomasum and subcutaneous fat suggest an increased duodenal flow of these PUFA, which might be induced either by reduced lipolysis through (physical) protection of the fatty acids, by a (partial) inhibition of microbial lipases or by reduced microbial "contact time" due to for e.g. higher outflow rates which might be the most probable reason. Indeed, the former are unlikely as differences in C18:2 n-6 and C18:3 n-3 proportions should then be obvious in the rumen also, which is not the case. Moreover, increased rumen outflow rates have been reported before for clover rich diets (Dewhurst et al., 2003a, b; Lee et al., 2003). Although our experimental design did not allow a quantitative evaluation of abomasal flows and rumen and abomasum contents have been sampled at one single time point at slaughter, this approach revealed valuable for a qualitative assessment of the rumen fatty acid metabolism.

Animals presented a low total amount of FA in the subcutaneous fat, compared with results of Enser et al. (1996), Wachira et al. (2002) and Cooper et al. (2004). This is most probably related to contamination of the subcutaneous fat by connective tissue. Concerning the FA metabolism, it is widely known that subcutaneous fat is more responsive to changes in the dietary fatty acid supply or changes in rumen metabolism than the intramuscular fat (Wachira et al., 2002; Demirel et al., 2004). This was also observed in the present study. Moreover, differences in intramuscular fat content additionally might complicate interpretation. Intramuscular fat of the BD pasture animals presented the highest proportions for most of the PUFA, in agreement with Ådnøy et al. (2005), who reported higher proportions of PUFA in intramuscular fat of lambs grazing mountain pastures, with a higher botanical diversity, than cultivated lowland pastures. Particularly, higher proportions of C20:4 n-6, C20:5 n-3 and C22:5 n-3 in intramuscular fat of the BD pasture animals were observed. Furthermore, indices for elongation and desaturation activity suggested some stimulation of the process involved in the production of these long chain PUFA in muscle. Nevertheless, a confounding effect with the lower intramuscular fat content of these animals and associated higher phospholipid/triacylglycerol ratios and long chain PUFA proportion cannot be excluded. This is confirmed by the considerably lower levels of C18:1 c9 in intramuscular fat of BD pasture animals compared to the other pasture groups. Indeed, oleic acid is the major FA present in the non-phospholipid fraction of meat, and proportions of oleic acid in the triacylglycerol and the polar lipid fraction were shown to increase with increasing fatness in beef cattle (Kazala et al., 1999; Itoh et al., 1999).

Obviously, the current experimental design only gives some indications of possible effects on rumen and intramuscular fatty acid metabolism as induced by botanically diverse pasture grazing. These effects and the interference with e.g. fat deposition merit further investigation.

CONCLUSIONS

From this study, we suggest that grazing different pastures induced changes in the rumen microbial population, which are most likely the reason for differences in biohydrogenation of PUFA. Furthermore, grazing a more diverse pasture might affect intramuscular FA metabolism as suggested from indices of PUFA desaturation and elongation, although differences between treatments in terms of absolute fat deposition might have provoked some confounding effect. Finally, higher PUFA proportions in abomasum, subcutaneous and intramuscular fat were observed in lambs grazing a leguminous rich pasture.

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Chapter 5

Effect of botanical composition of silages on rumen fatty acid metabolism and fatty acid composition in *Longissimus* muscle and subcutaneous fat of lambs

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Chapter 5

Effect of botanical composition of silages on rumen fatty acid metabolism and fatty acid composition in *Longissimus* muscle and subcutaneous fat of lambs

To study the effect of feeding silages with different botanical composition, on rumen and lamb fat, 30 male lambs were assigned to 5 different silage groups for 11 weeks: Botanically diverse (BDS); White clover (WCS); Red clover (RCS), Intensive Perennial ryegrass (IRS) and Maize silage and crushed linseed (MSL). Besides the silages, animals received organic wheat and barley and the MSL group additionally received bicarbonate (15g/d). Silages were sampled when the bales were opened and analysed for fatty acid (FA) content and chemical composition. At slaughter, ruminal contents were sampled and 24h after slaughter, *Longissimus* muscle and subcutaneous fat were sampled. All samples were analysed for FA composition. The MSL group ingested the highest amount of FA (35.8 g/d *vs.* 13.5, 19.4, 17.2 and 30.4 g/d for MSL *vs.* BDS, WCS, RCS and IRS respectively) and the sum of the major polyunsaturated FA, C18:2 n-6 and C18:3 n-3, was similar for groups BDS, WCS, RCS, and MSL (61.3, 62.3, 62.3, 63.7 g/100 g of FA methylesters (FAME), respectively), while group IRS ingested higher proportions of these FA (74.5 g/100 g of FAME). Rumen data showed that animals fed BDS presented higher proportions of biohydrogenation intermediates, particularly C18:1 t11 and CLA c9t11, suggesting partial inhibition of rumen biohydrogenation. In the MSL group, the content of C18:3 n-3 in the rumen was highest, most probably due to reduced lipolysis and hence biohydrogenation through the combined effect of esterified C18:3 n-3 and seed protection. Additionally, C18:3 n-3 proportions were higher in rumen contents of RCS animals compared with WCS animals, which could be due to the activity of the polyphenol oxidase enzyme in the RC silages. Proportions of C18:3 n-3 were similar between treatments both for subcutaneous and intramuscular fat, whereas CLA c9t11 content was higher in the subcutaneous fat of BDS animals and lower in the intramuscular fat of IRS animals compared to the other forage groups. No differences were found for C20:4 n-6, C20:5 n-3, C22:5 n-3 and C22:6 n-3 in the intramuscular fat of the animals. Nevertheless, indices for desaturation and elongation activity in muscle of BDS animals suggest some

stimulation of the first three steps of desaturation and elongation (Δ^6-desaturase, elongase and Δ^5-desaturase) of long chain FA.

Key words: Biohydrogenation, Botanical Composition, Fatty Acid Metabolism, Silages

OBJECTIVES

The objectives of this study were to describe the intramuscular and subcutaneous fatty acid (FA) composition in relation to (i) feeding intensive ryegrass *vs.* clover *vs.* botanically diverse silage; (ii) white *vs.* red clover silage feeding and (iii) C18:3 n-3 supply from forage silage *vs.* linseed. Moreover, rumen FA composition and muscle FA indices were used to assess some indicators for rumen and muscle FA metabolism.

MATERIALS AND METHODS

Animals

Thirty male lambs of similar genetic background ("Vlaams Kuddeschaap", a typical "herding" sheep breed), born from yearling ewes and originating from an organic farm (Berendrecht, Belgium) were used. Before the beginning of the trial, lambs were grazing with their mothers on pastures of the organic farm of origin. At weaning, animals were assigned based on their live weight and age to one of the five different groups (6 lambs per group), i.e. a group fed botanically diverse silage (BDS), rich white clover silage (WCS), rich red clover silage (RCS), intensive perennial ryegrass silage (IRS) or maize silage and crushed linseed (MSL). It is obvious that in botanically diverse silages not only the botanical composition is diverse but the maturity stages of the different plant species present differ. For better reading of this chapter we will refer to the latter silages as to 'botanically diverse silages' only. The average age and live weight at the onset of the experimental period was 118 (s.d. 8) days and 29.6 (s.d. 3.6) kg respectively, and did not differ significantly between groups. Animals of the same group were divided into two pens (three animals per pen).

Feeding and diets

The experiment lasted 11 weeks (July 5th 2005 until September 19th 2005). Animals were fed in the morning at 08:00 (700 g/kg DM of silage and 300 g/kg DM of a mixture of wheat and barley, separately). The amount of feed was adjusted per pen every 15 days to meet net energy (VEVI, Van Es, 1978; CVB, 2004) and protein (DVE,

Tamminga et al., 1994; CVB, 2004) requirements in accordance with the average growth rate of the three animals per pen. Essential minerals (Sodium (270 g/kg), Calcium (60 g/kg), Phosphorus (2 g/kg) and Magnesium (1 g/kg)) and micronutrients (Zinc (18 000 mg/kg), Manganese (2 000 mg/kg), Iodine (100 mg/kg), Cobalt (40 mg/kg) and Selenium (10 mg/kg)) were provided by a mineral block for sheep (Timac Potasco, Belgium).

Red clover, white clover and botanically diverse silages were provided in bales of approximately 270 kg. These silages were from natural grassland pastures situated at the farm of origin (Berendrecht, Belgium, 51º20 N/04º28 E, 14 m.a.s.l.) and without any type of fertilization. These silages were baled during the summer of 2004 (at the second and third cuts). The silages were wilted for 48h and no inoculum was used. Silage bales of intensive perennial ryegrass were made from a pasture with circa 70% of *Lolium perenne* – perennial ryegrass (the other 30% were mainly *Bromus hordeaceus* – soft brome – and *Lolium multiflorum* – Italian ryegrass) and fertilized with organic manure (30 to 40 ton/ha cow manure) at the end of February 2005, with 25 kg N/ha of on March 21st 2005 and with organic manure (25 ton/ha pig manure) after the first cut at the end of April 2005. Maize silage was produced from a maize crop fertilized with 30 to 40 ton/ha of cow or pig manure and 210 kg/ha of compound fertilizer (corresponding to 63 kg N/ha and 21 kg P_2O_5/ha). Both the perennial ryegrass pasture and the maize crop were situated at the experimental farm of Ghent University at Melle, Belgium (50º59 N/03º49 E, 11 m.a.s.l.).

In addition to the silages, all groups received organic ground wheat and barley grains. The ratio of wheat and barley was adapted during the experimental period to meet energy and protein requirements for growth. Animals in group MSL received extra crushed linseed in order to provide C18:3 n-3 in the range of the supply of the forage silages. The MSL group received also 15 g of sodium bicarbonate daily, in order to prevent rumen acidosis. Animals had free access to water.

Measurements and sampling

The silage portions were prepared per pen every time a bale was opened (on average every nine days for the groups BDS, WCS and RCS, and weekly for the IRS group). Maize silage portions were prepared weekly from the silo. All daily portions of silage for the different groups were kept in the fridge at 4ºC until fed to the animals. Wheat, barley and crushed linseed portions were prepared every three days. Leftovers of

silage were recovered daily and weighed to assess the average intake per pen. There were no leftovers of grains and linseed. The amount of silage and grains distributed and the barley/wheat ratio were adjusted according to the average weight and growth rate per pen in order to provide 110% of the energy (VEVI, Van Es, 1978; CVB, 2004) and protein (DVE, Tamminga et al., 1994; CVB, 2004) requirements.

All silages were sampled for FA analysis, dry matter (DM) determination and chemical composition at time of weighing. Wheat, barley and linseed were sampled every 4 weeks for FA analysis and chemical composition. Samples were taken directly to the lab where FA extraction and DM determination were performed immediately. Samples for chemical composition analysis were stored at -20ºC.

At the end of the experimental period, the lambs were transported to a private abattoir (Ronse, Belgium) without prior fasting and slaughtered according to conventional practice. Ruminal (1 L) contents were sampled into plastic pots after thorough mixing, and kept refrigerated until arrival in the lab. To ensure correct sampling, the pH of rumen contents was measured at three different locations. Rumen sub-samples (25 ml) were prepared for volatile fatty acid (VFA) analysis, as soon as they arrived in the lab. Samples were acidified with 0.5 ml of phosphoric/formic acid (10/1 vol/vol) and centrifuged for 15 min at 31 000 x *g*. The supernatant was recovered and 1 ml was transferred to vials and analysed by gas chromatography (Schimadzu GC-14A, Belgium), according to Van Nevel and Demeyer (1977). The rest of the rumen samples were freeze-dried and kept at -20ºC until analysis of FA.

Meat and subcutaneous (SC) fat samples were taken 24h after slaughter, from chilled carcasses (4ºC). Meat samples were taken from the *m. longissimus thoracis*, from the left side of the carcass (between T7 and T8). Meat and SC fat samples were stored vacuum packed at -20ºC until FA analysis. Meat samples were trimmed of external fat so that only intramuscular (IM) fat was extracted and analysed.

Chemical composition analysis

Silage samples for chemical composition determination were freeze-dried, ground through 1.5 mm mesh (Brabander, Duisburg, Germany) and further pooled per 4 weeks. Wheat, barley and crushed linseed were finely (0.5 to 1 mm) ground (Grindomix GM 200, Retsch, Germany) and further analysed. Chemical composition analysis consisted of determination of crude protein, according to the Kjeldahl method (European Community, 1993), acid detergent fibre (ADF) and neutral detergent fibre

(NDF) using the method of Van Soest et al. (1991), and crude fat with the Soxhlet method (International Organization for Standardization, 1973). Results are presented in Table 5.1.

Table 5.1. – Chemical composition of the five different silages (n=3) and of the grains (n=2) given to the animals

Feed	DM (g/kg)	NDF (g/kg DM)	ADF (g/kg DM)	Fat (g/kg DM)	Protein (g/kg DM)	Net energy (MJ/kg DM)
Silages						
Botanically diverse	668	579	383	26.4	94.4	0.324
White clover	609	530	348	32.5	113	1.19
Red clover	521	523	366	30.3	106	0.762
Intensive ryegrass	380	393	259	40.2	129	3.21
Maize	351	471	250	40.5	71.6	3.04
Grains						
Wheat	-	314	58.5	46.0	111	7.40
Barley	-	263	70.6	30.8	86.0	6.98
Linseed	-	336	175	394	186	5.37

Net energy (NE) of pastures was calculated as: *Net energy (MJ/kg DM)* = 0.55 x 0.82 x DE (MJ/kg DM), where DE is the digestible energy of the pastures. Digestible energy was calculated based on NRC (2000), using the formula: *Digestible energy (MJ/kg DM)* = [4.22 – 0.115 x (ADF %) + 0.0332 (CP%) + 0.00112 (ADF%)] x 4.184 MJ.

Fatty acid Analysis

- *Extraction*

Fatty acids of all silage samples were extracted in duplicate with chloroform/methanol (C/M; 2/1, vol/vol), as described in Chapter 4. Briefly, 5 g of fresh material were cut into 1 cm stripes and homogenised for 1 min (Ultra-Turrax T25, IKA-Labortechnik, Belgium). The endogenous water was determined (105ºC for 4h) in order to adjust the ratio of chloroform/methanol/water to 8/4/3 (vol/vol/vol). In all samples, 40 ml of C/M (2/1, vol/vol) was added, and 10 mg of nonadecanoic acid (C19:0; Sigma, Belgium) used as internal standard and samples were extracted overnight. The next morning, samples were centrifuged at 1821 x *g* for 10 min and the C/M layer was recovered. In the second and third extraction step, 30 ml and 20 ml of C/M (2/1,

vol/vol) respectively, were added and the samples were centrifuged at 1821 x *g* for 10 min for every extraction step. The extracts were combined and washed once with distilled water and the C/M layer recovered. Finally, the extracts were brought to a final volume of 100 ml with C/M (2/1, vol/vol).

Wheat, barley and linseed (finely – 0.5 to 1 mm – grinded (Grindomix GM 200, Retsch, Germany), and rumen samples (freeze-dried and finely grinded as for wheat, barley and linseed) were analysed in duplicate for FA as described in Chapter 3. Briefly, 2.5 g of sample was extracted overnight with 30 ml of C/M (2/1, vol/vol), 20 ml of distilled water and 10 mg of nonadecanoic acid (C19:0; Sigma, Belgium) as internal standard. Samples were then centrifuged at 1821 x *g* for 10 min and the C/M layer recovered. This procedure was repeated twice, adding 25 ml of C/M (2/1, vol/vol) in the second and 20 ml in the third extraction step. Finally, samples were washed with distilled water and the C/M layer was recovered. Extracts were brought to a final volume of 100 ml with C/M (2/1, vol/vol).

Meat samples were extracted in duplicate as described by Raes et al. (2001). Briefly, 5 g of meat was homogenised for 30 sec (Ultra-Turrax T25, IKA-Labortechnik, Belgium) and extracted overnight with 30 ml of C/M (2/1, vol/vol) and 3 ml of BHT in chloroform (0.1% w/vol). Samples were then filtered (Fiorini, S.A.) and the filtrate collected. The filter was washed twice with 10 ml of C/M (2/1, vol/vol). The filtrate was then transferred to the extraction tubes and 15 ml of distilled water added. Samples were centrifuged at 1821 x *g* for 10 min and the C/M layer recovered and evaporated with a rotavapor (Laborota 4000 WB, Germany) at 40ºC. The dry residue was then re-suspended in 10 ml of C/M (2/1, vol/vol).

Subcutaneous fat samples (1 g) were extracted using a similar procedure as described above for FA extraction of meat (Raes et al., 2001), however the bottom layer was recovered into volumetric flasks after washing with distilled water and was brought to a final volume of 100 ml with C/M (2/1, vol/vol).

- *Methylation*

For methylation of intramuscular (IM) and subcutaneous (SC) lipids, 2 ml of extract was taken and 1 ml of nonadecanoic acid (2 mg/ml; C19:0; Sigma, Belgium) was added. For methylation of silage, wheat, barley, linseed and rumen lipids, 10 ml of extract was used. Samples were methylated at 50ºC with NaOH in methanol (0.5M) followed by HCl/Methanol (1/1, vol/vol) according to Raes et al. (2001).

- *Gas chromatography*

Fatty acid methylesters (FAME) were analyzed on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Belgium) with a CP-Sil88 column for FAME (100m x 0.25mm x 0.2 µm; Chrompack Inc., The Netherlands). For more detailed information about the GC conditions for analysis of silage, wheat, barley, linseed, rumen, intramuscular and subcutaneous fat samples we refer to Raes et al. (2004b). Separation of the FA C16:1 t9 and *iso* C17:0 was not possible due to the status of the GC column. Conjugated linoleic acid *cis-cis* (CLA cc) isomers and CLA *trans-trans* (CLA tt) isomers are reported as the sum of all CLA isomers with two *cis* or *trans* double bounds, respectively, as with the GC method used it was not possible to separate all CLA cc and all CLA tt isomers.

Statistics

A one-way ANOVA was used to compare the feed FA content and composition of each group and to evaluate the effect of the different diets on rumen, intramuscular and subcutaneous fat FA and rumen VFA, according to $Y_i = \mu + B_i + \varepsilon_i$, where μ is the overall mean, B_i the effect of the different silages and ε_i the residual error. Five orthogonal contrasts were applied: (1) MSL diet *vs.* the 4 other diets to compare the supply of forage C18:3 n-3 (mainly unesterified) *vs.* linseed C18:3 n-3 (mainly in triacylglycerols); (2) BDS diet *vs.* WCS + RCS diets to compare botanical diversity with clover rich diets; (3) BDS diet *vs.* IRS diet; (4) WCS + RCS diets *vs.* IRS diet to compare clover rich diets with ryegrass; (5) WCS diet *vs.* RCS diet.

Principal component analysis (PCA), based on the correlation matrix, was conducted to determine components which account for most of the total variation in odd and branched chain FA (OBCFA). Each object (animal x treatment, n=30) was considered to be a data vector of 11 variables (*iso* C13:0, *anteiso* C13:0, C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *anteiso* C17:0, C17:0 and C17:1 c9 all expressed as g/100 g of FAME). The principal component scores are presented in a scatter plot to evaluate grouping of treatments (Figures 5.1. and 5.2.).

All statistical analyses were performed using SPSS 12.0 (SPSS software for Windows, release 12.0, SPSS inc., USA).

RESULTS

Live weight gain of the animals in group BDS tended to be lower compared to the animals of the other groups, with an average live weight at slaughter of 33.0 kg for the BDS group compared to 37.7, 35.1, 40.8 and 37.9 kg for the WCS, RCS, IRS and MSL group respectively. The total amount of BDS diet ingested by the lambs could not meet the energy requirements for lambs (CVB, 2004), 4.5 MJ NE/d vs. 4.7MJ NE/d for NE ingested and required, respectively.

Diets

In Table 5.2., total average individual FA and proportions of FA ingested by the animals is presented. For the first four diets, 96% of all FA is reported, whereas for diet MSL, 98% of all FA is reported.

Table 5.2. – Total average individual DM (kg/d) and fatty acid (g/d) intake, and proportions of FA (g/100 g FAME) ingested by the animals fed the five different diets (n=11)

	BDS	WCS	RCS	IRS	MSL	SEM[1]	Sign[2]	Sign[3]	Sign[4]	Sign[5]	Sign[6]
Total DMI	1.02	1.24	1.11	1.16	1,29	0.075	†	n.s.	n.s.	n.s.	n.s.
Total FA	13.5	19.4	17.2	30.4	35.8	3.10	***	***	***	***	n.s.
C12:0	0.232	0.139	0.288	0.162	0.075	0.020	***	n.s.	*	*	***
C14:0	0.500	0.462	0.679	0.465	0.210	0.030	***	†	n.s.	**	***
C16:0	18.5	17.7	17.7	14.9	11.5	0.237	***	**	***	***	n.s.
C16:1 c9	1.21	1.33	1.23	1.34	0.094	0.065	***	n.s.	n.s.	n.s.	n.s.
C17:0	0.283	0.270	0.275	0.158	0.129	0.009	***	n.s.	***	***	n.s.
C18:0	2.57	2.50	2.68	1.79	2.99	0.097	***	n.s.	***	***	n.s.
C18:1 c9	6.50	5.90	5.78	3.72	17.6	0.329	***	*	***	***	n.s.
C18:2 n-6	28.1	26.0	24.8	18.6	36.7	0.776	***	**	***	***	n.s.
C18:3 n-3	33.2	36.3	37.5	49.7	27.0	1.28	***	*	***	***	n.s.
Total C18	71.6	71.7	71.4	74.5	85.2	0.417	***	n.s.	***	***	n.s.
Total OBCFA	3.65	3.84	3.89	4.01	0.687	0.129	***	n.s.	*	n.s.	n.s.
Phytanic acid	0.077	0.071	0.009	0.019	0.151	0.011	***	**	***	n.s.	***

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † – 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001;
[2] – Orthogonal contrast MSL *vs.* Forages; [3] – Orthogonal contrast BDS *vs.* Clover diets; [4] – Orthogonal contrast BDS *vs.* IRS; [5] – Orthogonal contrast Clover diets *vs.* IRS; [6] – Orthogonal contrast WCS *vs.* RCS;
Total OBCFA – sum of all odd and branched FA: *iso* C13:0; *anteiso* C13:0; C13:0; *iso* C14:0; *iso* C15:0; *anteiso* C15:0; C15:0; *iso* C16:0; *iso* C17:0; *anteiso* C17:0; C17:0; C17:1 c9

Animals of the MSL group ingested the highest total amount of FA, followed by animals of group IRS, with animals fed the BDS diet ingesting the lowest amount.

Proportions of C16:0 in the MSL diet were significantly lower than for the four forage diets. Proportions of C18:2 n-6 were significantly higher for the group MSL, followed by BDS, WCS and RCS groups and were lowest for the IRS diet. On the other hand, proportions of C18:3 n-3 were lowest for the MSL group and highest for the IRS diet. Nevertheless, in terms of C18:3 n-3 intake, the MSL group was intermediate (9.67 g/d) between the IRS (15.1 g/d) and the clover diets (7.04 and 6.45 g/d, respectively), whereas the BDS group ingested the lowest C18:3 n-3 amount (4.48 g/d). Proportions of polyunsaturated FA (PUFA; C18:2 n-6 + C18:3 n-3) were similar for diets BDS, WCS, RCS and MSL (61.3, 62.3, 62.3 and 63.7 g/100 g of FAME for BDS, WCS, RCS and MSL respectively) while this was higher for diet IRS (74.5 g/100 g of FAME).

Fatty acid composition of rumen contents

Total rumen concentrations of VFA, proportions of individual VFA and VFA ratios are presented in Table 5.3. Animals in the MSL group presented a significantly lower proportion of acetate and a significantly higher proportion of propionate compared to the forage silage fed groups. Proportions of butyrate did not differ significantly between the five groups, with only a trend (P=0.060) for animals in BDS group to show higher butyrate proportions than animals in the IRS group. Lambs in the IRS group tended to have higher valerate proportions compared to lambs in the BDS (P=0.084) group and in the WCS+RCS group (P=0.083).

Table 5.3. – Total volatile fatty acid concentration (mmol/L) and relative proportions of VFA (mmol/mol total VFA) in the rumen of animals fed the five different diets (n=6)

	BDS	WCS	RCS	IRS	MSL	SEM[1]	Sign[2]	Sign[3]	Sign[4]	Sign[5]	Sign[6]
Total	128	136	147	120	112	9.00	*	n.s.	n.s.	†	n.s.
Relative proportions of VFA											
Acetate	698	720	722	685	592	22.1	***	n.s.	n.s.	n.s.	n.s.
Propionate	137	134	137	168	223	18.7	**	n.s.	n.s.	n.s.	n.s.
*Iso*butyrate	8.62	7.62	7.42	8.62	31.0	2.99	***	n.s.	n.s.	n.s.	n.s.
Butyrate	140	125	115	111	124	10.5	n.s.	n.s.	†	n.s.	n.s.
*Iso*valerate	7.84	5.52	7.04	6.84	14.7	1.55	***	n.s.	n.s.	n.s.	n.s.
Valerate	9.16	8.92	12.4	20.5	15.6	4.46	n.s.	n.s.	†	†	n.s.
Rumen pH	5.67	5.49	5.56	5.42	5.84	0.112	*	n.s.	n.s.	n.s.	n.s.

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † – 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001;
[2] – Orthogonal contrast MSL *vs.* Forages; [3] – Orthogonal contrast BDS *vs.* Clover diets; [4] – Orthogonal contrast BDS *vs.* IRS; [5] – Orthogonal contrast Clover diets *vs.* IRS; [6] – Orthogonal contrast WCS *vs.* RCS

Table 5.4. – Total concentration (mg/g DM) and proportions of individual FA (g/100 g FAME) in rumen contents of animals fed the five different diets (n=6)

Fatty acids	BDS	WCS	RCS	IRS	MSL	SEM[1]	Sign[2]	Sign[3]	Sign[4]	Sign[5]	Sign[6]
Total	24.6	33.6	33.5	50.2	75.1	3.54	***	*	***	**	n.s.
C12:0	0.186	0.175	0.166	0.117	0.122	0.028	n.s.	n.s.	†	n.s.	n.s.
C14:0	0.834	0.703	0.635	0.492	0.188	0.052	***	*	***	*	n.s.
C16:0	22.5	19.0	18.8	14.3	11.6	0.511	***	***	***	***	n.s.
C18:0	40.3	48.4	44.1	53.8	59.0	2.17	***	*	***	*	n.s.
C18:1 t9	0.098	0.115	0.156	0.163	0.139	0.017	n.s.	†	*	n.s.	n.s.
C18:1 t10	0.271	0.315	0.443	0.489	0.926	0.122	***	n.s.	n.s.	n.s.	n.s.
C18:1 t11	2.51	2.01	2.20	1.05	1.63	0.278	n.s.	n.s.	**	**	n.s.
C18:1 c9	5.70	4.05	4.64	5.03	5.81	0.467	†	*	n.s.	n.s.	n.s.
C18:1 c15	0.070	0.040	0.073	0.109	0.068	0.013	n.s.	n.s.	†	**	†
C18:2 t11c15	0.185	0.294	0.316	0.179	0.227	0.037	n.s.	*	n.s.	*	n.s.
CLA c9t11	1.31	0.788	0.589	0.330	0.517	0.123	†	***	***	*	n.s.
CLA t10c12	0.137	0.077	0.074	0.053	0.027	0.018	**	*	**	n.s.	n.s.
CLA cc	0.056	0.088	0.067	0.070	0.023	0.024	†	n.s.	n.s.	n.s.	n.s.
CLA tt	0.097	0.070	0.113	0.381	0.148	0.032	n.s.	n.s.	***	***	n.s.
C18:3 c9t11c15	0.141	0.171	0.148	0.158	0.091	0.077	n.s.	n.s.	n.s.	n.s.	n.s.
C18:2 n-6	5.91	4.29	4.97	2.92	5.46	0.753	n.s.	n.s.	*	†	n.s.
C18:3 n-3	1.00	1.62	2.61	1.76	4.87	0.386	***	*	n.s.	n.s.	†
Total C18	59.7	64.3	63.7	71.2	82.4	0.981	***	**	***	***	n.s.
Total MUFA	13.2	11.9	14.3	15.1	12.6	1.12	n.s.	n.s.	n.s.	n.s.	n.s.
Total OBCFA	8.00	6.27	6.65	4.99	2.94	0.371	***	**	***	**	n.s.
C16:1t9+*iso*C17:0	1.24	0.726	0.629	0.430	0.424	0.061	***	***	***	**	n.s.
iso C14:0	0.170	0.177	0.253	0.239	0.019	0.029	***	†	n.s.	†	n.s.
C18 Fatty acids relative to the sum of all C18-Fatty acids											
C18:0	67.5	75.2	69.1	75.5	71.5	2.54	n.s.	n.s.	*	n.s.	†
C18:1 t9	0.164	0.179	0.246	0.230	0.170	0.122	n.s.	n.s.	†	n.s.	†
C18:1 t10	0.449	0.490	0.697	0.688	1.13	0.151	**	n.s.	n.s.	n.s.	n.s.
C18:1 t11	4.23	3.12	3.46	1.48	1.99	0.396	*	†	***	**	n.s.
C18:1 c9	9.62	6.31	7.30	7.07	7.08	0.689	n.s.	**	*	n.s.	n.s.
C18:1 c15	0.118	0.063	0.116	0.153	0.082	0.018	n.s.	n.s.	n.s.	*	*
C18:2 t11c15	0.320	0.457	0.499	0.252	0.274	0.058	n.s.	*	n.s.	**	n.s.
CLA c9t11	2.22	1.23	0.930	0.465	0.628	0.204	*	***	***	*	n.s.
CLA t10c12	0.236	0.121	0.116	0.074	0.033	0.033	**	**	**	n.s.	n.s.
CLA cc	0.095	0.137	0.106	0.099	0.029	0.039	†	n.s.	n.s.	n.s.	n.s.
CLA tt	0.166	0.108	0.179	0.536	0.178	0.044	n.s.	n.s.	***	***	n.s.
C18:3 c9t11c15	0.231	0.261	0.228	0.219	0.109	0.112	n.s.	n.s.	n.s.	n.s.	n.s.
C18:2 n-6	9.74	6.68	7.78	4.10	6.66	1.06	n.s.	†	**	*	n.s.
C18:3 n-3	1.69	2.52	4.11	2.46	5.91	0.479	***	**	n.s.	n.s.	*

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † – 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001;

[2] – Orthogonal contrast MSL *vs.* Forages; [3] – Orthogonal contrast BDS *vs.* Clover diets; [4] – Orthogonal contrast BDS *vs.* IRS; [5] – Orthogonal contrast Clover diets *vs.* IRS; [6] – Orthogonal contrast WCS *vs.* RCS;

Total OBCFA – sum of all odd and branched FA: *iso* C13:0; *anteiso* C13:0; C13:0; *iso* C14:0; *iso* C15:0; *anteiso* C15:0; C15:0; *iso* C16:0; *iso* C17:0; *anteiso* C17:0; C17:0; C17:1 c9;

Total MUFA – Sum of monounsaturated FA: C14:1 c9, C15:1 c9, C16:1 t9, C16:1 c9, C17:1 c9, C18:1 t6-t8, C18:1 t9, C18:1 t10, C18:1 t11, C18:1 t12-t14, C18:1 c9, C18:1 c10, C18:1 c11, C18:1 c12, C18:1 c14, C18:1 c15 and C20:1 c9

Total FA concentration and proportions of FA of rumen contents are presented in Table 5.4. For rumen contents, the proportions of C18-FA are also expressed relative to the sum of all C18-FA identified, as this allows a better evaluation of rumen hydrogenation when dietary supply of C18-FA differs (Chow et al., 2004b). Rumen contents of MSL animals clearly had the highest amount of total FA, followed by animals in the IRS group, WCS and RCS group, whereas total rumen FA content was lowest for animals of the BDS group. Proportions of C18:2 n-6 were significantly lower in rumen contents of IRS animals compared to the BDS group and only a trend was found for lower proportions of C18:2 n-6 for the IRS group compared to the WCS+RCS groups (P=0.088). C18:3 n-3 proportions were higher in the rumen contents of the MSL group than in forage silage fed groups and were lower in animals of the BDS group than in animals of WCS+RCS groups. Further, lambs fed WCS tended (P=0.077) to have lower proportions of C18:3 n-3 in their rumen contents than lambs fed RCS. Rumen contents of BDS animals presented a higher sum of the proportions of biohydrogenation intermediates (C18:1 t11, C18:1 c15, C18:2 t11c15, CLA c9t11 and C18:3 c9t11c15) of the major rumen biohydrogenation pathways of C18:2 n-6 and C18:3 n-3 than the other groups (4.53 g/100 g of FAME *vs.* 3.88, 4.30, 3.60 and 3.44 g/100 g of FAME for BDS *vs.* WCS, RCS, IRS and MSL groups respectively). This is mainly due to the isomers C18:1 t11 and CLA c9t11. In addition, rumen contents of animals of the WCS and RCS groups contained higher proportions of C18:2 t11c15 compared to BDS and IRS groups and RCS animals had higher C18:1 c15 proportions than WCS animals. Rumen contents of animals of groups IRS and MSL had significantly higher proportions of C18:0 and lower accumulation of the major biohydrogenation intermediates compared to the other groups, except C18:1 c15 proportions which were highest for the IRS group. The differences seen between groups remain when these intermediates are expressed relative to the sum of all C18-FA (Table 5.4.). On the other hand, concerning intermediates of secondary biohydrogenation pathways, rumen contents of group MSL contained the highest proportions of C18:1 t10 compared to the forage silage fed groups, whereas BDS animals had unexpectedly significantly higher CLA t10c12 proportions in their rumen contents compared to the other groups.

Subcutaneous and intramuscular fatty acid composition

The FA acid pattern of the SC fat was a partial reflection of what was found in the rumen (Table 5.5.). Total concentration of FA in the SC fat was similar between

groups as well as the C18:0 and C18:3 n-3 proportions. Nevertheless, higher proportions of C18:2 n-6 were found in the SC fat of animals in group MSL and lower proportions in group IRS. The proportion of CLA c9t11 in the SC fat was significantly higher for animals of the BDS group compared with groups IRS and WCS+RCS.

Table 5.5. – Total concentration (mg/g fat) and proportions of individual FA (g/100 g FAME) in subcutaneous fat of animals fed the five different diets (n=6)

Fatty acids	BDS	WCS	RCS	IRS	MSL	SEM[1]	Sign[2]	Sign[3]	Sign[4]	Sign[5]	Sign[6]
Total	844	806	858	875	852	48.6	n.s.	n.s.	n.s.	n.s.	n.s.
C12:0	0.772	0.443	0.413	0.197	0.361	0.102	n.s.	*	***	†	n.s.
C14:0	6.38	4.73	4.48	3.77	4.00	0.504	n.s.	**	*	n.s.	n.s.
C16:0	24.4	26.8	23.6	26.3	22.5	1.11	*	n.s.	n.s.	n.s.	†
C18:0	20.8	21.1	21.9	22.2	21.9	1.74	n.s.	n.s.	n.s.	n.s.	n.s.
C18:1 t9	0.198	0.176	0.238	0.169	0.295	0.029	**	n.s.	n.s.	n.s.	n.s.
C18:1 t10	0.421	0.335	0.368	0.281	0.828	0.137	**	n.s.	n.s.	n.s.	n.s.
C18:1 t11	1.29	0.816	0.916	0.630	1.09	0.175	n.s.	†	*	n.s.	n.s.
C18:1 c9	28.3	28.4	26.7	29.0	30.8	0.984	*	n.s.	n.s.	n.s.	n.s.
C18:1 c15	0.068	0.070	0.081	0.082	0.062	0.010	n.s.	n.s.	n.s.	n.s.	n.s.
C18:2 t11c15	0.214	0.127	0.170	0.165	0.150	0.030	n.s.	†	n.s.	n.s.	n.s.
CLA c9t11	0.665	0.396	0.412	0.225	0.568	0.098	n.s.	*	**	n.s.	n.s.
CLA t10c12	0.043	0.033	0.042	0.005	0.010	0.008	*	n.s.	**	**	n.s.
CLA cc	0.042	0.034	0.041	0.060	0.024	0.007	*	n.s.	†	*	n.s.
CLA tt	0.037	0.033	0.031	0.041	0.030	0.005	n.s.	n.s.	n.s.	n.s.	n.s.
C18:3 c9t11c15	0.032	0.019	0.016	0.012	0.011	0.006	n.s.	†	*	n.s.	n.s.
C18:2 n-6	1.39	1.32	1.63	0.955	2.33	0.145	***	n.s.	*	**	n.s.
C18:3 n-3	1.04	1.14	1.42	1.22	1.24	0.125	n.s.	n.s.	n.s.	n.s.	n.s.
Total OBCFA	6.22	6.40	6.83	5.24	5.30	0.271	**	n.s.	*	***	n.s.
Total SFA	55.6	56.5	54.1	55.2	51.4	1.53	*	n.s.	n.s.	n.s.	n.s.
Total MUFA	35.3	34.0	32.7	35.5	38.8	1.16	**	n.s.	n.s.	n.s.	n.s.
Total PUFA	3.93	3.57	4.22	3.34	4.93	0.323	**	n.s.	n.s.	n.s.	n.s.
n-6/n-3 ratio	1.38	1.16	1.22	0.799	1.90	0.092	***	n.s.	***	**	n.s.
P/S ratio	0.047	0.047	0.061	0.042	0.074	0.005	***	n.s.	n.s.	†	†

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † – 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001;

[2] – Orthogonal contrast MSL *vs.* Forages; [3] – Orthogonal contrast BDS *vs.* Clover diets; [4] – Orthogonal contrast BDS *vs.* IRS; [5] – Orthogonal contrast Clover diets *vs.* IRS; [6] – Orthogonal contrast WCS *vs.* RCS;

Total OBCFA – sum of all odd and branched fatty acids: *iso* C13:0; *anteiso* C13:0; C13:0; *iso* C14:0; *iso* C15:0; *anteiso* C15:0; C15:0; *iso* C16:0; *iso* C17:0; *anteiso* C17:0; C17:0; C17:1 c9;

Total SFA – Sum of saturated FA: C10:0, C12:0, C13:0; C14:0, C15:0, C16:0, C17:0, C18:0 and C20:0;

Total MUFA – Sum of monounsaturated FA: C14:1 c9, C15:1 c9, C16:1 t9, C16:1 c9, C17:1 c9, C18:1 t6-t8, C18:1 t9, C18:1 t10, C18:1 t11, C18:1 t12-t14, C18:1 c9, C18:1 c10, C18:1 c11, C18:1 c12, C18:1 c14, C18:1 c15 and C20:1 c9;

Total PUFA – Sum of polyunsaturated FA: C18:2 t11c15, other C18:2 isomers; C18:2 n-6, C18:3 n-3, CLA c9t11, CLA t10c12, CLA cc, CLA tt and C18:3 c9t11c15;

n-6/n-3 ratio – Ratio between C18:2 n-6 and C18:3 n-3;

P/S ratio – Ratio between the sum of C18:2 n-6 and C18:3 n-3, and the sum of C14:0, C16:0 and C18:0

Concerning other CLA isomers, proportions of CLA t10c12 were highest for animals in groups BDS, WCS and RCS than for animals of IRS and MSL groups, whereas CLA cc proportions were higher in the SC fat for animals of the IRS group compared to the other groups and proportions of CLA tt were similar between groups.

Neither total concentration of FA nor the proportions of C18:3 n-3 in the IM fat differed between groups (Table 5.6.). Similarly to what was found for the rumen contents, lower proportions of C18:2 n-6 and CLA c9t11 were found in the IM fat of IRS animals, and proportions of CLA t10c12 were significantly higher for BDS, WCS and RCS animals compared to the IRS and MSL groups. Proportions of CLA cc tended to be higher for animals of IRS group (P=0.082) compared to BDS group and CLA tt proportions did not differ between groups. Proportions of C18:1 c9 tended to be higher in the IM fat of animals in the MSL group compared with the other groups (P=0.096) as seen for the rumen contents. Finally, proportions of C20:4 n-6, C20:5 n-3, C22:5 n-3 and C22:6 n-3 in the IM fat did not differ between groups. Nevertheless, in the muscle of BDS animals higher C20:5 n-3/C18:3 n-3 and C22:5 n-3/C18:3 n-3 indices for desaturation and elongation activity, as calculated by ratios of product to precursor FA, were observed than in the muscle of MSL and IRS animals. In the muscle of animals fed clover diets (WCS and RCS) higher C20:5 n-3/C18:3 n-3 and C22:5 n-3/C18:3 n-3 indices for desaturation and elongation activity were also observed than in the muscle of IRS animals.

Table 5.6. – Total concentration (mg/g meat) and proportions of individual FA (g/100 g FAME) in intramuscular fat of animals fed the five different diets (n=6)

Fatty acids	BDS	WCS	RCS	IRS	MSL	SEM[1]	Sign[2]	Sign[3]	Sign[4]	Sign[5]	Sign[6]
Total	17.9	17.6	18.5	23.8	25.4	3.19	n.s.	n.s.	n.s.	n.s.	n.s.
C12:0	0.422	0.403	0.349	0.226	0.273	0.053	n.s.	n.s.	*	*	n.s.
C14:0	3.58	3.39	3.17	2.71	3.00	0.297	n.s.	n.s.	*	n.s.	n.s.
C16:0	21.1	22.6	21.9	23.6	22.6	0.961	n.s.	n.s.	T	n.s.	n.s.
C18:0	15.5	16.8	17.2	17.3	17.5	0.939	n.s.	n.s.	n.s.	n.s.	n.s.
C18:1 t9	0.136	0.144	0.192	0.165	0.253	0.030	*	n.s.	n.s.	n.s.	n.s.
C18:1t10	0.345	0.380	0.411	0.247	0.445	0.081	n.s.	n.s.	n.s.	n.s.	n.s.
C18:1t11	0.567	0.596	0.645	0.353	0.550	0.095	n.s.	n.s.	n.s.	*	n.s.
C18:1c9	28.5	29.7	28.7	31.3	31.9	1.21	T	n.s.	n.s.	n.s.	n.s.
C18:1c15	0.048	0.031	0.015	0.047	0.078	0.016	*	n.s.	n.s.	n.s.	n.s.
C18:2 t11c15	0.109	0.112	0.148	0.118	0.116	0.024	n.s.	n.s.	n.s.	n.s.	n.s.
CLAc9t11	0.595	0.544	0.544	0.301	0.532	0.078	n.s.	n.s.	*	*	n.s.
CLAt10c12	0.038	0.035	0.036	0.019	0.022	0.005	T	n.s.	**	**	n.s.
CLAcc	0.029	0.035	0.035	0.040	0.026	0.004	T	n.s.	T	n.s.	n.s.
CLAtt	0.025	0.035	0.035	0.031	0.030	0.004	n.s.	T	n.s.	n.s.	n.s.
C18:3c9t11c15	0.039	0.038	0.042	0.029	0.039	0.005	n.s.	n.s.	n.s.	T	n.s.
C18:2 n-6	4.88	4.15	4.29	3.60	4.95	0.426	n.s.	n.s.	*	n.s.	n.s.
C18:3 n-3	1.53	1.62	1.82	1.62	1.57	0.151	n.s.	n.s.	n.s.	n.s.	n.s.
C20:4 n-6	2.70	1.90	1.78	1.70	1.46	0.429	n.s.	n.s.	n.s.	n.s.	n.s.
C20:5 n-3	1.08	0.867	0.840	0.826	0.655	0.170	n.s.	n.s.	n.s.	n.s.	n.s.
C22:5 n-3	1.31	1.02	1.03	0.941	0.756	0.185	n.s.	n.s.	n.s.	n.s.	n.s.
C22:6 n-3	0.272	0.245	0.252	0.273	0.233	0.035	n.s.	n.s.	n.s.	n.s.	n.s.
Total OBCFA	5.90	5.09	4.93	4.45	4.26	0.390	T	T	*	n.s.	n.s.
Total SFA	43.9	45.9	45.3	46.1	45.5	1.16	n.s.	n.s.	n.s.	n.s	n.s.
Total MUFA	34.0	35.1	34.4	36.4	37.2	1.13	T	n.s.	n.s.	n.s.	n.s.
Total PUFA	13.8	11.6	11.9	10.6	11.3	1.37	n.s.	n.s.	n.s.	n.s.	n.s.
n-6/n-3	1.98	1.79b	1.70	1.63	2.16	0.083	***	*	**	n.s.	n.s.
P/S	0.167	0.136	0.148	0.122	0.152	0.019	n.s.	n.s.	T	n.s.	n.s.
Indices for elongation and desaturation activity (calculated as ratios of FA)											
C20:4n-6/C18:2n-6	0.518	0.449	0.410	0.469	0.296	0.055	*	n.s.	n.s.	n.s.	n.s.
C20:5n-3/C18:3n-3	0.702	0.525	0.448	0.489	0.416	0.083	n.s.	*	T	n.s.	n.s.
C22:5n-3/C18:3n-3	0.868	0.628	0.557	0.570	0.489	0.102	n.s.	*	*	n.s.	n.s.
C22:6n-3/C18:3n-3	0.178	0.151	0.144	0.166	0.149	0.017	n.s.	n.s.	n.s.	n.s.	n.s.
C22:5n-3/C20:5n-3	1.25	1.21	1.26	1.19	1.20	0.068	n.s.	n.s.	n.s.	n.s.	n.s.
C22:6n-3/C20:5n-3	0.274	0.300	0.331	0.349	0.359	0.029	n.s.	n.s.	n.s.	n.s.	n.s.
C22:6n-3/C22:5n-3	0.221	0.246	0.261	0.293	0.308	0.021	*	n.s.	n.s.	n.s.	n.s.

[1] – Standard error of mean; n.s. – not significantly different (p>0.1); † – 0.1>p>0.05; * - 0.05>p>0.01; ** - 0.01>p>0.001; *** - p<0.001; [2] – Orthogonal contrast MSL *vs.* Forages; [3] – Orthogonal contrast BDS *vs.* Clover diets; [4] – Orthogonal contrast BDS *vs.* IRS; [5] – Orthogonal contrast Clover diets *vs.* IRS; [6] – Orthogonal contrast WCS *vs.* RCS; Total OBCFA – sum of all odd and branched fatty acids: *iso* C13:0; *anteiso* C13:0; C13:0; *iso* C14:0; *iso* C15:0; *anteiso* C15:0; C15:0; *iso* C16:0; *iso* C17:0; *anteiso* C17:0; C17:0; C17:1 c9; Total SFA – Sum of saturated FA: C10:0, C12:0, C13:0; C14:0, C15:0, C16:0, C17:0, C18:0 and C20:0; Total MUFA – Sum of monounsaturated FA: C14:1 c9, C15:1 c9, C16:1 t9, C16:1 c9, C17:1 c9, C18:1 t6-t8, C18:1 t9, C18:1 t10, C18:1 t11, C18:1 t12-t14, C18:1 c9, C18:1 c10, C18:1 c11, C18:1 c12, C18:1 c14, C18:1 c15 and C20:1 c9; Total PUFA – Sum of polyunsaturated FA: C18:2 t11c15, other C18:2 isomers, C18:2 n-6, C18:3 n-6, C18:3 n-3, CLA c9t11, CLA t10c12, CLA cc, CLA tt, C18:3 c9t11c15, C20:3 n-6, C20:3 n-3, C20:4 n-6, C20:5 n-3, C22:4 n-6, C22:5 n-3 and C22:6 n-3; n-6/n-3 ratio – Ratio between the sum of C18:2 n-6, C18:3 n.6, C20:3 n-6, C20:4 n-6 and C22:4 n-6, and the sum of C18:3 n-3, C20:3 n-3, C20:5 n-3, C22:5 n-3 and C22:6 n-3; P/S ratio – Ratio between the sum of C18:2 n-6 and C18:3 n-3, and the sum of C14:0, C16:0 and C18:0

DISCUSSION

Rumen fermentation patterns within the four groups fed forage silages were similar. Nevertheless, higher proportions of biohydrogenation intermediates, in particular CLA c9t11 and C18:1 t11 were found in the rumen of BDS animals, despite the similar precursor proportions for the different silages (except for the IRS which presented a higher feed C18:2 n-6 + C18:3 n-3 proportion). Microbial markers such as rumen OBCFA (Vlaeminck et al., 2005) could suggest a different microbial population for the BDS animals. Vlaeminck et al. (2006), observed a positive correlation between *iso* C17:0 and C18:1 t11, from which they suggested group B bacteria, responsible for the final hydrogenation step, to have lower *iso* C17:0 proportions. Further and similar to the results described in Chapter 3, reporting higher rumen *iso* C17:0 proportions in the rumen of BD animals, increased proportions of C16:1 t9 + *iso* C17:0 were observed in the rumen contents of BDS animals (Table 5.3.).

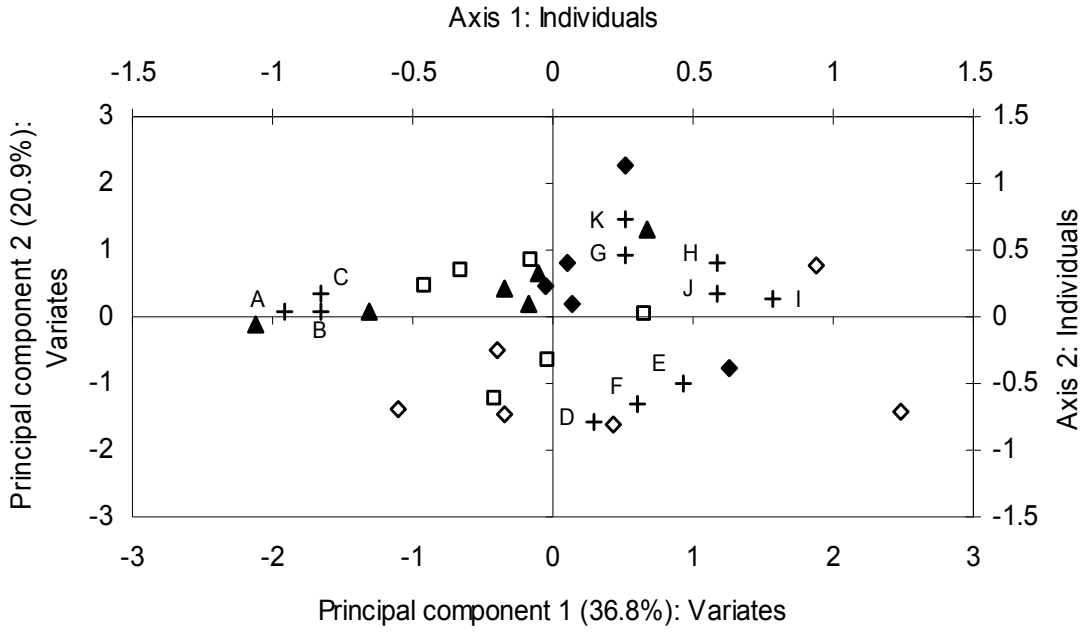

Figure 5.1. – Biplot representing both regression factor scores according to the silage groups (Botanical diverse silage (◊), White clover silage (□), Red clover silage (▲), Intensive ryegrass silage (♦)) and loadings (+) of the first two principal components, based on proportions (g/100 g of OBCFA) of rumen OBCFA. The letters refer to individual OBCFA: A – C15:0; B – *anteiso* C13:0; C – *iso* C14:0; D – *iso* C16:0; E – *iso* C15:0; F – *iso* C13:0; G – C17:1; H – *anteiso* C15:0; I – *anteiso* C17:0; J – C13:0; K – C17:0

In addition PCA analysis (Figure 5.1.), to determine the components which account for most of the variation in OBCFA, revealed a negative score on the second principal component for the BDS animals compared to animals fed the other forage diets (WCS, RCS and IRS), supporting the suggestion of a different microbial population for the BDS animals, based on the proportions of OBCFA observed in the rumen contents of the animals. The suggested different microbial population in the rumen of BDS animals may explain the changes observed in the rumen biohydrogenation intermediates. These suggested differences in microbial population and consequent differences in accumulation of some biohydrogenation intermediates may be due to the presence of compounds in the BDS plant species, which might have antimicrobial activity (Wallace, 2004) and affect the rumen fermentation pattern (Busquet et al., 2006).

Comparing the forages *vs.* linseed feeding, it was clear that rumen contents of MSL animals had the highest C18:1 t10 proportions. These higher proportions of C18:1 t10 have been reported to be associated with a lower rumen pH (Loor et al., 2003a, 2005), opposite to our results. However, these animals showed a different fermentation pattern in terms of increased propionate and lower acetate proportions when compared to the other four diets. This pattern was most probably due to the higher starch content of maize (increase of propionate at the expense of acetate, typical for starch-rich concentrate diets (France and Siddons, 1993)). Additionally, the supplementation of PUFA through linseed also might have a methane depressive effect, resulting in a shift of the VFA pattern towards increased propionate proportions (Chilliard et al., 2000; Owens et al., 2006). Moreover, shifts towards a more amylolytic population could also be suggested from changes in rumen OBCFA, in particular decreases of *iso* C14:0 (Table 5.3.). This FA has also been reported to be negatively correlated with C18:1 t10 by Vlaeminck et al. (2006), who suggested hydrogenating bacteria responsible for the appearance of C18:1 t10 in the rumen to have low proportions of *iso* C14:0. The suggestion for a different microbial population associated with the MSL diet is further illustrated by the PCA biplot (Figure 5.2.), which revealed the lowest first principal component score for the MSL animals compared to the other four groups, with the MSL animals clustering together, based on the proportions of OBCFA observed in the rumen contents of the animals.

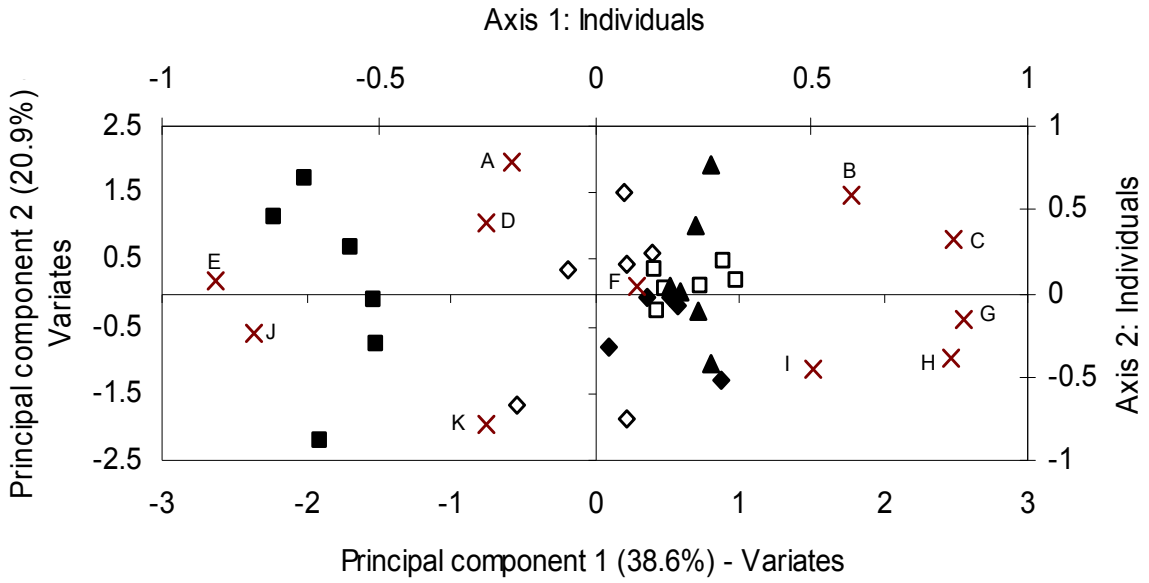

Figure 5.2. – Biplot representing both regression factor scores according to the silage groups (Botanical diverse silage (◊), White clover silage (□), Red clover silage (▲), Intensive ryegrass silage (♦), Maize silage and linseed (■)) and loadings (x) of the first two principal components, based on proportions (g/100 g of OBCFA) of rumen OBCFA. The letters refer to individual OBCFA: A – C15:0; B – *anteiso* C13:0; C – *iso* C14:0; D – *iso* C16:0; E – *iso* C15:0; F – *iso* C13:0; G – C17:1; H – *anteiso* C15:0; I – *anteiso* C17:0; J – C13:0; K – C17:0

The different bacterial populations in rumen contents of MSL group could be responsible for the shift of the hydrogenation of C18:2 n-6 from CLA c9t11 and C18:1 t11 to CLA t10c12 and C18:1 t10. Additionally, the higher proportions of C18:1 t10 could arise from the isomerization of C18:1 t11 or other C18:1 *trans* isomers (Proell et al., 2002; Loor et al., 2005) or from the isomerization of C18:1 c9 (Mosley et al., 2002; Loor et al., 2005).

Another important finding in this study was the higher C18:3 n-3 proportion in the rumen contents of MSL animals, despite the similar supply of C18 PUFA from the MSL diet compared to the other forage diets (Table 5.2.). This might be due to the presence of C18:3 n-3 in triacylglycerols in crushed linseed whereas the majority of FA in silages are unesterified. Indeed, Lourenço et al. (2005b) reported 51 % of the silage FA to be in the unesterified form. Additionally, C18:3 n-3 might have been physically protected against microbial attack by the coating of the linseed, which might be effective in impeding the access of the microbial lipases to the C18:3 n-3. Moreover, rumen contents of RCS animals also had significantly higher proportions of C18:3 n-3 compared to the rumen contents of WCS animals. Red clover silages have been described to increase omega-3 FA in milk of cows fed silages (Dewhurst et al., 2003b).

This has been hypothesized to be related to higher proportions of esterified FA being protected by the denaturation of plant lipases or to *o*-quinones (produced by polyphenol oxidase (PPO) activity (Jones et al., 1995)) linkages to nucleophilic amino acids of enzymes, e.g. lipases (Lee et al., 2004).

Intramuscular fat is known to be less responsive than SC fat to changes in the dietary supply of FA or changes in FA rumen metabolism (Demirel et al., 2004). In this study FA profile of the SC fat of the animals was a reflection of the rumen data and was more responsive to the changes observed in the rumen FA metabolism than the IM fat. IM fat of BDS animals had the highest, however not significantly different, proportions of most PUFA in line with former studies on pastured lambs (Chapter 4; Ådnøy et al., 2005). Although proportions of C20:4 n-6, C20:5 n-3, C22:5 n-3 and C22:6 n-3 did not differ between groups, indices for desaturation and elongation activity did, suggesting that tissue FA metabolism may be influenced by feeding botanically diverse silages, similarly to the results presented in Chapter 4 of this thesis. Moreover, results suggest that the activity of Δ^6-desaturase, elongase and Δ^5-desaturase (Figure 5.3.) might be affected as BDS animals had higher C20:5 n-3/C18:3 n-3 and C22:5 n-3/C18:3 n-3 indices than the IRS animals, and a higher C20:4 n-6/C18:2 n-6 indices than the WCS and RCS animals.

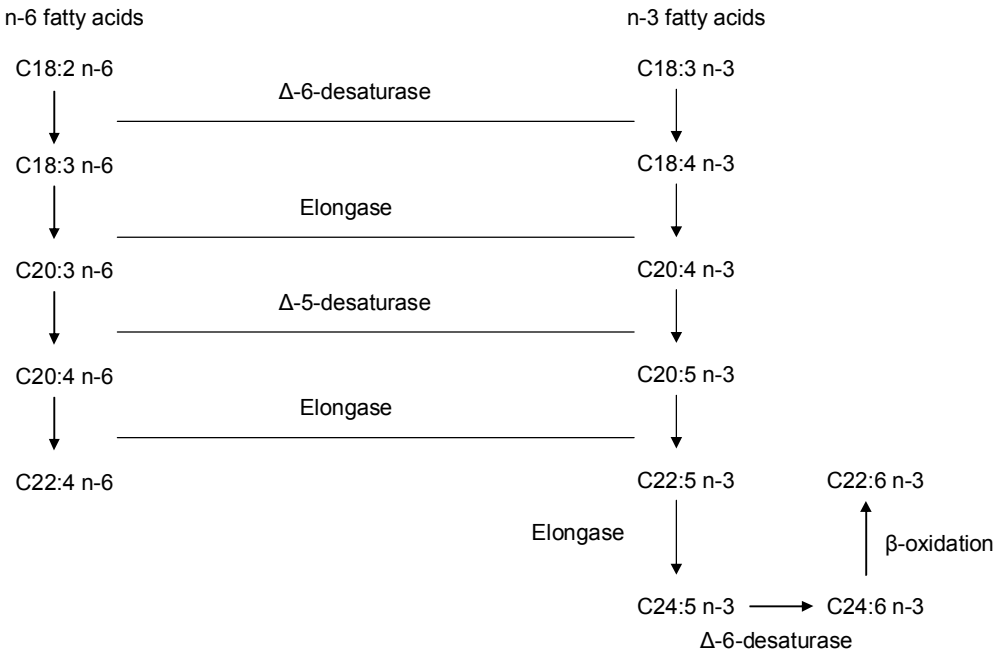

Figure 5.3. – Conversion of C18:2 n-6 and C18:3 n-3 into their long chain fatty acid products (adapted from Sprecher, 2000)

The indices representing the last steps of elongation and desaturation of long chain FA (Figure 5.3.) in muscle of BDS animals did not differ between WCS, RCS and IRS animals (C22:6 n-3/C18:3 n-3, C22:5 n-3/C20:5 n-3, C22:6 n-3/C20:5 n-3 and C22:6 n-3/C22:5 n-3 indices). This could be partially due to substrate competition between C18:3 n-3 and C24:5 n-3 for the Δ^6-desaturase enzyme. Portolesi et al. (2007) has shown that this enzyme has a higher affinity for C18:3 n-3, which may limit the accumulation of C22:6 n-3. Nevertheless, a confounding effect with the lower IM fat content of these animals and associated higher phospholipid/triacylglycerol ratios and long chain PUFA proportion cannot be excluded.

CONCLUSIONS

This study suggested that feeding botanically different silages induced changes in the rumen FA metabolism which might be related to differences observed in the extent of rumen biohydrogenation of PUFA. The higher rumen C18:3 n-3 concentrations of linseed supplemented animals might be related to its presence in triacylglycerols and a possible physical protection against microbial lipases through the seed coating. Additionally, higher proportions of C18:3 n-3 in the rumen contents of RCS animals were hypothesized to be due to the action of its PPO enzyme. Finally, feeding silages from more botanically diverse pastures could affect tissue FA metabolism as suggested from the indices for desaturation and elongation of PUFA. Overall, these results suggest that animals consuming more botanically diverse silages offer opportunities to produce a healthier FA profile from a human health perspective.

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Chapter 6

General Discussion

Redrafted after Lourenço, M., Vlaeminck, B., Van Ranst, G., De Smet, S., Fievez, V., 2007d. Influence of different dietary forages on the fatty acid composition of rumen digesta and ruminant meat and milk. *Animal Feed Science and Technology*, in press.

Chapter 6

General discussion

In this chapter of the present PhD thesis, we intend to review literature on effects of dietary forages on milk and tissue fatty acid (FA) composition of cattle and sheep, with particular emphasis on changes induced by leguminous and biodiverse forages *vs*. intensive ryegrass. Differences are discussed in relation to changes in rumen or duodenal digesta to explain the origin of the differences as, in most cases, increased omega-3 polyunsaturated FA (PUFA; i.e., C18:3 n-3 and/or long chain omega-3 PUFA) in milk and intramuscular fat (IMF) due to feeding of red or white clover and botanically diverse forages could not be attributed to increased dietary supply of linolenic acid (C18:3 n-3). Hence, increased forestomach outflow of C18:3 n-3 has been suggested to originate from reduced rumen lipolysis, with literature providing some evidence for the role of polyphenol oxidase (PPO), which is particular active in red clover, to inhibit rumen lipolysis. Increased proportions of c9t11 CLA in milk and IMF of ruminants fed botanically diverse forages have been associated with increased forestomach outflow of *trans*-vaccenic acid (C18:1 t11), which is the main precursor of endogenous CLA c9t11 production. Despite the lack of direct evidence, some plant secondary metabolites, present in herbs of botanically diverse forages, are suggested to be potential modifiers of rumen biohydrogenation based on their effects on rumen methanogenesis.

EFFECT OF THE BOTANICAL COMPOSITION OF DIETARY FORAGE ON MILK FATTY ACID PROFILE

Data and statistical analysis

In order to describe quantitatively overall trends in the different dairy cattle studies on the effects of type and diversity of forages on milk FA profile, three data sets were created: 1) studies reporting red clover *vs*. ryegrass based diets; 2) studies

reporting white *vs.* red clover forages and 3) studies reporting botanically diverse *vs.* grass based diets.

Table 6.1. – Overview of the studies used for the statistical comparisons in this chapter and the dietary groups considered

Reference	Control diet	Treatment diet
Red clover *vs.* ryegrass based diets		
Al-Mabruk et al., 2004	Grass silage with or without vitamin E (n=2)	Red clover silage with or without vitamin E (n=2)
Dewhurst et al., 2003b	Grass silage (exp.1); grass silage (n=1) + 4 or 8 kg/d concentrate (exp.2) (n=2)	Red clover silage (exp. 1); red clover silage (n=1) + 4 or 8 kg/d concentrate (exp.2) (n=2)
Van Doorland, 2006	Grass silage (exp.2) (n=1)	Red clover silage (exp.2) (n=1)
Red *vs.* white clover forages		
Dewhurst et al., 2003b	Red clover silage; red clover mixed with grass silage (50:50 DM basis) (exp.1); red clover silage (n=2) + 8 kg/d concentrate (exp.2) (n=1)	White clover silage; white clover mixed with grass silage (50:50 DM basis) (exp.1); white clover silage (n=2) + 8 kg/d concentrate (exp.2) (n=1)
Van Dorland, 2006	Fresh red clover (exp.1) (n=1); red clover silage (exp.2) (n=1)	Fresh white clover (exp.1) (n=1); white clover silage (exp.2) (n=1)
Steinshamn et al., 2006	Red clover silage with or without concentrate (n=2)	White clover silage with or without concentrate (n=2)
Botanically diverse *vs.* grass based diets		
Collomb et al., 2002	Lowland forage (n=1)	Highland pastures (n=1)
Ferlay et al., 2006	Ryegrass hay (n=1)	Natural grassland hay (n=1)
Kraft et al., 2003	Organic farming pastures (n=1)	Swiss Alp pastures; L'Etivaz pastures (n=2)
Leiber et al., 2005	Lowland period (Barn and pasture groups) (n=2)	Alpine period (Barn and pasture groups) (n=2)
Chapter 3	Intensively managed ryegrass silage (n=1)	60% Species-rich grassland silage (n=1)

Four studies were found describing the effects of feeding red clover compared to ryegrass silages (Dewhurst et al., 2003b; Al-Mabruk et al., 2004; Van Dorland, 2006; Vanhatalo et al., 2006). From these four studies only those reporting individual FA were used for the statistical analysis (Table 6.1.). Three studies were found comparing the milk FA profile of dairy cattle upon feeding red and white clover forages (Dewhurst et al., 2003b; Steinshamn et al., 2006; Van Dorland, 2006). In the available literature, eight studies reported the effect of feeding botanically diverse forages on the milk FA profile (Bugaud et al., 2001; Collomb et al., 2002; Innocente et al., 2002; Kraft et al., 2003; Leiber et al., 2005; Ferlay et al., 2006; Žan et al., 2006; results presented in Chapter 3 of this thesis). From these eight studies, only five were retained for statistical analysis (Table 6.1.) according to the following criteria: milk of dairy cattle was studied; individual FA were reported; a control diet, mainly composed of 60% or more of grasses (*Poaceae* family) and a botanically diverse forage with 30% or more of herbs (generally from the *Asteraceae*, *Rosaceae* and *Ranunculaceae* families) were compared. All the data were expressed as proportion of the total FA content of milk. A mixed model was used to compare the above mentioned comparisons as described by St-Pierre (2001). Study was considered a random effect (St-Pierre, 2001) and variables were weighted by the number of observations in each study. The values reported in the tables are the estimated marginal means. The intake of C18:2 n-6 and C18:3 n-3 were not reported in all studies included in the data set (Tables 6.2. and 6.3.). Due to the limited number of observations available for the meta-analysis of this Chapter, this approach has been considered to describe overall trends, but details of individual studies will be discussed in order to indicate possible reasons for deviations in individual studies from these generally observed tendencies.

In most of the tables, C18:1 t10+t11 is reported as in some of the studies included in this review, the separation of these two isomers was not possible. However, with the dietary treatments reported in this review, it is most likely that this sum mainly corresponds to C18:1 t11. Indeed, in those studies included in this review which reported C18:1 t10 and C18:1 t11 separately in milk and/or rumen samples (Kraft et al., 2003; Chapter 3; Loor et al., 2003a; Lee et al., 2006b; Chapter 5), C18:1 t11 represented 89 % of the sum of C18:1 t10+t11.

Milk

Red clover *vs.* ryegrass based diets – The results presented in Table 6.2. show that animals fed red clover forages produce milk with higher C18:3 n-3 proportions compared to animals fed ryegrass forages, despite the similar C18:3 n-3 supply. This is further reflected in higher recoveries of dietary C18:3 n-3 in milk (Table 6.2.) for animals fed red clover forages than for animals fed ryegrass forages. *De novo* synthesized FA did not differ in milk of animals fed red clover or ryegrass forages, nor the C18:1 t10+t11 and conjugated linoleic acid (CLA) c9t11 proportions.

Table 6.2. – Milk fatty acid profile (g/100 g FA), C18:2 n-6 and C18:3 n-3 intake (g/d), and recovery (g/100 g) of dietary C18:2 n-6 and C18:3 n-3 in the milk of dairy cattle fed either intensive ryegrass, red clover (RC) or white clover (WC)

	Ryegrass[2]	RC[2]	SEM[3]	Sign.	RC[2]	WC[2]	SEM[3]	Sign.
Fatty acids								
n	6	6			7	7		
C4:0	4.43	4.76	0.617	n.s.	4.10	3.88	0.749	n.s.
C6:0	2.55	2.65	0.122	n.s.	2.50	2.54	0.216	n.s.
C8:0	1.43	1.45	0.161	n.s.	1.46	1.53	0.164	n.s.
C10:0	2.89	2.80	0.209	n.s.	2.91	3.13	0.239	n.s.
C12:0	3.53	3.40	0.289	n.s.	3.52	3.77	0.361	n.s.
C14:0	11.3	11.2	0.410	n.s.	10.8	11.4	0.612	n.s.
C14:1	1.04	0.938	0.134	n.s.	0.979	1.04	0.111	n.s.
C16:0	31.5	31.1	0.640	n.s.	29.5	30.3	0.977	n.s.
C16:1	1.62	1.46	0.229	n.s.	1.49	1.56	0.193	n.s.
C18:0	10.8	10.7	0.600	n.s.	10.5	10.1	0.335	n.s.
C18:1 t10+t11	1.46	1.40	0.213	n.s.	1.97	1.71	0.448	n.s.
C18:1 c9	19.5	18.8	1.07	n.s.	19.5	18.5	4.55	n.s.
C18:2 n-6	1.26	1.56	0.104	n.s.	1.43	1.43	0.124	n.s.
CLA c9t11	0.546	0.501	0.120	n.s.	0.812	0.753	0.183	n.s.
C18:3 n-3	0.555	1.05	0.119	*	0.956	0.893	0.098	n.s
Intake								
n	6	6			5[1]	5[1]		
C18:2 n-6	83.0	100.4	11.50	n.s.	92.1	97.8	8.70	n.s.
C18:3 n-3	92.1	115.6	14.51	n.s.	136	171	17.5	†
Recovery								
n	6	6			5[1]	5[1]		
C18:2 n-6	15.2	15.2	2.39	n.s.	17.1	18.0	2.30	n.s.
C18:3 n-3	5.62	8.92	0.732	*	7.6	6.43	1.07	n.s.

[1] – Steinshamn et al. (2006) did not report intakes and hence recoveries could not be calculated;
[2] – For more details on which studies were used for which comparisons see Table 6.1.;
[3] – Standard error of mean; n.s. – not significantly different ($p>0.1$); † – $0.1>p>0.05$; * – $p<0.05$

Red *vs.* white clover forages – Feeding red or white clover forages generally does not affect milk FA profiles (Table 6.2.). Nevertheless, individual studies reported both higher (Steinshamn et al., 2006) and lower (Van Dorland, 2006) C18:3 n-3 in milk of red clover than in white clover fed animals which might be related to differences in clover proportions in the forage. Indeed, in the red clover diet studied by Steinshamn et al. (2006), clover represented 58% of the clover/grass silage, whereas the clover/grass silage of the white clover diet consisted of 40% white clover only.

Table 6.3. – Milk fatty acid profile (g/100 g of FA), C18:2 n-6 and C18:3 n-3 intake (g/d) and recovery (g/100 g) of dietary C18:2 n-6 and C18:3 n-3 in the milk of dairy cattle fed a less diverse forage (with 60% or more grasses from *Poaceae* family – control) or a botanically diverse forage (with 30% or more herbs from the *Asteraceae*, *Rosaceae* and *Ranunculaceae* families)

	Control[2]	Botanical diverse[2]	SEM[3]	Sign.
Fatty acids				
n	6	7		
C4:0	3.79	3.84	0.207	n.s.
C6:0	2.32	2.01	0.120	*
C8:0	1.31	1.03	0.062	*
C10:0	2.79	2.01	0.180	*
C12:0	3.41	2.67	0.438	n.s.
C14:0	10.3	8.98	0.751	T
C14:1	1.11	0.930	0.157	*
C16:0	26.8	26.0	2.17	n.s.
C16:1	1.34	1.20	0.080	*
C18:0	9.18	9.00	0.555	n.s.
C18:1 t10+t11	1.99	2.84	0.642	n.s.
C18:1 c9	17.2	18.8	0.795	n.s.
C18:2 n-6	1.07	1.22	0.113	n.s.
CLA c9t11	0.866	1.43	0.322	n.s.
C18:3 n-3	0.788	1.06	0.102	*
Intake				
n	4[1]	4[1]		
C18:2 n-6	65.4	64.0	14.0	n.s.
C18:3 n-3	147	116	45.6	n.s.
Recovery				
n	4[1]	4[1]		
C18:2 n-6	8.40	9.39	1.47	n.s.
C18:3 n-3	4.34	5.80	1.14	†

[1] – Kraft et al. (2003) and Ferlay et al. (2006) did not report intakes and hence recoveries could not be calculated;
[2] – For more details on which studies were used for the comparison see Table 6.1.;
[3] – Standard error of mean; n.s. – not significantly different ($p>0.1$); † – $0.1>p>0.05$; * – $p<0.05$

On the other hand, lower milk C18:3 n-3 proportions for animals fed fresh red clover in the study of Van Dorland (2006) were associated with lower clover proportions compared to the fresh white clover group.

Botanically diverse *vs.* grass based diets – It is evident from Table 6.3. that feeding botanically diverse forages changed the milk FA profile towards lower proportions of *de novo* synthesized FA. Further, higher milk C18:3 n-3 proportions were found for animals fed botanically diverse forages *vs.* animals fed grass based diets, despite the similar C18:3 n-3 intake for the two dietary groups. This was further associated with a trend for higher recovery of dietary C18:3 n-3 in milk of animals fed botanically diverse forages compared to animals fed grass based diets. The proportions of CLA c9t11 and C18:1 t10+t11 were numerically higher for milk of botanically diverse forages than for milk of grass based forages (1.43 *vs.* 0.87 and 2.84 *vs.* 1.99 g/100 g of total FA, respectively) although differences did not reach significance. Nevertheless, a significantly higher milk C18:1 t10+t11 and CLA c9t11 upon feeding botanically diverse forages was observed in three (Collomb et al., 2002; Kraft et al., 2003; results presented in Chapter 3) of the five studies included in the statistical analysis, whereas the other two studies (Leiber et al, 2005; Ferlay et al., 2006) reported lower milk C18:1 t10+t11 and CLA c9t11 when animals were fed botanically diverse forages compared to grass based diets. However, the control group of the latter two studies already showed high milk CLA c9t11 concentrations (1.7 and 0.87 g/100 g total FA for grazing and conserved ryegrass in the studies of Leiber et al. (2005) and Ferlay et al. (2006), respectively *vs.* 0.81, 0.87 and 0.26 g/100 g of total FA for grazing and conserved ryegrass in the studies of Collomb et al., 2002; Kraft et al., 2003; results presented in Chapter 3). Inter-experimental differences might be due to variation within the available literature in terms of e.g. intake of C18:2 n-6 and C18:3 n-3, which both are precursors of rumen C18:1 t11. However, C18:2 n-6 + C18:3 n-3 concentrations in the biodiverse forages tended to be lower compared to the control group both in studies showing a positive effect as well as a negative effect. Another reason for inter-experimental differences might be related to the proportions of botanically diverse forages in the diet, which varied from 20.4 to 87%. However, proportions of milk CLA were not correlated with the proportion of botanically diverse forages in the diet ($r_{pearson}$= -0.109; $p>0.05$; n=4). In addition, "botanically diverse" refers to different extents of diversity in the different studies. Hence, differences in the number of plant species in the botanical

diverse diet could be associated with changes in the milk C18:1 t10+t11 and CLA c9t11 proportions. However, the botanically diverse Alpine pastures of Leiber et al. (2005) (71 plant species, of which 46 herbs) resulted in lower milk C18:1 t10+t11 and CLA c9t11 proportions compared to the control group, opposite to the studies of Collomb et al. (2002) and of the study presented in Chapter 3, reporting higher milk C18:1 t10+t11 and CLA c9t11 proportions with less diverse pastures (55 and 42 plant species of which 13 and 22 herbs, respectively). It is most probable that a complex interaction of different factors determines CLA c9t11 and C18:1 t11 proportions in milk rather than a monofactorial mechanism, as suggested by Leiber et al. (2005).

EFFECT OF THE BOTANICAL COMPOSITION OF DIETARY FORAGE ON MEAT FATTY ACID PROFILE

Red clover *vs.* ryegrass forages – Studies describing the effects of feeding red clover or ryegrass on the meat FA metabolism include both lambs, fed either fresh (Fraser et al., 2004) or conserved forages (Chapter 5), and steers fed conserved forages (Scollan et al., 2006a). Figure 6.1. shows the effect of feeding red clover on C18:3 n-3, total PUFA, C18:1 t11 and CLA c9t11 proportions in IMF compared with ryegrass based forages.

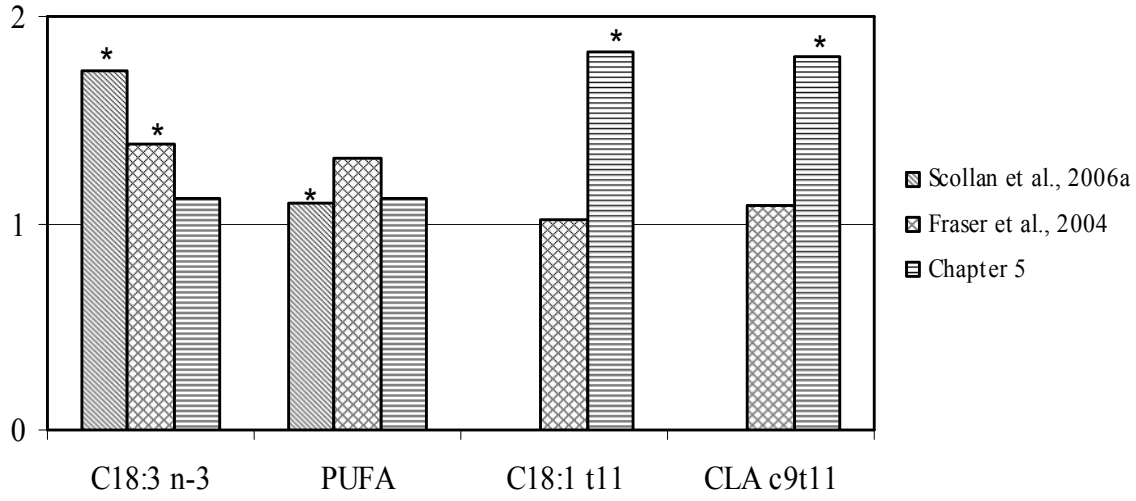

Figure 6.1. - Ratio of the proportions of C18:3 n-3, total PUFA, C18:1 t11 and CLA c9t11 in the IM fat of steers (Scollan et al., 2006a) and lambs (Fraser et al., 2004; Chapter 5 of the present thesis) fed red clover to ryegrass forages (* – Significant differences ($p<0.05$) reported between red clover and ryegrass forages)

From this figure, it is evident that lambs finished on fresh red clover (Fraser et al., 2004) and steers fed red clover silage (Scollan et al., 2006a) have higher C18:3 n-3 proportions than animals fed ryegrass forages, whereas no differences were found in Chapter 5 of the present thesis between the two dietary groups. Similarly, higher IMF C18:2 n-6 proportions were reported in animals fed red clover *vs*. ryegrass forages by Fraser et al. (2004) and Scollan et al. (2006a) (data not shown). Fraser et al. (2004) and in Chapter 5 of the present thesis no differences were found between animals fed red clover *vs.* ryegrass forages in the IMF proportions of PUFA, including the long-chain FA (C20:4 n-6; C20:5 n-3; C22:5 n-3 and C22:6 n-3), resulting from the metabolism of C18:2 n-6 and C18:3 n-3 in the muscle, (9.20 *vs.* 12.1 and 10.6 *vs.* 11.9 g/100 g total FA, for Fraser et al., 2004 and Chapter 5, respectively). However, Scollan et al. (2006a) reported a significant effect in the total PUFA proportions of steers fed red clover silage, although absolute differences between both groups were not higher compared to the former studies. Hence, significant differences were due to the relatively small variation within the dietary groups, but are of minor biological importance. Higher C18:1 t11 and CLA c9t11 proportions in the IMF of lambs fed red clover *vs.* ryegrass forages were reported in Chapter 5, but not by Fraser et al. (2004). This could be due to the fact that feeding fresh forages resulted in higher IMF CLA c9t11 proportions, both in the control and treatment diets, which might have masked possible effects of feeding red clover forages. Hence, it is most likely that the IMF CLA proportions in the animals fed fresh ryegrass (1.23 g/100 g total FA; Fraser et al., 2004) reached a relatively high value, as CLA c9t11 proportions in lamb muscle were reported to vary between 0.2 and 1 g/100 g of total FA in the review of Raes et al. (2004a).

Red *vs.* white clover silages – Comparing the effect of feeding red or white clover silages to growing lambs, in Chapter 5 no differences were reported in the muscle FA content or profile between the two dietary groups, in accordance with the minor changes observed in milk FA pattern when feeding either red or white clover.

Botanical diverse *vs.* grass based diets – Feeding fresh (Whittington et al., 2006; Chapter 4) or conserved (Chapter 5) botanically diverse forages to lambs did not affect the IMF C18:3 n-3 proportions (Figure 6.2.). Nevertheless, a general tendency for higher PUFA proportions (Ådnøy et al., 2005; Whittington et al., 2006; Chapter 4) when lambs were fed botanically diverse forages is evident, compared to the control

group. Further, the difference between total PUFA proportions and the sum of C18:2 n-6 and C18:3 n-3 (4.7 *vs.* 7.1 g/100 g FA for grass based diets and botanically diverse forages, respectively) suggests that lambs fed botanically diverse forages could have higher proportions of long chain PUFA (C20:4 n-6, C20:5 n-3, C22:5 n-3 and C22:6 n-3) in their IMF than lambs fed grass based diets, which has been reported in Chapter 4 of the present thesis. Moreover, indices for desaturation and elongation activity reported in Chapters 4 and 5 suggest the tissue FA metabolism to be influenced by feeding botanically diverse forages.

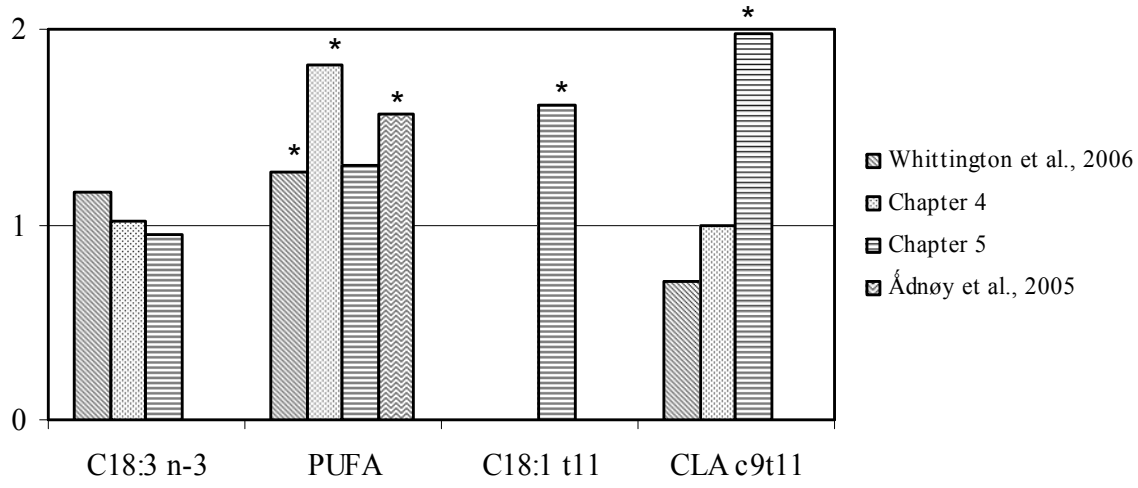

Figure 6.2. – Ratio of the proportions of C18:3 n-3, total PUFA, C18:1 t11 and CLA c9t11 in the intramuscular fat of lambs fed botanically diverse forages to grass based forages (* – Significant differences ($p<0.05$) reported between red clover and ryegrass forages)

Differences in C18:1 t11 and CLA c9t11 proportions were reported in Chapter 5 only, with CLA c9t11 proportions being almost double for lambs fed botanically diverse silage compared to lambs fed grass silage (Figure 6.2.). Although differences in meat FA composition might be a result of changes in the rumen and IMF FA metabolism, as suggested by the desaturation and elongation indices, a confounding effect of variable IMF content and phospholipid/triacylglycerol ratio cannot be excluded. Indeed, feeding regimes with more botanical diverse forages often result in reduced growth rate and leaner animals (e.g. Chapters 4 and 5), with increasing importance of the phospholipid fraction which preferentially contains long chain PUFA (De Smet et al., 2004) and

decreasing proportions of triacylglycerols, which are enriched in CLA c9t11 (De La Torre et al., 2006).

EFFECT OF THE BOTANICAL COMPOSITION OF DIETARY FORAGE ON RUMEN FATTY ACID COMPOSITION

Literature describing the effects of clover and botanically diverse forages on rumen FA metabolism include studies in which FA outflows from the rumen were measured (both in animals with duodenal cannulae (Dewhurst et al., 2003a; Lee et al., 2003, 2006b) and in continuous culture fermenters (Loor et al., 2003a)) as well as studies where rumen contents were analysed (both in rumen pools (Chapter 3) and in rumen spot samples at slaughter (Chapters 4 and 5)). In the study of Loor et al. (2003a), apparent biohydrogenation was calculated from C18:2 n-6 and C18:3 n-3 proportions in dietary and effluent C-18 FA, assuming that the total C18-FA input equals the total C18-FA output (Wu and Palmquist, 1991). For comparative purposes between the treatments reported in Chapters 4 and 5, the same method to calculate the apparent biohydrogenation was used. Due to the single rumen spot sampling at slaughter, values should not be compared with other studies.

Red clover *vs.* ryegrass based diets – Higher C18:3 n-3 flows and ruminal proportions were reported for red clover forages *vs.* ryegrass based diets for most of the studies presented in Tables 6.4. and 6.5. However, care should be taken when interpreting absolute duodenal flows of individual FA as this might be, to some extent, a reflection of differences in FA intake. Apparent biohydrogenation values, which express disappearance relative to intake, are more meaningful in terms of assessing changes in rumen FA metabolism. Indeed, C18:3 n-3 apparent biohydrogenation was lower for red clover forages compared to ryegrass based diets. Further, higher CLA c9t11 flows and ruminal proportions were described, suggesting a less extensive rumen biohydrogenation for red clover *vs.* ryegrass forages, except for Lee et al. (2006b). However, in the latter study very low duodenal CLA c9t11 flows were reported which might question the reliability of the data for this FA.

Table 6.4. – Fatty acid intake (g/d), FA outflow (mg/d) from a continuous *in vitro* system (Loor et al., 2003), FA duodenal flow (g/d) of dairy cattle (Dewhurst et al., 2003a) and steers (Lee et al., 2003, 2006) and apparent biohydrogenation (g/100 g)

	Loor et al. (2003a)		Dewhurst et al. (2003a)			Lee et al. (2003)			Lee et al. (2006b)	
	G	RC	G	RC	WC	G	RC	WC	G	RC
Intake										
C18:2 n-6	141	117	98	122*	139†	10.6	27.8*	38.0†	15.5	22.4*
C18:3 n-3	439	235	84	77*	203†	35.7	67.8*	91.9†	41.3	53.4*
Outflow										
C18:0	330	141*	210	202	273†	61.6	75.3	104	40.2	40.8
C18:1 t11	174	90.1*	16.8	20.3	21.5	3.23	4.63	6.89	4.60	3.83
CLA c9t11	0.62	1.29*	1.84	3.93*	1.71†	0.740	1.43*	2.66	0.041	0.030
C18:2 n-6	9.50	22.3*	11.6	17.0*	23.7†	1.44	4.37*	6.60	1.29	2.76*
C18:3 n-3	12.1	22.2*	4.75	10.4*	14.2†	2.69	11.0*	11.4	2.33	7.86*
Apparent biohydrogenation										
C18:2 n-6	94	82*	89	87*	83†	86	84	83	92	88
C18:3 n-3	98	90*	95	89*	93†	92	84*	88	95	85*

G – ryegrass forage; RC – Red clover forage; WC – White clover forage;
* – Significant differences ($p<0.05$) reported between red clover and ryegrass forages in the different studies; † – Significant differences ($p<0.05$) reported between red and white clover silages in the different studies

The effect of red clover forages on C18:1 t11 was not consistent for all studies. Lower C18:1 t11 amounts in the continuous culture outflow were reported by Loor et al. (2003a), whereas in Chapter 5 higher rumen C18:1 t10+t11 proportions for red clover forages were reported compared to ryegrass based diets. Other studies observed no differences in the C18:1 t11 flow (Dewhurst et al., 2003a; Lee et al., 2003, 2006b).

Red *vs.* white clover forages – Differences in rumen FA metabolism when feeding red or white clover forages were not so clear. Dewhurst et al. (2003a) found a higher duodenal C18:3 n-3 flow for red *vs.* white clover forages, whereas Chapter 5 lower rumen C18:3 n-3 proportions for red *vs.* white clover forages were reported and Lee et al. (2003) observed no differences between the two dietary treatments. However, a general tendency for lower C18:3 n-3 apparent biohydrogenation in red clover forages compared to white clover forages is evident from Tables 6.4. and 6.5.. C18:1 t11 duodenal flows and ruminal proportions were similar for red and white clover forages, whereas only Dewhurst et al. (2003a) reported higher duodenal CLA c9t11 flow for red *vs.* white clover. These observations suggest an inhibition of lipolysis to occur through

red clover feeding rather than an inhibition of intermediate steps in the rumen biohydrogenation process.

Table 6.5. – Forage fatty acid content (mg/g DM), individual fatty acid intake (g/d), total rumen fatty acid content (mg/g DM), proportions of individual fatty acids (g/100 g fatty acid methylesters) and apparent biohydrogenation (g/100 g)

	Chapter 3		Chapter 4		Chapter 5			
	IMS	SPR	IR	BD	IRS	BDS	RCS	WCS
	Forage				*Intake*			
C18:2 n-6	1.75	1.93	3.54	3.37*	18.6	28.1*	24.8†	26.0
C18:3 n-3	7.63	5.10	14.6	9.56*	49.7	33.2*	37.5†	36.3
Rumen FA								
Total FA	41.1*	26.8	63.6	45.2*	50.2	24.6*	33.5†	33.6
C18:0	45.2	39.4*	57.6	53.1*	53.8	40.3*	44.1†	48.4
C18:1 t10+t11	2.14	3.71*	4.47	7.29*	1.54	2.78*	2.64†	2.33
CLA c9t11	0.068	0.077	0.057	0.113*	0.330	1.31*	0.589†	0.788
C18:2 n-6	2.45	2.84	1.27	1.53	2.92	5.91*	4.97†[1]	4.29
C18:3 n-3	2.96	2.30*	1.89	1.79	1.76	1.00	2.61	1.62‡
Apparent biohydrogenation								
C18:2 n-6	n.r.	n.r.	91	91	84	75	78	82
C18:3 n-3	93	94	96	97	96	96	92	95

IMS – Intensively managed ryegrass silage; SPR – 60% species rich silage and 40% IR silage; IR – Intensive ryegrass pasture; BD – Botanical diverse pasture; L – Leguminous rich pasture; IRS – Intensive ryegrass silage; BDS – Botanical diverse silage; RCS – Predominantly red clover silage; WCS – Predominantly white clover silage; n.r. – not reported;
* – Significant differences ($p<0.05$) reported between botanical diverse and ryegrass forages in the different studies; † – Significant differences ($p<0.05$) reported between red clover and ryegrass silages; †[1] – Tendency for differences ($0.1>p>0.05$) between red clover and ryegrass silages; ‡ – Significant differences ($p<0.05$) reported between red and white clover silages

Botanically diverse *vs.* grass based diets – Lower rumen C18:3 n-3 proportions upon feeding botanically diverse forage were reported in Chapter 3, but for both dietary groups apparent biohydrogenation values of C18:3 n-3 were similar, which also has been observed in the other studies (Chapters 4 and 5; Table 6.5.). Higher proportions of C18:1 t10+t11 (all studies) and CLA c9t11 (Chapters 4 and 5) observed in the rumen contents suggest some inhibition of complete rumen biohydrogenation in animals fed botanically diverse forages compared to animals fed grass based diets.

ORIGIN OF HIGHER PUFA PROPORTIONS IN MILK, MEAT AND RUMEN FATTY ACID PATTERN WHEN FEEDING RED OR WHITE CLOVER AND BOTANICALLY DIVERSE FORAGES

Higher C18:3 n-3 outflow was described upon feeding of red clover forages, which was further reflected in higher milk and meat C18:3 n-3 proportions. Nevertheless, long chain PUFA in IMF remained similar in animals fed red clover and ryegrass based diets. Feeding botanically diverse forages *vs.* feeding grass based diets resulted in higher C18:3 n-3 milk proportions and IMF long chain PUFA proportions, although rumen C18:3 n-3 apparent biohydrogenation did not differ between dietary treatments. Unfortunately, only rumen samplings at slaughter and no duodenal flow studies have been reported with botanically diverse forages.

Increased rumen outflow of C18:3 n-3 might be explained by a reduced rumen lipolysis as accumulation of unesterified C18:2 n-6 and C18:3 n-3 is limited in the rumen (Harfoot and Hazlewood, 1997). Rumen circumstances known to reduce lipolysis are associated with for example a low pH (Van Nevel and Demeyer, 1996), which is rather unlikely with pasture based diets. However, some plant metabolites, such as polyphenol oxidase (PPO) present in the red clover was suggested to reduce the activity of plant lipases (Lee et al., 2004; Lourenço et al., 2005b). PPO is a phenol and diphenol oxidising enzyme (Mayer and Harel, 1979). As such, it forms diphenols from phenols and it oxidises diphenols to quinones. Both phenols and diphenols are naturally occurring in plants. The formed quinones are very reactive structures and react with nucleophilic binding sites, which are found in other quinones, forming a polymer network of quinones. However, quinones can also bind with certain amino acids (e.g. lysine, methionine and cysteine), thereby including proteins in the polymer network. The polymerised proteins are denaturated and as a consequence enzyme activity is inhibited. More recently, Lee et al. (2007) have shown that lower rumen biohydrogenation of C18:3 n-3 is associated with red clover clonal lines with high PPO activity. The lower biohydrogenation could be due to three factors or their combination: firstly, it could result from a lower plant lipase activity, due to the binding of the quinones, resulting in a lower degree of lipolysis in the silo which could have reduced further biohydrogenation in the rumen, as lipolysis is a prerequisite for ruminal biohydrogenation. Secondly, quinones could be hypothesized to react with microbial enzymes in the rumen and thus inhibit microbial lipolysis as well as other rumen

microbial processes. This hypothesis is most unlikely as quinones are very reactive structures and will have bound to other proteins or quinones to form the polymer network prior to their appearance in the rumen. Further, formation of quinones in the silo or in the rumen is limited due to the anaerobic conditions in these environments, impairing oxidation of diphenols (Theodorou et al., 2006). Finally, red clover FA could have been protected against plant or microbial lipolysis through their presence in the plant oleosomes embedded in a layer of phenol bound proteins. This possible mechanism is analogous to the protection of lipids against rumen biohydrogenation through formaldehyde treatment of proteins as described by Gulati et al. (2005). The PPO enzyme is present not only in red clover species, but also in other plant species such as grasses like *Lolium perenne* (perennial ryegrass) and *L. multiflorum* (Italian ryegrass), *Dactylis glomerata* (Orchardgrass or Cocksfoot) and *Festuca pratensis* (Meadow fescue) (Theodorou et al., 2006), but it has not yet been investigated in herb species present in botanically diverse forages. Although the latter grass species have PPO enzyme present, the activity is not the same for all species. For e.g. *Festuca pratensis*, *Lolium perenne* and *Lolium multiflorum* only have 0.6, 14 and 22% of the PPO activity in red clover. On the other hand, PPO activity levels of *Dactylis glomerata* and red clover were rather similar (Theodorou et al., 2006). Although white clover does not contain significant amounts of PPO (Theodorou et al., 2006), similar or higher C18:3 n-3 proportions in milk and duodenal fat and similar dietary C18:3 n-3 recoveries compared to red clover fed animals were reported (Table 6.2. and 6.4.), except for the spot rumen samples taken at slaughter of Chapter 5 (Table 6.5.). In Chapter 3 of this PhD thesis as well as in the studies of Dewhurst et al. (2003a) and Lee et al. (2003) it was suggested the stimulatory effect of white clover on rumen outflow rate to be responsible for a reduced microbial contact time and hence apparent biohydrogenation. However, from continuous culture studies, increased rumen outflow rates were suggested rather to increase apparent biohydrogenation (Qui et al., 2004; Martin and Jensen, 2002), which has been attributed to increased microbial activity. Moreover, Lourenço et al. (2005b) reported reduced lipolysis in white clover silage compared to ryegrass silage. In this study, clover saponins have been suggested to be possibly involved in this inhibitory process, as saponins from tea extracts have been suggested to inhibit pancreatic lipases (Han et al., 2001). Direct effects of other plant secondary metabolites on plant lipases or lipases of rumen microbes have not yet been studied. Nevertheless, there are some indications for other compounds affecting lipases, as for

e.g. condensed tannins from grape seed extracts (Moreno et al., 2003) and tea (Ikeda et al., 2005) that also have been described as inhibitors of pancreatic lipases. Shimura et al. (1992) and Sharma et al. (2005) tested the inhibitory effect on lipase activity of several extracts of medicinal plants. Sharma et al. (2005) reported 35.6 to 71.7%, 20.9 to 69.3%, 20.5 % and 35.9% inhibition of lipase activity for plant extracts from the *Fabaceae*, *Asteraceae*, *Ranunculaceae* and *Rosaceae* families, respectively. However, the inhibitory effect on lipase activity described for the latter plant families might not be the only reason for the observed lower rumen lipolysis when feeding botanically diverse forages (Chapter 3), as Sharma et al. (2005) also reported 41 to 83% inhibition of lipase activity by extracts of plants from the *Poaceae* family.

ORIGIN OF HIGHER ACCUMULATION OF BIOHYDROGENATION INTERMEDIATES IN MILK AND RUMEN FATTY ACID PATTERN WHEN FEEDING BOTANICALLY DIVERSE FORAGES

Red clover feeding did not affect C18:1 t11 duodenal flow, whereas CLA c9t11 duodenal flow was increased. However, as endogenous synthesis from C18:1 t11 is the main source of milk and IMF CLA c9t11 (Loor et al., 2003b), neither milk nor IMF C18:1 t11 and CLA c9t11 proportions were affected by feeding red clover forages to the animals. Feeding botanically diverse forages increased rumen C18:1 t11 and CLA c9t11 contents, which was reflected in higher CLA c9t11 and C18:1 t11 proportions in milk and IMF in most of the studies reported in this Chapter. This higher rumen C18:1 t11 is caused by a reduction of the conversion of C18:1 t11 to C18:0, which might be due to shifts in the rumen microbial population. Indeed, in Chapters 4 and 5 botanically diverse forages were suggested to be associated with changes in microbial populations from a principal component analysis based on rumen proportions of odd and branched chain FA which can be used as rumen microbial markers (Vlaeminck et al., 2005). These changes in the microbial population could have been induced by changes in the chemical composition of the diet, but most probably this is a factor of minor importance as the chemical composition of botanical diverse forages was not significantly different from grass based diets in Chapters 4 and 5. The suggested differences in the microbial population and accumulation of some biohydrogenation intermediates in the rumen

contents of animals might have been due to the presence of specific herbs and/or plant metabolites in these forages. Some plant metabolites with antimicrobial activity (Lai and Roy, 2004) have been described to be present in some plant species of botanically diverse forages as for e.g. thymol in the *Ranunculaceae* family (Lai and Roy, 2004), flavonols in the *Asteraceae* family (Broudiscou et al., 2000) and triterpene saponins in the *Fabaceae* and *Rosaceae* families (Hess et al., 2003 and Pen et al., 2006, respectively).

Table 6.6. – Plant secondary metabolites, their effect on rumen methanogenesis and plant families in which these metabolites occur

Plant metabolite	Origin	Methane	References	Plant families
Essential oils				
Allicin	Synthetic	↓	Busquet et al., 2005b; Calsamiglia et al., 2007	*Liliaceae*
Carvacrol	Synthetic	↓	Noirot & Bayourthe, 2006	*Labiatae*; *Lamiaceae*
Thymol	Synthetic	↓	Calsamiglia et al., 2007	*Labiatae*; *Lamiaceae*; *Ranunculaceae*
Cinnamaldehyde	Synthetic	↓	Calsamiglia et al., 2007	*Lauraceae*
Saponins				
Steroidal	*Yucca schidigera* extract	↓ or ≈ (depends on diet & dose)	Sliwinski et al., 2002; Lila et al., 2005; Wina et al., 2005; Pen et al., 2006	*Agavaceae*
Triterpene	*Trifolium repens* extract	≈	Mbanzamihigo et al., 2002	*Fabaceae*
	Sapindus saponaria extract	↓	Hess et al., 2003, 2004a; Wina et al., 2005	*Sapindaceae*
	Albizia sp. extract	↓	Babayemi et al., 2004	*Fabaceae*
		≈	Hess et al., 2003	*Fabaceae*
	Tea extract	↓	Hu et al., 2005; Wina et al., 2005	*Theaceae*
	Quillaja saponaria extract	≈	Pen et al., 2006	*Rosaceae*

↓ – Reduced methanogenesis; ≈ – no effect on methanogenesis

Table 6.6. (*cont.*)

Plant metabolite	Origin	Methane	References	Plant families
Flavonoids				
Luteolin-7-glucoside	*Lavandula officinalis* and *Salvia officinalis* extracts	↓	Broudiscou et al., 2000	*Lamiacea*
	Achillea millefolium extract	↓	Broudiscou et al., 2000	*Asteraceae*
Isoquercetrin	*Equisetum arvense* extract	↓	Broudiscou et al., 2000	*Equisetaceae*
Rutin	*Solidago virgaurea* extract	↓	Broudiscou et al., 2000	*Asteraceae*
Quercetin	*Gingko biloba* extract	↓	Broudiscou et al., 2000	*Ginkgoaceae*
Phenolic acids	*Terminalia chebula* extract	↓	Patra et al., 2006	*Combretaceae*
	Terminalia belerica extract	≈	Patra et al., 2006	*Combretaceae*
	Emblica officinalis extract	≈	Patra et al., 2006	*Euphorbiaceae*
Tannins				
Condensed tannins	*Lespedeza cuneata* extract	↓	Puchala et al., 2005	*Fabaceae*
	Lotus pedunculatus extract	↓	Tavendale et al., 2005; Mueller-Harley, 2006	*Fabaceae*
	Calliandra calothyrsus extract	↓	Hess et al., 2004b	*Fabaceae*
	Salix caprea extract	↓	Mueller-Harley, 2006;	*Salicaceae*

↓ – Reduced methanogenesis; ≈ – no effect on methanogenesis

To our knowledge, no studies are available in the literature describing the effect of secondary plant metabolites on the conversion of C18:1 t11 to C18:0. However, there are some studies (summarized in Table 6.6.) reporting plant secondary metabolite groups, present in herbs of biodiverse forages to affect rumen methanogenesis. Concomitant inhibition of rumen methanogenesis and rumen biohydrogenation has been described for other compounds, such as ionophores (e.g. monensin (e.g. Fellner et al. (1997) and Sauer et al. (1998)), medium chain FA, like C10:0 (Fievez et al., 2006), C12:0 and C14:0 (Soliva et al., 2004), fish oil (Fievez et al, 2003a; Chow et al, 2004b)

and microalgae (Boeckaert et al., 2007; Fievez et al., 2007). Hence, further research towards the effect of these secondary plant metabolites on rumen biohydrogenation might be of interest. From Table 6.6. it can be suggested that essential oils, triterpene saponins and flavonoids are potential candidates to be studied for their effect on rumen biohydrogenation, as decreased methane production in the presence of these metabolites has been described consistently in literature (Table 6.6.).

CONCLUSIONS

It is evident from this discussion that feeding botanically diverse forages affects the FA metabolism in the rumen, towards higher rumen outflow of CLA c9t11 and C18:1 t11 proportions, which are further reflected in higher CLA c9t11 proportions of ruminant products. These changes in the rumen FA metabolism were not always associated to differences in the chemical composition of the pastures nor to their PUFA content. Other factors might play a role in the modified rumen FA metabolism. Plant secondary metabolites are good candidates to explain the observed differences in rumen FA metabolism. Only a few plant secondary metabolites have been described to modify the rumen FA metabolism and particularly lipolysis, such as PPO through the production of phenol complexes. This might explain the higher C18:3 n-3 proportions in fat of ruminant digesta and end products of red clover fed animals. Other plant compounds present in white clover and herbs might exert similar effects. Secondary plant metabolites reported to decrease rumen methanogenesis are possible candidates to provoke increased rumen outflow of C18:1 t11 and merit further investigation.

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PART III

Plant secondary metabolites and their effect on rumen fatty acid metabolism *in vitro*

Chapter 7

Screening the effect of four plant secondary metabolites on rumen fatty acid metabolism using dual-flow continuous culture fermenters

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Chapter 7

Screening the effect of four plant secondary metabolites on rumen fatty acid metabolism using dual-flow continuous culture fermenters

Four different plant secondary metabolites were screened for their effect on rumen biohydrogenation of long chain fatty acids, using dual-flow continuous culture fermenters. Treatments were: control (no additive), positive control (12 mg/L of monesin) and plant extracts (1000 mg/L and 500 mg/L triterpene saponin; 500 mg/L and 250 mg/L quercetin; 250 mg/L eugenol; 500 mg/L cinnamaldehyde). Monensin caused shifts in the fermentation pattern (increased propionate and decreased acetate and butyrate proportions) and inhibited the complete biohydrogenation to the saturated end product although, compared to literature, intermediates of earlier steps of the biohydrogenation process (C18:2 t11c15 rather than C18:1 t11) occurred. Cinnamaldehyde decreased microbial activity, based on the reduction of volatile fatty acids (VFA) and the proportions of odd and branched chain fatty acids (OBCFA) in total fat effluent. Apparent biohydrogenation of C18:2 n-6 and C18:3 n-3 was also lower. In addition, cinnamaldehyde induced a shift from the major known to a secondary biohydrogenation pathway of C18:2 n-6, as suggested from the accumulation of C18:1 t10 and CLA t10c12. Both quercetin treatments resulted in increased total VFA concentrations, suggesting a stimulation of the microbial activity. Eugenol resulted in the accumulation of C18:1 t15 and C18:1 c15, end products of an alternative biohydrogenation pathway of C18:3 n-3. Triterpene saponins did not result in changes of the microbial activity nor in shifts in the fermentation pattern or pathways or extent of biohydrogenation. However, these are results of a first *in vitro* screening test. More detailed studies on the effect of plant secondary metabolites on rumen fermentation and fatty acid biohydrogenation are of interest in future research.

Key words: Biohydrogenation, Cinnamaldehyde, Eugenol, Flavonoids, *In vitro*, Saponins

INTRODUCTION

Some studies (Chapters 3, 4 and 5 of the present PhD thesis) have recently reported an inhibition in the rumen of the complete biohydrogenation of unsaturated to saturated fatty acids upon feeding botanically diverse forages, mainly resulting in the accumulation of intermediates of the last steps of the polyunsaturated long chain fatty acids (LCPUFA) biohydrogenation (C18:1 t11 and CLA c9t11) This was linked with changes in the rumen microbial population, and suggested to be due to the presence in the biodiverse forages of specific herbs or plant secondary metabolites or both (Chapters 3, 4 and 5 of the present PhD thesis). Indeed, some plant secondary metabolites with known antimicrobial activity (Lai and Roy, 2004) have been described to be present in some plant species of biodiverse forages, e.g. thymol in the *Ranunculaceae* family (Lai and Roy, 2004), flavonols in the *Asteraceae* family (Broudiscou et al., 2000) and triterpene saponins in the *Fabaceae* and *Rosaceae* families (Hess et al., 2003 and Pen et al., 2006, respectively). To our knowledge, the effect of these plant secondary metabolites on rumen long chain fatty acid (LCFA) metabolism has not yet been studied but some reports, both *in vivo* and *in vitro*, on reduced rumen methanogenesis and modified rumen fermentation through supplementation of these compounds are available (Table 6.6., Chapter 6; Calsamiglia et al., 2007). Their effect on rumen methanogenesis might be of particular interest as concomitant inhibition of rumen methanogenesis and rumen biohydrogenation of LCPUFA has been described for other compounds, such as ionophores (Fellner et al., 1997), medium chain FA (Soliva et al., 2004), long chain FA from fish oil (Fievez et al., 2003) and microalgae (Boeckaert et al., 2007; Fievez et al., 2007).

With this study we intended to screen the effect of four plant secondary metabolites, possessing different chemical and functional groups, on rumen fermentation and fatty acid (FA) biohydrogenation by means of a dual-flow continuous culture fermenter system.

MATERIALS AND METHODS

Apparatus

Eight 1320 ml dual-flow continuous culture fermenters (Hoover et al., 1976) were used in 4 replicated periods of 5 days (2 days for adaptation and 3 days for sample collection). On the first day of each period, rumen fluid diluted with artificial saliva (1/2, vol/vol) was inoculated in all fermenters. Rumen fluid was obtained from 2 rumen fistulated Holstein dairy cows (650-kg BW) fed a total mixed diet (35.7 g/kg DM alfalfa hay, 7.6 g/kg DM ryegrass hay, 15.5 g/kg DM ground corn, 11.6 g/kg DM barley grain, 11.9 g/kg DM corn gluten feed, 8.1 g/kg DM cottonseed, 4.6 g/kg DM molasses, 1.6 g/kg DM soybean meal, 1.3 g/kg DM calcium soaps of fatty acids, and 2.1 g/kg DM mineral and vitamin mixture). Artificial saliva was prepared according to Weller and Pilgrim (1974) and contained 0.4 g/L urea to simulate recycled N. The saliva was continuously infused into the fermenters. Temperature (38.5ºC), pH (6.4 ± 0.05), and liquid (10%/h) and solid (5%/h) dilution rates were maintained constant, and fermentation conditions were monitored with LabView Software (FieldPoint; National Instruments, Austin, USA). Solid and liquid fermenter effluents were collected separately and collection vessels were maintained at 4ºC to impede microbial action. Anaerobic conditions in the fermenters were maintained by infusion of 40 ml N_2/min.

Diets and Treatments

Lolium perenne (perennial ryegrass) was cut, dried (70ºC-80ºC for 48h) and ground through 1 mm mesh (Brabander, Duisburg, Germany) and stored at -20ºC until used for fermenter feeding and FA analysis. Fermenters were fed 60 g dried *Lolium perenne* in 2 equal portions daily (at 10h00 and 22h00).

Plant secondary metabolites used in this study were chosen based on their effect on rumen fermentation and their presence in biodiverse forages (Table 6.6., Chapter 6). Treatments were: control (CON, no additive), positive control (monensin, MON (12 mg/L); Sigma Chemical, St. Louis, USA) and four plant secondary metabolites: triterpene saponins from Quillaja Bark (QB1000 (1000 mg/L) and QB500 (500 mg/L); sapogenin content ~25%; Sigma Chemical, St. Louis, USA); quercetin (Q500 (500 mg/L) and Q250 (250 mg/L); $C_{15}H_{10}O_7 \cdot 2H_2O$, 98% purity; Sigma-Aldrich Chemical, Steinheim, Germany); eugenol (E250 (250 mg/L); $C_{10}H_{12}O_2$, 98% purity; Pancosma

S.A., Bellegarde-sur-Valserine Cedex, France) and cinnamaldehyde (C500 (500mg/L); C_9H_8O, 98% purity; Pancosma S.A., Bellegarde-sur-Valserine Cedex, France). Doses of monensin cinnamaldehyde and eugenol were chosen based on previous *in vitro* research (Calsamiglia et al., 2007). Doses of saponin and quercetin were chosen based on literature. The daily dose of the additives was divided in 2 equal fractions and added into the fermenters at the same time of feeding to achieve the expected average concentration.

Sampling

Each experimental period lasted 5 days, with 2 days for adaptation and 3 days for sample collection. For sample collection, solid and liquid effluents were mixed and homogenized for 1 min, and a 250 ml sample was removed via aspiration, from which a sub-sample (100 ml) was collected for FA analysis. The rest of the sample was then filtrated through 1 layer of cheese cloth and subsamples were collected for the determination of ammonia N (4 ml) and volatile FA (VFA) concentration (4 ml). One ml of a solution composed of 0.2 % (w/w) of mercuric chloride, of 0.2 % (w/w) 4-methylvaleric acid (used as internal standard) and 2% (vol/vol) orthophosphoric acid was added to samples for VFA analysis. Samples for ammonia determination were acidified with 4 ml of 0.2 N HCl. All samples were frozen immediately after collection and kept at -20ºC until analysis. Samples for FA analysis were freeze-dried without shelf heat (Dura-Dry MP, FTS Systems, New York, USA) and kept at -20ºC until analysis.

Analysis

- *Fatty acid analysis*

Fatty acids of all fermenters effluents of the 3rd, 4th and 5th experimental days were extracted with C/M (2/1, vol/vol) as described in Chapter 3 with some adaptations for smaller sample aliquots. Briefly, 1.25 g of freeze-dried sample was extracted overnight with 15 ml of C/M (2/1, vol/vol), 10 ml of distilled water and 10 mg of tridecanoic acid (C13:0; Sigma, Belgium) as internal standard. Samples were then centrifuged at 1821 x *g* for 10 min and the C/M layer recovered. This procedure was repeated twice, adding 10 ml of C/M (2/1, vol/vol) in the second and third extraction steps. Finally, samples were washed with distilled water and the C/M layer was recovered. Extracts were brought to a final volume of 50 ml with C/M (2/1, vol/vol).

Fatty acids of dried perennial ryegrass samples of each experimental period were extracted with C/M (2/1, vol/vol) as described in Chapter 3. Briefly, 2.5 g of dried sample was extracted overnight with 30 ml of C/M (2/1, vol/vol), 20 ml of distilled water and 10 mg of tridecanoic acid (C13:0; Sigma, Belgium) as internal standard. Samples were then centrifuged at 1821 x *g* for 10 min and the C/M layer recovered. This procedure was repeated twice, adding 25 ml of C/M (2/1, vol/vol) in the second and 20 ml in the third extraction steps. Finally, samples were washed with distilled water and the C/M layer was recovered. Extracts were brought to a final volume of 100 ml with C/M (2/1, vol/vol). For preparation of FA methylesters (FAME) of effluent and dried perennial ryegrass, 10 ml of extract was used. Samples were methylated at 50ºC with NaOH in methanol (0.5M) followed by HCl/Methanol (1/1, vol/vol) according to Raes et al. (2001). Fatty acid methylesters were analyzed on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard Co., Belgium) with a CP-Sil88 column for FAME (100m x 0.25mm x 0.2 µm; Chrompack Inc., The Netherlands). For more detailed information about the GC conditions we refer to Raes et al. (2004b). The FA profile of dried perennial ryegrass is presented in Table 7.1.

Table 7.1. – Total FA (mg/g DM) and proportions of individual FA (g/100g FAME) of the dried perennial ryegrass fed to the fermenters (n=4)

Fatty acids	Perennial ryegrass	SD
Total fatty acids	29.3	0.869
Individual fatty acids		
C12:0	0.599	0.026
C14:0	2.34	0.096
C16:0	13.2	0.156
C16:1 c9	1.35	0.005
C18:0	1.64	0.052
C18:1 c9	3.49	0.070
C18:2 n-6	11.8	0.063
C18:3 n-3	57.6	0.459

SD – Standard deviation of mean

- *Volatile fatty acid analysis*

Samples for VFA analysis (from all 5 days of each experimental period) were prepared as described by Jouany (1982). Samples were centrifuged at 15 000 x *g* for 15 min at 7ºC and 1 ml of the supernatant was diluted with distilled water (1/1, vol/vol).

Volatile FA were analysed on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, USA) with a polyethylene glycol nitroterephtalic acid-treated capillary column for VFA (BP21; SGE, Europe Ltd., Buckinghamshire, UK). For more detailed information about the GC conditions we refer to Busquet et al. (2005a). Data of samples from the 3rd, 4th and 5th experimental days were further used for statistical analysis.

- *Ammonia N analysis*

Ammonia N concentration (mg/100 ml) was determined according to Chaney and Marbach (1962). Samples (from all 5 days of each experimental period) were centrifuged at 15 000 x *g* for 15 min at 7ºC and 100 µl of the supernatant was collected and analysed by visible spectrophotometry (Libra S21; Biochrom, Cambridge, UK). Data of samples from the 3rd, 4th and 5th experimental days were further used for statistical analysis.

Calculations

Apparent biohydrogenation was calculated from C18:2 n-6 and C18:3 n-3 proportions in dietary and effluent C18-FA, assuming that the total C18-FA input equals the total C18-FA output (Wu and Palmquist, 1991).

Statistics

A split plot analysis for repeated measures (days 3, 4 and 5 of each experimental period) was used to evaluate the effect of dietary treatments on rumen VFA, long-chain FA and ammonia N, variations between periods and the interaction between diets and periods. The effect of sampling day was evaluated from the "within-subject-effect". The dietary treatments (CON, MON, QB1000, QB500, Q500, Q250, E2500 and C500) and periods (1, 2, 3 and 4) were introduced as "between-subject-factors" and their effects evaluated from the "between-subject-effects". Comparison of means was done using Duncan as post-hoc test.

All statistical analyses were performed using SPSS 12.0 (SPSS software for Windows, release 12.0, SPSS inc., USA).

RESULTS

Two days were sufficient for the adaptation of the rumen microbial population to the fermenter conditions (data not shown), similarly to what has been reported by Busquet et al. (2005a, c).

Volatile fatty acids and ammonia N concentrations

Total VFA concentrations and proportions of individual VFA are presented in Table 7.2. Although CON and MON did not differ in terms of total VFA concentrations, addition of MON resulted in higher proportions of propionate and in lower proportions of acetate and butyrate compared to the CON.

Table 7.2. – Total VFA (mM), proportions of individual VFA (mol/100 mol) and ammonia N (mg/100 ml) according to the different dietary treatments tested (n=12)

	CON	MON	QB1000	QB500	Q500	Q250	E250	C500	SEM[1]	Sign.
Total VFA	67.0^{cd}	63.9^{c}	69.5^{bc}	67.0^{cd}	76.8^{a}	72.9^{ab}	64.6^{c}	24.7^{e}	1.53	***
Acetate	61.2^{b}	50.4^{c}	62.6^{b}	62.0^{b}	67.0^{a}	63.7^{b}	61.4^{b}	64.6^{ab}	1.06	***
Propionate	22.7^{b}	37.2^{a}	21.9^{b}	22.2^{b}	20.5^{b}	22.2^{b}	22.0^{b}	13.7^{c}	0.885	***
Butyrate	12.0^{b}	7.43^{c}	12.0^{b}	12.2^{b}	10.4^{bc}	11.1^{b}	12.2^{b}	17.4^{a}	1.09	***
Valerate	2.51^{b}	4.16^{a}	2.37^{b}	2.38^{b}	1.52^{c}	2.06^{bc}	2.52^{b}	3.59^{a}	0.266	***
BCVFA	1.50^{ab}	0.830^{bc}	1.11^{bc}	1.25^{bc}	0.599^{c}	1.01^{bc}	1.95^{a}	0.773^{c}	0.213	**
Acet/Prop	2.71^{b}	1.36^{c}	2.87^{b}	2.81^{b}	3.30^{b}	2.88^{b}	2.82^{b}	5.03^{a}	0.217	***
NH_3-N	5.49^{c}	7.92^{b}	4.04^{c}	4.85^{c}	5.04^{c}	5.34^{c}	5.30^{c}	14.8^{a}	0.568	***

QB1000 – Quillaja bark (1000 mg/L); QB500 – Quillaja bark (500 mg/L); Q500 – Quercetin (500 mg/L); Q250 – Quercetin (250 mg/L); E250 – Eugenol (250 mg/L); C500 – Cinnamaldehyde (500 mg/L); MON – Monensin (12 mg/L); CON – control;
BCVFA – Branched chain volatile fatty acids (*iso*-butyrate and *iso*-valerate); NH_3-N – Ammonia N;
[1] – Standard error of mean; ** - $0.01>p>0.001$; *** - $p<0.001$; [a, b, c] – Within a row, means without a common superscript letter differ ($p<0.05$)

In contrast, Q250 and Q500 increased total VFA concentrations, whereas C500 reduced approximately 3-fold total VFA concentrations compared with the CON. Treatment C500 increased butyrate proportions and decreased propionate proportions compared with both the CON and MON. For the other plant metabolites, only minor changes were observed. Treatment Q500 increased acetate proportions and decreased valerate and branched chain VFA concentrations compared with the CON. The proportions of individual VFA for Q250 were intermediate between Q500 and the CON

but did not significantly differ from either of the two. The fermentation pattern in terms of both total VFA concentrations and individual VFA proportions in treatments QB1000, QB500 and E250 remained similar to the CON.

The concentration of ammonia N (Table 7.2.) was highest for C500, followed by MON. The CON and all other treatments showed similar and lower ammonia N concentrations.

Fatty acids

Total amount of FA and proportions of individual FA are presented in Table 7.3. Compared to the CON, addition of Q500 increased total amount of FA whereas C500 and MON decreased total amount of FA. MON increased total odd and branched chain FA (OBCFA) proportions compared with the CON (Table 7.3.), whereas E250 and C500 reduced total OBCFA proportions, compared with the CON, with C500 having the strongest effect. For the other plant secondary metabolites (QB1000 and QB500), only minor changes were observed in the proportions of some individual OBCFA compared with the CON.

Total saturated FA (SFA) proportions of E250, C500 and MON were lower compared with the CON, which was mainly due to the low proportion of C18:0. Treatment E250 increased C18:1 t15 and C18:1 c15 proportions, whereas total monounsaturated FA (MUFA) proportions remained similar to the CON. Treatments C500 and MON increased C18:1 t10 proportions, but total MUFA proportions were decreased compared with the CON (Table 7.3.). On the other hand, C500 had higher CLA t10c12 proportions compared with the CON. Proportions of C18:1 intermediates of major known C18:2 n-6 and C18:3 n-3 biohydrogenation pathways, such as C18:1 t11, C18:1 t15 and C18:1 c15 were always lower for C500 and MON compared with the CON. Proportions of C18:2 t11c15, a biohydrogenation intermediate of an earlier step in the process, were markedly increased in the fermenters effluent of C500 and MON. However, for none of the plant metabolites nor for MON, proportions of CLA c9t11 differed from the CON. The proportions of C18:2 n-6 and C18:3 n-3 in the C500 and MON fermenters effluent were higher compared with the CON and all other treatments, further reflected in the higher total polyunsaturated FA (PUFA) proportions in C500 and MON and the lower apparent biohydrogenation of C18:2 n-6 and of C18:3 n-3 (Table 7.3.).

Table 7.3. – Total FA (mg/g), proportions of individual FA (g/100g FAME) in the effluent and apparent biohydrogenation (g/100g) according to the different dietary treatments tested (n=12)

	CON	MON	QB1000	QB500	Q500	Q250	E250	C500	SEM	Sign.
Total FA	24.0^{b}	21.3^{c}	22.2^{bc}	24.4^{ab}	26.6^{a}	24.5^{ab}	22.3^{bc}	18.5^{d}	0.805	***
C13:0 iso	0.265	0.224	0.193	0.216	0.233	0.212	0.213	0.249	0.037	n.s.
C13:0 anteiso	0.163^{c}	0.276^{ab}	0.188^{c}	0.202^{c}	0.294^{a}	0.218^{bc}	0.184^{c}	0.077^{d}	0.021	***
C14:0 iso	0.106^{b}	0.247^{a}	0.093^{b}	0.106^{b}	0.205^{a}	0.126^{b}	0.085^{b}	0.025^{c}	0.016	***
C15:0 iso	0.341	0.533	0.282	0.337	0.397	0.415	0.177	0.329	0.081	n.s.
C15:0 anteiso	0.870^{ab}	1.09^{a}	0.917^{ab}	0.892^{ab}	0.592^{c}	0.743^{bc}	0.696^{bc}	0.222^{d}	0.084	***
C16:0 iso	0.094^{c}	0.223^{a}	0.082^{cd}	0.096^{c}	0.159^{b}	0.102^{c}	0.071^{cd}	0.050^{d}	0.012	***
C17:0 iso	0.123^{abc}	0.141^{ab}	0.105^{c}	0.116^{bc}	0.151^{a}	0.154^{a}	0.072^{d}	0.012^{e}	0.011	***
C17:0 anteiso	0.209^{b}	0.283^{a}	0.188^{bc}	0.218^{b}	0.192^{bc}	0.210^{b}	0.143^{c}	0.165^{bc}	0.017	***
C15:0	1.30^{bc}	1.84^{a}	1.47^{ab}	1.31^{bc}	0.951^{cd}	1.08^{bcd}	0.808^{d}	0.365^{e}	0.128	***
C17:0	0.417^{bc}	0.455^{ab}	0.468^{a}	0.438^{abc}	0.411^{cd}	0.405^{cd}	0.369^{d}	0.176^{e}	0.014	***
Total OBCFA	4.17^{b}	5.33^{a}	4.35^{b}	4.17^{b}	3.60^{bc}	3.93^{b}	2.95^{c}	2.12^{d}	0.276	***
C12:0	0.209^{b}	0.248^{a}	0.235^{ab}	0.255^{a}	0.266^{a}	0.240^{ab}	0.235^{ab}	0.246^{a}	0.010	*
C14:0	0.745^{abc}	0.686^{c}	0.806^{a}	0.785^{b}	0.836^{a}	0.816^{a}	0.695^{bc}	0.423^{d}	0.031	***
C16:0	11.9^{b}	12.4^{b}	11.9^{b}	11.9^{b}	12.1^{b}	11.8^{b}	10.9^{c}	14.1^{a}	0.225	***
C18:0	30.8^{ab}	2.45^{d}	29.2^{bc}	30.3^{ab}	32.3^{a}	30.7^{ab}	27.7^{c}	2.52^{d}	0.734	***
Total SFA	45.4^{ab}	18.3^{d}	44.1^{b}	45.1^{ab}	47.0^{a}	45.1^{ab}	40.8^{c}	17.9^{d}	0.783	***
C18:1 t6-t8	0.610^{b}	0.128^{c}	0.708^{b}	0.610^{b}	0.717^{b}	0.641^{b}	0.933^{a}	0.039^{c}	0.068	***
C18:1 t9	0.408^{a}	0.110^{b}	0.435^{a}	0.384^{a}	0.447^{a}	0.414^{a}	0.478^{a}	0.092^{b}	0.037	***
C18:1 t10	0.394^{b}	1.31^{a}	0.493^{b}	0.414^{b}	0.457^{b}	0.410^{b}	0.487^{b}	1.52^{a}	0.168	***
C18:1 t11	13.7^{a}	8.20^{b}	13.3^{a}	13.5^{a}	14.8^{a}	13.7^{a}	14.0^{a}	5.23^{b}	1.50	**
C18:1 t15	1.05^{b}	0.182^{d}	1.27^{a}	1.03^{b}	0.825^{c}	1.03^{b}	1.39^{a}	0.132^{d}	0.060	***
C18:1 c9	5.95^{cd}	4.10^{e}	6.96^{a}	6.25^{abc}	5.31^{d}	5.99^{bcd}	6.73^{ab}	4.34^{e}	0.243	***
C18:1 c11	0.811^{abc}	0.254^{d}	0.849^{ab}	0.834^{abc}	0.703^{c}	0.762^{bc}	0.913^{a}	0.161^{d}	0.045	***
C18:1 c15	0.916^{bc}	0.283^{d}	1.09^{ab}	0.851^{bc}	0.509^{cd}	0.782^{bc}	1.47^{a}	0.101^{d}	0.136	***
Total MUFA	29.0^{a}	18.1^{b}	30.6^{a}	29.3^{a}	29.0^{a}	28.9^{a}	32.5^{a}	15.1^{b}	1.27	***
C18:2 t11c15	2.21^{c}	25.5^{a}	2.19^{c}	2.29^{c}	1.91^{c}	2.48^{c}	3.45^{c}	15.7^{b}	0.847	***
CLA c9t11	0.203	0.282	0.146	0.231	0.267	0.245	0.249	0.467	0.072	n.s.
CLA t10c12	0.036^{bc}	0.089^{b}	0.032^{bc}	0.044^{bc}	0.018^{c}	0.030^{bc}	0.052^{bc}	0.208^{a}	0.021	***
CLA t11c13	0.602^{ab}	0.275^{cd}	0.433^{bc}	0.628^{a}	0.674^{a}	0.744^{a}	0.317^{c}	0.098^{d}	0.062	***
C18:2 n-6	2.66^{b}	6.76^{a}	2.68^{b}	2.47^{b}	2.49^{b}	2.71^{b}	2.85^{b}	7.89^{a}	0.417	***
C18:3 n-3	8.23^{c}	17.8^{b}	8.36^{c}	7.50^{c}	7.92^{c}	8.38^{c}	9.27^{c}	30.3^{a}	1.35	***
Total PUFA	10.9^{b}	36.1^{a}	10.5^{b}	10.4^{b}	10.8^{b}	11.4^{b}	12.3^{b}	36.0^{a}	1.71	***
Total C18-FA	71.5^{b}	68.4^{d}	71.4^{b}	70.4^{bc}	72.0^{b}	71.9^{b}	74.0^{a}	69.2^{cd}	0.554	***
Apparent biohydrogenation										
C18:2 n-6	76.4^{a}	36.8^{b}	76.1^{a}	77.7^{a}	78.0^{a}	76.0^{a}	75.5^{a}	33.4^{b}	0.036	***
C18:3 n-3	85.1^{a}	66.3^{b}	84.8^{a}	86.2^{a}	85.8^{a}	84.9^{a}	83.8^{a}	43.1^{c}	0.027	***

QB1000 – Quillaja bark (1000 mg/L); QB500 – Quillaja bark (500 mg/L); Q500 – Quercetin (500 mg/L); Q250 – Quercetin (250 mg/L); E250 – Eugenol (250 mg/L); C500 – Cinnamaldehyde (500 mg/L); MON – Monensin (12 mg/L); CON – control;

[1] – Standard error of mean; n.s. – not significantly different ($p>0.1$); * - $0.05>p>0.001$ ** - $0.01>p>0.001$; *** - $p<0.001$; [a, b, c, d, e] – Within a row, means without a common superscript letter differ ($p<0.05$)

Total OBCFA – Sum of all odd and branched chain FA: *iso* C13:0; *anteiso* C13:0; *iso* C14:0; *iso* C15:0; *anteiso* C15:0; *iso* C16:0; *iso* C17:0; *anteiso* C17:0, C15:0 and C17:0;

Total SFA – Sum of all saturated FA: C12:0, C14:0, C16:0, C18:0 and C20:0;

Total MUFA – Sum of all monounsaturated FA: C14:1 c9, C16:1 c9, C18:1 t6-t8, C18:1 t9, C18:1 t10, C18:1 t11, C18:1 t12-t14, C18:1 t15; C18:1 c9, C18:1 c11, C18:1 c12, C18:1 c13, C18:1 c14 and C18:1 c15;

Total PUFA – Sum of all polyunsaturated FA: C18:2 t11c15, CLA c9t11, CLA t10c12, CLA t11c13, C18:2 n-6, C18:3 n-6 and C18:3 n-3

DISCUSSION

In the current paper, indicators of reduced microbial activity in rumen which will be considered are 1) decreased total VFA concentrations in the effluent; 2) decreased proportions in effluent fat of OBCFA, as flow of OBCFA has been suggested as a marker of microbial biomass (Vlaeminck et al., 2005); 3) decreased biohydrogenation of dietary PUFA and 4) decreased fat proportions in the effluent. Indeed, fermentation results in the partial conversion of non-fat organic matter into gaseous end products which escape from the fermenters. Hence, when degradation is reduced, higher amounts of OM are recovered in the effluent, therefore diluting the concentration of fat. The constant level of rumen undegradable dietary fat, combined with reduced *de novo* synthesis of OBCFA by rumen bacteria and the significantly higher recovery of the dietary organic matter result in a decreased concentration per g of DM of total fat.

Shifts in the fermentation pattern (Table 7.2.) and a reduced apparent biohydrogenation of C18:2 n-6 and C18:3 n-3 (Table 7.3.) were observed in the presence of MON, in agreement with what has been reported in literature (Fellner et al., 1997; Jenkins et al., 2003; Wang et al., 2005) for *in vitro* systems. However, in our study MON resulted in an accumulation of C18:2 t11c15 and C18:1 t10 rather than C18:1 t11, as observed in other studies (Jenkins et al., 2003; Wang et al., 2005), suggesting the inhibition, retardation or both, of biohydrogenation to occur at earlier steps of the process rather than at the last step as observed by Jenkins et al. (2003) and Wang et al. (2005), and a shift from the major known (via C18:1 t11) to a secondary biohydrogenation pathway of C18:2 n-6 biohydrogenation (via C18:1 t10).

The reduction in total VFA concentrations, biohydrogenation of dietary PUFA and the proportions of total OBCFA in C500 suggests that microbial activity was inhibited. This overall reduction in the rumen processes further results in a decrease in the total fat concentration of the effluent (Table 7.3.). Additionally, some microbial populations seem even more sensitive to the presence of cinnamaldehyde than others, which is reflected in shifts in the OBCFA proportions (Table 7.3.), shifts in the proportions of individual VFA (Table 7.2.) and in some shifts in the biohydrogenation pathway as suggested from the accumulation of C18:1 t10 and CLA t10c12 (Table 7.3.). Moreover, an inhibition of the biohydrogenation process is suggested from the accumulation of other intermediates (C18:2 t11c15) and the reduced apparent

biohydrogenation of C18:2 n-6 and C18:3 n-3. Accumulation of these FA reduced precursor supply for later steps in the biohydrogenation process, explaining the lower C18:1 t11, C18:1 t15 and C18:1 c15 proportions. The dramatic overall reduction of the rumen microbial activity as observed here has been reported in literature with high doses of cinnamaldehyde (3000 mg/L; Busquet et al., 2006), while moderate (31.2 and 312 mg/L; Busquet et al., 2005a) doses, comparable to the current applied dose, were reported to cause some shifts in the proportions of individual VFA. A possible reason for the different outcome of this study compared to the one of Busquet et al. (2005a) could be linked to an interaction of the plant secondary metabolite with the basal diet used. Busquet et al. (2005a) fed the fermenters a 50:50 alfalfa hay:concentrate diet whereas in our study a complete forage diet was used. Moreover, using moderate doses of cinnamaldehyde, Busquet et al. (2005a) reported a different fermentation pattern, with higher butyrate and propionate proportions, whereas in our study, only the proportion of butyrate increased in the presence of cinnamaldehyde and the propionate proportion decreased. Further, Castillejos et al. (2005) reported different fermentation patterns for a high concentrate diet (10:90 forage:concentrate ratio) compared with a high forage diet (60:40 forage:concentrate ratio) in combination with a blend of essential oil compounds (Crina® for ruminants).

Although total OBCFA proportions in the presence of eugenol (E250) were reduced compared with the CON (Table 7.3.), suggesting a reduced total microbial biomass, total amounts of VFA and apparent biohydrogenation of C18:2 n-6 and C18:3 n-3 did not differ from the CON. Moreover, no difference in the end products of rumen fermentation were found (Table 7.2.), opposite to the results of Busquet et al. (2006) and Castillejos et al. (2006). Nevertheless, the biohydrogenation process seemed to be slightly inhibited by the presence of E250 based on the increased accumulation of C18:1 t15 and C18:1 c15, end products of an alternative biohydrogenation pathway of C18:3 n-3 and on the lower C18:0 proportions compared with the CON (Table 7.3.). The concentrations used might explain why some studies report an effect of eugenol on the fermentation pattern (Busquet et al., 2006; Castillejos et al., 2006) and other studies not (Busquet et al., 2005c (2.2 mg/L rumen fluid); the present study). Further, Busquet et al. (2006), reported a different fermentation pattern in the presence of eugenol, with a dose of 3000 mg/L rumen fluid compared with the results of Castillejos et al. (2006), where a dose of 500 mg/L was used. With high doses of eugenol (3000 mg/L), total VFA, butyrate and BCVFA proportions decreased and propionate proportions increased

(Busquet et al., 2006), whereas with moderate doses (500 mg/L), total VFA, acetate and BCVFA proportions decreased and propionate and butyrate proportions increased (Castillejos et al., 2006).

The higher total amount of total VFA in quercetin supplemented treatments (Q500 and Q250; Table 7.2.) could suggest a stimulation of the microbial activity. However, proportions of OBCFA remained similar to the CON and no shifts in the biohydrogenation pathways or extent were observed.

Triterpene saponins (QB1000 and QB500) did not affect the rumen total microbial biomass or activity, based on the similar total OBCFA proportions and total amounts of rumen VFA compared with the CON. Additionally, neither the FA metabolism nor the fermentation pattern were affected by triterpene saponins. Secondary plant metabolite concentrations were based on literature data, but observed changes in te rumen fermentation pattern and rumen microbial activity differed from what has been expected from literature. This divergence from literature data might have different reasons, e.g. interaction with basal diet, applied concentration or origin of the plant secondary metabolites. Indeed, triterpene saponins studied in literature were derived from a variety of plants including *Albizia sp.* (Hess et al., 2003; Babayemi et al., 2004), *Quillaja saponaria* (Pen et al., 2006), *Sapindus saponaria* (Hess et al., 2003, 2004a, Wina et al., 2005), *Trifolium repens* (Mbanzamihigo et al., 2002) and tea extract (Hu et al., 2005; Wina et al., 2005). Effects on rumen methanogenesis provoked by triterpene saponins are not unequivocal even with saponins with the same origin (*Albizia sp.*), e.g. Babayemi et al. (2004) *vs.* Hess et al. (2003). Additionally, the fast adaptation of the microbial population to saponins by its conversion to sapogenins, as reported in literature for plant secondary metabolites (Busquet et al., 2005a) could mask possible effects of the plant metabolite on the rumen processes.

This is the first study, to our knowledge, reporting the effect of plant secondary metabolites on rumen FA metabolism and no direct comparison with literature is possible. Previous *in vivo* studies (Chapters 3, 4 and 5 of the present PhD thesis) reported a partial inhibition of the ruminal biohydrogenation process upon feeding botanically diverse forages compared with feeding intensive ryegrass forages. This inhibition of the ruminal biohydrogenation of forage PUFA was reflected in the increased C18:1 t11 and CLA c9t11 proportions in the rumen contents of the animals fed botanically diverse forages. This inhibition of the last steps of dietary PUFA biohydrogenation was associated with changes in the microbial population, and the

latter was suggested to be due to the presence of specific herbs or plant secondary metabolites or both in these biodiverse forages (Chapters 3, 4 and 5 of the present PhD thesis). Hence, this *in vitro* screening test was a first attempt to get more insight in the origin of the observed *in vivo* effects (Chapters 3, 4 and 5 of the present PhD thesis). Differences between *in vivo* effects upon feeding botanically diverse forages (Chapters 3, 4 and 5 of the present PhD thesis) and *in vitro* (present study) could be due to 1) the combined action of different plant secondary metabolites present in botanically diverse forages *vs.* single plant secondary metabolites screened in this study; 2) the action of other plant metabolites of botanically diverse forages not screened in this study or 3) the interaction between the basal diet and the plant secondary metabolite. In Chapters 3 and 5, of this PhD thesis forage diets were supplemented with concentrate (75:25 and 70:30 forage:concentrate ratio for Chapters 3 and 5, respectively) and rumen contents of botanically diverse forage fed animals were reported to have increased proportions of C18:1 t11 and CLA c9t11 (intermediates of later steps of the biohydrogenation process of dietary PUFA) compared with animals fed intensive ryegrass forages. Opposite, in Chapter 4 of this PhD thesis no concentrate was provided to the animals and intermediates of earlier steps of the biohydrogenation process (C18:2 t11c15) were increased, besides the increased C18:1 t11 proportions in the rumen contents of botanically diverse forage fed animals.

CONCLUSIONS

Monensin induced shifts in both the fermentation pattern and in the biohydrogenation, without effect on total VFA concentrations. Eugenol caused some minor inhibition of the biohydrogenation process, without effects on the fermentation process. Supplementation of quercetin resulted in higher total VFA concentrations, suggesting a stimulation of the microbial activity. However, shifts in the pathways or extent of biohydrogenation did not occur for both quercetin and triterpene saponins. Cinnamaldehyde suggested to cause an overall inhibition of the microbial biomass and activity, based on the reduced VFA and OBCFA proportions. Moreover, cinnamaldehyde caused a shift from the major to a secondary biohydrogenation pathway of C18:2 n-6 as suggested by the higher proportions of C18:1 t10 and CLA

t10c12 in the fermenter effluent. More detailed studies on the effect of plant secondary metabolites on rumen fermentation and FA biohydrogenation are of interest in future research.

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IMPLICATIONS TO HUMAN NUTRITION

Implications to human nutrition

Results of this PhD thesis showed the potential of botanically diverse pastures to change the fatty acid (FA) profile of the ruminant end product: increased polyunsaturated FA (PUFA) proportions in meat and conjugated linoleic acid (CLA) proportions in milk. However, the significance of this improvement is not always easy to assess in terms of human nutrition.

In Chapter 3 of this PhD thesis, CLA content was almost double in milk of animals fed species rich silages (60SPR) compared with milk of animals fed intensive ryegrass (100IM) silages (0.19 and 0.11 mg/g milk for 60SPR and 100IM, respectively). No recommendations for the intake of CLA have been established yet, but increased CLA consumption has been shown to have positive effects on human health (Yaqoob et al., 2006). Based on animal studies, Ip et al. (1994) suggested 3g/d of CLA would be required for cancer protection. In addition, to prevent osteoporosis, the WHO (2003) recommends a daily intake of 2 to 3 servings from the dairy products group, corresponding to 600 ml of milk. In light of these suggestions, consuming 600 ml of milk from 60SPR fed animals would provide 4% (122 mg CLA) of the suggested daily intake of CLA, compared with only 2% (67 mg CLA) with milk from 100IM fed animals. Despite this considerable increase in CLA intake, the contribution to suggested CLA intake is still rather marginal, although dairy products have been suggested to be the major natural source of CLA. On the other hand, no significant difference was observed in milk total PUFA proportions of 60 SPR and 100IM fed animals (2.32 *vs.* 1.95 g/100 g total FA, for 60SPR and 100IM, respectively). At the daily recommended intake of 600 ml of milk, and with 1g of fat corresponding to 9 kcal, the PUFA from milk of 60SPR fed animals would supply 5.3 kcal, and the PUFA from milk of 100IM fed animals would supply 4.3 kcal to the total daily energy intake. This represents only 0.3 and 0.2 % of the total energy intake (based on 2000 kcal/d), for milk of 60SPR and 100IM fed animals, respectively. Taking into account the daily 6 to 10% of total energy intake from PUFA recommended by the WHO (2003), the achieved levels can be considered low.

In Chapter 4 of this PhD thesis, total CLA content was not improved in the lamb intramuscular fat (IMF) by grazing a botanically diverse (BD) pasture compared with

grazing an intensive ryegrass (IR) pasture (15 *vs.* 18 mg total CLA/100 g meat). Total PUFA content was only slightly increased in the IMF of BD pasture animals compared with IR pasture animals (346 *vs.* 233 mg total PUFA/100 g meat). The contribution to the total energy intake of PUFA is 0.05% higher with the IMF of BD pasture animals compared with IR pasture animals (0.15 *vs.* 0.10% of total energy intake, based on 2000 kcal/day). Similar to Chapter 3, the achieved levels of energy contribution from PUFA are low, in light of the 6 to 10% of total energy intake from PUFA suggested by the WHO (2003).

It has been suggested the health benefits of increased C18:3 n-3 intake to be associated with an increased synthesis of EPA (Givens et al., 2006). Nevertheless, endogenous synthesis of EPA and DHA in the human body is low and does not meet the requirements of long chain n-3 PUFA. Hence, research has focused on the direct supply of these FA through the diet. EPA and DHA contents were significantly higher in the IMF of BD pasture animals than of IR pasture animals (51 *vs.* 33 mg of EPA+DHA/100 g meat). At a daily intake of 100g, muscle of BD pasture animals would contribute with 10% for the minimum recommended daily intake of EPA+DHA (500 mg/d; International Society for the Study of Fatty Acids and Lipids (ISSFAL), 2004) compared with 7% contribution of the muscle of IR pasture animals.

Finally, in Chapter 5 of this PhD thesis, total CLA contents were reported to be 1.5 times higher in the IMF of animals fed botanically diverse silages (BDS) than in the IMF of animals fed intensive ryegrass silages (IRS; 11.4 *vs.* 7.7 mg/100 g meat, for BDS and IRS animals, respectively). Nevertheless, compared to milk, meat from BDS fed animals would only supply 0.4% of the suggested daily intake of CLA by Ip et al. (1994). These results show the higher efficiency of dietary strategies to manipulate the milk CLA composition compared to the meat CLA composition. Total PUFA contents in the IMF of BDS and IRS animals were similar and their contribution to the intake of PUFA as % of total energy would be the same (0.11% of total energy, based on 2000 kcal/d), similar to calculations based on results of Chapter 4 of the present PhD thesis. However, a small decrease in the EPA+DHA contents in the IMF was observed for BDS animals compared with IRS animals (24 *vs.* 26 mg/100 g meat for BDS and IRS animals, respectively).

In summary, the contribution of the end products (milk and meat) of botanically diverse forage fed animals to human nutrition can be pointed as marginally improved. Nevertheless, the latter ruminant end products can be considered to have a healthier FA

profile in light of the human nutritional recommendations relative to fat intake (WHO, 2003). As mentioned before, the assessment of improved FA profile in ruminant products in terms of human nutrition and health is difficult. Factors such as the contribution of a particular food item to the overall human diet and its fat content affect the significance of an improved FA profile of that particular food item. The FA metabolism and partitioning in the animal body also affects the FA profile of the edible parts. Indeed, PUFA will deposit in the phospholipid fraction of the muscle fat, while CLA will preferentially deposit in the triacylglycerol fraction of the muscle fat (De Smet et al., 2004). Thus, animals with poor growth, as in Chapters 4 and 5 of the present PhD thesis, can have a lower muscle fat content and proportions of PUFA might be overestimated and CLA proportions underestimated, compared with animals with normal growth, due to the confounding effect of a higher phospholipid/triacylglycerol ratio.

Further research to improve animal production using botanically diverse forages and to better assess the impact on human nutrition is of interest and necessary.

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SUMMARY - SAMENVATTING

Summary

Nowadays, consumer demands and nutritional recommendations in terms of dietary fats emphasize on an increased consumption of polyunsaturated fatty acids (PUFA), in particular n-3 fatty acids (FA), and on a decrease in dietary saturated FA (SFA). Ruminant products have been criticized for their high content in SFA, resulting from *de novo* synthesis in the mammary gland, mainly of SFA, and the active rumen biohydrogenation of dietary PUFA. On the other hand, the rumen microbial population produces FA, among which conjugated linoleic acid (CLA) or their precursors that are relatively unique to ruminant products, and were suggested to possess health-supporting properties. Hence, to improve the FA composition of ruminant meat and dairy products and to increase the human consumption of non-SFA, research on the potential to manipulate the ruminant endogenous FA metabolism, in particular rumen FA metabolism, is necessary. To some extent, this manipulation is possible through modification of the animal diet.

Farming systems based on high dietary forage proportions increased n-3 PUFA in ruminant fat compared to conventional farming systems that rely more on concentrate feeding. Further, diets rich in leguminous plant species, in particular the white and red clover, have been shown to improve the ruminant milk and meat FA profile in terms of human nutritional recommendations. It is obvious that not only the forage proportion of the diet affects the FA profile of milk and meat, but also the botanical composition of those forages. Alpine/mountain systems, characterized by high forage proportions and botanical diversity, also have been described to increase the n-3 PUFA and CLA c9t11 contents in ruminant fat. Less studies are available reporting the effect of more botanically diverse forage based systems in Belgium or The Netherlands, despite the governmental policy to stimulate the creation of semi-natural grasslands within agricultural areas. Moreover, the “origin” of the observed differences is still unknown.

The main objectives of this PhD research were to assess 1) the FA composition of leguminous rich and botanically diverse forages, both fresh and ensiled, and produced under Dutch and Flemish conditions; 2) the effect of feeding these forages on the FA composition of dairy milk and lamb fat and 3) the potential “origin” of

differences in the latter based on precursor supply and changes in the endogenous ruminant FA metabolism. Forage FA analysis is vital in terms of assessing the dietary PUFA supply (C18:2 n-6 and C18:3 n-3), the first objective of this PhD research. Hence, appropriate extraction solvents and correct handling procedures and storage conditions are of major importance, but only few studies compared these aspects, in particular for fresh forage. In the first part of this PhD thesis, proper handling procedures were studied in order to minimize the FA losses (Chapter 1) from harvest until FA analysis. Fatty acids were extracted from grass samples either immediately after harvest, after 3h in liquid N_2, after 24h frozen storage at -20ºC, -80ºC and -20ºC in the extraction solvent or after freeze-drying the grass samples. Results showed that FA analysis should be performed as soon as possible after harvesting and, if not possible, short term conservation in liquid N_2 is the best option. Different extraction solvents were also compared in order to assess the most effective solvent for FA analysis of plant tissues (Chapters 1 and 2). An adapted procedure of Folch et al. (1957), using a mixture of chloroform/methanol (2/1, vol/vol) was compared to the recommended method for lipid extraction of plant tissues, a pre-extraction with *iso*propanol, proposed to deactivate plant enzymes, and to ethanol, a less toxic solvent used by other groups to extract FA from plant tissues. Chloroform/methanol revealed to be the most powerful solvent mixture to guarantee complete extraction of both galactolipids, less polar esterified FA and unesterified FA both from fresh and conserved forage material (Chapters 1 and 2). Moreover, regarding the high proportions of unesterified FA in ensiled forages, a methylation step in an acidified environment seems an additional prerequisite for appropriate forage FA analysis (Chapter 2).

In the second part of this PhD thesis, the second and third objectives are addressed. In the first *in vivo* experiment of this PhD research (Chapter 3), four lactating and rumen canulated Holstein cows were used in a 4x4 Latin square design. Cows were fed four different diets: 100% intensively managed silage (100 IMS); 80% intensively managed silage and 20% semi-natural species poor silage (20 SPP); 40% intensively managed silage and 60% semi-natural species poor silage (60SPP) and 40% intensively managed silage and 60% semi-natural species rich silage (60 SPR). Milk CLA c9t11 proportions of cows fed diet 60 SPR were almost doubled compared to the other diets, despite the reduced dietary supply of C18:3 n-3 from diet 60 SPR. The higher CLA c9t11 proportions were associated with a partial inhibition of rumen biohydrogenation

of C18:2 n-6 and/or C18:3 n-3, suggested by the accumulation of some biohydrogenation intermediates (C18:2 t11c15 and C18:1 t11) and the reduced C18:0 proportions in the rumen pool samples. Differences in milk C18:3 n-3 were small, which reflected higher recoveries of dietary C18:3 n-3. This higher C18:3 n-3 recovery was most probably related to a higher transfer efficiency of C18:3 n-3 from the duodenum to the mammary gland, as C18:3 n-3 biohydrogenation did not differ between the dietary treatments.

In order to study the effect of grazing pastures with different botanical composition on rumen and intramuscular FA metabolism (Chapter 4), 21 male lambs were assigned at weaning to three botanically different pastures: botanically diverse (BD); leguminosa rich (L) and intensive Perennial ryegrass (IR), and were grazing exclusively on these pastures for 84 days on average before slaughter and sampling. Higher PUFA proportions, in particular of C20:4 n-6, C20:5 n-3 and C22:6 n-3 associated with higher indices for elongation and desaturation activity in the intramuscular fat of BD grazing animals were observed, suggesting some stimulation of elongation and desaturation to long chain FA. Higher proportions of biohydrogenation intermediates (C18:2 t11c15, CLA c9t11 and C18:1 t11) were observed in the spot rumen samples, taken at slaughter from BD animals, suggesting an inhibition of the biohydrogenation process. These changes were associated with shifts in the rumen microbial population, as suggested from the rumen volatile FA pattern and microbial odd and branched chain FA.

A final *in vivo* experiment (Chapter 5) aimed at assessing the effect on intramuscular and subcutaneous fat in relation to 1) feeding intensive ryegrass (IRS) *vs.* clover *vs.* botanically diverse silages (BDS); 2) white (WCS) *vs.* red (RCS) clover silage feeding and 3) C18:3 n-3 supply from forage silages *vs.* linseed in combination with maize silage (MSL). Thirty male lambs per group were fed one of these silages and an additional amount of concentrate for 76 days before slaughter and sampling. Although proportions of long chain PUFA were similar between groups, indices for desaturation and elongation activity in muscle of BDS animals suggested some stimulation of elongation and desaturation to long chain PUFA. Additionally, proportions of CLA c9t11 were higher in the muscle of BDS animals compared to the other forage groups. These changes in the muscle FA composition were associated with higher proportions of biohydrogenation intermediates (CLA c9t11and C18:1 t11) in the rumen contents of BDS animals, suggesting partial inhibition of rumen biohydrogenation. Moreover,

rumen C18:3 n-3 proportions of RCS animals were higher compared to WCS animals. This was suggested to be related to the activity of the polyphenol oxidase enzyme in the red clover silages. However, rumen proportions of C18:3 n-3 were highest in the MSL group. This might be a result of reduced lipolysis and hence biohydrogenation, due to the combined effect of esterified C18:3 n-3 supply and seed protection.

A general discussion, including both experimental data of this PhD research and literature data is presented in Chapter 6. Changes in rumen FA were used to explain the higher C18:3 n-3 levels in milk and meat of ruminants upon feeding clover and botanically diverse forages and the increased proportions of C18:1 t11 in the rumen of animals fed botanically diverse forages. The origin of the general increase in PUFA and CLA contents of milk and meat through botanically diverse forage feeding could not always be explained by differences in the dietary precursor supply of PUFA (i.e. C18:2 n-6 and C18:3 n-3) or by differences in fibre content or rumen pH, which are known to affect rumen biohydrogenation. This indicates other factors to play a role. We suggested plant secondary metabolites present in specific herbs of botanically diverse forages as possible candidates to modify the hydrogenating community of the rumen microbial population. Plant metabolites with methane inhibitory properties were suggested of particular interest, as a concomitant inhibitory effect on rumen methanogenesis and PUFA biohydrogenation has been observed before for synthetic additives, such as monensin or dietary supplements, such as fish oil or micro algae (Chapter 6).

As no response studies describing the effect of plant secondary metabolites on rumen PUFA biohydrogenation are available, a first *in vitro* screening with continuous cultures fermenters was performed (Chapter 7). Four different plant secondary metabolites (cinnamaldehyde, eugenol, triterpene saponin and quercetin) were screened for their effect on the biohydrogenation of C18:2 n-6 and C18:3 n-3. Doses tested were based on literature data and for triterpene saponin and quercetin, two doses were tested. A positive control (monensin) and a negative control were included. Addition of cinnamaldehyde resulted in a strong decrease of the microbial activity and microbial biomass, based on the reduced total amounts of volatile FA and proportions of odd and branched chain FA. This was further reflected in a reduction of the apparent biohydrogenation of C18:2 n-6 and C18:3 n-3. In addition, cinnamaldehyde resulted in higher proportions of C18:1 t10 and CLA t10c12, suggesting a shift from the major to a

secondary C18:2 n-6 biohydrogenation pathway. At the doses tested in this study, none of the other plant metabolites modified microbial activity or caused shifts in the pathways or extent of rumen biohydrogenation.

In summary, results of this PhD thesis showed that FA analysis of forage samples should be performed immediately after harvesting using a mixture of chloroform/methanol (2/1, vol/vol), and when not possible, short term storage in liquid N_2 is the best solution. Results of this PhD thesis also showed the potential of botanically diverse forages to increase the PUFA content of milk and meat of ruminants. This increase of the PUFA content of ruminant products was shown to be associated with changes in the rumen FA metabolism, which are most probably induced by shifts in the rumen microbial population. The latter might be linked to the presence of plant secondary metabolites in specific herb plant species of botanically diverse forages. Nevertheless, although evidence exists, there are still many unknown factors in these interactions, and hence more research is needed to elucidate the effects of plant secondary metabolites on rumen biohydrogenation.

Samenvatting

Heden ten dage is bekend dat een dieet met een hoog gehalte polyonverzadigde vetzuren (PUFA) en een laag gehalte verzadigde vetzuren gezondheidsbevorderende effecten kan hebben. Herkauwersproducten hebben op dit vlak echter geen goede reputatie. Ze staan bekend om hun hoog gehalte verzadigde vetzuren als gevolg van enerzijds *de novo* synthese van hoofdzakelijk verzadigde vetzuren in de uier, en anderzijds een actieve biohydrogenatie in de pens van de PUFA in het voeder. Naast verzadigde vetzuren, geeft biohydrogenatie echter ook aanleiding tot het ontstaan van een hele reeks geconjugeerde vetzuren, waaronder geconjugeerd linolzuur (CLA). Deze zogenaamde hydrogenatie intermediairen komen bijna uitsluitend voor in producten van herkauwers. Er wordt aangenomen dat ze belangrijk kunnen zijn voor de menselijke gezondheid. Bijgevolg is onderzoek om de vetzuur-samenstelling van vlees en zuivel van herkauwers te verbeteren, en dan vooral door onderzoek van de manipulatie van het vetzuurmetabolisme in de pens, nodig. Het is namelijk tot op zekere hoogte mogelijk het pensmetabolisme te beïnvloeden door aanpassingen in het rantsoen. Rantsoenen met een hoog percentage ruwvoeder verhogen het gehalte PUFA in vergelijking met rantsoenen die meer krachtvoeder bevatten. Wanneer het ruwvoeder rijk is aan vlinderbloemigen, vooral rode en witte klaver, wordt een hoger gehalte PUFA in de melk en vlees vastgesteld. Het is bijgevolg duidelijk dat niet enkel het aandeel dat het ruwvoeder van het rantsoen uitmaakt van belang is, maar ook de botanische samenstelling van dit ruwvoeder. Zo leveren ook productiesystemen die gebruik maken van alpenweiden, gekarakteriseerd door een grote soortenrijkdom, een hoger gehalte linoleenzuur en CLA c9t11 in vergelijking met conventionele systemen. Er zijn echter weinig studies beschikbaar die het effect van soortenrijke weides en ruwvoeders in België of Nederland bespreken. Dit ondanks een stimulans van de overheid om grasland extensief uit te baten. Ook de ‘oorsprong’ van de gevonden verschillen is nog onduidelijk.

De belangrijkste doelen van dit doctoraatsonderzoek waren het nagaan van 1) de vetzuur-samenstelling van ruwvoeders met veel vlinderbloemigen en soortenrijke ruwvoeders onder Vlaams-Nederlandse omstandigheden, zowel vers als ingekuild; 2)

het effect van het voederen van deze gewassen op de vetzuursamenstelling van koemelk en subcutaan en intramusculair vet van lammeren; 3) de mogelijke oorsprong van verschillen in vetzuursamenstelling van deze dierlijke eindproducten op basis van verschillen in de aanvoer van vetzuren en het vetzuurmetabolisme in de herkauwer. Vetzuuranalyses in ruwvoeder zijn bijgevolg van groot belang om de PUFA inname te bepalen. Hiervoor zijn de gebruikte extractiesolventen, handelingen tijdens de extractieprocedure en bewaarmethodes van het uitgangsmateriaal van groot belang. Dit geldt zeker voor verse gewassen met een hoge activiteit van plantenzymen. Aangezien slechts een beperkt aantal studies de invloed van deze verschillende aspecten vergelijken worden in het eerste deel van deze thesis verschillende behandelingen en bewaarmethodes bestudeerd om het verlies aan vetzuren vanaf de staalname tot aan de werkelijke detectie te minimaliseren. Vetzuren werden geëxtraheerd uit grasstalen onmiddellijk na staalname, na 3u in vloeibare N_2, na 24u bevroren bij -20°C, -80°C, bij -20°C in het extractiesolvent of na vriesdrogen. De resultaten tonen dat de extractie zo snel mogelijk dient plaats te vinden, indien niet mogelijk is een korte bewaring in vloeibare N_2 het beste alternatief. Verschillende extractriesolventen werden vergeleken, om zo het meest effectieve solvent voor vetzuurextractie uit plantenmateriaal te vinden (Hoofdstukken 1 en 2). Een aangepaste procedure volgens Folch et al. (1957), waarin gebruik wordt gemaakt van een mengsel van chloroform en methanol (2/1, vol/vol) werd vergeleken met de aangeraden methoden voor vetextractie uit plantenmateriaal. Een pre-extractie met isopropanol in deze laatste methode zou de plantenenzymen deactiveren. Verder werden deze twee methodes vergeleken met een procedure gebruik makend van ethanol, een minder toxisch solvent, gebruikt door andere onderzoeksgroepen om vetzuren uit plantenmateriaal te extraheren. Hieruit kwam chloroform/methanol als meest krachtige solvent voor de extractie van vetzuren uit plantenmateriaal onder de vorm van galactolipiden, triglyceriden en vrije vetzuren, zowel uit vers als uit ingekuild materiaal (Hoofdstukken 1 en 2). Daarnaast blijkt dat, gezien het hoge gehalte vrije vetzuren in geconserveerde ruwvoeders een bijkomende zure methylatie noodzakelijk is voor een accurate vetzuuranalyse bij ruwvoeders (Hoofstuk 2).

In het tweede deel van deze thesis, wordt het tweede en het derde doel beoogd. In het eerste *in vivo* experiment (Hoofdstuk 3), worden vier lacterende en pens gecannuleerde Holstein koeien ingezet in een 4x4 Latijns vierkant. De koeien werden

vier verschillende rantsoenen gegeven: silages van 100% intensief uitgebaat grasland (100 IM); 80% intensief uitgebaat grasland en 20% soortenarme beheersweide (20 SPP); 40% intensief uitgebaat grasland en 60% soortenarme beheersweide (60 SPP); 40% intensief uitgebaat grasland en 60% soortenrijke beheersweide (60 SPR). Het aandeel CLA c9t11 in de melk van koeien die het 60 SPR rantsoen kregen was bijna dubbel zo hoog vergeleken met de andere rantsoenen, ondanks een lagere opname van C18:3 n-3 met 60 SPR. Het hogere CLA c9t11 aandeel was gerelateerd met een gedeeltelijke inhibitie van de biohydrogenatie in de pens van C18:2 n-6 en /of C18:3 n-3. Dit kon worden afgeleid uit de accumulatie van biohydrogenatie intermediairen, zoals C18:2 t11c15 en C18:2 t11, samen met een lager aandeel C18:0 in de pensinhoud. De verschillen in het gehalte linoleenzuur in de melk waren klein, wat, door een lagere opname van linoleenzuur uit het 60 SPR rantsoen, resulteerde in een hogere overdracht van voeder naar melk. Deze hogere overdracht is waarschijnlijk het gevolg van een hogere efficiëntie van de overdracht van het duodenum naar de uier, aangezien de biohydrogenatie van linoleenzuur niet verschillend was voor de verschillende rantsoenen.

Om het effect van het grazen van weiden met een verschillende botanische samenstelling op pens en intramusculair vetzuurmetabolisme na te gaan (Hoofdstuk 4), werden 21 mannelijke lammeren bij het spenen verdeeld over 3 verschillende weiden: een soortenrijke weide (BD), een weide rijk aan vlinderbloemigen (L) en een intensieve Engels raaigras weide (IR). De lammeren hebben gedurende 84 dagen voor het slachten en staalname enkel op deze weide gegraasd. Hoge aandelen PUFA, meer specifiek C20:4 n-6, C20:5 n-3 en C22:6 n-3 in combinatie met verhoogde elongase en desaturase indexen, in het intramusculair vet van de dieren die op de BD weide graasden, duiden op één of andere vorm van activatie van elongase en desaturase. Hoge aandelen biohydrogenatie intermediairen (C18:2 t11c15, CLA c9 t11 en C18:1 t11) werden teruggevonden in de pens stalen van de BD grazende dieren, die eenmalig werde genomen bij het slachten werden genomen. Dit duidt op een inhibitie van het biohydrogenatieproces. Deze veranderingen waren geassocieerd met een verschuiving in de microbiële populatie in de pens, afgeleid uit het vluchtige vetzuurpatroon van de pens en de microbiële oneven en vertakteketen vetzuren.

Een laatste *in vivo* experiment (Hoofdstuk 5) had tot doel het effect op intramusculair en onderhuids vet na te gaan van 1) voederen van silages van intensief raaigras (IRS) vs. klaver silages vs. botanisch diverse silages (BDS) 2) voederen van

witte klaver silages (WCS) vs. rode klaver silages (RCS) en 3) input van linoleenzuur afkomstig van het ruwvoeder vs. lijnzaad in combinatie met mais (MSL). Dertig mannelijke lammeren per groep werden met één van deze 4 silages samen met een bepaalde hoeveelheid krachtvoer gevoederd gedurende 76 dagen voor ze geslacht werden en de stalen werden genomen. Ondanks een gelijkaardig aandeel PUFA in het intramusculair vet van de verschillende groepen, duiden de indexen voor desaturatie en elongatie in het spierweefsel van BDS gevoederde dieren op een stimulatie van de elongase en desaturase activiteit. Bovendien was het aandeel CLA c9 t11 hoger in het spierweefsel van BDS gevoederde dieren in vergelijking met dieren die de andere silages kregen. Deze verschillen in de vetzuursamenstelling van het spierweefsel waren geassocieerd met hogere aandelen biohydrogenatie intermediairen (CLA c9 t11 en C18:1 t11) in de pensinhoud van de BDS gevoederde dieren, wat duidt op een inhibitie van de biohydrogenatie. Ook was het aandeel linoleenzuur hoger in de pensstalen van de RCS gevoederde dieren in vergelijking met de WCS gevoederde dieren. Dit zou te wijten kunnen zijn aan de activiteit van polyfenol oxidase in de silages van rode klaver. Het gehalte linoleenzuur in de pensstalen was echter het hoogst in de MSL gevoederde groep. Dit kan dan weer te wijten zijn aan een verminderde lipolyse en bijgevolg ook biohydrogenatie als gevolg van een gecombineerd effect van de aanvoer van veresterde vetzuren uit zaden en de bescherming van de vetzuren door inkapseling in de zaden.

Een algemene discussie met daarin zowel de experimentele data van dit doctoraatsonderzoek als data uit de literatuur wordt gegeven in Hoofdstuk 6. Veranderingen in pensvetzuren werden gebuikt om hogere gehalten linoleenzuur in melk en vlees van herkauwers bij het voederen van klaver- en soortenrijke ruwvoeders te verklaren. De stijging in gehalte PUFA en CLA in melk en vlees door het voederen van soortenrijk ruwvoeder kon niet in alle gevallen verklaard worden door een hogere input van precursoren van PUFA (d.i. C18:2 n-6 en C18:3 n-3) en/of een verschil in vezelinhoud van de pens of pens pH, factoren die de pens biohydrogenatie kunnen beïnvloeden. Dit duidt erop dat andere factoren een rol spelen. Secundaire plant metabolieten, die aanwezig zijn in bepaalde kruiden van soortenrijke weides, werden als mogelijk kandidaten aangeduid voor het veroorzaken van een verschuiving in de populatie hydrogenerende micro-organismen. Plantmetabolieten met een inhiberend effect op de methaanproductie lijken interessant te zijn, daar bij sommige synthetische additieven die de methanogenese inhiberen (bv. monensin) ook een inhibitie van de

biohydrogenatie van PUFA werd vastgesteld. Hetzelfde fenomeen werd vastgesteld bij supplementen als visolie en micro-algen (Hoofdstuk 6).

Gezien er geen studies in de literatuur beschikbaar zijn die het effect van secundaire plant metabolieten op de pens PUFA biohydrogenatie beschrijven, werd een eerste *in vitro* screening met countinue culturen uitgevoerd (Hoofdstuk 7). Vier verschillende secundaire plantmetabolieten (cinnamaldehyde, eugenol, triterpene saponines en quercetin) werden gescreend op hun effect op de biohydrogenatie van linol- en linoleenzuur. De toegepaste dosis werd gebaseerd op gegevens uit de literatuur, waarbij voor triterpeen saponines en quercetin twee dosissen werden uitgetest. Daarnaast werd ook een positieve (monensine) en een negatieve controle uitgevoerd. Toevoeging van cinnamaldehyde resulteerde in een sterke daling van de microbiële activiteit en biomassa, af te leiden uit de reductie in hoeveelheid vluchtige vetzuren en het aandeel oneven en vertakte keten vetzuren. Dit werd verder weerspiegeld in een vermindering van de biohydrogenatie van C18:2 n-6 en C18:3 n-3. Daarenboven resulteerde cinnamaldehyde in een hoger aandeel C18:1 t10 en CLA t10c12, wat suggereert dat er een verschuiving plaatsvindt van de belangrijkste biohydrogenatieweg naar een secundaire pathyway in de biohydrogenatie van C18:2 n-6. Bij de dosissen die getest werden in dit experiment veroorzaakte geen van de andere secundaire metabolieten een verandering in de microbiële activiteit, een verschuiving in de biohydrogenatiepathway of verandering in de biohydrogenatie.

Samenvattend kan gesteld worden dat deze doctoraatsthesis aantoont dat voor vetzuuranalyses op ruwvoeder de extractie onmiddellijk na staalname of indien niet mogelijk na een korte bewaring in vloeibare N_2 dient te worden uitgevoerd door gebruik te maken van een mengsel van chloroform en methanol (2/1, vol/vol). De resultaten in deze doctoraatsthesis geven ook aan dat soortenrijke ruwvoeders het PUFA gehalte in melk en vlees van herkauwers verhogen. Er werd aangetoond dat deze stijging in PUFA gehalte samen gaat met veranderingen van het vetzuurmetabolisme in de pens, wat waarschijnlijk het gevolg is van verschuivingen in de microbiële populatie in de pens. Deze verschuiving zou gerelateerd kunnen worden aan de aanwezigheid van secundaire metabolieten die in specifieke kruiden aanwezig zijn in soortenrijke ruwvoeders. Ondanks aanwijzingen voor deze hypothese, zijn er nog steeds vele onbekende factoren.

Bijgevolg is meer onderzoek in dit domein nodig om het effect van secundaire metabolieten op de pens biohydrogenatie op te helderen.

CURRICULUM VITAE

Short overview of the curriculum vitae of DVM. Marta Lourenço

Marta Lourenço was born in Lisbon, Portugal on May 19th 1979. In 2003, she completed her studies on Veterinary Medicine at the Faculty of Veterinary Medicine of the Technical University of Lisbon, Portugal. From January 2003 until April 2003, to obtain her degree of Veterinary Medicine, she did her scientific stage on "*Study of the influence of time of soybean oil supplementation on meat fatty acid profile of lambs*", at the Department of Animal Nutrition of the National Zootechnical Station, Santarém, Portugal. From June 2003 until September 2003, she worked as a volunteer in research studies on CLA content and profile of Portuguese beef breeds. In October 2003 she started her PhD research entitled "*Effects of the botanical composition of forages on rumen, milk and meat fatty acid metabolism*", within the project "*Merkerprofielen voor functioneel vlees*" founded by IWT – Institute for the Promotion of Innovation by Science and Technology in Flanders, with a grant from the Foundation for Science and Technology – Portugal. Within her PhD research, she stayed at the Animal Nutrition, Management and Welfare Research Group (Department de Ciència Animal I dels Aliments, Universitat Autònoma de Barcelona, Spain) for a period of 2 months. At the moment she received a post-doctoral grant from the Foundation for Science and Technology – Portugal, for one year to study the "*Effect and identification of plant secondary metabolites on rumen lipolysis and biohydrogenation*".

She is the main author or co-author of different publications, participated actively and passively in many international symposia, congresses and study-days.

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