UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

MICROORGANISMS AND DIETARY FACTORS AFFECTING BIOHYDROGENATION AND CONJUGATED LINOLEIC ACID PRODUCTION IN THE RUMEN ECOSYSTEM

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TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS
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To those who never stop believing

Acknowledgements

First, I would like to acknowledge the Faculdade de Medicina Veterinária of Universidade Técnica de Lisboa for accepting me as a PhD student, and the Estação Zootécnica Nacional, Instituto Nacional de Investigação Agrária e das Pescas, and the Rowett Research Institute for accepting me into their research groups and for providing all means and facilities which enabled me to carry out the experimental work.

To Professor Apolinário Vaz Portugal, Director of Estação Zootécnica Nacional when I started my PhD studies, I wish to leave a special word of appreciation for having accepted me in his Institute but especially for his always kind words and inspiration to follow up my dreams in science.

I would like to express my gratitude to Professor João Ramalho Ribeiro, as head of Departamento de Nutrição Animal of Estação Zootécnica Nacional, for providing all conditions for my research to take place and, as my supervisor, for having accepted to take me under his guidance and for all the support given throughout the years.

I wish to acknowledge my appreciation to Professor Luís Ferreira, from Faculdade de Medicina Veterinária, for having accepted to be my co-supervisor and for all the inspiring conversations, support and encouragement given.

To Professor Rui Bessa I wish to express my profound acknowledgement and gratitude for all the guidance, critical discussions and endless encouragement given throughout the years as well as the friendship ever shown, which were essential for the accomplishment of this thesis.

To Professor John Wallace I wish to express my sincere gratitude for taking me under his guidance during my stay at the Rowett Research Institute, for critical assessment of my progress, knowledge transfer, and the invaluable support. I would also like to thank all members of the Gut Microbial Biochemistry group for everything that I have learnt, all the

help provided and their friendship, namely to Dr. Neil McEwan, Ms. Freda McIntosh, Ms. Nadine Thomas, Mr. Tony Richardson, Dr. Stefan Muetzel, Dr. Delphine Paillard, Dr. Tracy Wood, Dr. Shahid Mahmood, Dr. Estelle Devillard, Mr. Kenneth Young and especially to Mrs. Nest McKain and Dr. Nicola Walker. A special thanks to Mrs. Maureen Annand and Mr. David Brown for the technical assistance provided in fatty acid analysis.

To Dr. Olga Moreira I wish to express my gratefulness for all the knowledge transfer during my stay at the Estação Zootécnica Nacional, and for the help provided in the conduction of the *in vitro* experiments. I would also like to thank all members of the Departamento de Nutrição Animal for everything that I have learnt, all the precious help provided and their friendship, especially to Dr. Ana Teresa Belo, Mrs. Isabel Almas, Mrs. Deolinda Carvalho, Mrs. Teresa Dentinho, Mrs. Maria Manuela Salgueiro, Mrs. Madalena Coelho, Mr. José Batista, Mrs. Alexandrina Soares, Mrs. Ana Sá, Mrs. Guilhermina Inácio, Mrs. Ilda Gonçalves, Mrs. Nulita Álvaro, Mr. Fernando Rodrigues, Mrs. Edite Silva, Mr. António José Madeira, Mrs. Ana Batista and Mrs. Rosália Ferreira. A special thanks to Mr. António Camilo Leite for the help provided in the *in vivo* experiment and sample preparation.

I also would like to thank Professor António Mira da Fonseca and Professor Ana Rita Cabrita for the help provided in the formulation of diets for the *in vivo* experiment, critical discussion of results and endless encouragement.

Completion of this project would not have been possible without the support offered and help provided by my friends, especially to Dr. Andrea Olvera-Ramirez, Dr. Abdi Guliye, Dr. Rusmana Ningrat, Ms. Collete Walsh, Dr. Mariette Barbier, Mr. José-Luiz Gonzalez, Dr. Susana Chamorro, Dr. Cisca Kimbeke, Dr. Adamo Valle, Dr. Cedric Charrier, Ms. Susana Alves, Ms. Eliana Jerónimo, Mrs. Ana Paula Portugal, Ms. Ingrid Santos, Dr. Maria do Rosário Marques, and many others....

I would also like to take this opportunity to thank the Fundação para a Ciência e Tecnologia for the financial support given. This research was only possible with the attribution of a PhD grant (SFRH/BD/6976/2001) and the Project (POCTI/CVT/2000/2002) supported by this Institution.

Last but not least, I would like to thank my parents, grandparents, and brother for their life-long support and constant encouragements, which have had such an essential impact in my achievements.

To Diogo, Margarida and Miguel for making every single day special.

Maia, M.R.G. (2010). *Microrganismos e factores alimentares que afectam a bioidrogenação e a produção de isómeros conjugados do ácido linoleico no ecossistema ruminal*. Tese de Doutoramento. Lisboa: Faculdade de Medicina Veterinária - Universidade Técnica de Lisboa.

A crescente incidência de doenças cardiovasculares, obesidade e diabetes, tem sido associada com o elevado consumo de gorduras, nomeadamente de origem animal. Os produtos edíveis dos ruminantes, leite e carne, são ricos em gordura saturada, devido à bioidrogenação dos ácidos gordos (AG) da dieta pela população microbiana do rúmen. No entanto, a bioidrogenação origina, igualmente, AG com efeitos benéficos para a saúde, como sejam os isómeros conjugados do ácido linoleico (CLA) e o ácido vacénico (18:1 *trans*-11). Leite e carne com melhores perfis em AG poderão contribuir para a promoção da saúde dos consumidores mas, para isso, é essencial um conhecimento mais aprofundado do metabolismo lipídico ruminal. Assim, o objectivo geral desta tese foi estudar alguns microrganismos envolvidos na bioidrogenação e factores que afectam o metabolismo lipídico no rúmen.
Avaliou-se o metabolismo dos AG polinsaturados pelo *Butyrivibrio fibrisolvens* (Capítulo 2). A toxicidade dos AG polinsaturados aumentou com o número de carbonos, número de insaturações e a configuração *cis* das duplas ligações. O papel da bioidrogenação como um mecanismo de detoxificação foi mostrado e sugerido um efeito protector da acumulação de 18:1 *trans*-11 face à toxicidade dos AG. Os efeitos do pH e nível de inclusão de amido, em dietas suplementadas com óleo, no padrão de bioidrogenação, designadamente no aumento de *trans*-10 associado com a depressão da gordura no leite, foram testados em RUSITEC (Capítulo 3). O aumento de *trans*-10 observou-se em dietas com elevado teor em amido, sendo o efeito independente do pH da incubação (6 ou 7). A modificação no padrão de bioidrogenação sugere mudanças na população microbiana. Os efeitos do tipo de azoto da dieta e da suplementação com óleo de soja na composição em AG do conteúdo ruminal e fracções bacterianas foram avaliados *in vivo* (Capítulo 4). O padrão de bioidrogenação não foi afectado pelo tipo de azoto mas a suplementação de óleo a dietas com proteína verdadeira aumentou os CLA. O aumento do ácido *cis*-vacénico (18:1 *cis*-11) em dietas ricas em azoto não proteico sugere um possível mecanismo compensatório para regulação da fluidez das membranas bacterianas face a baixas proporções de AG ímpares e ramificados.

Palavras-chave: Bioidrogenação; Rúmen; Suplementação lipídica; Isómeros conjugados do ácido linoleico; *Butyrivibrio fibrisolvens*; Toxicidade; *Trans*-10; pH; Amido; Fonte de azoto.

Abstract

Consumption of animal fat has been associated with an increased incidence of chronic diseases, as cardiovascular disease, obesity and diabetes. Ruminant products, milk and meat, have high saturated fatty acids (FA) content as a result of biohydrogenation by microbial metabolic activity in the rumen. Biohydrogenation also originates FA with health promoting or disease preventing properties, including conjugated linoleic acids (CLA) and vaccenic acid (18:1 *trans*-11). Improvement of FA composition of milk and meat might promote human health but research is needed on ruminal lipid metabolism, which plays a major role in FA profile. The overall aim of this thesis was to investigate on specific microorganism and factors affecting the rumen lipid metabolism. Different methodological approaches were used, ranging from *in vitro* studies, with pure and mixed cultures, to *in vivo* studies.

Polyunsaturated FA metabolism by *Butyrivibrio fibrisolvens*, a major hydrogenating rumen bacterium, was evaluated (Chapter 2). Toxicity of non-esterified unsaturated FA to *B. fibrisolvens* increased with FA length, number of unsaturations, and *cis* geometrical configuration. Biohydrogenation was shown to be a detoxifying mechanism, although mechanisms by which toxicity occur were not identified. Results suggest a protective role of 18:1 *trans*-11 to FA toxicity. A semi-continuous *in vitro* fermentation system was used to determine the effects of pH and starch inclusion level, in diets supplemented with oil, on the biohydrogenation pattern, namely in identifying the factor responsible for the *trans*-10 shift, associated with milk fat depression (Chapter 3). The *trans*-10 shift was observed to be associated with high starch level, independently of pH incubation level (6 or 7). Modification of the biohydrogenation pathway was suggested to be associated with changes in microbial population. Dietary nitrogen source and soybean oil supplementation effects on rumen contents and bacterial fractions FA composition were evaluated *in vivo* (Chapter 4). Biohydrogenation pattern was not affected by nitrogen source, yet CLA was promoted in true protein diets with soybean oil. The increase in *cis*-vaccenic acid (18:1 *cis*-11) in non-protein nitrogen diets may suggest a regulatory mechanism of bacterial membrane fluidity in the presence of lower proportions of odd- and branched-chain FA.

Keywords: Biohydrogenation; Rumen; Oil supplementation; Conjugated linoleic acids; *Butyrivibrio fibrisolvens*; Toxicity; *Trans*-10 shift; pH; Starch; Nitrogen source.

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List of abbreviations and symbols

Abbreviations

ADFom - Acid detergent fibre expressed without residual ash;
ADL - Acid detergent lenhin;
AG - Ácidos gordos;
ATCC - American Type Culture Collection;
ATP - Adenosine triphosphate;
BCFA - Branched-chain fatty acids;
BHT - Butylated hydroxytoluene;
C16:1 - Hexadecenoic acids;
C18 - Fatty acids with 18 carbon chain length;
C18:1 - Octadecenoic acids;
C18:2 - Octadecadienoic acids;
C18:3 - Octadecatrienoic acids;
ca. - *Circa*;
CH_3 - Methyl;
CLA - Conjugated linoleic acids; isómeros conjugados do ácido linoleico;
CO_2 - Carbon dioxide;
CoA - Coenzyme A;
COOH - Carboxyl;
CP - Crude protein;
$DiOC_2(3)$ - 3,3'-Diethyloxacarbocyanine iodide;
DM - Dry matter;
DMOX - 4,4-Dimethyloxazoline;

DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen;

DTT - Dithiothreitol;

EE - Ether extract;

FA - Fatty acids;

FAO - Food and Agriculture Organization;

FCCP - Carbonyl cyanide p-trifluoromethoxyphenylhydrazone;

HDL - High density lipoprotein;

HpH - High pH;

HS - High starch;

HSHpH - High starch diet incubated at high pH;

HSLpH - High starch diet incubated at low pH;

i.e. - *Id est;*

LAB - Liquid associated bacteria;

LDL - Low density lipoprotein;

LpH - Low pH;

LS - Low starch;

LSHpH - Low starch diet incubated at high pH;

LSLpH - Low starch diet incubated at low pH;

LSM - Least square means;

N - Nitrogen;

N_2 – Gaseous nitrogen;

NAN - Non-ammonia nitrogen;

NDF - Neutral detergent fibre;

NDFom - Neutral detergent fibre expressed without residual ash;

$N\text{-}NH_3$ - Ammonia nitrogen;

NP - Norma Portuguesa;

O_2 - Oxygen

OBCFA - Odd-and branched-chain fatty acids;

OCFA - Odd-chain fatty acids;

OD - Optical density;

OJEC - Official Journal of the European Communities;

OM - Organic matter;

PI - Propidium iodide;

PPAR - Peroxisome proliferator-activated receptors;

PUFA - Polyunsaturated fatty acids;

RUSITEC - Rumen simulation technique;
SAB - Solid associated bacteria;
SEM - Standard error of the mean;
SM - Oat hay plus sunflower meal;
SMO - Oat hay plus sunflower meal with soybean oil;
TNF - Tumour necrosis factor;
TRC - Total rumen contents;
U - Oat hay plus urea concentrate;
UO - Oat hay plus urea concentrate with soybean oil;
VFA - Volatile fatty acids;
vs. - *Versus*;
WHO - World Health Organization.

Fatty acids

12:0 - Dodecanoic acid = Lauric acid;
anteiso-13:0 - 10-Methyldodecanoic acid;
iso-13:0 - 11-Methyldodecanoic acid;
13:0 - Tridecanoic acid;
iso-14:0 - 12-Methyltridecanoic acid;
14:0 - Tetradecanoic acid = Myristic acid;
anteiso-15:0 - 12-Methyltetradecanoic acid;
iso-15:0 - 13-Methyltetradecanoic acid;
15:0 - Pentadecanoic acid;
iso-16:0 - 14-Methylpentadecanoic acid;
16:0 - Hexadecanoic acid = Palmitic acid;
anteiso-17:0 - 14-Methylhexadecanoic acid.
iso-17:0 - 15-Methylhexadecanoic acid;
16:1 *cis*-9 - *cis*-9 Hexadecenoic acid = Palmitoleic acid;
17:0 - Heptadecanoic acid = Margaric acid;
18:0 - Octadecanoic acid = Stearic acid;
18:1 *trans*-9 - *trans*-9 Octadecenoic acid = Elaidic acid;
18:1 *trans*-10 - *trans*-10 Octadecenoic acid
18:1 *trans*-11 - *trans*-11 Octadecenoic acid = Vaccenic acid;
18:1 *cis*-9 - *cis*-9 Octadecenoic acid = Oleic acid;

18:1 *cis*-11 - *cis*-11 Octadecenoic acid = *Cis*-vaccenic acid;

18:2 *n*-3 - *cis*-12,*cis*-15 Octadecadienoic acid;

18:2 *n*-6 - *cis*-9,*cis*-12 Octadecadienoic acid = Linoleic acid;

18:2 *cis*-9,*trans*-11 - *cis*-9,*trans*-11 Octadecadienoic acid = Rumenic acid;

18:2 *cis*-9,*trans*-13 - *cis*-9,*trans*-13 Octadecadienoic acid;

18:2 *trans*-9,*trans*-11 - *trans*-9,*trans*-11 Octadecadienoic acid;

18:2 *trans*-9,*trans*-12 - *trans*-9,*trans*-12 Octadecadienoic acid = Linoelaidic acid;

18:2 *trans*-10,*cis*-12 - *trans*-10,*cis*-12 Octadecadienoic acid;

18:3 *n*-3 - *cis*-9,*cis*-12,*cis*-15 Octadecatrienoic acid = α-Linolenic acid;

18:3 *n*-6 - *cis*-6,*cis*-9,*cis*-12 Octadecatrienoic acid = γ-Linolenic acid;

18:4 *n*-3 - *cis*-6,*cis*-9,*cis*-12,*cis*-15 Octadecatetraenoic acid = Stearidonic acid;

20:3 *n*-6 - *cis*-8,*cis*-11,*cis*-14 Eicosatrienoic acid = Dihomo-γ-linolenic acid;

20:4 *n*-3 - *cis*-8,*cis*-11,*cis*-14,*cis*-17 Eicosatetraenoic acid = Eicosatetraenoic acid;

20:4 *n*-6 - *cis*-5,*cis*-8,*cis*-11,*cis*-14 Eicosatetraenoic acid = Arachidonic acid;

20:5 *n*-3 - *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17 Eicosapentaenoic acid;

22:5 *n*-3 - *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 Docosapentaenoic acid;

22:6 *n*-3 - *cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 Docosahexaenoic acid;

Symbols and units

% - Percentage;

λ_{EM} - Emission wavelength;

λ_{EX} - Excitation wavelength;

< - Less than;

> - Greater than;

± - standard error;

Ca - Calcium;

cm - Centimetre;

d - Day;

dL - Decilitre;

g - Gram;

g - Gravitational force unit;

h - Hour;

kg - Kilograms;

L - Litre;

Mg - Magnesium;

mg - Milligram;

min - Minute;

mL - Millilitre;

mm - Millimetre;

mM - Millimolar;

N - Normality;

nm - Nanometre;

ºC - Degree centigrade;

rpm - Revolutions *per* minute;

s - Second;

U - Enzyme activity unit;

v/v - volume/volume;

wt/v - Weight/volume;

wt/wt - Weight/weight;

µg - Microgram;

µL - Microlitre.

Chapter 1

General introduction

1.1. Introduction

Nutrition plays an important role in human health. Increasing evidences imply nutrition in the development of chronic diseases with high social and economical impact, such as obesity, diabetes, cancer and cardiovascular disease. Dietary lipids are often associated to metabolic disorders and diseases, having a pejorative connotation next to consumers as well as nutritionists. Human studies have implied the consumption of high saturated fats and *trans* fatty acids (FA) with the increased incidence of cardiovascular disease and increased risk of developing metabolic syndrome (Hu et al., 1997; Nugent, 2004). Therefore, nutritional guidelines suggesting a reduction of the overall fat intake as well as the intake of saturated FA and *trans* FA have been widely disseminated. Latest nutritional guidelines were established in 2008 by a Joint FAO/WHO Expert Consultation on Fats and Fatty Acids in Human Nutrition after revision of available data from previous 15 years of research. The FAO/WHO Expert Consultation recommended for adults a total fat intake varying between 20 and 35% of total energy, with a minimum of 15% total energy to prevent deficiency of essential FA and lipid-soluble vitamins (Elmadfa & Kornsteiner, 2009). In order to reduce the risk of cardiovascular disease and maintain cholesterol levels within normal ranges, intakes lower than 10 and 1% of total energy for saturated FA and *trans* FA, respectively, were also recommended (Elmadfa & Kornsteiner, 2009).

Ruminant meat, milk and dairy products are important sources of fat in human diets, namely of saturated FA and *trans* FA. Consumption of ruminant-derived products has largely increased over the last years in developed and developing countries, and prospects are to

continue to increase. By 2020, production and consumption of ruminant meat are estimated to be nearly 70 million metric tonnes higher than in 2000, reaching 300 million metric tonnes worldwide (Wolmarans, 2009). In view of its importance in diets, meat and dairy products have been seen as a 'public enemy' to human health. Nutritional guidelines promoted the decrease of milk, dairy products and red meat consumption and its replacement by foods with high polyunsaturated FA (PUFA) content (Elmadfa & Kornsteiner, 2009). Dairy products and meat have been related to the incidence of cardiovascular disease and increase of plasma cholesterol (Menotti et al., 1999; Williams, 2000; Givens, 2005). Nonetheless, consumption of ruminant-derived products may confer beneficial health effects to humans. Milk and dairy products possess minerals, proteins and lipids with bioactive properties in prevention of atherosclerosis, degenerative disorders, osteoporosis and cancer (Shingfield, Chilliard, Toivonen, Kairenius & Givens, 2008).

Ruminant fat contains FA with potential health-promoting effects, among which two are of particular interest; conjugated linoleic acids (CLA) and vaccenic acid (18:1 *trans*-11). In fact, ruminant products are the main source of CLA for human consumption (Chin, Liu, Storkson, Ha & Pariza, 1992; Lawson, Moss & Givens, 2001). Dietary CLA have been shown to prevent cancer, decrease atherosclerosis, improve immunity and modify energy metabolism in animal models (Park, Storkson, Albright, Liu & Pariza, 1999; Parodi, 1999; Belury, 2002; Pariza, 2004; Palmquist, Lock, Shingfield & Bauman, 2005). Although *trans* FA in general are negatively associated with health, 18:1 *trans*-11 is converted in animal tissues by the action of the enzyme Δ9-desaturase to rumenic acid (18:2 *cis*-9,*trans*-11; Griinari et al., 2000), a CLA isomer, and therefore considered to be health-promoter. Furthermore, FA composition of ruminant products can be improved by decreasing their saturated FA content while increasing PUFA content, particularly *n*-3 FA (Scollan et al., 2006), meeting the FAO/WHO recommendations.

Ruminant feedstuff lipids are rich in unsaturated FA, yet ruminant fat has long been known to be more saturated than that of non-ruminants (Banks & Hilditch, 1931). The transformation of unsaturated FA towards more saturated FA, or biohydrogenation, occurs in the rumen as consequence of microbial activity. Paradoxically, biohydrogenation also results in the formation of the health-promoting CLA and 18:1 *trans*-11 FA. Unsaturated FA leaving the rumen have great potential to improve the nutritional value of ruminant-derived products, either those that escaped rumen microbial metabolism as the biohydrogenation intermediates formed (Or-Radish, Wright & McBride, 2009). The improvement of ruminant milk and meat FA profile represents a new insight and added value to the livestock sector as well as to

human nutrition. Improving FA profile of everyday consumed foods as meat, milk and dairy products, might be the most efficient and effective way to promote healthier dietary fat and FA profile intake, as it would not represent an effort to consumers or an alteration of their consumption habits.

Considering the importance and impact of lipid content and FA profile of ruminant products, effects of different classes of FA in human health will be discussed firstly, followed by a review of the faith of feed lipids in the rumen, their saturation and origin of health-promoting FA, as well as the importance and significance of those processes on promotion of healthfulness of milk, meat and dairy products to humans.

1.2. Health effects of dietary fatty acids on humans

Dietary lipids have generally a negative denotation, being linked to obesity, cardiovascular diseases and cancer. However, lipids play an important role in meeting human daily energy requirements as well as absorption of fat-soluble vitamins. The basic building blocks of lipids are FA. Saturated FA and *trans* FA have been negatively associated to human health effects, including the consumption of milk, dairy products and red meat. Recently, the Joint FAO/WHO Expert Consultation have acknowledge specific effects of particular FA and isomers on human health, rather than the bulk labelling of good or bad by degree of FA saturation or geometric configuration (Elmadfa & Kornsteiner, 2009). Thus, the effects of saturated, PUFA and *trans* FA on human health will be shortly reviewed.

1.2.1. Saturated fatty acids versus polyunsaturated fatty acids

The typical structure of FA is a backbone of carbon atoms with hydrogen atoms linked to the carbon string, with a methyl group ($-CH_3$) at one end (*n*- end) and a carboxyl group (-COOH) at the other end (Δ end). Fatty acids can vary in length, usually ranging from 2 to 80 carbon atoms. When all carbon atoms are linked by single bonds the FA is referred as saturated, whereas FA with at least one double bond are designated by unsaturated. Polyunsaturated FA are unsaturated FA with two or more double bonds. Accordingly to the position of the first double bond in the FA chain, PUFA are classified into families; *n*-3 and *n*-6 being the main PUFA. Linoleic acid (18:2 *n*-6) and α-linolenic acid (18:3 *n*-3) are the two most important

PUFA from the *n*-6 and *n*-3 families, respectively. Many PUFA can be synthesised in mammalian tissues by a series of FA chain elongation and desaturation steps. However, desaturases of vertebrate animals, including humans, only have the ability to insert double bonds at specific positions until Δ9 carbon. Thus, 18:2 *n*-6 and 18:3 *n*-3 have to be ingested to meet the required levels for normal physiological functions in human body tissues (Lunn & Theobald, 2006). In body tissues, these essential FA undergo a series of FA chain elongation and desaturation steps in the Δ6-desaturase pathway, being converted to a multitude of longer and more unsaturated FA. 18:2 *n*-6 is metabolised to γ-linolenic acid (18:3 *n*-6), dihomo-γ-linolenic acid (20:3 *n*-6), and finally arachidonic acid (20:4 *n*-6). Similarly, 18:3 *n*-3 is metabolised to stearidonic acid (18:4 *n*-3), eicosatetraenoic acid (20:4 *n*-3), eicosapentaenoic acid (20:5 *n*-3), docosapentaenoic acid (22:5 *n*-3), and finally docosahexaenoic acid (22:6 *n*-3; Napier & Sayanova, 2005).

High intakes of saturated FA have been associated with adverse effects on cardiovascular disease and cholesterol (Williams, 2000; Givens, 2005). The individual FA identified as having marked effects on increasing total cholesterol and low density lipoprotein (LDL) were lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:0) whereas stearic acid (18:0) had neutral effects (Givens, 2008). Saturated FA were also related to reduced insulin sensitivity, a key factor for metabolic syndrome development (Nugent, 2004). Replacement of saturated FA by PUFA has beneficial health effects. Higher intakes of PUFA, namely of long-chain *n*-3 PUFA 20:5 *n*-3 and 22:6 *n*-3, were described to increase clinical benefits and improve mechanisms related to cardiovascular health, growth, development, inflammatory processes, neurodegenerative diseases, and cancer (Seo, Blaner & Deckelbaum, 2005; Calder & Yaqoob, 2009).

Health effects of *n*-6 PUFA in cardiovascular disease were the first to be evaluated, but concerns on the intake of high *n*-6 to *n*-3 ratio arose (Simopoulos, Leaf & Salem, 1999; Simopoulos, 2008). Indeed, *n*-6 and *n*-3 PUFA compete for the enzymes involved in the FA elongation and desaturation, with some enzymes having more affinity for *n*-6 or *n*-3 PUFA (Lunn & Theobald, 2006). Moreover, the *n*-3 metabolism in body tissues is particularly limited. It has been estimated that less than 4% and 8% of 18:3 *n*-3 is metabolised to 20:5 *n*-3 and 22:6 *n*-3, respectively (Vermunt, Mensink, Simonis & Hornstra, 2000; Burdge, Jones & Wootton, 2002). Fatty acids 20:4 *n*-6 and 20:5 *n*-3 are in the basis of eicosanoids formation. Eicosanoids are a group of molecules that acts as important regulatory signals, including prostaglandins, prostacyclins, thromboxanes, and leukotrienes (Lunn & Theobald, 2006). Eicosanoids formed from *n*-3 and *n*-6 PUFA have distinct biological effects. Whereas 20:4 *n*-

6 leads to the formation of pro-inflammatory and pro-thrombotic eicosanoids, 20:5 *n*-3 and 22:6 *n*-3 are associated to less inflammatory and even anti-inflammatory eicosanoids (Calder, 2009; Calder & Yaqoob, 2009). Nevertheless, Harris (2010) recently reviewed *n*-6 PUFA effects and reported a protective effect as they decrease the risk of coronary heart disease. Health beneficial roles of *n*-6 PUFA on brain growth and cognitive development, blood pressure, and factors involved in insulin resistance have been also described (Harris et al., 2009). Moreover, potent anti-inflammatory lipoxins are produced from 20:4 *n*-6 (Calder, 2009).

The usefulness of *n*-6 to *n*-3 ratio in nutritional guidelines has been questioned recently (Harris, 2006; Stanley et al., 2007; Harris, 2010), as its use, or of any other ratio, may mask very high or low intakes of both *n*-6 and *n*-3 PUFA. Another addressed issue was which FA should be included to calculate the *n*-6 to *n*-3 ratio, e.g., 18:2 *n*-6 to 18:3 *n*-3, 20:4 *n*-6 to 20:5 *n*-3 or total *n*-6 to total *n*-3 FA (Stanley et al., 2007).

1.2.2. Trans fatty acids

The term *trans* FA includes all unsaturated FA with at least one double bond in *trans* geometric configuration. Octadecenoic (C18:1) isomers comprise 80 to 90% of total *trans* FA in most foods but others can be found as hexadecenoic (C16:1), octadecadienoic (C18:2) and octadecatrienoic (C18:3) acids, and long-chain PUFA (Weggemans, Rudrum & Trautwein, 2004; Gebauer et al., 2007). Most CLA are *trans* FA. Nevertheless, and due to their uniqueness, CLA effects on health will be issued separately (**section 1.2.3**).

Ruminant-derived foods and industrially-hydrogenated foods are the two major sources of *trans* FA in human diet. Both sources vary in the profile and content of *trans* FA (**Figure 1.1**). Dietary *trans* FA are mainly found in partially hydrogenated vegetable oils, which contains 10 to 40% *trans* FA of total fat. In dairy products and ruminant meat, the concentration of *trans* FA ranges from 3 to 8% of total fat (Gebauer, Psota & Kris-Etherton, 2007). The major *trans* isomer in hydrogenated foods is usually elaidic acid (18:1 *trans*-9) and in ruminant foods 18:1 *trans*-11, although 18:1 *trans*-10 can be a major component is both sources (Weggemans et al., 2004).

Epidemiologic studies have shown a strong association between intake of *trans* FA and risk of coronary heart disease, even stronger than that with intake of saturated FA (Hu et al., 1997). However, studies investigating the association between *trans* FA from ruminant sources and

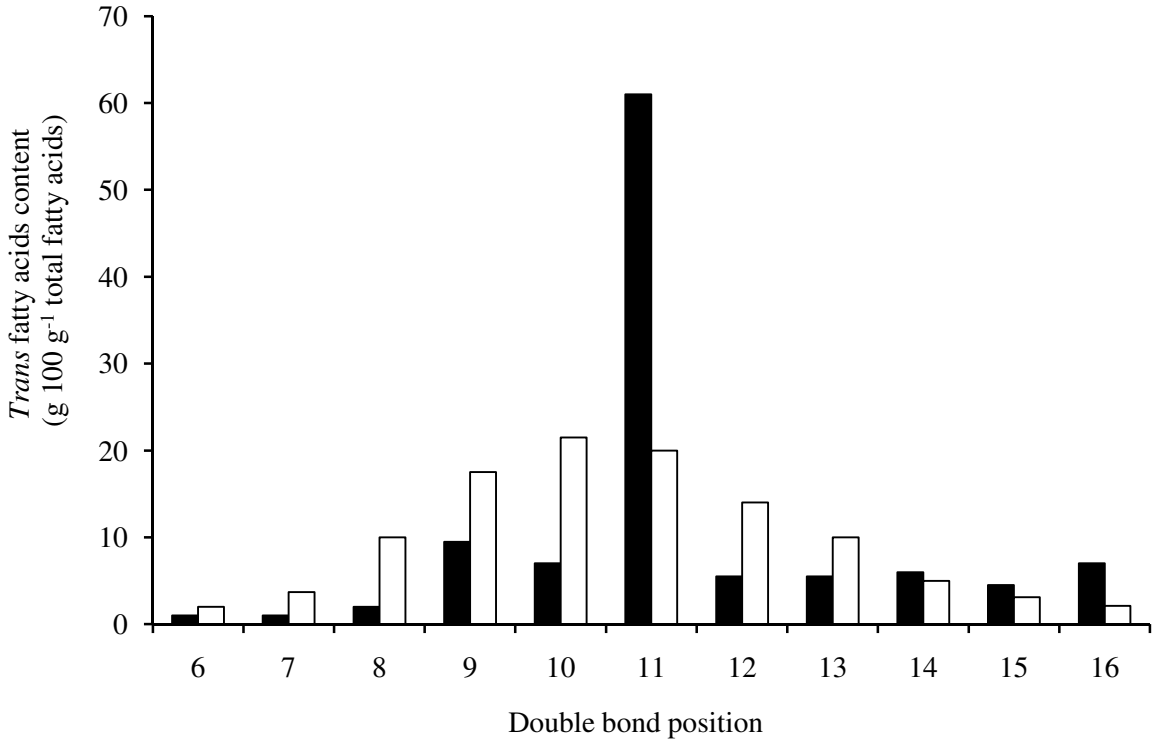

Figure 1.1. Typical distribution of *trans* fatty acids in ruminant-derived products and partially hydrogenated vegetable oils. Ruminant foods (■), hydrogenated oils (□). Adapted from Bauman, Perfield and Lock (2004).

coronary heart disease risk have indicated no association or a potentially beneficial association (Willett et al., 1993; Ascherio et al., 1994). Jakobsen et al. (2006) noted that most comparisons were based on relative intake data. When absolute amounts of *trans* FA intake were calculated, no significant differences in the risk of coronary heart disease were found for different sources of *trans* FA (Weggemans et al., 2004). Gebauer et al. (2007) estimated that at intakes greater than 3 g d^{-1}, total *trans* FA were associated with an increased risk of coronary heart disease. As intakes of *trans* FA from ruminant products were below this amount, no conclusions were possible to make.

It has been estimated that reducing *trans* FA intake by 2% of total energy would result in a decrease in triacylglicerol levels of approximately 3 mg dL^{-1} (Ascherio, Katan, Zock, Stampfer & Willett, 1999). Furthermore, increasing amounts of *trans* FA resulted in an increase in small dense LDL particles in a dose-dependent manner, when compared with a diet rich in saturated FA (Ascherio et al., 1999). Intervention studies demonstrating specific effects of individual *trans* FA isomers on blood lipids are lacking (Gebauer et al., 2007). In a randomized study with healthy young men, a 18:1 *trans*-11 rich diet (3.6 g d^{-1} 18:1 *trans*-11) lowered total cholesterol and high density lipoprotein (HDL) by 6 and 9%, respectively, when

compared with a control diet (Tholstrup et al., 2006). However, it was concluded that these differences may have been due to higher monoenoic FA and lower saturated FA profile of the 18:1 *trans*-11 rich diet rather than due to 18:1 *trans*-11 by itself.

Trans FA intake was positively associated with tumour necrosis factor (TNF) receptor levels in healthy women, and with levels of C-reactive protein and interleukin-6 in women with higher body mass index (Mozaffarian et al., 2004).

Dietary *trans* FA were associated with an increased risk of developing type 2 diabetes (Salmeron et al., 2001). Nevertheless, no significant difference were found in insulin sensitivity on healthy individuals when comparing a *trans* FA diet (5% total energy) to a monounsaturated FA diet (5% total energy as oleic acid (18:1 *cis*-9); Louheranta, Turpeinen, Vidgren, Schwab & Uusitupa, 1999), and a *trans* FA diet (9% total energy) to a saturated FA diet (9% of energy as 16:0; Lovejoy et al., 2002). Overall data suggest that *trans* FA do not have a significant effect on insulin sensitivity in healthy normal weight individuals and overweight individuals with elevated cholesterol. In subjects with type 2 diabetes, high *trans* FA intake could increase insulin resistance; however, more studies with larger numbers of participants are needed (Gebauer et al., 2007).

In a series of animal studies, the anticarcinogenic potential of 18:1 *trans*-11 was evaluated in rat mammary glands (Banni et al., 2001; Corl, Barbano, Bauman & Ip, 2003; Lock, Corl, Barbano, Bauman & Ip, 2004). Methylnitrosourea-induced tumour growth in rat mammary glands was attenuated when rats were fed diets supplemented with 2% (wt/wt) 18:1 *trans*-11 (Banni et al., 2001). It was hypothesised that this effect was mediated through the Δ9-desaturation of 18:1 *trans*-11 to 18:2 *cis*-9,*trans*-11. In a followup study, sterculic oil was used to block the Δ9-desaturase activity, resulting in an attenuated anticarcinogenic effect of 18:1 *trans*-11 supplementation. These results indicate that reduction in tumour growth was primarily mediated by 18:1 *trans*-11 conversion to 18:2 *cis*-9,*trans*-11 (Lock et al., 2004). Therefore, the divergent data regarding 18:1 *trans*-11 and its effects on breast cancer may be related to differences in its conversion to 18:2 *cis*-9,*trans*-11. Δ9-desaturase polymorphisms have been identified and a number of different transcription factors, including peroxisome proliferator-activated receptors (PPAR), are involved in the regulation of this enzyme (Risérus et al., 2005; Warensjö et al., 2007). It was speculated that a decrease in the functionality of the Δ9-desaturase enzyme would decrease the conversion of 18:1 *trans*-11 to the anticancer FA, 18:2 *cis*-9,*trans*-11 (Voorrips et al., 2002; Rissanen, Knekt, Jarvinen, Salminen & Hakulinen, 2003).

Most case-control studies associate *trans* FA with increased prostate, colon and breast cancers risk (Astorg, 2005; King, Kristal, Schaffer, Thornquist & Goodman, 2005). In contrast, large (>10,000 subjects) prospective cancer studies of prostate (Schuurman, van der Brandt, Dorant, Brants & Goldbohm, 1999), colon (Limburg et al., 2008) and breast (Byrne, Rockett & Holmes, 2002) showed no relationship between *trans* FA and cancer risk. Smith, Robinson, Nam and Ma (2009) noted that differences in analytical determination of *trans* FA may explain differences between studies. Long (100 m), highly polar capillary columns are needed for resolving the numerous *trans* FA isomers. However, most studies use simple GC methods and short columns, being only able to report total *trans* FA content or a limited number of *trans* FA isomers, which may mask individual isomer effects. Thus, the role of *trans* FA in prostate, breast and colon cancer across different experimental models and human studies remains inconclusive. Although a number of studies have evaluated the individual effects of *trans* FA, human and animal studies have shown both positive and negative associations with specific and total *trans* FA. Moreover, scarce information is available on the potential effects of *trans* FA on pre-existing cancers (Awad, Herrmann, Fink & Horvath, 1995; Eitsuka, Nakagawa, Suzuki & Miyazawa, 2005; Lampen, Leifheit, Voss & Nau, 2005).

1.2.3. Conjugated linoleic acid on human health

The term CLA refers to a group of 28 positional and geometric isomers of 18:2 *n*-6. Instead of typical methylene interrupted double bonds, conjugated FA have the double bonds separated by one single bond. CLA isomers may occur in *cis*,*cis*, *cis*,*trans*, *trans*,*cis* or *trans*,*trans* configuration, predominantly present at positions 8 and 10, 9 and 11, 10 and 12 or 11 and 13 (Bessa, Santos-Silva, Ribeiro, Portugal, 2000; Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006).

Dairy products and ruminant meat are the primary sources of CLA in foods (Chin et al., 1992; Lin, Boylston, Chang, Luedecke & Schultz, 1995; Parodi, 1997), as a result of the ruminal biohydrogenation. The main isomer present in ruminant-derived foods, corresponding to over 80% of total CLA isomers (Chin et al., 1992), is 18:2 *cis*-9,*trans*-11, which is formed both during biohydrogenation of 18:2 *n*-6 in the rumen (Polan, McNeill & Tove, 1964; Kepler, Hirons, McNeill & Tove, 1966) and by Δ-9 desaturation of 18:1 *trans*-11 in tissues (Corl et al., 2003; Kay, Mackle, Auldist, Thomson & Bauman, 2004).

Since elucidation of 18:2 *n*-6 biohydrogenation pathway by rumen bacteria that CLA was known (Polan et al., 1964; Kepler et al., 1966). However, major interest in these FA arose two

decades ago when Pariza and Hargraves (1985) first describe their anticarcinogenic effects. During the investigation of the carcinogenic properties of grilled beef, FA were found to exhibited anticancer properties rather than procancer properties; these FA were identified as being CLA (Pariza & Hargraves, 1985; Ha, Grimm & Pariza, 1987).

Several beneficial health effects have been described in animal studies; but in humans' effects of CLA are less conclusive (Tricon, Burdge, Williams, Calder & Yaqoob, 2005; Bhattacharya et al., 2006; Salas-Salvado, Marquez-Sandoval & Bullo, 2006; Park, 2009). The two predominant CLA isomers with known bioactive properties are 18:2 *cis*-9,*trans*-11, and 18:2 *trans*-10,*cis*-12 (**Figure 1.2**). Anticarcinogenic properties have been implied to both isomers (Ip et al., 2002, Park, 2009). The isomer 18:2 *cis*-9,*trans*-11 was shown to be involved in the inhibition of TNF-α, growth promotion in rodents, and improvement of lipoprotein profiles (Cook, Miller, Park & Pariza, 1993; Yang & Cook, 2003; Valeille et al., 2004). On the other hand, 18:2 *trans*-10,*cis*-12 was shown to be involved in body weight, fat composition, and inhibition of Δ9-desaturase activity, protein and/or mRNA (Park et al., 1999; Park et al., 2000).

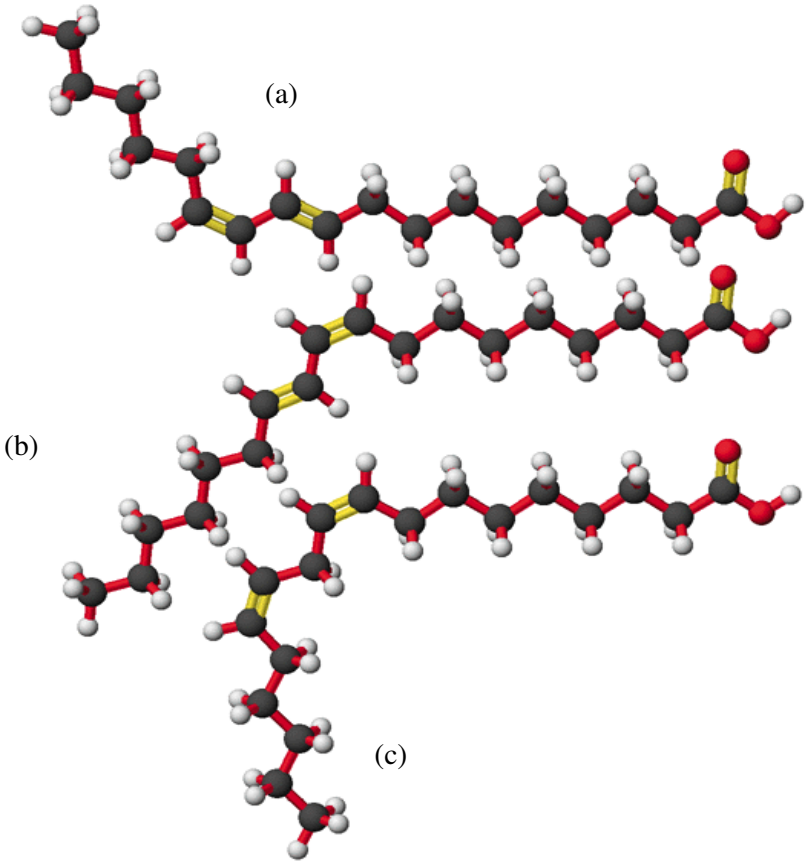

Figure 1.2. Chemical structures of the two predominant conjugated linoleic acid isomers with known bioactive properties and of linoleic acid. 18:2 *trans*-10,*cis*-12 (a); 18:2 *cis*-9,*trans*-11, rumenic acid (b); 18:2 *n*-6, linoleic acid (c). Adopted from Steinhart (1996).

Most research has been done with mixtures of both main CLA isomers, usually at equal amounts. Nevertheless, minor CLA isomers may also play biological roles. Indeed, 18:2 *trans*-9,*trans*-11 has been shown to inhibit platelet aggregation (Al-Madaney, Kramer, Deng & Vanderhoek, 2003; Li, Barnes, Butz, Bjorling & Cook, 2005) and to exert antiproliferative effects (Lai, Yin, Li, Zhao & Chen, 2005).

In animal models, CLA have been shown to reduce various forms of cancer, including breast, forestomach, colon, skin, liver, and prostate cancer (Lee, Lee, Cho & Kim, 2005; Bhattacharya et al., 2006; Kelley, Hubbard & Erickson, 2007), either by reducing its initiation, promotion, progression and/or metastasis (Park, 2009). Compared to animal studies, only a limited number of human clinical studies investigating the effect of CLA on cancer development have been reported. The first report suggesting the involvement of CLA in cancer was based on indirect evidences. Knekt, Jarvinen, Seppanen, Pukkala and Aromaa (1996) noted an inverse relation between milk intake and the incidence of breast cancer, and pointed out CLA as one of the potential compounds responsible. Clinical studies have shown no clear association between dietary CLA, adipose or serum levels of CLA, and reduced risk for breast cancer (Voorips et al., 2002; Chajes et al., 2002; Aro et al., 2000; McCann et al., 2004). A 15 years study reported an inverse correlation between intake of high-fat dairy foods and CLA, and the incidence of colorectal cancer in women (Larsson, Bergkvist & Wolk, 2005).

Promising effects of CLA on body fat were reported in animal studies. Growing mice fed 1% CLA mixture decreased by 60% the body fat (Terpstra et al., 2002). Further research clarified that the responsible for this effect was the isomer 18:2 *trans*-10,*cis*-12 (Park et al., 1999). 18:2 *trans*-10,*cis*-12 was suggested to reduce body fat by increasing energy expenditure, reducing lipid accumulation in adipose tissue, increasing adipocyte apoptosis, modulation of adipokines and cytokines, and increasing FA β-oxidation in skeletal muscle (Park & Pariza, 2007). Effects of CLA on body weight and body fat reduction in humans was less effective than in animal models (Wang & Jones, 2004; Terpstra, 2004; Bhattacharya et al., 2006; Salas-Salvado et al., 2006, Park & Pariza, 2007; Park, 2009). Recently, reduced body fat mass was described in overweight individuals supplemented with 3.4 g d^{-1} CLA for 6 months (Gaullier et al., 2007) and in overweight/obese individuals supplemented equal amounts for 12 months (Gaullier et al., 2005). Yet, Larsen, Toubro, Gudmundsen and Astrup (2006) found that a similar CLA supplementation for one year did not prevent weight or fat mass regain in moderately obese individuals. Differences in the effects of CLA supplementation on body fat reduction in humans compared to animal models may be due to metabolic differences

between species, different dietary regimens, differences in energy balance status (positive energy balance in animals *vs*. negative energy balance in humans), and lower CLA doses used in human studies (Park, 2009).

Dietary supplementation of CLA reduced the development of atherosclerosis in animal studies (Lee, Kritchevsky & Pariza, 1994; Nicolosi, Rogers, Kritchevsky, Scimeca & Huth, 1997; Kritchevsky, Tepper, Wright, Tso & Czarnecki, 2000; Wilson, Nicolosi, Chrysam & Kritchevsky, 2000; Kritchevsky, Wright & Czarnecki, 2002). Changes in both total plasma cholesterol and individual lipoprotein cholesterol concentrations have been implicated as major determinants of atherosclerosis risk. However, the reduced incidence of atherosclerosis in animals fed CLA was not accompanied by an improvement of the plasma lipid profile (Wilson et al., 2000). Results from studies investigating the effects of CLA supplementation on lipids and lipoproteins have been mixed (Gebauer et al., 2007, Park, 2009). Moreover, different effects of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12 have been reported (Risérus, Arner, Brismar & Vessby, 2002; Tricon et al., 2004). Noone, Roche, Nugent and Gibney (2002) reported an improved plasma triacylglycerol and LDL metabolism in healthy subjects supplemented a 50:50 CLA mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12 isomers, whereas a 80:20 mixture reduced the concentration of LDL-cholesterol. Further studies with pure isomers showed that 18:2 *cis*-9,*trans*-11 exert stronger effects on lowering blood lipids, plasma triacylglycerol, total plasma cholesterol, LDL-cholesterol, and LDL:HDL-cholesterol compared to 18:2 *trans*-10,*cis*-12 (Tricon et al., 2004). Moreover, CLA isomers were incorporated into plasma and cellular lipids to a similar extent and in a dose-dependent manner (Burdge et al., 2004).

Effects of CLA on glucose metabolism, bone metabolism and metabolic syndrome in humans are, at present, unclear and inconclusive (Bhattacharya et al., 2006; Tricon & Yaqoob, 2006; Park, 2009).

In summary, multiple factors are known to affect human health, diet being one important factor. Therefore it is easy to understand that the establishment of a correlation between health effects and a specific dietary FA or FA group is extraordinarily difficult. To alleviate this difficulty, nutritionist commonly base dietary guidelines in terms of PUFA (*n*-3 and *n*-6), *trans* FA, and saturated FA intakes. However, it should be kept in mind that different FA within each of these groups have specific effects on health, including beneficial and detrimental effects. Moreover, further research is needed to clarify the effects of dietary FA in humans and the required amounts for health promotion to occur. Additionally, the use of *n*-6 to *n*-3 ratio, or other ratios, must be reconsidered.

1.3. Lipid metabolism in the rumen

The rumen hosts a complex ecosystem with an extremely high microbial density, including bacteria, protozoa, archaea and fungi, with bacteria alone being present at up to 10^{11} mL^{-1} of rumen fluid (Stewart, Flint & Bryant, 1997). Individual genera or species seldom have a unique role and overlapping of nutrient requirements and fermentation end-products often occurs. Also numerous interrelationships occur among the various ruminal microbes, such as symbiosis, competition for nutrients, antagonism and predation, resulting in a highly competitive microbial community (Firkins, Karnati & Yu, 2008). The rumen microbial population digests the feed ingested by the ruminant host, constituting the products of microbial digestion the majority of nutrients that sustain the animal itself.

Modifying the microbial FA metabolism and profile by diet manipulation is an effective way to improve the quality of ruminant-derived products, such as milk and meat. However, the ruminal transformation of dietary lipid plays a major role in determining the FA composition of ruminant products. Dietary lipids are readily and extensively transformed in the rumen by two main processes, lipolysis and biohydrogenation, although others may occur. The main processes of lipid metabolism in the rumen, the microorganisms responsible, and the mechanisms involved will be discussed below.

1.3.1. Lipolysis

The form of dietary lipids entering the rumen depends on the feedstuff ingested by the animal. In grazing animals, forage lipids are mainly galactolipids, sulpholipids and phospholipids whereas in animals fed concentrates, cereals and seed oils, triacylglycerols are the most abundant lipids (Harfoot & Hazlewood, 1997). Once in the rumen, the hydrolysis of ester linkages of dietary acyl lipids to non-esterified FA, or lipolysis, occurs rapidly (Garton, Hobson & Lough, 1958; Dawson & Hemington, 1974; Dawson, Hemington & Hazlewood, 1977).

Microbial lipases are the main responsible for the lipolytic activity in the rumen (Dawson & Hemington, 1974; Dawson et al., 1977). Nevertheless, plant enzymes may play a role in lipolysis as they are rich in galactolipases and phospholipases. Omar Faruque, Jarvis and Hawke (1974) suggested that hydrolysis of galactolipids and triacylglycerols from grass was due to plant enzyme activity, as plant lipases remained active in the rumen after ingestion.

Using autoclaved ^{14}C-labelled grass as substrate, Dawson et al. (1977) observed a rapid hydrolysis of grass galactolipids, concluding that microbial lipases were more important than plant enzymes, as these were inactivated. Moreover, when these authors inactivated microbial lipases by boiling the rumen fluid and used grass as substrate, galactolipids were not hydrolyzed. Although contribution of plant endogenous lipases to the overall lipolytic activity in the rumen seems weak, their role on lipolysis external to the rumen deserves to be investigated (Jenkins, Wallace, Moate & Mosley, 2008). Dierick and Decuypere (2002) found that 25 to 65% of milled cereals fat was lipolysed after 56 days of storage, showing that plant lipases may play an important role on lipolysis prior to the rumen.

1.3.1.1. Lipase activity of rumen microorganisms

Using selective media with emulsified linseed oil as substrate, the most active ruminal lipolytic species isolated was *Anaerovibrio lipolytica* (Hobson & Mann, 1961; Hobson, 1965). Two hydrolytic enzymes were found to be produced; one cell bound esterase and one cell surface or extracellular lipase (Harfoot, 1978). The lipase hydrolysed triacylglycerols and tributyrin, with diacylglycerols being more rapidly degraded than triacylglycerols (Hobson & Mann, 1961). Phospholipids and galactolipids were not hydrolysed. Thus, *A. lipolytica* would be expected to be particularly important and prevalent in lipase activity of animals fed concentrate feeds or oil supplemented but not on grazing animals. Nevertheless, Prins, Lankhorst, Van der Meer and Van Nevel (1975) found *A. lipolytica* to be present at around 10^7 mL^{-1} in rumen contents of grazing animals, suggesting that other roles may be played by this microorganism.

The most active bacteria isolated with phospholipase and galactolipases activities were from the genus *Butyrivibrio*, a non-cellulolytic strain of *Butyrivibrio fibrisolvens* and a FA auxotrophic *Butyrivibrio* strain (Hazlewood & Dawson, 1975 and 1979). Triacylglycerols were not hydrolysed, however. The *Butyrivibrio* spp. lipase activity indicated the presence of phospholipases A and C, lysophospholipase, phosphodiesterase and phosphomonoesterase activities, similar to those found *in vivo* (Harfoot & Hazlewood, 1997). Phospholipases A and C and galactolipases were located in the cytoplasmatic membrane (Hazlewood, Cho, Dawson & Munn, 1983).

The ability of a small group of rumen bacteria to hydrolyse lipids as well as to biohydrogenate the released unsaturated FA, suggest that carrying out both processes may be "advantageous to the biochemical economy of an organism" (Harfoot & Hazlewood, 1997, pp. 387). Lipase

activity is essential for FA auxotrophic bacteria, such as *Butyrivibrio* strain S2. Polyunsaturated FA of plant lipids are hydrolysed by *Butyrivibrio* S2 and the released non-esterified FA incorporated in its membrane lipids (Hazlewood & Dawson, 1979).

Rumen ciliate protozoa were described to be responsible for 30 to 40% of total lipolytic activity in the rumen (Wright, 1961; Latham, Storry & Sharpe, 1972), with *Epidinium* spp. being the most active protozoa. Nevertheless, the role of protozoa in lipase activity in the rumen is not yet clear. As it is impossible, to date, to grow protozoa cultures free of intracellular or surface-associated bacteria, lipolytic activity of protozoa cannot be dissociated from lipolytic activity of the associated bacteria (Harfoot & Hazlewood, 1997).

The rumen anaerobic flagellated fungi appear to play no role in the hydrolysis of dietary lipids.

Most studies on lipase activity in the rumen are dated from middle 60's to 80's. Recent interest on lipid metabolism arose with the discovery of the health promoting effects of rumen derived FA, namely of CLA. Over the last decade a large number of groups have focus the research on the biohydrogenation mechanisms and strategies to improve the quality, FA profile and healthfulness of ruminant products, as milk and meat. More recently, metagenomic techniques have been used to characterise the microbial population and attempt to establish a link to the ruminal metabolism and by-products. Despite recent advances made in this area, little research has been made on lipase activity. Therefore, as metagenomic libraries will be constructed and lipase enzymes determined, new insight may be provided on the lipase activity, substrate specificity and microorganisms involved in the process.

1.3.2. Biohydrogenation

Tissue lipids of ruminants are long being known to be more saturated than those of non-ruminants. When first observed by Banks and Hilditch (1931), it was suggested that the saturation, or biohydrogenation, of forage lipids was conducted in those tissues. After two decades, evidence of ruminal biohydrogenation was first reported by Reiser (1951).

The major unsaturated FA entering the rumen of grazing animals is 18:3 *n*-3, whereas 18:2 *n*-6 usually predominates in animals fed concentrates or oil supplemented. Before hydrogenation to occur, dietary galactolipids, phospholipids, and triacylglycerols are hydrolysed and the non-esterified FA released. Lipolysis is a crucial step for biohydrogenation of unsaturated FA, as the presence of a free carboxyl group is an absolute requirement for biohydrogenation to take place (Hawke & Silcock, 1970; Kepler, Tucker &

Tove, 1970; Dawson, Hemington, Grime, Lander & Kemp, 1974; Hazlewood, Kemp, Lander & Dawson, 1976; Hazlewood & Harfoot, 1997).

Reiser (1951) incubated linseed oil with sheep ruminal contents and found the 18:3 *n*-3 content to decrease from 30 to 5%. 18:3 *n*-3 was converted to C18:2 and C18:1 FA and to 18:0 as a result of microbial metabolic activity (Shorland, Weenick & Goldfine, 1955). Wood, Bell, Grainger and Teekel (1963) administrated ^{14}C-labelled 18:2 *n*-6 intra-ruminally and observed that 85 to 96% of the dose that remained in the rumen after 48 h, only 3 to 6% was 18:2 *n*-6. Of the remaining, 33 to 55% was hydrogenated to 18:1 *cis*-9 or 18:1 *trans*-9, and 46% was fully saturated to 18:0. A rapid hydrogenation of ^{14}C-labelled 18:3 *n*-3 and 18:1 *cis*-9 incubated with whole rumen contents was noted by Ward, Scott and Dawson (1964). When 18:3 *n*-3 was used as substrate it was hydrogenated to C18:2, C18:1 and 18:0 whereas 18:1 *cis*-9 was mainly converted to 18:0 with only small radioactivity detected in C18:1 *trans* FA (Ward et al., 1964). The first biohydrogenation scheme of 18:3 *n*-3 was designed by Wilde and Dawson (1966). Incubations of ^{14}C-labelled 18:3 *n*-3 with whole rumen contents elucidated the initial isomerisation of the *cis*-12 bond to either the carbon 11 or the carbon 13 position. One of the three double bonds was then hydrogenated to C18:2. Another hydrogenation of one of the double bonds occurred producing an C18:1 that was further hydrogenated to 18:0, the final biohydrogenation product.

Elucidation of the biohydrogenation metabolic schemes was provided by pure culture studies. Biohydrogenation pathway of 18:2 *n*-6 was characterized by using *B. fibrisolvens* (Polan et al., 1964; Kepler et al., 1966). These researchers observed that 18:2 *n*-6 was initially isomerised to a conjugated C18:2 FA isomer, putatively 18:2 *cis*-9,*trans*-11, and then hydrogenated to 18:1 *trans*-11. The 18:1 *trans*-11 was not further metabolised. Kepler and Tove (1967) confirmed the initial isomerisation of 18:2 *n*-6 to 18:2 *cis*-9,*trans*-11 and further hydrogenation to a mixture of C18:1 *trans* isomers. When 18:3 *n*-3 was incubated with *B. fibrisolvens*, it was initially isomerised to the conjugated trienoic 18:3 *cis*-9,*trans*-11,*cis*-15 and then hydrogenated to a non-conjugated dienoic acid. However, in mixed ruminal bacteria, 18:0 was the end-product of C18 unsaturated FA (Ward et al., 1964).

A bacterium with the ability to hydrogenate C18 unsaturated FA to 18:0 was isolated by White, Kemp and Dawson (1970). When 18:2 *n*-6 or 18:1 *cis*-9 were used as substrate, approximately 80% of the added FA was converted to 18:0 within 72 h of incubation. Traces of C18:1 *trans* and C18:1 and C18:2 FA were also present with 18:1 *cis*-9 and 18:2 *n*-6 incubations, respectively. When 18:3 *n*-3 was used as substrate, 80% was converted to a mixture of C18:1 FA but not to 18:0. Traces of a non-conjugated dienoic acid were also found. Kemp, White and Lander (1975) isolated 5 bacteria from the rumen of sheep with

hydrogenating activity, including two *Eubacterium* spp., *Ruminococcus albus*, and two *Fusocillus* spp. *Eubacterium* spp. and *R. albus* were able to metabolise 18:2 *n*-6 and 18:3 *n*-3 to a mixture of C18:1 FA. Both *Fusocillus* spp. hydrogenated 18:2 *n*-6 and 18:1 *cis*-9 to 18:0, and 18:3 *n*-3 to 18:1 *cis*-15.

These earlier studies were followed by many others showing that dietary C18 unsaturated FA are extensively biohydrogenated in the rumen after lipolysis, yielding a variety of FA in different proportions. Generally, C18 PUFA biohydrogenation pathways involve an initial isomerisation followed by hydrogenation of the *cis* double bound until the formation of the saturated 18:0 (**Figure 1.3**). Therefore, 18:2 *n*-6 metabolism involves the transient formation of 18:2 *cis*-9,*trans*-11 which is hydrogenated to 18:1 *trans*-11 and further hydrogenated to 18:0. Metabolism of 18:3 *n*-3 is similar, involving the isomerisation to 18:3 *cis*-9,*trans*-11,*cis*-15 which is hydrogenated to 18:2 *trans*-11,*cis*-15 and further hydrogenated to 18:1 *trans*-11 or 18:1 *cis*-15 and 18:1 *trans*-15. 18:1 *trans*-11 is finally converted to 18:0 but not 18:1 *cis*-15 and 18:1 *trans*-15 (Harfoot & Hazlewood, 1997). Evidence of these pathways arose from incubations of 18:2 *n*-6 and 18:3 *n*-3 FA with rumen contents *in vivo* and *in vitro*, and pure culture studies with bacteria isolated from the rumen or non-ruminal sources (Harfoot & Hazlewood, 1997).

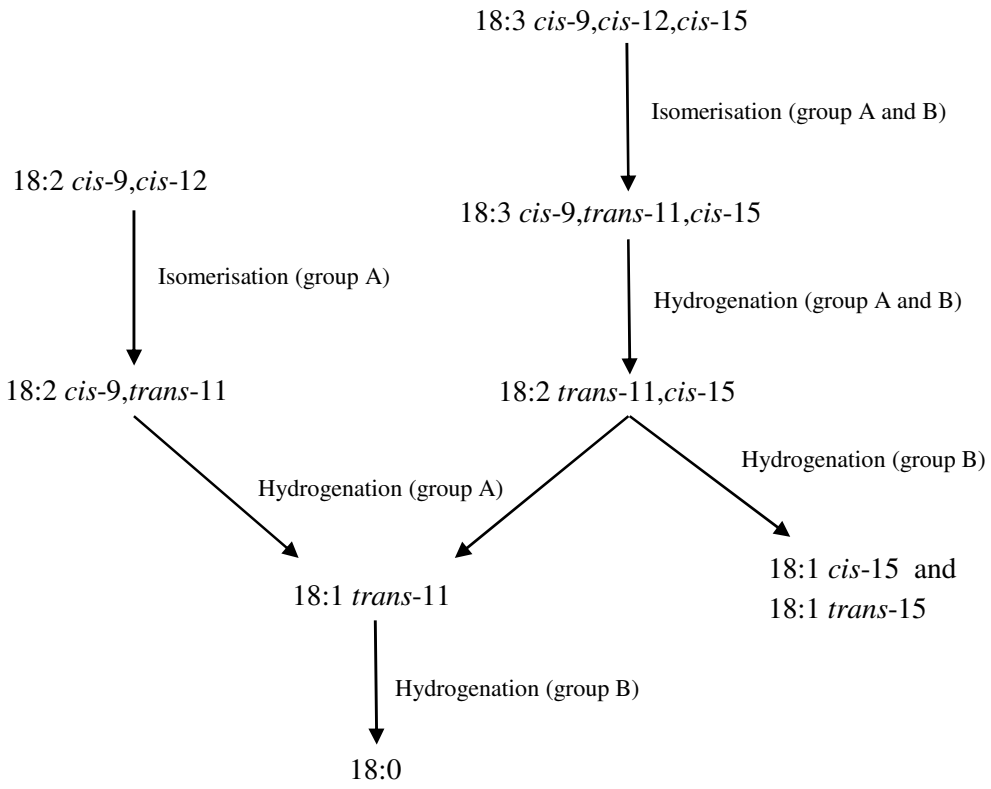

Figure 1.3. Biohydrogenation pathways of α-linolenic acid (18:3 *cis*-9,*cis*-12,*cis*-15) and linoleic acid (18:2 *cis*-9,*cis*-12). Adapted from Harfoot and Hazlewood (1997).

1.3.2.1. Role of bacteria

Ruminal biohydrogenation occurs as a result of the microbial metabolic activity (Shorland et al., 1955), bacteria being the main microorganisms responsible for this process. For many years, the only bacterium known to carry out biohydrogenation was *B. fibrisolvens* (Polan et al., 1964). Other bacteria with the ability to biohydrogenate C18 unsaturated FA were later isolated and identified, including species of the genera *Butyrivibrio*, *Ruminococcus*, *Treponema-Borrelia, Micrococcus*, *Eubacterium*, and *Fusocillus* (Kemp et al., 1975; Hazlewood et al., 1976; Harfoot & Hazlewood, 1997). Nevertheless, scarce information on the relative activity and significance of the different bacteria was provided as concentrations of the labelled FA used as substrates were very low (Jenkins et al., 2008). More recently, two new hydrogenating species of the genus *Butyrivibrio* were isolated (van de Vossenberg & Joblin, 2003; Wallace et al., 2006) and hydrogenation activity detected in *Megasphaera elsdenii* (Kim, Liu, Rychlik & Russell, 2002) and *Propionibacterium acnes* (Wallace et al., 2006; Wallace, McKain, Shingfield & Devillard, 2007). Despite the attempts, the number of biohydrogenating species known to date is quite small. Difficulties in isolating these microorganisms are related to the fact that no specific isolation pressure can be used in the process (Harfoot and Hazlewood, 1997), as biohydrogenators are sensitive to the toxic effects of unsaturated FA present in the isolation media.

The most active biohydrogenating species is *B. fibrisolvens* (Polan et al., 1964). The *B. fibrisolvens* species encloses a group of genetically and functionally diverse group of motile bacteria, mainly isolated from the rumen of cattle and sheep (Stewart et al., 1997) but also present in the faecal flora of man, rabbits and horses (Wedekind, Mansfield & Montgomery, 1988; Hespell, 1992; Rumney, Duncan, Henderson & Stewart, 1995; Eckburg et al., 2005). Due to its extraordinary thin cellular membrane, this species stains Gram-negative although being Gram-positive (Cheng & Costerton, 1977). Several are the ruminal functions where *B. fibrisolvens* is involved, such as fibre breakdown (Hespell, Wolf & Bothast, 1987), proteolysis (Hazlewood et al., 1983; Wallace & Brammall, 1985; Strydom, Mackie & Woods, 1986), and FA biohydrogenation (Polan et al., 1964; Kepler et al., 1966; Wallace et al., 2006). Metabolism of 18:2 *n*-6 by *B. fibrisolvens* produces 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11 as biohydrogenation intermediates (Polan et al., 1964; Kepler et al., 1966). However, no 18:0 was formed during the process. Kemp et al. (1975) screened over 200 bacteria isolates from the rumen of sheep and isolated two bacteria with the ability to biohydrogenate 18:2 *n*-6 and 18:1 *cis*-9 to 18:0, but not 18:3 *n*-3, being identified as *Fusocillus* spp. The two *Fusocillus*

spp. were motile, strictly anaerobic, non-sporing bacilli, with a single sub-polar flagellum, and stained Gram-negative. The isolates were deposited with the National Collection of Industrial Bacteria but were later shown to be non-viable. As the cultures, neither did the genus description survived (Wallace et al., 2006). Thus, no further studies could be carried out to characterise *Fusocillus* biohydrogenation activity and pathways or the genus itself by modern metagenomic techniques available our days. The pursuit for 18:0-producers was achieved many years later by van de Vossenberg and Joblin (2003). These researchers isolated a bacterium with the ability to hydrogenate 18:2 *n*-6 to 18:0 from the rumen of a grazing cow. The bacterium was phenotypically similar to *Fusocillus*, although molecular analysis showed it to be phylogenetically close to *Butyrivibrio hungatei*. This identification was proven to be wrong. Using a larger number of 16S rDNA sequences, Paillard et al. (2007) showed that the strain Su6 identified as *B. hungatei* by van de Vossenberg and Joblin (2003) was most closely clustered with *Clostridium proteoclasticum*. More recently, Wallace et al. (2006) screened 400 fresh isolates from the rumen of grazing sheep and identified one isolate, P-18, as a 18:0-producer. The isolate was named *C. proteoclasticum* and was morphologically and metabolically similar to *Fusocillus*. The main difference found was the ability of *C. proteoclasticum* P-18 to hydrogenate 18:3 *n*-3 to 18:0 whereas *Fusocillus* could only convert it to 18:1 *cis*-15 (Kemp et al., 1975). Despite its name, *C. proteoclasticum* is closely related to *Butyrivibrio* spp. (Attwood, Reilly & Patel, 1996; Paillard et al., 2007). Indeed, it was recently renamed as *Butyrivibrio proteoclasticus* based on the phylogenetical analysis of its 16S rDNA (Moon et al., 2008).

All *Butyrivibrio* strains are members of cluster XIVa of the *Clostridium* subphylum (Willems, Amatmarco & Collins, 1996). The majority of *Butyrivibrio* isolates include species names as *B. hungatei*, *Butyrivibrio crossotus*, *Pseudobutyrivibrio ruminis*, *Pseudobutyrivibrio xylanivorans*, and *C. proteoclasticum* (Moore, Johnson & Holdeman, 1976; van Gylswyk, Hippe & Rainey, 1996; Kopečný, Zorec, Mrázek, Kobayashi & Marinšek-Logar, 2003; van de Vossenberg & Joblin, 2003).

Paillard et al. (2007) evaluated the phylogenetic position of different *Butyrivibrio*-like bacteria from the rumen (**Figure 1.4**). Lipase activity, biohydrogenation products and sensitivity to FA concentration were found to correspond with the phylogenetic position, but not with linoleate isomerase activity. All *Butyrivibrio* strains across the phylogenetic tree formed 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11 from 18:2 *n*-6, but only a small group closely related to *B. proteoclasticus* formed 18:0. Butyrate metabolism was also related with the phylogenetic position. All bacteria producing 18:2 *cis*-9,*trans*-11, 18:1 *trans*-11 or both from

18:2 *n*-6 were butyrate producers. The opposite was not true, however (Wallace et al., 2006; Paillard et al., 2007).

Butyrate is the main fermentation product of *B. fibrisolvens* after acetate and its synthesis involves a central metabolic pathway and two terminal alternative routes (**Figure 1.5**; Bennett & Rudolph, 1995; Diez-Gonzalez, Bond, Jennings & Russell, 1999). One route consists of the conversion of butyryl-CoA to butyryl-phosphate by the phosphobutyryl-transferase, followed by the formation of butyrate by the butyrate kinase, generating one molecule of ATP for each molecule of butyrate synthesised. The alternative pathway involves the enzyme butyryl-CoA:acetate-CoA transferase which transfers the CoA moiety from butyryl-CoA onto acetate (Diez-Gonzalez et al., 1999).

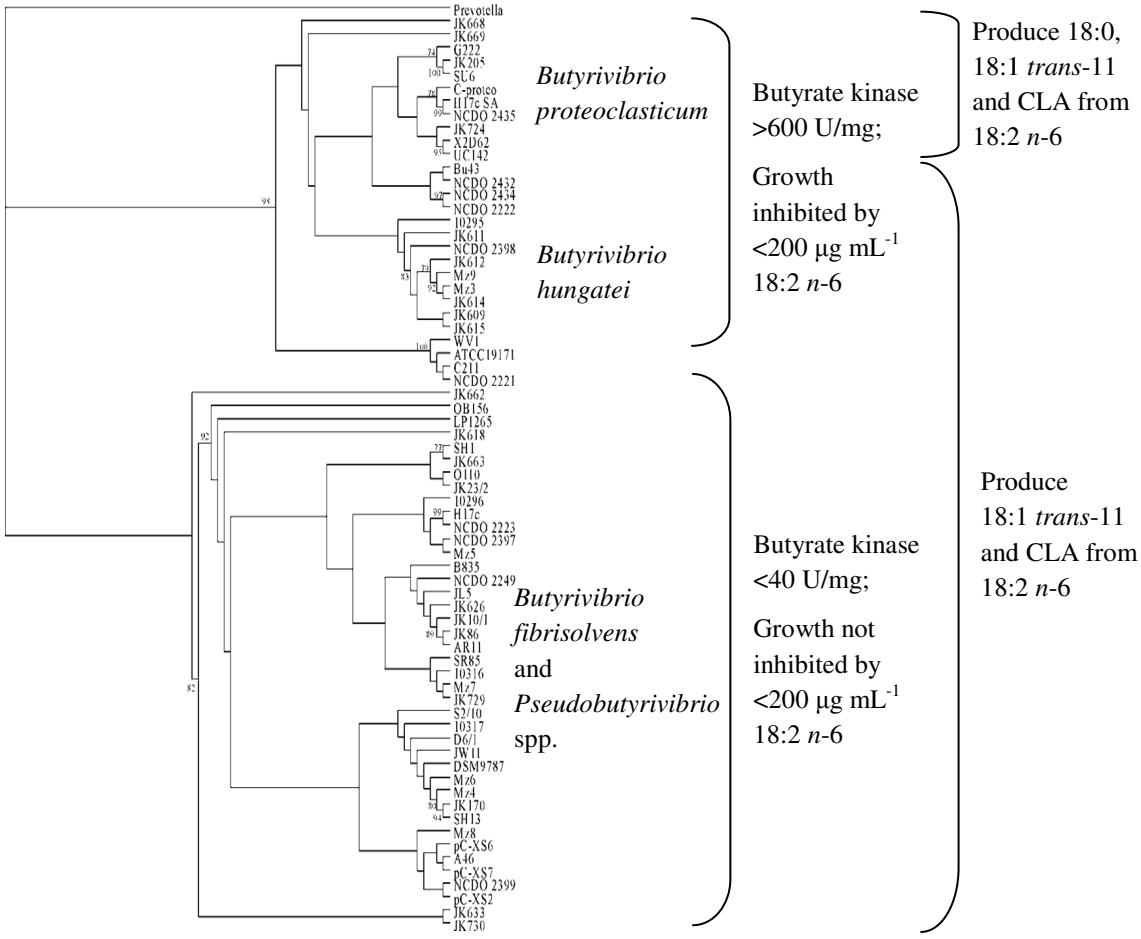

Figure 1.4. Phylogenetic tree of *Butyrivibrio*-like bacteria based on 16S rRNA sequence analysis and relation to butyrate formation mechanism, sensitivity to growth inhibition and linoleic acid biohydrogenation products. CLA, conjugated linoleic acids; 18:2 *n*-6, linoleic acid; 18:1 *trans*-11, vaccenic acid. Adapted from Lourenço, Ramos-Morales and Wallace (2010).

Both pathways have been identified in different strains of the genus *Butyrivibrio* (Diez-Gonzalez et al., 1999). Indeed, based on the mechanism by which butyrate is formed, two groups can be distinguished on the *Butyrivibrio*-like bacteria phylogenetic tree (**Figure 1.4**).

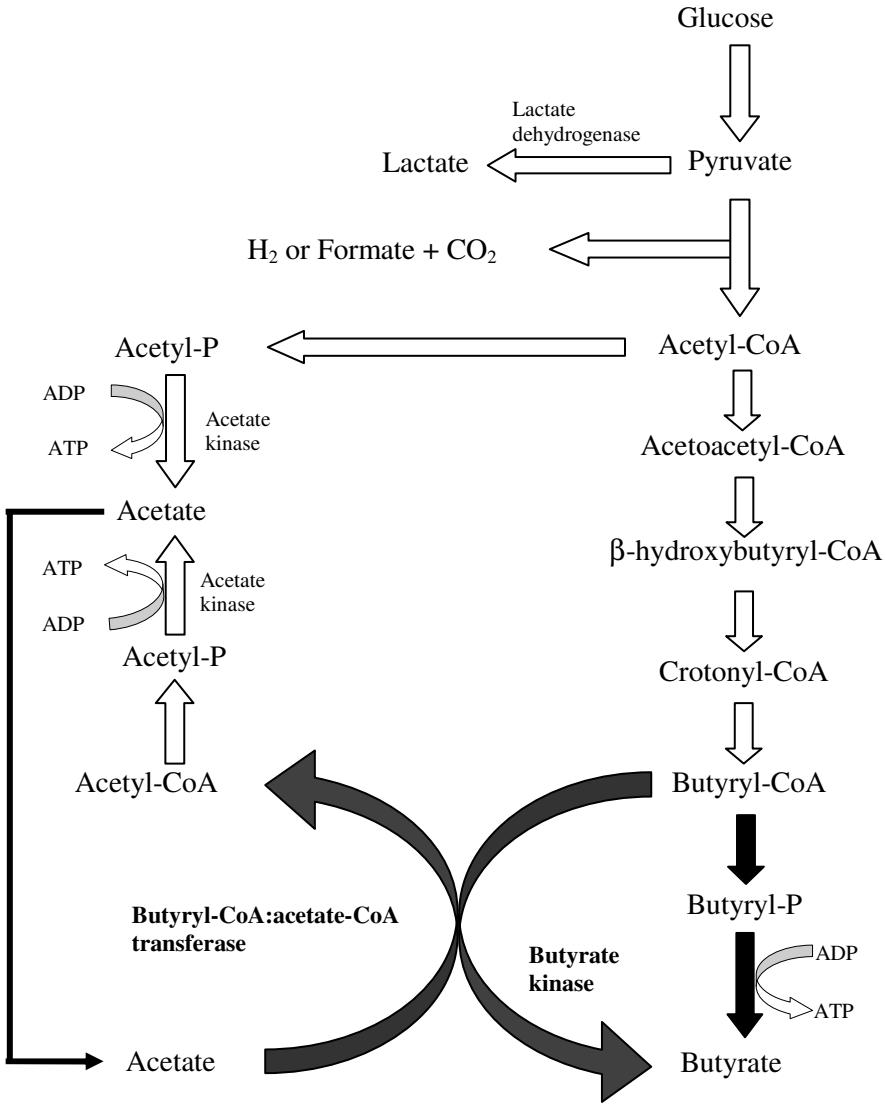

Figure 1.5. Schematic representation of butyrate formation from glucose in *Butyrivibrio fibrisolvens* by butyrate kinase and butyryl-CoA:acetate-CoA transferase. Open arrows represent enzymes common to all *Butyrivibrio* strains. Adapted from Diez-Gonzalez, Bond, Jennings and Russell (1999).

Butyrivibrio hungatei and *B. proteoclasticus* groups had a butyrate kinase activity higher than 600 U mg^{-1} protein, while the rest of the *Butyrivibrio/Pseudobutyrivibrio* cluster had an activity lower than 40 U mg^{-1} protein (Paillard et al., 2007). The two groups forming butyrate via butyrate kinase, *B. hungatei* and *B. proteoclasticus*, were more sensitive to toxic effects of C18 unsaturated FA than strains forming butyrate via butyryl-CoA:acetate-CoA transferase.

The correlation observed by Paillard et al. (2007) led these researchers to speculate that the enzymatic mechanism by which butyrate is produced may be linked to the toxic effects of unsaturated FA, with butyrate kinase producers being more sensitive than those with butyryl-CoA:acetate-CoA transferase mechanism.

1.3.2.1.1. Biohydrogenating bacteria classification

Kemp and Lander (1984) proposed the classification of biohydrogenating bacteria into two different groups, group A and group B, based on their biohydrogenation products. Members of group A mostly hydrogenate 18:3 *n*-3 and 18:2 *n*-6 to 18:1 *trans*-11. No C18:1 FA is further reduced by group A bacteria, however. Members of group B bacteria mostly hydrogenate 18:2 *n*-6 and C18:1 FA, as 18:1 *cis*-9 and 18:1 *trans*-11, to 18:0. An example of a group A bacteria is *B. fibrisolvens* whereas group B is represented by *Fusocillus* and *B. proteoclasticus*. Hazlewood et al. (1976) considered the separation into three groups of bacteria. Members of group 1 bacteria hydrogenated the *cis*-9 double bond of 18:2 *cis*-9,*trans*-11, producing 18:1 *trans*-11 as end-product. Members of group 2 bacteria had the ability to hydrogenate the *cis*-9 double bond of 18:2 *cis*-9,*trans*-11 as well as the *cis*-15 double bond of 18:3 *cis*-9,*trans*-11,*cis*-15. Group 1 and group 2 bacteria of Hazlewood et al. (1976) comprised group A bacteria of Kemp and Lander (1984) whereas group 3 was similar to group B.

No isolate of any group had the capacity to further hydrogenate 18:1 *cis*-15 and 18:1 *trans*-15 formed during biohydrogenation of 18:3 *n*-3, however. The reason why this incomplete biohydrogenation pathway is chosen is not understood (Harfoot & Hazlewood, 1997). Nevertheless, 18:1 *cis*-15 and 18:1 *trans*-15 are often found in *in vitro* incubations, even as major end-products (White et al., 1970; Body, 1976).

Classification of biohydrogenating bacteria into groups A and B (Kemp & Lander, 1984) is commonly used up to date. However, it was recently clarified the phylogenetic relation between members of both groups in the *Butyrivibrio*/*Pseudobutyrivibrio* cluster (Paillard et al., 2010). Therefore, Lourenço, Ramos-Morales and Wallace (2010) proposed the description of biohydrogenating bacteria based on taxonomy rather than on biohydrogenation end-products formation.

1.3.2.2. Role of protozoa

Wright (1959, 1960) incubated fractionated rumen contents and concluded that protozoa and bacteria were both responsible for biohydrogenation. However, the extensive ingestion of bacteria by protozoa was considered by Dawson and Kemp (1969) to cause confusion in experimental results. Dawson and Kemp (1969) eliminated protozoa from sheep rumen contents using detergent and compared the biohydrogenation rates of 18:3 *n*-3 and 18:1 *cis*-9 between defaunated and mixed faunated animals. Biohydrogenation rates decreased slightly after defaunation, however protozoa were not necessary for biohydrogenation to occur. Girard and Hawke (1978) and Singh and Hawke (1979), using suspensions of washed protozoa, found little biohydrogenating activity associated with protozoa while bacterial suspensions rapidly hydrogenated the substrates. The results obtained led the researchers to suggest that the contribution of protozoa to biohydrogenation was due to the activity of ingested or associated bacteria.

Following the emerging interest of CLA isomers and 18:1 *trans*-11 production in the rumen, biohydrogenation activity of protozoa was revisited by Devillard, McIntosh, Newbold and Wallace (2006). Protozoal lipids are known to contain more unsaturated FA than bacterial lipids (Katz & Keeney, 1966; Harfoot & Hazlewood, 1997). Among these unsaturated FA were recently described the presence of CLA and 18:1 *trans*-11 in levels more than eight and three times higher in protozoa than in bacteria, respectively (Devillard et al., 2006). Fatty acids composition varied within different protozoal species, with entodiniomorphs containing higher unsaturation levels than holotrich. The only holotrich studied, *Isotricha prostoma*, had low concentration of CLA and 18:1 *trans*-11. Among entodiniomorphs, larger species, such as *Ophryoscolex caudatus*, contained a concentration of CLA and 18:1 *trans*-11 more than 10 times higher than smaller species, as *Entodinium nannelum* (Devillard et al., 2006). The metabolism of 18:2 *n*-6 by fractionated ruminal digesta was also observed and the results found were similar to those previously described for 18:3 *n*-3 metabolism (Girard & Hawke, 1978; Singh & Hawke, 1979). Strained rumen fluid and bacterial fractions had similar activities whereas protozoal activity was much lower. Recently, Boeckaert et al. (2009) showed that *I. prostoma* did not biohydrogenate 18:2 *n*-6. Nevertheless, extrapolations cannot be made to other protozoal species, namely entodiniomorphs, due to *I. prostoma* low CLA and 18:1 *trans*-11 concentrations.

Although protozoa are rich in CLA and 18:1 *trans*-11 their biohydrogenating activity appears to be scarce or inexistent. Could these FA be desaturated by protozoa instead? Labelled ^{14}C-

18:0 was incubated with protozoal fraction but no radioactivity was incorporated into CLA or 18:1 *trans*-11 (Devillard et al., 2006). Moreover, no FA desaturase genes were found in cDNA libraries from ruminal protozoa with gene sequences similar to FA desaturases from other organisms (Jenkins et al., 2008; Lourenço et al., 2010). Therefore, protozoa do not appear to biohydrogenate 18:2 *n*-6 nor desaturate 18:0 to CLA or 18:1 *trans*-11 despite their high concentration in protozoal lipid composition. Jenkins et al. (2008) suggested that high concentrations of CLA and 18:1 *trans*-11 in protozoa might be explained by the preferential incorporation of these FA from ingested bacteria or, more likely, by the lower activity of 18:1 *trans*-11 reductase compared to isomerase and even CLA reductase, that would decrease to such levels in digested bacteria that 18:0 formation would not be significant. An increased vulnerability of the bacteria responsible for the hydrogenation of 18:1 FA to 18:0 to protozoal activities was further proposed by Lourenço et al. (2010) as an explanation for the high concentrations of CLA and 18:1 *trans*-11 in protozoa.

Protozoa are an important source of FA to the host animal as protozoal lipids may comprise up to approximately 75% of total microbial FA (Keeney, 1970). In a recent study, Huws et al. (2009) showed evidences that high PUFA content of protozoa was a consequence of chloroplast uptake, using chlorophyll and 18:3 *n*-3 as markers. Ciliate protozoa are selectively retained within the rumen by a migration/sequestration mechanism that depends on chemotaxis (Abe, Iriki, Tobe & Shibui, 1981). Consequently, protozoal biomass reaching the duodenum is less than would be expected if a similar flow with the ruminal digesta occurred (Hungate, Reichl & Prins, 1971; Weller & Pilgrim, 1974). Enhancing the flow of protozoa to the duodenum, without altering microbial density within the rumen, would increase PUFA absorption by the host animal for incorporation into milk and meat (Or-Rashid, Odongo & McBride, 2007), as these may be more dependent on the flow of protozoa from the rumen than of bacteria (Jenkins et al., 2008). Yáñez-Ruiz, Scollan, Merry and Newbold (2006) determined that 30 to 43% CLA and 40% 18:1 *trans*-11 reaching the duodenum were of protozoal origin, whereas protozoal contribution to 16:0 and 18:0 flows was less than 20 and 10%, respectively. Thus, protozoa may play an important role on the concentration of CLA and 18:1 *trans*-11 leaving the rumen, even though they lack the ability to produce these FA by biohydrogenation or desaturation. Nevertheless, further research is needed regarding the effects of protozoal FA contribution to the host animal. In earlier studies, concentrations of PUFA in the blood tended to increase in defaunated sheep compared to mixed faunated sheep, while no differences were found on concentration of saturated FA (Klopfenstein, Purser & Tyznik, 1966; Abaza, Abouakkada & Elshazly, 1975).

1.3.2.3. Role of anaerobic fungi

High content of 18:1 FA were described by Kemp, Lander and Orpin (1984b) in anaerobic flagellated ruminal fungi, and later confirmed by Body and Bauchop (1985). After incubations of *Piromyces communis* with ^{14}C-labelled 18:0 radioactivity was detected on 18:1 *cis*-9, suggesting a Δ9-desaturase activity in *P. communis* (Kemp et al., 1984b). Kemp et al. (1984b) also found the production of conjugated FA when *P. communis* was incubated with 18:2 *n*-6 and 18:3 *n*-3 FA.

Recently, Nam and Garnsworthy (2007a and 2007b) demonstrated the production of 18:2 *cis*-9,*trans*-11 from 18:2 *n*-6 by mixed rumen fungi. Maia, Chaudhary, Figueres and Wallace (2007) evaluated the biohydrogenation activity of two ruminal fungi species, *Neocallimastix frontalis* and *P. communis*. After 96 h incubation in a complete medium with 50 μg mL^{-1} of 18:2 *n*-6, no growth was observed with *P. communis* whereas *N. frontalis* had grown and metabolised nearly half of the 18:2 *n*-6 content to 18:2 *cis*-9,*trans*-11. Nevertheless, the contribution of *N. frontalis* to overall 18:2 *n*-6 hydrogenating activity in the rumen is extremely small. A similar conversion of 50 μg mL^{-1} is achieved in a few minutes by *B. fibrisolvens* (Maia et al., 2007; Jenkins et al., 2008).

1.3.2.4. Biohydrogenation pathways and intermediates

Early microbiological studies, combined with *in vitro* and *in vivo* studies, have established the biohydrogenation pathways and FA intermediates of 18:3 *n*-3 and 18:2 *n*-6 metabolism in the rumen (**Figure 1.3**), which have been widely recognised, accepted and used up to date. Although the number of different biohydrogenation intermediates determined in those studies, the need to identify major hydrogenation routes led to a simplification of the biohydrogenation pathway representation. Recent advances in chemical analysis of FA involving the use of very long (100 m or more) and highly polar capillary columns combined with gas-chromatography with or without mass spectrometry, emerged new interest on biohydrogenation pathways and intermediates.

Most development in rumen lipid research over the last decades has been achieved in animal production and nutrition areas, mainly focused on modification of lipid composition and profile of muscle tissues and milk. Several *in vivo* studies showed an extremely complex FA profile of digesta contents, including several C18:1 *trans* FA and CLA isomers. The amount

and complexity of intermediates found in those studies could not be explained by the established pathways. Determining the origin of a specific biohydrogenation intermediate can be a rather difficult process, however. Although manipulation of the rumen metabolic activity and fermentation provided new insights on intermediates of PUFA biohydrogenation, no major breakthroughs were made on the elucidation of microbiological and underlying biochemical mechanisms (Harfoot & Hazlewood, 1997).

Formation of CLA isomers by the rumen microbial population led to more research and interest on the identification of biohydrogenation intermediates and pathways of 18:2 *n*-6 metabolism, whereas less is known on 18:3 *n*-3 metabolism in the rumen. Nevertheless, although less studied, conjugated trienoic acids may be just as important, or even more, on health promotion as CLA (Tsuzuki, Tokuyama, Igarashi & Miyazawa, 2004).

1.3.2.4.1. α-Linolenic acid biohydrogenation

According to classical biohydrogenation pathway (Harfoot & Hazlewood, 1997), 18:3 *n*-3 is initially isomerised to 18:3 *cis*-9,*trans*-11,*cis*-15 (**Figure 1.3**), although other C18:3 FA have been described (**Figure 1.6**). Wilde and Dawson (1966) proposed the isomerisation of 18:3 *n*-3 to the conjugated trienoic 18:3 *cis*-9,*cis*-11,*cis*-15 or 18:3 *cis*-9,*cis*-13,*cis*-15, which were further hydrogenated to non-conjugated C18:2 *cis*,*cis* and C18:2 *cis*,*trans* or *trans*,*cis* FA. Dawson and Kemp (1969) reported the isomerisation of 18:3 *n*-3 into different conjugated trienoic acids, even when hydrogenation was completely suppressed by addition of detergents. Wąsowska et al. (2006) demonstrated the isomerisation of 18:3 *n*-3 to 18:3 *cis*-9,*trans*-11,*cis*-15 and 18:3 *trans*-9,*trans*-11,*cis*-15 and hydrogenation of the trienoic conjugated acids to the non-conjugated 18:2 *trans*-11,*cis*-15 acid. 18:3 *cis*-9,*trans*-11,*cis*-15 was identified as the main trienoic acid formed in ruminal mixed digesta (Wąsowska et al., 2006), in agreement with previous suggestions of Kepler and Tove (1967) and Kemp and Dawson (1968).

Further evidences of 18:3 *n*-3 metabolism in the rumen and biohydrogenation intermediates formed were provided from studies other than on ruminal contents. Loor, Ueda, Ferlay, Chilliard and Doreau (2004) reported the duodenal flow of 18:3 *cis*-9,*trans*-12,*cis*-15, 18:3 *cis*-9,*trans*-12,*trans*-15 and 18:3 *trans*-9,*trans*-12,*trans*-15 isomers in dairy cows fed conventional diets, which increased with linseed oil supplementation. It was suggested that these three non-conjugated FA were originated from biohydrogenation of 18:3 *n*-3, as precursors with 3 or more double bonds would be necessary to form C18:3 intermediates (Loor et al., 2004). In milk fat, Destaillats, Trottier, Galvez and Angers (2005) reported the

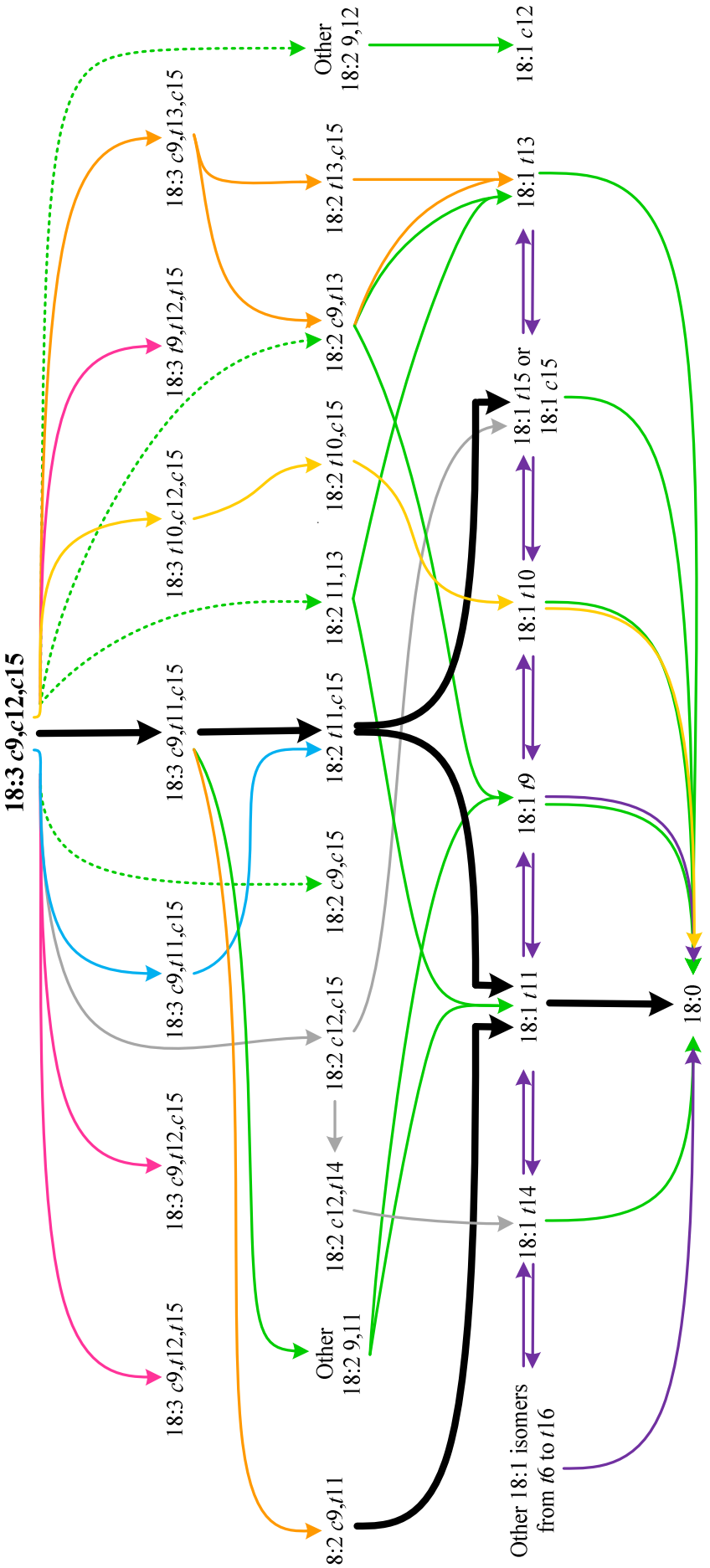

Figure 1.6. Biohydrogenation pathway of α-linolenic acid (18:3 *cis*-9,*cis*-12,*cis*-15). Thick arrows represent the main biohydrogenation pathway (Harfoot & Hazlewood, 1997) and thin arrows the putative pathways, with unknown octadecatrienoic acids formation as dotted lines. When geometric configuration is not mentioned, the several *cis,cis, cis,trans, trans,cis* and *trans,trans* configurations may exist. *C, cis* double bond configuration; *t, trans* double bond configuration. Adapted from Grinarii and Bauman (1999; yellow), Loor, Ueda, Ferlay, Chilliard and Doreau (2004; pink), Destaillats, Trottier, Galvez and Angers (2005; orange), Wąsowska et al. (2006; blue); Bessa et al. (2007; grey), Chilliard et al. (2007; green), Mosley, Powell, Riley and Jenkins (2002; purple), and Proell, Mosley, Powell and Jenkins (2002; purple).

occurrence of two conjugated C18:3 isomers, 18:3 *cis*-9,*trans*-11,*cis*-15 and 18:3 *cis*-9,*trans*-13,*cis*-15, and proposed an alternative pathway based on their occurrence. The proposed pathway for 18:3 *n*-3 biohydrogenation includes the initial isomerisation to the trienoic acids 18:3 *cis*-9,*trans*-11,*cis*-15 and 18:3 *cis*-9,*trans*-13,*cis*-15, and further hydrogenation to the non-conjugated dienoic isomers 18:2 *trans*-11,*cis*-15 and 18:2 *cis*-9,*trans*-13 but also to the CLA isomers 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-13,*cis*-15 (Destaillats et al., 2005).

Alves and Bessa (2007) identified an unusual *cis*-*cis* methylene-interrupted diene, 18:2 *cis*-12,*cis*-15 (18:2 *n*-3), in muscle samples of lambs fed lucerne supplemented with linseed oil, which was further proposed as an intermediate of 18:3 *n*-3 biohydrogenation (Bessa et al., 2007). Unlike most pathways, Bessa et al. (2007) did not propose an initial isomerisation step but rather a direct hydrogenation of 18:3 *n*-3 to 18:2 *n*-3, by saturation of the double bond at carbon 9. The 18:2 *n*-3 intermediate was suggested to be hydrogenated to 18:1 *cis*-15 or isomerised into 18:2 *cis*-12,*trans*-14 and further hydrogenated to 18:1 *trans*-14 (Bessa et al., 2007).

Griinari and Bauman (1999) proposed that 18:3 *n*-3 could also be converted to the conjugated triene 18:3 *trans*-10,*cis*-12,*cis*-15, which would be hydrogenated to 18:2 *trans*-10,*cis*-15, further hydrogenated to 18:1 *trans*-10, and finally converted to 18:0.

1.3.2.4.2. Linoleic acid biohydrogenation

Since discovery of anticarcinogenic activity in fried ground beef extracts (Pariza & Hargraves, 1985) and subsequent identification of CLA isomers as being the responsible for the exerted properties (Ha et al., 1987), new interest emerged on investigation of 18:2 *n*-6 biohydrogenation. Evidences of the multiple intermediates of 18:2 *n*-6 arose from different *in vitro* studies with pure and mixed rumen bacteria and *in vivo* studies on digesta contents, milk and tissues (**Figure 1.7**). Nevertheless, no major changes have been made on the classical biohydrogenation pathway representation, as previously noted for 18:3 *n*-3.

The 18:2 *n*-6 biohydrogenation pathway used up to date (Harfoot & Hazlewood, 1997) involves an initial isomerisation to 18:2 *cis*-9,*trans*-11, followed by a hydrogenation with the formation of 18:1 *trans*-11 and further hydrogenation to 18:0 (**Figure 1.3**), similarly to the pathway proposed by Garton (1977) over 30 years ago. Chilliard et al. (2007) recently presented a more complex representation of 18:3 *n*-3 and 18:2 *n*-6 biohydrogenation pathways. This representation, besides the main biohydrogenation pathway, included putative pathways suggested by the wide range of intermediates isomers determined in *in vitro batch*

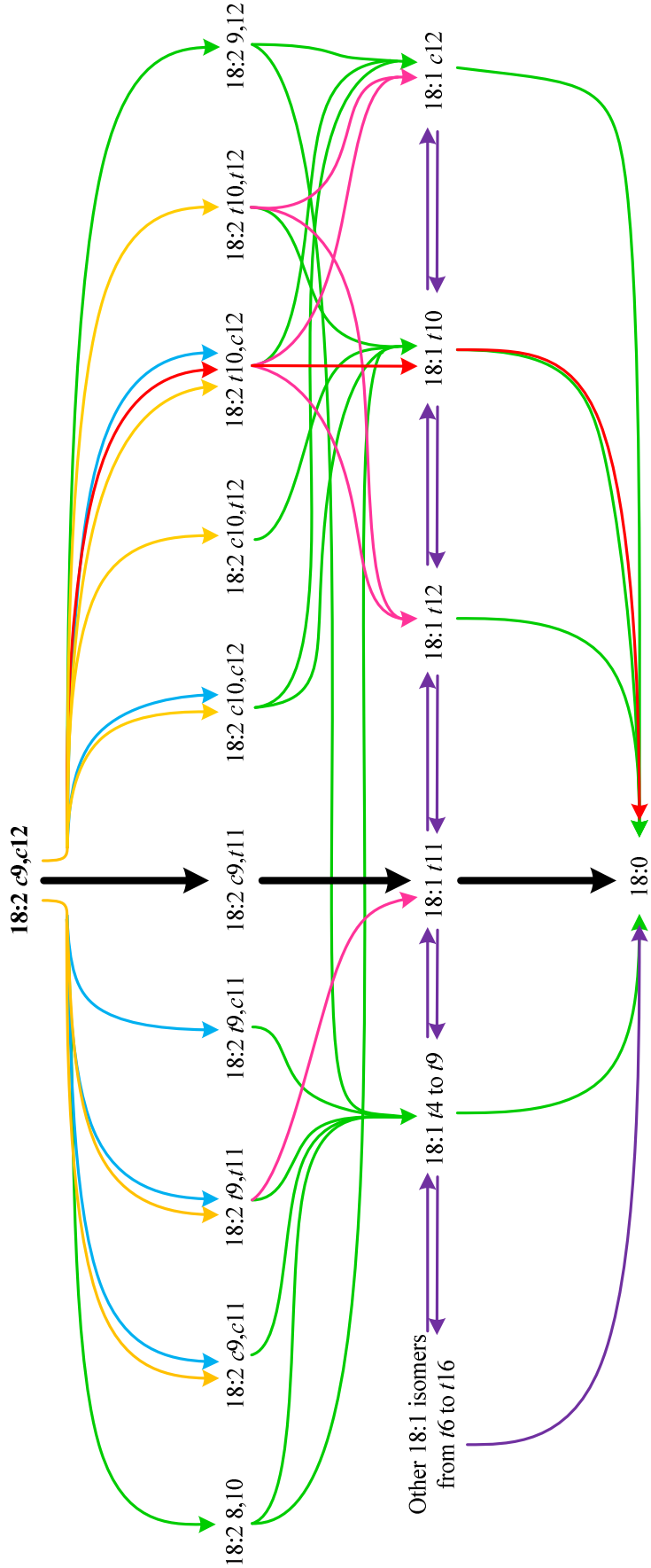

Figure 1.7. Biohydrogenation pathway of linoleic acid (18:2 *cis*-9,*cis*-12). Thick arrows represent the main biohydrogenation pathway (Harfoot & Hazlewood, 1997) and thin arrows the putative pathways. When geometric configuration is not mentioned, the several *cis,cis*, *cis,trans*, *trans,cis* and *trans,trans* configurations may exist. *C*, *cis* double bond configuration; *t*, *trans* double bond configuration. Adapted from Grinarii and Bauman (1999; red), Jenkins, Wallace, Moate and Mosley (2008; yellow), Wallace, McKain, Shingfield and Devillard (2007; blue), Chilliard et al. (2007; green), Mosley et al. (2002; purple), Proell et al. (2002; purple), and McKain, Shingfield and Wallace (2010; pink).

incubations of mixed rumen cultures with 18:2 *n*-6, 18:3 *n*-3 and linseed oil (Jouany, Lassalas, Doreau & Glasser, 2007).

The conjugated diene 18:2 *cis*-9,*trans*-11, or rumenic acid (Kramer et al., 1998), is usually the predominant CLA isomer found in the rumen, milk and meat. Nevertheless, several other CLA isomers are found, 18:2 *trans*-9,*trans*-11 being the most outstanding isomer (Fritsche et al., 1999; Shingfield et al., 2003; Palmquist et al., 2005).

In animals fed high-concentrate diets supplemented with oils, 18:2 *trans*-10,*cis*-12 isomer becomes a major biohydrogenation intermediate in ruminal digesta and consequently in ruminant products (Offer, Marsden & Phipps, 2001; Daniel, Wynn, Salter & Buttery, 2004). Griinari and Bauman (1999) proposed an alternative pathway for 18:2 *n*-6 biohydrogenation involving the initial isomerisation to 18:2 *trans*-10,*cis*-12 and further hydrogenation to 18:1 *trans*-10, similarly to the established pathway for 18:2 *cis*-9,*trans*-11. The formation of 18:2 *trans*-10,*cis*-12 was later shown to occur in enriched cultures with starch by activity of large cocci identified as *Megasphaera elsdenii* (Kim et al., 2002). Maia et al. (2007) attempted to recreate the findings of Kim et al. (2002) but none of the *M. elsdenii* strains studied, including the A type, produced 18:2 *trans*-10,*cis*-12 or metabolised 18:2 *n*-6. Ruminal bacteria *Propionibacterium acnes* may be the responsible for 18:2 *trans*-10,*cis*-12 production (Wallace et al., 2006; Lourenço et al., 2010). In fact, species of the genus *Propionibacterium* occur in considerable numbers in the rumen of animals fed high-concentrate diets (Stewart et al., 1997), which seems to be in agreement with the high 18:2 *trans*-10,*cis*-12 production found under those dietary conditions.

Recent incubations of mixed rumen contents with labelled substrates provided new information on the conjugated dienes formation and hydrogenation. Jenkins et al. (2008) reported the isomerisation of ^{13}C-labelled 18:2 *n*-6 to 7 ^{13}C-enriched CLA isomers with double bonds determined at 9,11 or 10,12 positions. All 9,11 and 10,12 *cis*,*cis*, *cis*,*trans*, *trans*,*cis*, and *trans*,*trans* geometric isomers were identified, except for 18:2 *trans*-9,*cis*-11 (Jenkins et al., 2008). Using ^{2}H-labelled water, Wallace et al. (2007) determined the production of 6 CLA isomers by mixed ruminal digesta. The major isomer was 18:2 *cis*-9,*trans*-11, followed by 18:2 *trans*-10,*cis*-12 and 18:2 *trans*-9,*trans*-11, and trace amounts of 18:2 *trans*-9,*cis*-11, 18:2 *cis*-9,*cis*-11, and 18:2 *cis*-10,*cis*-12. Moreover, Wallace et al. (2007) reported a different formation mechanism of 18:2 *trans*-10,*cis*-12 and 18:2 *cis*-9,*trans*-11 isomers, based on ^{2}H-enrichment of 18:2 *cis*-9,*trans*-11 but not of 18:2 *trans*-10,*cis*-12 .

Metabolism of CLA isomers by the ruminal bacteria *B. fibrisolvens*, *B. proteoclasticus* and *P. acnes* was further investigated by McKain, Shingfield and Wallace (2010). *Butyrivibrio fibrisolvens* metabolised extensively 18:2 *cis*-9,*trans*-11 to 18:1 *trans*-11, while 18:2 *trans*-

9,*trans*-11 was only partly hydrogenated to 18:1 *trans*-11. 18:2 *trans*-10,*cis*-12 was mainly converted to 18:1 *trans*-10 and to lesser extent to 18:1 *cis*-12 and 18:1 *trans*-12. *Butyrivibrio proteoclasticus* was unable to growth in the presence of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12, and metabolised partly 18:2 *trans*-9,*trans*-11 to 18:0, with slight intermediates formed. No CLA isomer was metabolised by *P. acnes*, however.

McKain et al. (2010) suggested the FA metabolism in ruminal biohydrogenating bacteria to be composed by three systems: i) reduction of 18:2 *cis*-9,*trans*-11 geometric isomers to 18:1 *trans*-11; ii) reduction 18:2 *trans*-10,*cis*-12 geometric isomers to 18:1 *trans*-10 and 18:1 *cis*-12; and iii) reduction of C18:1 FA, including 18:1 *trans*-10, to 18:0.

1.3.2.4.3. Oleic acid biohydrogenation

Biohydrogenation of 18:1 *cis*-9 is usually represented as a direct conversion to 18:0, although isomerisation to other C18:1 isomers had been reported (**Figure 1.8**). Kemp et al. (1975) observed the ability of *Fusocillus* T344 to convert 18:1 *cis*-9 to 18:0 and detected trace amounts of 18:1 *trans*-11. Further studies with *Fusocillus* spp. elucidated the biohydrogenation ability of different C18:1 *cis* and *trans* isomers to 18:0 (Kemp, Lander & Gunstone, 1984a). All C18:1 isomers with double bonds from position 5 to 13 were hydrogenated to some extent after 3 h incubation, independently of the geometric conformation. Isomers 18:1 *cis*-5 to *cis*-11 were converted to 18:0 between 73 and 79%, whereas 18:1 *cis*-12 and 18:1 *cis*-13 were only hydrogenated up to 30% and 5%, respectively.

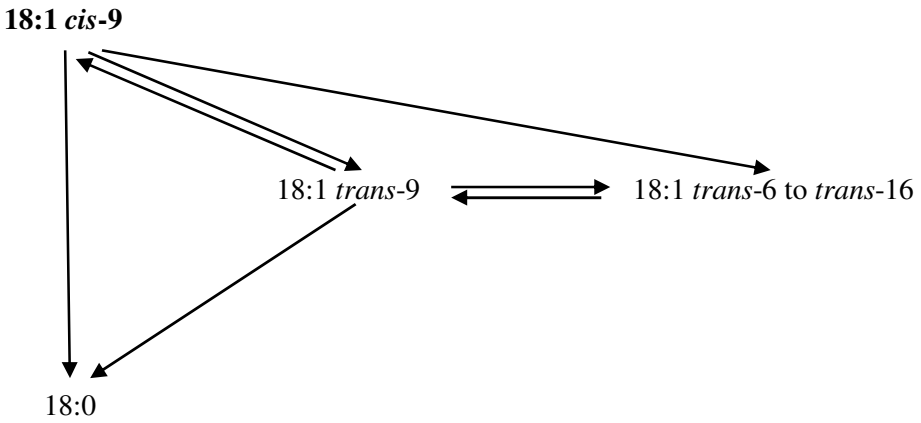

Figure 1.8. Biohydrogenation pathway of oleic acid (18:1 *cis*-9). Adapted from Jenkins, AbuGhazaleh, Freeman and Thies (2006).

Biohydrogenation of 18:1 *cis*-9 was recently revisited by Mosley, Powell, Riley and Jenkins (2002). *In vitro* incubations of rumen fluid with ^{13}C-labelled 18:1 *cis*-9 produced ^{13}C-labelled 18:0 as well as several C18:1 *trans* isomers with double bond ranging from carbon 6 to carbon 16. *Cis* to *trans* isomerisation of C18:1 FA isomers was clearly shown to occur *in vitro*. More evidences are needed to determine the extent to which ^{13}C-label was transferred directly from 18:1 *cis*-9 to 18:0 or if it occurred through a C18:1 *trans* isomer.

Proell, Mosley, Powell and Jenkins (2002) provided further insight to C18:1 FA isomerisation in the rumen. When ^{13}C-labelled 18:1 *trans*-9 was incubated with rumen fluid, it was isomerised to a wide range of C18:1 *trans* isomers having double bond positions between carbons 6 and 16, demonstrating the occurrence of *trans* to *trans* isomerisation. After 48 h incubation, nearly 18% of 18:1 *cis*-9 present was estimated to be derived from the labelled 18:1 *trans*-9. Such results suggested a *trans* to *cis* isomerisation to occur as well in the rumen, but the unusually high concentration of 18:1 *trans*-9 added to the culture was pointed out as an explanation for this peculiar occurrence (Proell et al., 2002). Conversion of 18:1 *trans*-9 to 18:0 or other C18:1 *trans* isomers was not observed in the absence of ruminal bacteria, suggesting the isomerisation to be an enzymatic process. Yet, Kemp et al. (1984a) noted some *cis* to *trans* isomerisation in incubations without bacterial inoculation.

Isomerisation of 18:1 *cis*-9 in the rumen might be conditioned by environmental conditions. In continuous culture, lower pH and decreasing dilution rates restricted the isomerisation of ^{13}C-labelled 18:1 *cis*-9 to *trans* isomers with double bond positions above carbon 10 (AbuGhazaleh, Riley, Thies & Jenkins, 2005).

1.3.2.4.4. Very long-chain polyunsaturated fatty acids biohydrogenation

The most important PUFA entering the rumen, apart from 18:3 *n*-3 and 18:2 *n*-6, are the fish oil and marine algal very long-chain 20:5 *n*-3 and 22:6 *n*-3 FA. These FA are extensively biohydrogenated at the rumen. Over 70% biohydrogenation of 20:5 *n*-3 and 22:6 *n*-3 were reported in dairy cattle (Doreau & Chilliard, 1997) and sheep (Wachira et al., 2000). Mixed rumen microorganisms were found to have the ability to biohydrogenate 20:5 *n*-3 and 22:6 *n*-3 *in vitro* in a dose dependent response (AbuGhazaleh & Jenkins, 2004a).

Although biohydrogenation of 20:5 *n*-3 and 22:6 *n*-3 occurs extensively in the rumen, the pathways and intermediates formed during its metabolism are not yet known. By analogy to C18 unsaturated FA biohydrogenation pathway, metabolism of 20:5 *n*-3 and 22:6 *n*-3 in the rumen would be expected to originate multiple intermediates until the formation of the

saturated FA 20:0 and 22:0, respectively, as end-products. Initial FA intermediates produced by isomerisation would be expected to be isomers with 5 or 6 double bonds with at least one double bond with *trans* configuration, further hydrogenated to isomers with 4 or 5 double bonds (Jenkins et al., 2008). Although none of these intermediates have been identified in digesta of fish oil fed animals (Jenkins et al., 2008), Vlaeminck, Harynuk, Fievez and Marriott (2007) reported multiple C22 FA intermediates in milk fat analysed by two-dimensional GC, providing indications of 22:6 *n*-3 biohydrogenation in the rumen.

Complete biohydrogenation of C18 unsaturated FA is inhibited by 20:5 *n*-3 and 22:6 *n*-3 supplementation, leading to an accumulation of 18:1 *trans*-11 in ruminal digesta. *In vitro* incubations of mixed ruminal cultures with 22:6 *n*-3 promoted the accumulation of 18:1 *trans*-11 in the presence of 18:2 *n*-6 (AbuGhazaleh & Jenkins, 2004b). Moreover, *in vivo* studies reported increased omasal flows of 18:1 *trans*-11 along with 18:2 *trans*-9,*trans*-11 and several non-conjugated dienes, but not 18:2 *cis*-9,*trans*-11, in dairy cows (Shingfield et al., 2003) and growing steers (Shingfield et al., 2010) fed fish oil.

At the present, not enough information is available on the putative biohydrogenation intermediates formed by microbial metabolism of 20:5 *n*-3 and 22:6 *n*-3 to establish the biohydrogenation pathways for these very long-chain *n*-3 PUFA.

1.3.2.5. Role of biohydrogenation

Biohydrogenation of C18 unsaturated FA in the rumen is a sequential enzymatic process with the consequent formation of the saturated FA 18:0, isomerases and reductases being the two microbial enzyme classes involved in the process. The underlying reason for biohydrogenation to be carried out by ruminal microorganisms is not entirely clear. Although the auxotrophic FA *Butyrivivrio* S2 hydrogenate 18:3 *n*-3 and 18:2 *n*-6 to 18:1 *trans*-11 and incorporate the latter FA into its membrane lipids (Hazlewood & Dawson, 1979), other *Butyrivibrio* with similar biohydrogenating activity do not incorporate FA intermediates or end-products (Kepler et al., 1970). Understanding the role of biohydrogenation in the rumen is therefore crucial to predict the extent and biohydrogenation intermediates pattern, and ultimately to manipulate the lipid metabolism towards a healthier FA profile of ruminant products for human consumption.

Two are the main theories proposed for biohydrogenation activity to occur in the rumen. Lennarz (1966) suggested that biohydrogenation was a mechanism to dispose of the excess reducing power. Disposal of hydrogen is essential for bacterial survival in an anaerobic

reduced environment as the rumen. The main mechanism for hydrogen disposal in the rumen ecosystem is methanogenesis. Comparatively to methanogenesis, the amount of hydrogen equivalents removed by biohydrogenation is tiny. Although unsaturated FA inhibit methanogenesis (Demeyer & Hendrickx, 1967; Prins, Van Nevel & Demeyer, 1972), they do it at millimolar concentrations, which is too low for them to be major competitors for hydrogen (Harfoot & Hazlewood, 1997). Nevertheless, the reductase that converts 18:2 *cis*-9,*trans*-11 to 18:1 *trans*-11 correspond to 0.5% of total cell protein in *B. fibrisolvens* (Hughes, Hunter & Tove, 1982), which represents a significant expenditure of cellular resources on a enzyme whose benefits to the microorganism are not evident. Harfoot and Hazlewood (1997) noted that *B. fibrisolvens* possesses a hydrogenase, which would dispose more efficiently the excess reducing power than biohydrogenation. The reducing power hypothesis seems, therefore, unlikely.

An alternative theory is that biohydrogenation is a detoxification mechanism (Kemp & Lander, 1984; Kemp et al., 1984a). Unsaturated FA are known for long to be toxic to many microorganisms (Nieman, 1954), including those of the rumen (Prins et al., 1972). In fact, non-esterified FA have been widely described to exert antimicrobial effects in a diversity of organisms, including algae, bacteria, fungi, protozoa, virus, animals and plants. Most reported effects are antibacterial, which ranged from bacteriostatic (inhibition of growth) to bactericidal (bacterial death) in Gram-negative and Gram-positive bacteria across different ecosystems (Desbois & Smith, 2010).

In pure culture study, *Butyrivibrio* spp. growth was inhibited by addition of 14:0, 16:0, 18:0, and 18:1 *cis*-9, the latter being the most inhibitory (Henderson, 1973). Marounek, Skrivanova and Savka (2002) found caprylic (8:0) and capric (10:0) acids to be more toxic to ruminal and rabbit caecal bacteria than other chain lengths, but the study was only of saturated FA and 18:1 *cis*-9. In non-ruminal bacteria, 18:2 *n*-6 and 18:3 *n*-3 were much more toxic than saturated or 18:1 FA, and *cis* unsaturated FA had stronger toxic effects than *trans* isomers on bacterial strains (Galbraith, Miller, Paton & Thompson, 1971). Maczulak, Dehority and Pamquist (1981) reported the effects of 16:0, 18:0, 18:1 *cis*-9, and 18:1 *trans*-11 on growth of 7 ruminal bacteria. Although marked differences in sensitivities were observed among bacterial species and strains, growth was strongly inhibited by 18:1 *cis*-9.

More recently, different sensitivities to 18:2 *n*-6 were described within isolates of the *Butyrivibrio*-like phylogenetic tree, with *B. proteoclasticus* group being much more sensitive than *B. fibrisolvens* isolates (Paillard et al., 2007). Moreover, different sensitivities to C18 unsaturated FA suggest to be related to biohydrogenation extent. *Butyrivibrio proteoclasticus* is sensitive to 18:1 *trans*-11, thus hydrogenation to 18:0 might be explained by the need of

bacteria to remove 18:1 *trans*-11 in order to grow. Other *Butyrivibrio* isolates are sensitive to 18:2 *n*-6 and 18:2 *cis*-9,*trans*-11 but not 18:1 *trans*-11. Similarly, metabolism of PUFA by *B. fibrisolvens* and *Pseudobutyrivibrio* to 18:1 *trans*-11 might be explained by the need to detoxify those FA in order to grow. Further hydrogenation to 18:0 by these isolates would only represent an energy expenditure. In face of the present data, unsaturated FA seem to inhibit growth whereas biohydrogenation appears to reverse the process (Jenkins et al., 2008). Thus, the detoxification hypothesis seems more plausible.

1.3.2.5.1. Mechanisms of fatty acid toxicity

The effects of non-esterified FA on bacteria appear to depend on carbon chain length, degree of saturation, configuration of double bonds, and FA concentration, although mechanisms underlying FA toxicity remain unclear.

Galbraith et al. (1971) noted differences on inhibitory effects of FA in non-ruminal bacteria and hypothesised that relative inhibitory effects could be partially explained by the capacity of FA to complex with calcium and form soaps. Thus, the extent of soap formation in mixed rumen cultures would be ordered as 18:0 > 18:1 *trans*-11 > 16:0 > 18:1 *cis*-9, corresponding to a minor effect of 18:0 on bacteria whereas 18:1 *cis*-9 presented the higher effect (Jenkins & Palmquist, 1982).

Gram-positive bacteria were found to be more susceptible to monounsaturated and PUFA than Gram-negative (Galbraith et al., 1971; Miller, Brown & Morse, 1977, Maczulak et al., 1981). Susceptibility differences might lay on membrane cell structure. Gram-negative bacteria outer membrane has a lipopolysaccharide layer, which may prevent FA adherence or penetration and thus decrease susceptibility to unsaturated FA (Sheu & Freese, 1973).

Differences in inhibitory effects of 18:1 geometrical isomers were described in ruminal pure cultures (Maczulak et al., 1981) and mixed cultures (Demeyer & Hendrickx, 1967), with *trans* configuration being less inhibitory than *cis* one. Hence, a relation between geometrical configuration of the unsaturated FA and the degree of inhibition was suggested, but studies were of monounsaturated geometrical isomers only.

In nature, unsaturated FA occur usually in the *cis* configuration. Apart from the rumen ecosystem, *trans* FA are usually described as an exception (Keweloh & Heipieper, 1996). However, biosynthesis of *trans* FA was demonstrated in the non-ruminal bacteria *Pseudomonas atlantica*, even though neither of the known routes of unsaturated FA formation, the aerobic or the anaerobic pathways, could explain its formation (Guckert,

Ringelberg & White, 1987). Further studies with *P. putida* clarified the existence of a direct *cis* to *trans* isomerase, by which *trans* FA were formed from *cis* FA without a shift in the double bond position (Keweloh & Heipieper, 1996). The *cis* to *trans* isomerase activity was observed in growing cells, as reaction to addition of toxic compounds (Heipieper, Diefenbach & Keweloh, 1992), and in non-growing cells, when lipid synthesis was suppressed (Diefenbach, Heipieper & Keweloh, 1992; Heipieper & de Bont, 1994). Guckert et al. (1987) noted increased amounts of *trans* FA in starving cells of *Vibrio cholerae* and suggested the use of a *trans* to *cis* ratio higher than 0.1 in environmental samples as an index for starvation or stress. However, studies with *P. putida* and *Schewanella putrefaciens* showed no relation between *trans* FA content and starvation (Diefenbach et al., 1992; Nichols, McMeekin & Nichols, 1994). Experiments with *P. putida* under adverse growth conditions demonstrated that the *cis* to *trans* isomerisation was an adaptation mechanism to modify membrane fluidity. Members of the genus *Pseudomonas* have the ability to grow in the presence of organic solvents. Heipieper, Loffeld, Keweloh and de Bont (1995) noted that the *cis* to *trans* FA isomerisation on *P. putida* was related to the organic solvents toxicity to cells, and to its concentration. Organic solvents partition cell membranes, causing an increase of membrane fluidity and a non-specific membrane permeabilization (Heipieper, Keweloh & Rehm, 1991; Kitagawa, Orinaka & Hiratan, 1993; Heipieper, Weber, Sikkema, Keweloh & de Bont, 1994; Sikkema, de Bont & Poolman, 1995).

Most bacteria regulate the fluidity of their cell membrane by altering the degree of saturation of the FA present in the lipid bilayer (Russell, 1984; Keweloh, Diefenbach & Rehm, 1991; Suutari & Laakso, 1994). Cell membranes with high content in saturated FA have highly packed FA structure and thereby a more rigid membrane. A similar, although not so tight, membrane might be obtained with the incorporation of *trans* FA (**Figure 1.9**).

Unsaturated FA with *trans* configuration have an extended structure which is similar to that of saturated FA. This conformation is preferred when the fluidity of the membrane is low (Keweloh & Heipieper, 1996). The presence of *cis* FA in membranes alters the bilayer FA order, increasing the cell envelope fluidity. Double bonds with *cis* conformation have a non-moveable bend with a 30 º angle in the acyl chain. This bend disturbs the acyl packing in membranes and leads to lower phase transition temperature (Cronan & Gelman, 1975), disruption of the cell integrity, growth inhibition or even death (Galbraith & Miller, 1973). The replacement of a *cis* by a *trans* unsaturated FA may increase the phase transition temperature by 18 to 31 ºC, which would result in a reduction of cell membrane fluidity. Bacteria using the anaerobic pathway of FA biosynthesis can only change membrane fluidity by *de novo* synthesis. As FA and lipid synthesis is closely related to growth, in environmental

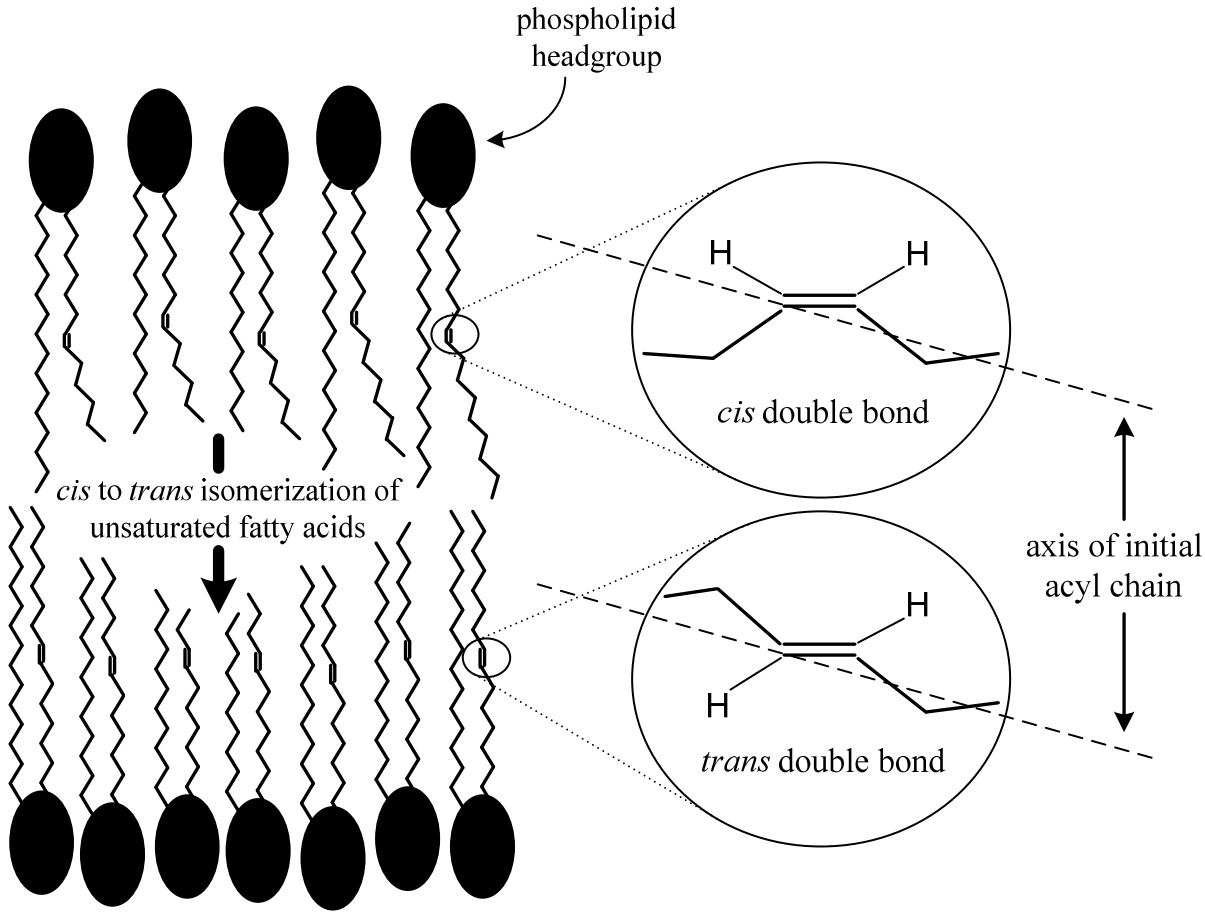

Figure 1.9. *Cis* to *trans* isomerisation of monounsaturated fatty acids and molecular packing of phospholipids with *cis* and *trans* fatty acids. Adapted from Keweloh and Heipieper (1996).

condition when growth is inhibited, bacteria are unable to form new FA and thereby to alter their membrane fluidity. Bacteria possessing the *cis* to *trans* isomerase are thereby able to regulate the membrane fluidity by changing the unsaturated FA configuration in the cell envelope bilayer, even under extreme adverse environmental conditions (Keweloh & Heipieper, 1996).

The existence of a direct *cis* to *trans* isomerase in the rumen was never reported, however its possibility cannot be ruled out. To date, besides biohydrogenation, the only isomerisation reported in the rumen was of 18:1 *cis*-9 (Mosley et al., 2002) and 18:1 *trans*-9 (Proell et al., 2002) to a diversity of other 18:1 *trans* FA with double bonds between carbon 6 and carbon 16. Yet, formation of *trans* FA during biohydrogenation pathway may be on its own a strategy of survival for ruminal bacteria under adverse conditions. Indeed, increased 18:1 *trans* FA concentration was reported with low pH (Kalscheur, Tetter, Piperova & Erdman, 1997), presence of ionophores (Fellner, Sauer & Kramer, 1997), and high unsaturated FA

concentration (Harfoot, Noble & Moore, 1973; Bateman & Jenkins, 1998). Thus, Bessa et al. (2000) suggested that high concentrations of 18:1 *trans* FA in the rumen might be involved in an adaption response of the rumen ecosystem to stress induction factors, as unsaturated FA toxicity.

Although it remains unclear how FA exert their antibacterial effects, the main target appears to be the bacterial cell membrane. The inner membrane of Gram-positive and Gram-negative bacteria is an important site for energy production, with the carriers in the electron transport chain being embedded within the membrane (Mitchell, 1961). During the electron transport chain process, the protons are exported from the inside to the outside of the cell, while the concentration of electrons increases in the cytosol. This generates a proton gradient and membrane potential, which are crucial for the production of ATP by bacteria (Mitchell, 1961). The diffusion of FA across the bacterial membrane may cause chemiosmotic difficulties, either by uncoupling the proton-motive force (Mitchell, 1979), dissipating the membrane potential (Nichols, 1982) or decoupling intramembrane pathways (Rottenberg & Hashimoto, 1986; Rottenberg & Steiner-Mordoch, 1986).

Many other effects may contribute to bacteriostatic or bactericidal effects of non-esterified FA (**Figure 1.10**), including i) cell lysis, ii) inhibition of enzymes, at the bacterial membrane or cytosol, crucial for the microorganism survival, iii) reduction of the nutrient uptake by directly disruption of the membrane transporter proteins or indirectly reduction of the proton-motive force required for the energy active transport, and iv) the generation of FA secondary degradation products responsible for peroxidation and auto-oxidation (Desbois & Smith, 2010).

1.3.2.5.2. Factors affecting biohydrogenation

Several factors affect FA biohydrogenation in the rumen, with the dietary composition, amount of diet, lipid supplementation, and interactions between these factors playing the main role in this process. Diet-induced changes alter the predominant ruminal biohydrogenation pathways resulting in changes of FA intermediates profile, along with modifications in microbial population profiles.

Gerson, John, Shelton and Sinclair (1982) reported different concentrations of unsaturated FA in the rumen when dietary nitrogen was supplemented at different levels. Increased proportions of dietary nitrogen were further shown to increase rates of lipolysis and biohydrogenation (Gerson, John & Sinclair, 1983). Decreased lipolysis and biohydrogenation

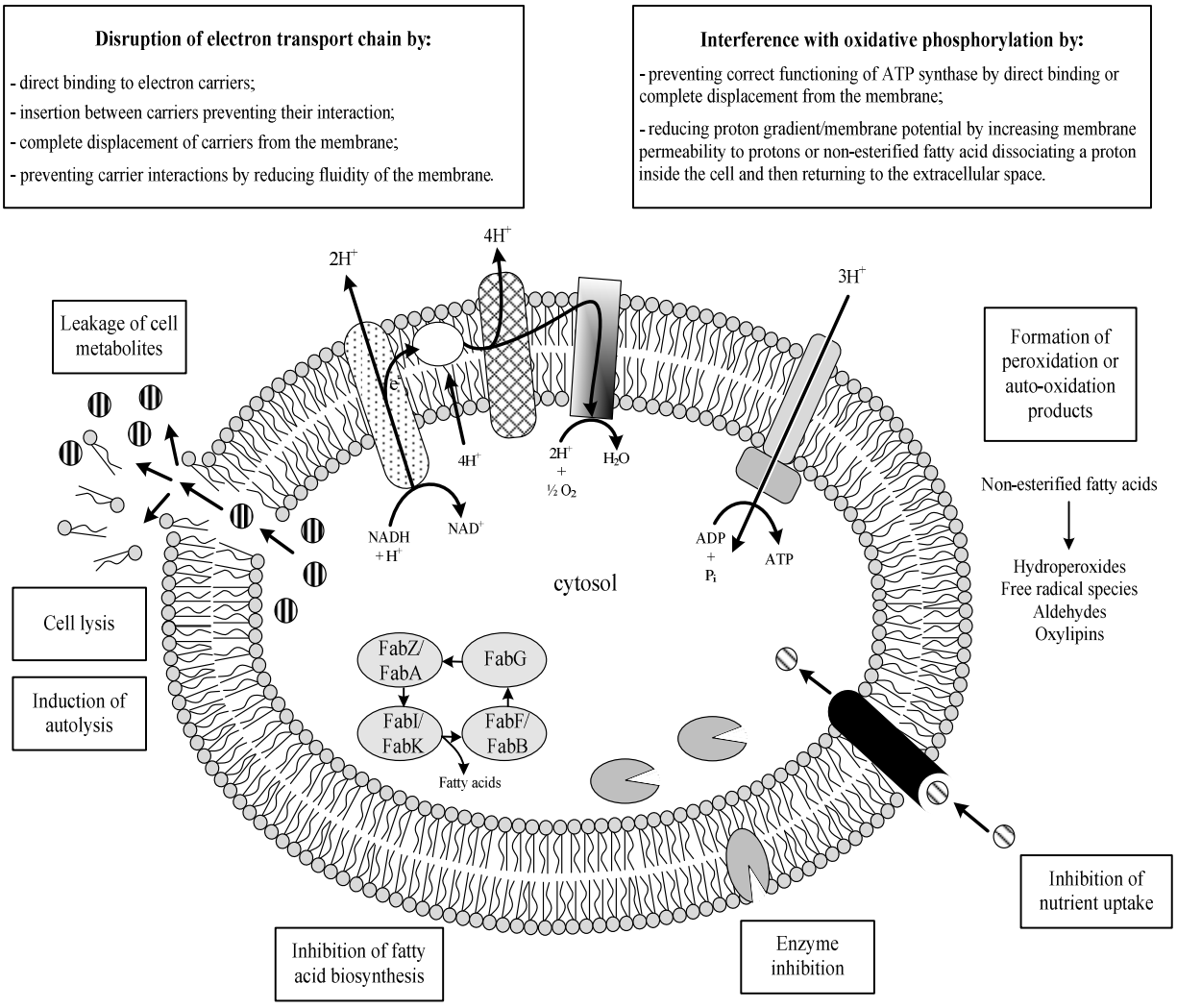

Figure 1.10. Schematic representation of possible membrane targets and mechanisms of antibacterial action of non-esterified fatty acids. Adapted from Desbois and Smith (2010).

were observed in low-roughage diets (Latham et al., 1972; Leat, 1977) and when fibre was replaced by starch (Gerson, John & King, 1985). Addition of readily fermentable carbohydrates did not inhibit either lipolysis or biohydrogenation, however (Gerson et al., 1985). Low pH inhibited both processes, with lipase activity being more strongly inhibited than biohydrogenation, however (Van Nevel & Demeyer, 1996).

Lipid supplementation affects the extent of biohydrogenation. This inhibition might be caused by an inhibitory effect of unsaturated FA or an eventual lipid coat of microorganism with a hydrophobic film, which would interfere with adherence to feed particles and thereby cellulose hydrolysis (Jenkins, 1993). Oilseeds and plant oils supplementation leads to a less complete biohydrogenation, promoting the accumulation of 18:1 *trans*-11 (Kennelly, 1996; Bu, Wang, Dhiman & Liu, 2007; Glasser, Ferlay & Chilliard, 2008). Fish oil strongly inhibits

the last step of biohydrogenation, i.e., the conversion of 18:1 *trans*-11 to 18:0 (Lee et al., 2005; Wąsowska et al., 2006; Chilliard et al., 2007). An alternative source of 20:5 *n*-3 and 22:6 *n*-3 is microalgae. Boeckaert, Vlaeminck, Mestdagh and Fievez (2007) reported an inhibition of the biohydrogenation when 22:6 *n*-3-edible microalgae were incubated *in vitro*, whereas *in vivo* CLA and 18:1 *trans*-11 content of milk increased (Boeckaert et al., 2008).

A high forage diet may minimise the negative effects of unsaturated FA, as forage has the ability to promote a normal rumen function for maximum biohydrogenation. The dietary forage to concentrate ratio, the type and physical form of the forage fed to animals affect lipid metabolism (French et al., 2000). Prolonged lipid supplementation and high fermentable carbohydrates cause a shift in the biohydrogenation pathway towards the formation of 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10, which were involved in milk fat depression (Bauman & Griinari, 2001; Shingfield et al., 2006; Shingfield & Griinari, 2007).

Recently, an interest on botanical species and plant secondary compounds emerged, because of their potential use as natural manipulators of the rumen metabolism, including lipid metabolism. Polyphenol oxidase in red clover has been related to inhibition of plant lipases activity (Lee et al., 2004; Lourenço, Van Ranst & Fievez, 2005; Van Ranst, Fievez, Vandewalle, De Riek & Van Bockstaele, 2009), and biohydrogenation of 18:3 *n*-3 (Lee, Parfitt, Scollan & MinChin, 2007; Van Ranst et al., 2009).

Essential oils are steam-volatile or organic solvent extracts of plants, usually herbs (Lourenço et al., 2010). The effects of essential oils on biohydrogenation have been contradictory, varying from no reported effects on milk FA (Benchaar, Petit, Berthiaume, Whyte & Chouinard, 2006; Benchaar et al., 2007) to ruminal accumulation of 18:2 *cis*-9,*trans*-11 (Lourenço et al., 2010).

Tannins are polyphenolic substances that bind to protein. The inhibitory effect of tannins on ruminal biohydrogenation was only recently shown. Results suggest an inhibition of 18:1 *trans*-11 hydration to 18:0 in biohydrogenation of 18:2 *n*-6 (Vasta, Makkar, Mele & Priolo, 2009; Vasta et al., 2010) and 18:3 *n*-3 (Khiaosa-Ard et al., 2009) by tannins.

Palmquist et al. (2005) reviewed the effects of nutrition on lipid metabolism in the rumen and resumed them into five main points, these are: i) the biohydrogenation of dietary PUFA is most extensive on high-forage diets based on grass silages; ii) diets containing high proportions of rapidly fermented carbohydrates, low amount of fibre, and/or plant oils or oilseeds promote a less extent biohydrogenation and the accumulation of 18:1 *trans* FA; iii) the conversion 18:1 *trans* intermediates to 18:0 is inhibited more strongly by fish oil or marine lipids rich in 20:5 *n*-3 and 22:6 *n*-3 than by plant oils and oilseeds; iv) changes in the composition of the basal diet lead to minor effects on biohydrogenation; and v) modifications

in the carbohydrate composition and lipid content of the diet markedly affect biohydrogenation FA intermediate profile.

Biohydrogenation may also be affected by the overall ruminal metabolism. Indeed, lipid metabolism is strictly linked to other areas of ruminal metabolism, as microbial species involved in biohydrogenation are also involved in other metabolic processes (Lourenço et al., 2010).

1.3.3. Hydration in the rumen

Dietary PUFA are extensively transformed by microorganisms in the rumen. The main FA modification is biohydrogenation of C18 PUFA (Harfoot & Hazlewood, 1997), but other transformations also occur, such as hydration.

Hydration of unsaturated FA in non-ruminal ecosystems was first reported by Wallen, Jackson and Benedict (1962). The ability to hydrate 18:1 *cis*-9 to 10-hydroxy 18:0 was demonstrated across bacteria (Wallen et al., 1962; Seo, Yamada, Takada & Okada, 1981; Koritala, Hosie, Hou, Hesseltine & Babgy, 1989; Elsharkawy, Yang, Dostal & Rosazza, 1992), yeast (Elsharkawy et al., 1992) and fungi (Elsharkawy et al., 1992) species. The hydration of 18:1 *cis*-9 in *Pseudomonas* sp. was proposed to be the first step to its utilization as an energy source (Wallen et al., 1962). Later work with *Pseudomonas* strain NRRL 3266 showed that 18:1 *cis*-9 was converted to a mixture of 10-hydroxy 18:0 and 10-keto 18:0 under aerobic conditions, whereas only 10-hydroxy 18:0 was produced under anaerobic conditions (Davis, Wallen, Goodwin, Rohwedde & Rhodes, 1969). In aerobic conditions, Lanser (1993) noted a 90% conversion of 18:1 *cis*-9 to 10-keto 18:0 in *Staphylococcus* sp.

Ruminal bacteria are able to hydrogenate unsaturated FA, but also to hydrate them to hydroxy and keto 18:0 (Katz & Keeney, 1966; Hudson, MacKenzie & Joblin, 1995 and 1996; Morvan & Joblin, 1999; McKain et al., 2010). The 18:0-producer *Fusocillus babrahamensis* P2/2 was found to equally hydrate 18:1 *cis*-9 to 10-hydroxy 18:0 (Kemp et al., 1975; Hazlewood et al., 1976). Similar production of 10-hydroxy 18:0 was described when 18:1 *cis*-9 was incubated with strained rumen fluid (Payne, 1974). More recently, Hudson et al. (1995) reported hydration activity in strains of *Selenomonas ruminantium* and *Enterococcus faecalis*. However, these rumen bacteria had no biohydrogenation activity (Hudson et al., 1995). Conversion of 18:1 *cis*-9 to hydroxy and keto 18:0 was further demonstrated (Morvan & Joblin, 1999) and may account for 6 to 10% of 18:1 *cis*-9 net losses in rumen *batch* cultures, whereas in continuous cultures it might arise up to 30% (Jenkins, AbuGhazaleh, Freeman &

Thies, 2006). Katz and Keeney (1966) reported the formation of 10-keto 18:0 by oxidation of 10-hydroxy 18:0 in ruminal *in vitro* cultures. A different mechanism was demonstrated in a *Flavobacterium* sp. grown anaerobically, which converted 10-hydroxy 18:0 to 10-keto 18:0 by a secondary alcohol dehydrogenase (Hou, 1994).

Hydrogenation of 18:1 *cis*-9 to 10-hydroxy 18:0 to 10-keto 18:0 has been the most reported hydrogenation mechanism. Nevertheless, hydrogenation of other unsaturated FA may occur. Ogawa, Matsumura, Kishino, Omura and Shimizu (2001) reported the formation of 10-hydroxy 18:1 *cis*-12 from 18:2 *n*-6 by *Lactobacillus acidophilus*. 10-hydroxy 18:1 *cis*-12 was proposed to be the first step of 18:2 *n*-6 biohydrogenation in lactic acid bacteria. Thus, 18:2 *cis*-9,*trans*-11 would be formed by subsequent oxidation of the 10-hydroxy 18:1 *cis*-12 intermediate. However, Kepler, Tucker and Tove (1971) ruled out the possibility of a similar biohydrogenating mechanism to occur in *B. fibrisolvens* metabolism of 18:2 *n*-6. In mixed ruminal digesta, ricinoleic acid [(*R*)-12-hydroxy 18:1 *cis*-9] was hydrated to 10-hydroxy 18:0, along with other unidentified products, but not biohydrogenated (Wallace et al., 2007). A recent study reported the formation of 10-hydroxy 18:0 and 10-keto 18:0 acids from the hydration of 18:1 *cis*-9 and 18:1 *trans*-10 by *P. acnes* (McKain et al., 2010). Different hydration mechanisms were suggested to occur in *P. acnes*. Hydration of 18:1 *cis*-9 to 10-hydroxy 18:0 involved the incorporation of one deuterium atom, while two deuterium atoms were incorporated during 18:1 *trans*-10 hydration.

Hydroxyl FA were also reported in rumen holotrich protozoa, although in small amounts (Katz & Keeney, 1967).

The formation of hydroxyl FA in the rumen might have implications on human health, as these 18:0 derivatives will entry into the human food supply via meat and milk consumption. If accumulation of hydroxyl 18:0 in the tissues of ruminants occurs to some extent, beneficial effects to human health might be observed. Indeed, the reported biological and physiological effects of hydroxyl 18:0 include potent cytotoxic effects, inhibition of cell proliferation, and increased cell viability (Jenkins et al., 2006).

1.4. Conclusions

Increasing concerns on the role of nutrition in human health have emerged over the last years, as incidence of chronic diseases was correlated to higher dietary fat intake. Red meat, milk and dairy products are among the main sources of fat, saturated FA, and *trans* FA consumption in developed and developing countries, particularly in most Western diets. As chronic diseases have high financial and social implications in society, modification of diet composition may play an important role in human health promotion and disease prevention.

Most recent nutritional guidelines promote a partial replacement of ruminant-derived products consumption by vegetables and fish products, in order to increase PUFA, and consequently decrease saturated FA, intake. However, ruminant meat and milk have several beneficial nutrients, including the health-promoting FA CLA and 18:1 *trans*-11. These FA are formed by microbial activity in the rumen. Lipids of forages and feedstuffs, mainly rich in 18:3 *n*-3 and 18:2 *n*-6, once in the rumen are readily hydrolysed to non-esterified FA by microbial lipases. After hydrolysis, the released non-esterified PUFA undergo a series of enzymatic steps leading to the formation of more saturated FA, until 18:0 formation. This process is named biohydrogenation. During biohydrogenation multiple FA intermediates are formed such as CLA isomers and 18:1 *trans*-11.

It might be argued that inhibiting lipase or biohydrogenation activities would increase the PUFA content of milk and meat. Yet, if the ruminal lipid metabolism is inhibited no CLA or 18:1 *trans*-11 FA would be formed in the rumen, absorbed and transfer to ruminant products. Moreover, it should be considered that the role played by these FA on human health may be more important or beneficial than an eventual increased PUFA content obtained by inhibiting lipid metabolism in the rumen.

Manipulation of the ruminant diet appears to be the most effective way to modify and improve the FA content and profile of the ruminal digesta and ruminant products, and therefore to improve human health. The rumen is a complex microbial ecosystem, however. Several are the microorganisms involved, the underlying mechanisms and the factors affecting ruminal lipid metabolism. A complete and integrate view of the rumen metabolism and ecosystem is the key to achieve promising FA profiles in ruminant products for improved human health.

1.5. Objectives

Ruminant products, such as meat and milk, constitute an important part of human diets. Unlike plants, the basis of ruminant diets, lipid composition of milk and meat is highly saturated. This is mainly due to microbial lipolysis and biohydrogenation of PUFA in the rumen. The overall aims of the present study were to evaluate the metabolism of FA in the rumen, identify mechanisms by which biohydrogenation occurs and to elucidate factors leading to changes in biohydrogenation pathways. To achieve these objectives a diversity of experiments were conducted, ranging from *in vitro* studies with pure cultures and mixed ruminal cultures to *in vivo* studies.

Subsequent to **Chapter 1**, which constitutes a general overall scientific background, the outlines of the research can be summarized as follows:

Chapter 2 studies the metabolism of PUFA by the ruminal bacterium *Butyrivibrio fibrisolvens*. Biohydrogenation was evaluated as a detoxification process. Additionally, mechanisms by which detoxification occurred were studied.

Chapter 3 discusses the effects of starch and pH on the biohydrogenation pattern *in vitro*. The main factor responsible for biohydrogenation shift towards *trans*-10 was examined in a semi-continuous fermentation system (RUSITEC). More specifically, biohydrogenation extent, intermediates and fermentation pattern at different pH values and starch levels will be discussed.

Chapter 4 describes the effects of nitrogen source on biohydrogenation profile of rumen digesta *in vivo*. Moreover, rumen contents were fractionated into liquid associated bacteria and solid associated bacteria, their FA profile determined, and effects of nitrogen source and oil supplementation discussed.

Chapter 5 integrates the results found in Chapters 2, 3, and 4 and provides an overall discussion. Furthermore, future research is proposed.

Chapter 6 provides an overview of the research outcomes.

1.6. References

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Chapter 2

Biohydrogenation of polyunsaturated fatty acids as a detoxification mechanism to the ruminal bacterium *Butyrivibrio fibrisolvens*

2.1. Introduction

Plant lipids are particularly rich in polyunsaturated fatty acids (PUFA) such as α-linolenic acid (18:3 *n*-3) and linoleic acid (18:2 *n*-6). Although PUFA are abundant in ruminant feedstuffs, their concentration in meat and milk is low. In fact, tissue lipids of ruminants have been known for a long time to be more saturated than those of non-ruminants (Banks & Hilditch, 1931). Due to the more saturated fatty acids (FA) profile, the consumption of milk, dairy products and ruminant meat has been related to an increasing incidence of coronary heart disease in man (Menotti, Kromhout, Blackburn, Fidanza & Buzina, 1999). More recently, a new insight on ruminant-derived products has emerged due to the potential positive effects of specific FA on health-promotion and disease prevention. In fact, milk, dairy products, and ruminant meat have garnered appreciation as functional (health-promoting) foods due to their beneficial physiological effects on humans' health (Harvantine, Boisclair & Bauman, 2009).

The biohydrogenation, or transformation of unsaturated FA into more saturated FA, has long been known to occur in the rumen as the result of microbial metabolic activity (Shorland, Weenink & Johns, 1955; Viviani, 1970). In middle 60's, the ruminal bacterium *Butyrivibrio fibrisolvens* was identified to undertake the biohydrogenation of 18:2 *n*-6 (Polan, Tove & McNeill, 1964) and to form rumenic acid (18:2 *cis*-9,*trans*-11) and vaccenic acid (18:1 *trans*-

11) as intermediates in the process (Kepler, Hirons, McNeill & Tove, 1966). However, stearic acid (18:0) was not formed from 18:2 *n*-6 by *B. fibrisolvens*. The bacteria responsible for 18:0 formation were later identified as *Fusocillus* spp. (Kemp, White & Lander, 1975). Recently, 18:0-producing bacteria were re-isolated from the rumen (Wallace et al., 2006) and renamed *Butyrivibrio proteoclasticus* (Moon et al., 2008).

Kim, Liu, Bond and Russell (2000) observed that 18:2 *n*-6 inhibited growth of *B. fibrisolvens*, an effect that depended both on the concentration of 18:2 *n*-6 and the growth status of the bacteria, with growing bacteria being more tolerant. Also *B. proteoclasticus* was highly sensitive to the toxic effects of 18:2 *n*-6 (Wallace et al., 2006). Differential toxicity could be envisaged as a strategy to manipulate ruminal biohydrogenation, which would improve the healthiness of ruminant meats and milk by increasing their unsaturated FA composition in general and PUFA in particular (Scollan et al., 2001). One of the unsaturated FA which concentration appears most desirable to increase is the conjugated linoleic acid (CLA) isomer 18:2 *cis*-9,*trans*-11, because of its anticarcinogenic and other health-promoting properties (Kritchevsky, 2000; Whigham, Cook & Atkinson, 2000). Although a few studies have examined the toxicity of saturated FA, and monoenoic FA to ruminal bacteria (Henderson, 1973; Marounek, Skrivanova & Savka, 2002), no studies have related PUFA toxicity and biohydrogenation.

The present study aimed to investigate the mechanisms of PUFA toxicity towards *B. fibrisolvens*, the metabolic responses and biohydrogenation activity. Toxicity of 18:2 *n*-6 to some of the most common species of ruminal bacteria was also evaluated.

2.2. Material and Methods

2.2.1. Rumen bacteria

All bacterial strains were held at the Rowett Research Institute collection and routinely checked for bacterial purity by Gram staining. Most strains were isolated at the Rowett Research Institute although some were purchased from culture collections or gifted by international colleagues. *Butyrivibrio fibrisolvens* JW11 and SH13, and *Prevotella albensis* M384 were originally isolated from sheep as proteolytic species (Wallace & Brammall, 1985). *Butyrivibrio proteoclasticus* P-18 was recently isolated as a 18:0-producing bacterium from the ruminal digesta of grazing sheep and is described in Wallace et al. (2006).

Mitsuokella multiacidus 46/5 was originally isolated at the Rowett by S.O. Mann in 1970 from sheep fed dried grass plus concentrates. *Butyrivibrio hungatei* JK614 and JK615, and *B. proteoclasticus* UC142 were a gift from J. Kopečný and are described in Kopečný, Zorec, Mrázek, Kobayashi and Marinšek-Logar (2003). *Lachnospira multipara* D15d, *B. fibrisolvens* A38 and *Ruminococcus flavefaciens* FD1 were kindly gifted by B.A. Dehority, J.B. Russell and M.P. Bryant, respectively. *Pseudobutyrivibrio ruminis* A12-1 and *Prevotella bryantii* $B_1$4 were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) while *Prevotella brevis* GA33, *Fibrobacter succinogenes* S85 and *Anaerovibrio lipolytica* 5S were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All bacteria held in the collection culture at the Rowett were isolated from the rumen of either sheep or cattle.

2.2.2. Media culture

The growth medium used was the liquid form of Hobson's M2 medium (Hobson, 1969). M2 medium is a general purpose medium containing (per litre) 2 g glucose, 2 g maltose, 2 g cellobiose, 10 g bacto casitone, 2.5 g yeast extract, 4 g $NaHCO_3$, 200 mL clarified rumen fluid, 150 mL mineral solution I (3 g K_2HPO_4 L^{-1}), 150 mL mineral solution II (3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4$, 0.6 g $CaCl_2$ L^{-1}), 1 mL resazurin solution (1 g L^{-1}), 10 mL DL-lactic acid sodium salt, and 0.1 g L-cysteine-hydrochloride.

Bacto casitone and yeast extract were supplied by Difco Laboratories (Detroit, MI, USA) and Oxoid (Basingstoke, Hampshire, UK). All other chemicals were supplied by Sigma-Aldrich Inc. (Poole, Dorset, UK). Rumen contents were collected *post-mortem* from cows, filtrated through two layers of linen cloth and centrifuged at 13,000 rpm for 30 min to obtain the clarified rumen fluid.

M2 medium was prepared by adding all chemicals, but cysteine, and take to boil twice, under continuous magnetic stirring. The reducing agent cysteine was then added and the medium take to boil again. After boiled, the medium was bubbled with O_2-free CO_2 until cool. M2 medium was dispensed under an O_2-free CO_2 atmosphere into Hungate-type tubes (Bellco Biotechnology Inc., Vineland, NJ, USA) or Wheaton bottles (Sigma-Aldrich Inc., Poole, Dorset, UK), sealed with butyl septum stoppers (Bellco Biotechnology Inc., Vineland, NJ, USA) and screwed or cramped, respectively, to maintain anaerobic conditions, and autoclaved at 121 ºC for 15 min.

2.2.3. Fatty acids metabolism

Metabolism of FA by *B. fibrisolvens* JW11 was determined in M2 medium containing 50 μg FA mL^{-1}. The FA used in these experiments were the non-esterified FA and FA methyl esters of docosahexaenoic acid (22:6 *n*-3), eicosapentaenoic acid (20:5 *n*-3), γ-linolenic acid (18:3 *n*-6), α-linolenic acid (18:3 *n*-3), linoleic acid (18:2 *n*-6), conjugated linoleic acid (CLA; a mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12), vaccenic acid (18:1 *trans*-11), oleic acid (18:1 *cis*-9), and stearic acid (18:0). Elaidic acid (18:1 *trans*-9), *cis*-vaccenic acid (18:1 *cis*-11) and linoelaidic acid (18:2 *trans*-9,*trans*-12) non-esterified FA were also used. All FA, non-esterified and methyl esters, were supplied by Sigma-Aldrich Inc. (St. Louis, MO, USA). Fatty acids were prepared as a separate solution by sonication for 4 min in 4 mL of distilled water, taken from the volume needed to prepare the M2 medium. After sonication, FA solution was added to the medium before dispensing and autoclaving.

2.2.4. Growth conditions

All transfers and incubations were carried out under O_2-free CO_2 and in Hungate-type tubes (Hungate, 1969) incubated in a water-bath at 39 ºC, in triplicate. Inoculum volumes were 5% (v/v) of a fresh overnight grown culture. Growth of bacteria was determined at different incubation times by measuring the optical density (OD) at 650 nm, using a Novaspec II spectrophotometer (Amersham Biosciences, Amersham, Buckinghamshire, UK).
In growth experiments with *B. fibrisolvens* JW11, FA concentrations as well as growth were determined at different incubation times. At each sampling time, three tubes were removed from the water bath at 39 ºC, and the turbidity determined. Tubes were immediately placed in a boiling water bath for 5 min to stop growth and any metabolic process, left to cool down and kept at -20 ºC until further analysis. After thawed, 1 mL sample was taken for protein analysis and for FA extraction and esterification.

2.2.5. Analytical procedures

2.2.5.1. Fatty acids extraction and analysis

Long-chain FA were extracted from samples (Bligh & Dyer, 1959) and converted to methyl esters by a short, mild acid catalysed esterification to minimise isomerization of unsaturated FA as proposed by Christie (2003).

One mL of sample was added to 1.25 mL acidified salt solution (17 mM sodium chloride in 1 mM sulphuric acid), 100 µL of 200 µg mL^{-1} heptadecanoic acid (17:0; internal standard) in methanol and 2.5 mL of methanol. The mixture was vortexed for 1 min, then 2.5 mL of chloroform containing 200 µg mL^{-1} butylated hydroxytoluene (BHT) was added and the mixture vortexed for 2 min. Tubes were centrifuged at 2,000 *g* for 10 min and the upper phase removed by aspiration. The lower phase was dried by passing through anhydrous sodium sulphate and taken to dryness in a centrifugal evaporator for 1 h at 43 °C (Savant AES2010, Thermo Electron Corporation, Basingstoke, Hampshire, UK). Dried lipid extract was resuspended in 0.5 mL toluene and briefly vortexed. One hundred microlitre of 200 µg mL^{-1} pentadecanoic acid (15:0) in methanol was added as a second internal standard to account for potential lipid losses during esterification. Then, 1 mL of fresh made 1% methanolic sulphuric acid (v/v, concentrated sulphuric acid in methanol) was added, the tube flushed with N_2 and incubated at 50 °C for 1 h. After cooled, 2.5 mL of 5% (wt/v) sodium chloride was added and the tube vortexed for 30 s. One millilitre of iso-hexane was added, the tube was briefly vortexed and allowed to settle until the separation into two layers. The upper layer was transferred to a new tube and the iso-hexane extraction repeated. The iso-hexane fractions were combined, 1.5 mL of 2% (wt/v) potassium hydrogen carbonate added, the mixture was vortexed for 30 s and allowed to settle. The upper phase was transferred to a new tube and take to dryness in a centrifugal evaporator, as previously described. Dried FA methyl esters extract was resuspended in 200 µL of iso-hexane containing 200 µg mL^{-1} BHT, transferred to a vial and kept at -20 ºC until gas chromatography analysis.

Fatty acid methyl esters were separated and quantified using a gas chromatograph HP6890 series (Agilent Technologies UK Ltd, Stockport, UK) equipped with a flame ionization detector and using a fused silica capillary column (CP-Sil 88; Chrompack, Varian Analytical Instruments, Walton-on-Thames, Surrey, UK) with 60 m, 0.25 mm internal diameter and 0.2 µm film thickness. Injector and detector temperatures were 250 and 275 ºC, respectively.

Column initial temperature of 80 ^{o}C was held for 1 min, increased to 160 ºC at a rate of 25 C^{o}/min and held for 3 min, then increased to 190 ºC at a rate of 1 C^{o}/min and held for 5 min, and finally increased to 230 ºC at a rate of 2 C^{o}/min and held for 25min. Helium was used as carrier gas at flow rate of 0.5 mL min^{-1}.

Fatty acids identification was done by comparison with retention times of methyl esters standards obtained from Sigma-Aldrich Inc. (Poole, Dorset, UK) and Matreya Inc. (Pleasant Gap, PA, USA). In some cases, co-elution with standards was also done. During incubation of 18:3 *n*-3, several chromatographic peaks appeared which could not be identified by comparison with known standards by gas chromatography or gas chromatography-mass spectrometry. Structural identification of those FA was achieved by comparing elution profiles and mass spectra to those previously identified from 4,4-dimethyloxazoline (DMOX) FA derivatives by Wąsowska et al. (2006).

2.2.5.2. Protein determination

Protein content was measured by alkaline hydrolysis of samples followed by reaction with the Folin-Ciocalteu reagent (Herbert, Phipps & Strange, 1971).

2.2.6. Measurement of cell integrity using propidium iodide

Methods used to assess the influence of FA on cell integrity of different species were based on work of Ben Amor et al. (2002). One mL of fresh overnight culture was inoculated into 10 mL of M2 medium and incubated at 39 ºC, for approximately 4 h, until mid-exponential phase (OD_{650} = 0.4). Bacterial cultures were centrifuged at 3,000 *g* for 10 min at 4 ºC. The pellet was washed twice with anaerobic potassium phosphate buffer (100 mM; pH 7.0) containing 1 mM dithiothreitol (DTT). Anaerobic conditions were maintained by carrying out all transfers in an anaerobic chamber with a gas phase of 80% N_2, 10% CO_2 and 10% H_2, at a temperature of 39 ºC.

Cells were resuspended in 15 mL of the same buffer, and non-esterified FA or FA methyl esters were added to the cell suspension to a final concentration of 50 µg mL^{-1}. Stock solutions (1 mg mL^{-1}) of non-esterified FA and FA methyl esters were prepared immediately before use by sonication for 4 min in anaerobic potassium phosphate buffer (100 mM, pH 7.0,

containing 1 mM DTT). Untreated and heat-treated cells (100 ºC for 20 min) served as control samples.

After 30 min incubation of cell suspensions with FA, cell integrity was determined by measuring the fluorescence of propidium iodide (PI; Sigma-Aldrich Inc., St. Louis, MO, USA). Ten microlitre of each sample were added to 985 µL of anaerobic potassium phosphate buffer and 5 µL of 1.5 mM PI. Propidium iodide solution was prepared in distilled water and stored at 4 °C in the dark. The mixtures were incubated for 15 min at 39 ºC in the anaerobic chamber. After incubation, were immediately transferred to an ice-water slurry and kept in the dark for up to 15 min before being analysed for fluorescence. Fluorimetry measurements were made using a Baird Nova spectrofluorimeter (Baird-Atomic Ltd, Braintree, Essex, UK) set at λ_{EX} = 488 nm and λ_{EM} = 650 nm or a Spectramax GeminiXS spectrofluorimeter (Molecular Devices, Wokingham, Berkshire, UK) set at λ_{EX} = 530 nm and λ_{EM} = 620 nm.

2.2.7. Membrane potential measurements

Membrane potential was measured by a red to green fluorescence ratio analysis (Novo, Perlmutter, Hunt & Shapiro, 2000) using $DiOC_2(3)$ (3,3'-diethyloxacarbocyanine iodide; Molecular Probes, Leiden, The Netherlands). The procedure was based on methods of Ben Amor et al. (2002). Mid-exponential phase cells were prepared as for cell integrity measurements. In the anaerobic chamber, 950 µL of cell suspension was added to tubes containing 50 µL of 1 mg mL^{-1} FA solution, vigorously vortex for 30 s, and incubated for 15 min at 39 ºC. After incubation, 250 µL was added to flow cytometry tubes containing 746 µL of staining buffer and 4 µL of 3 mM $DiOC_2(3)$. Working solution of $DiOC_2(3)$ was prepared in dimethyl sulphoxide and stored at -20 ºC in the dark. The staining buffer contained 60 mM Na_2HPO_4 and 60 mM NaH_2PO_4 mixed to produce a solution with pH 7.0, and supplemented with 5 mM KCl, 130 mM NaCl, 1.3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1 mM DTT and 10 mM glucose (Novo, Perlmutter, Hunt & Shapiro, 1999). Tubes were incubated at 39 ºC for 2 min. After incubation, tubes were immediately transferred to an ice-water slurry and analysed by flow cytometry within 30 min.

Flow cytometry was carried out with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with an air-cooled argon ion laser emitting 15 mW of blue light at 488 nm. Emission filters at 530 nm (green) and >600 nm (red) were used. FACSFlow solution (Becton Dickinson) was used as sheath fluid. The

analyses were done using the low rate settings (12 µL min^{-1}). The red to green ratio was calculated by subtracting the log of the green fluorescence response from the log of the red fluorescence response and adding a constant (384) as proposed by Shapiro (1995).

2.3. Results

2.3.1. Fatty acids metabolism by Butyrivibrio fibrisolvens JW11

Metabolism of FA was measured during the growth cycle of one of the less sensitive *Butyrivibrio* strains to PUFA, *B. fibrisolvens* JW11.

When *B. fibrisolvens* JW11 was inoculated with 18:2 *n*-6 (**Figure 2.1**), the concentration of 18:2 *n*-6 in the medium dropped from 31 µg mL^{-1} at 0 h to undetectable traces at 3 h. 18:2 *n*-6 was immediately metabolized to 18:2 *cis*-9,*trans*-11, which was present at the highest

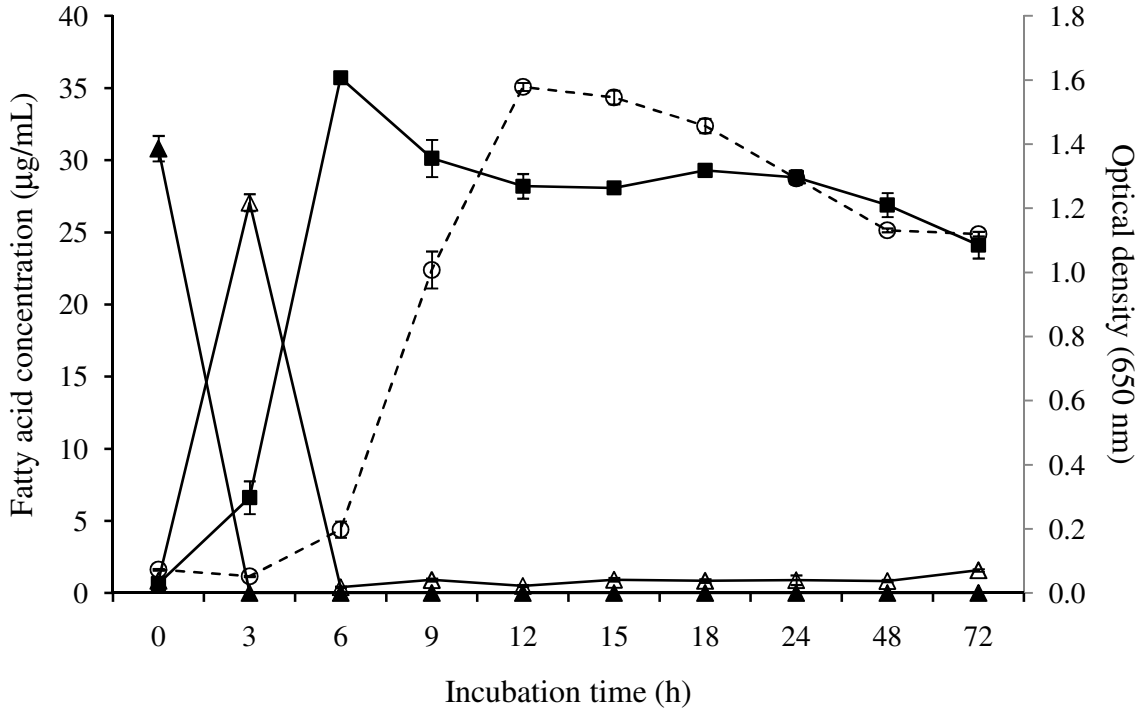

Figure 2.1. Concentration of fatty acids in the medium following inoculation of *Butyrivibrio fibrisolvens* JW11 into medium with 50 µg mL^{-1} linoleic acid (18:2 *n*-6). Growth (○, OD_{650}), 18:2 *n*-6 (▲), 18:2 *cis*-9,*trans*-11 (△), 18:1 *trans*-11 (■). Results are means and standard deviations from three cultures.

concentration at 3 h but rapidly decreased to basal levels after 6 h of incubation, as it was converted to 18:1 *trans*-11. No 18:0 was formed (data not shown). No growth was observed until 6 h. Growth only occurred after dienoic FA were extensively converted to the monoenoic 18:1 *trans*-11.

A longer lag phase was observed in incubations of *B. fibrisolvens* JW11 with 18:3 *n*-3 (**Figure 2.2a**). Similarly, 18:3 *n*-3 was rapidly metabolized during early lag phase to the conjugated 18:3 *cis*-9,*trans*-11,*cis*-15. Besides the formation of the main conjugated trienoic acid, a low concentration of the 18:3 *trans*-9,*trans*-11,*cis*-15 isomer was also detected. The main dienoic acid formed was 18:2 *trans*-11,*cis*-15, which was subsequently converted to 18:1 *trans*-11. Variations were observed in the time taken for different replicate tubes to escape the lag phase. These differences lead to a misleading average concentration across the three tubes and are in the basis of the high standard deviations observed. For example, at 32 h, replicate tubes contained 0.125, 0.140 and 0.193 mg bacterial protein mL^{-1}, indicating that the culture in the third tube had begun to grow earlier than the others. The concentrations of 18:3 *cis*-9,*trans*-11,*cis*-15 were 23.0, 21.1 and 0 μg mL^{-1}, respectively, while the concentrations of 18:2 *trans*-11,*cis*-15 were 0, 0 and 24.5 μg mL^{-1}.

An analysis comparing bacterial protein concentrations and FA concentrations in the same tubes (**Figure 2.2b**) demonstrated that bacterial protein was low while 18:3 *n*-3, 18:3 *cis*-9,*trans*-11,*cis*-15 and 18:3 *trans*-9,*trans*-11,*cis*-15 were present in the medium. Higher bacterial concentrations occurred only when these FA were removed from individual cultures. High concentrations of 18:1 *trans*-11 did not affect growth, while 18:2 *trans*-11,*cis*-15 also appeared to permit growth. No 18:0 was formed in any 18:3 *n*-3-containing culture (data not shown).

No growth was observed in incubations of *B. fibrisolvens* JW11 with 18:3 *n*-6 (**Figure 2.3**). During lag phase, 18:3 *n*-6 was metabolized, although not extensively. After 72 h incubation, over 39% of the initial 18:3 *n*-6 concentration was still present in the medium. A trienoic acid was formed from 18:3 *n*-6 metabolism, which according to its elution time in gas chromatography might be conjugated, probably 18:3 *cis*-6,*cis*-9,*trans*-11. This putative conjugated trienoic acid was not further metabolized. The concentration of 18:1 *trans*-11 was similar throughout the incubation period, not being formed from 18:3 *n*-6 metabolism. No 18:0 was formed from 18:3 *n*-6 biohydrogenation (data not shown).

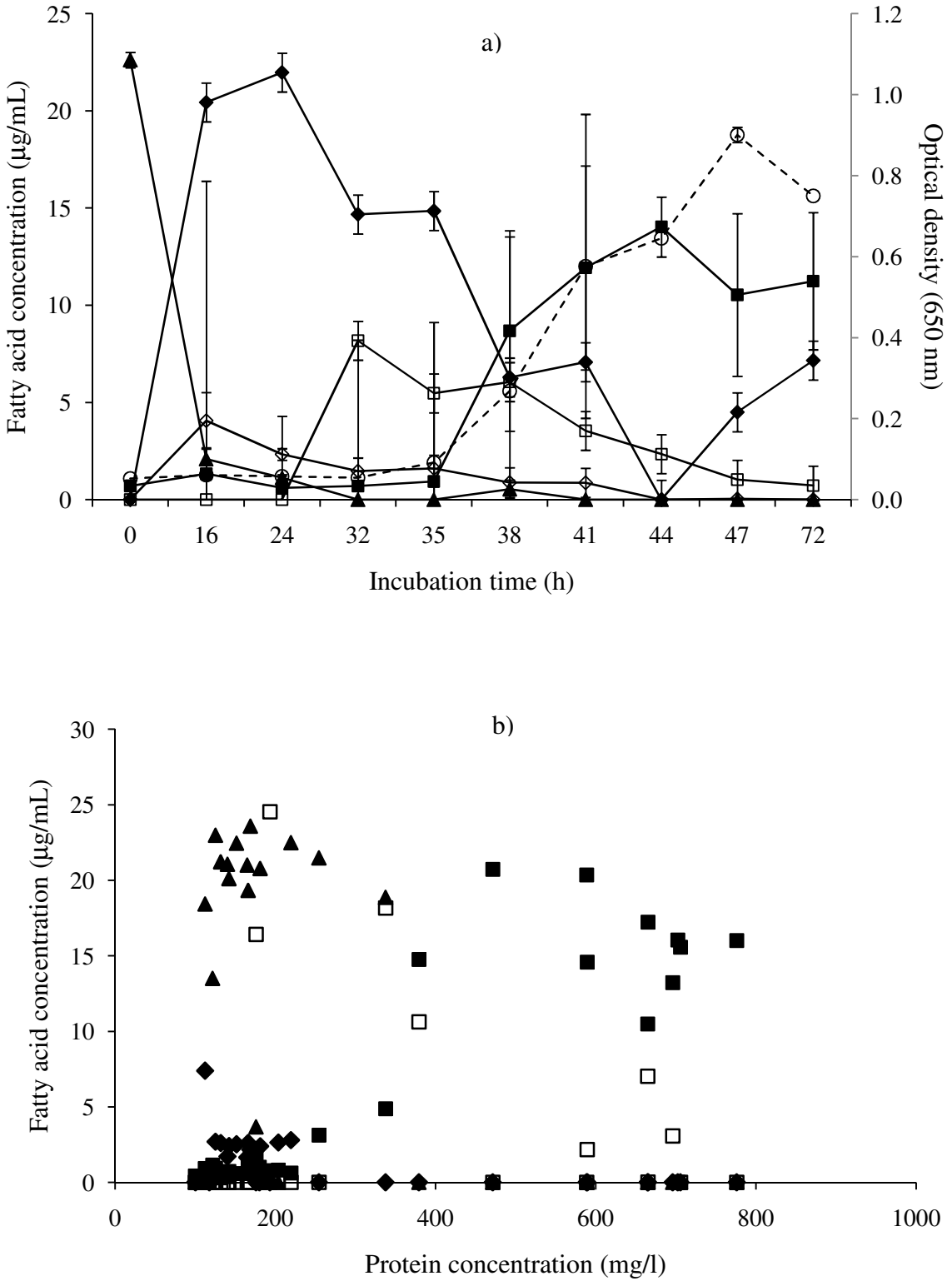

Figure 2.2. (**a**) Concentration of fatty acids in the medium following inoculation of *Butyrivibrio fibrisolvens* JW11 into medium with 50 µg mL^{-1} α-linolenic acid (18:3 *n*-3). Growth (○, OD_{650}), 18:3 *n*-3 (▲), 18:3 *cis*-9,*trans*-11,*cis*-15 (◆),18:3 *trans*-9,*trans*-11,*cis*-15 (◇), 18:2 *trans*-11,*cis*-15 (□), 18:1 *trans*-11 (■). Results are means and standard deviations from three cultures. (**b**) Fatty acid concentrations *vs.* bacterial protein concentration.

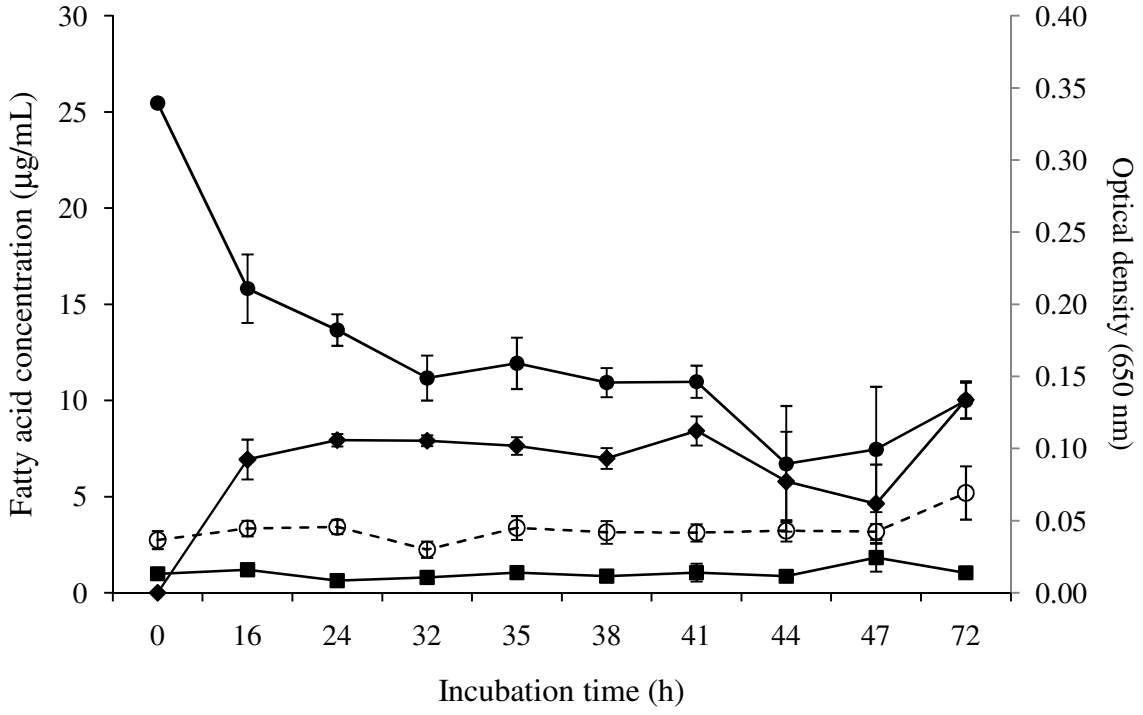

Figure 2.3. Concentration of fatty acids in the medium following inoculation of *Butyrivibrio fibrisolvens* JW11 into medium with 50 µg mL^{-1} γ-linolenic acid (18:3 *n*-6). Growth (○, OD_{650}), 18:3 *n*-6 (●), presumed conjugated trienoic acid, possibly 18:3 *cis*-6,*cis*-9,*trans*-11 (◆), 18:1 *trans*-11 (■). Results are means and standard deviations from three cultures.

2.3.2. Non-esterified fatty acids and fatty acids methyl esters metabolism by Butyrivibrio fibrisolvens JW11 and effects on growth

The effects of various FA, in the form of non-esterified or methyl esters, on growth of *B. fibrisolvens* and on the biohydrogenation products in M2 medium, are summarized in **Table 2.1**. Incubations were carried out similarly to those previously described. The more unsaturated non-esterified FA were the longer was the lag phase observed. Oppositely to the toxicity exhibit by PUFA 18:1 *trans*-11 and 18:0 had no growth-inhibitory activity, compared to cultures grown in FA-free M2 medium. Neither 22:6 *n*-3 or 20:5 *n*-3 were metabolized nor growth was detected. 18:3 *n*-6 was metabolized probably to a conjugated trienoic acid, possibly 18:3 *cis*-6,*cis*-9,*trans*-11, which was not further metabolized. *B. fibrisolvens* JW11 was not able to grow in the presence of 18:3 *n*-6 or its metabolized product. Incubations with 18:3 *n*-3 induced a lag phase of 35 h while 18:2 *n*-6 and CLA, a mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12, showed a lower toxicity, as the lag phase lasted for 6 h. Growth

Table 2.1. Effects of non-esterified fatty acids and their methyl esters (50 μg mL^{-1}) on growth and fatty acid metabolism by *Butyrivibrio fibrisolvens* JW11 in M2 medium.

	Fatty acids[1]								
	DHA	EPA	γLNA	αLNA	LA	CLA	VA	OA	SA
Non-esterified fatty acids									
Lag phase (h)	>72	>72	>72	35	6	4	0	2	0
BH[2]	No	No	Yes	Yes	Yes	Yes	No	No	No
End product[3]	ND[4]	ND	Conjugated 18:3	VA	VA	VA	ND	ND	ND
Fatty acid methyl esters									
Lag phase (h)	0	0	0	0	0	0	0	0	0
BH	No	No	Yes	Yes	Yes	Yes	No	No	No
End product	ND	ND	Conjugated 18:2	VA	VA	VA	ND	ND	ND

[1] DHA – 22:6 *n*-3; EPA – 20:5 *n*-3; γLNA – 18:3 *n*-6; αLNA - 18:3 *n*-3; LA - 18:2 *n*-6; CLA – a mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12; VA – 18:1 *trans*-11; OA – 18:1 *cis*-9; SA – 18:0;

[2] BH – biohydrogenation occurrence;

[3] End product – major end product after 72 h incubation;

[4] ND – none detected.

only occurred after these PUFA where biohydrogenated to 18:1 *trans*-11. None of the monoenoic FA studied was metabolized. However, 18:1 *cis*-9 showed some toxicity as the lag phase lasted for 2 h.

None of the FA methyl esters studied caused a lag phase, yet they were converted to the same end product as non-esterified FA, with the exception of methyl-18:3 *n*-6, which formed as end product a FA putatively identified as being a conjugated dienoic acid. Additionally to the extraction and esterification procedures previously described, samples from FA methyl esters incubations were directly analysed by gas chromatography after extraction. By comparison of both results, it was observed that added FA methyl esters retained their methyl group, but the isomerisation and biohydrogenation products did not (data not shown).

2.3.3. Geometrical and positional C18 fatty acids effects on growth of Butyrivibrio fibrisolvens JW11

Effects of double bond position and isomer configuration of C18 FA on growth of *B. fibrisolvens* were evaluated (**Figure 2.4**). Incubations with *cis* and *trans* isomers of monoenoic and dienoic acids were carried out as those previously described.

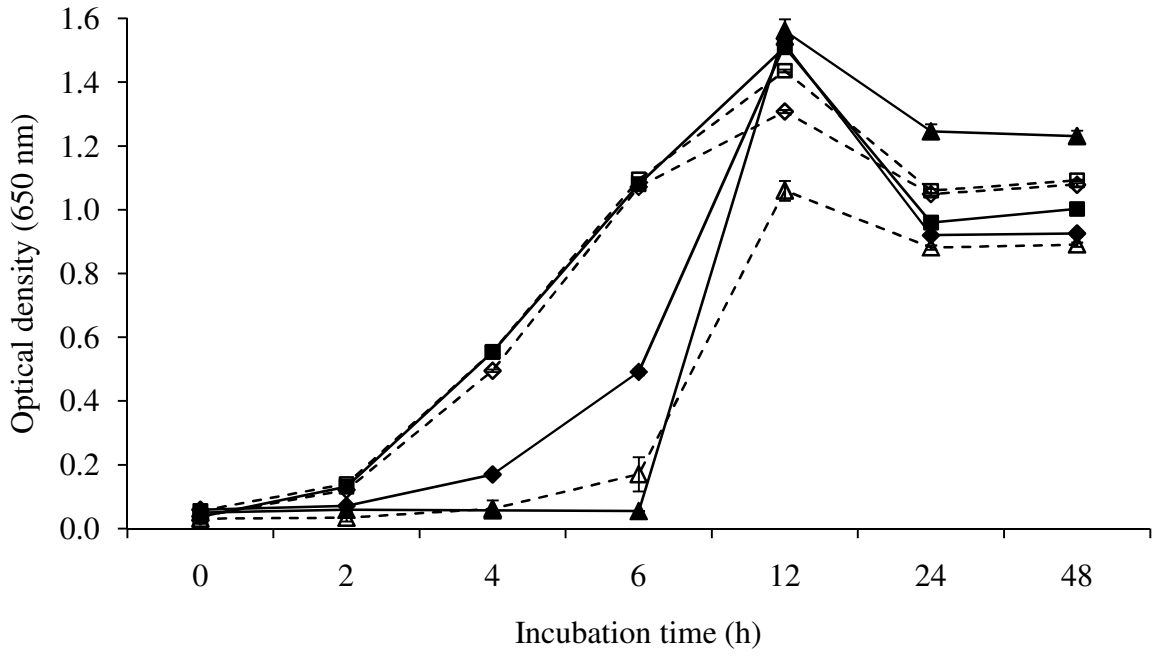

Figure 2.4. Influence of fatty acids (50 µg mL^{-1}) on growth of *Butyrivibrio fibrisolvens* JW11. 18:2 *n*-6 (▲); 18:2 *trans*-9,*trans*-12 (△); 18:1 *cis*-11 (■); 18:1 *trans*-11 (□); 18:1 *cis*-9 (◆); 18:1 *trans*-9 (◇). Results are mean values and standard deviations from three cultures.

No lag phase was observed with incubations of *trans* monoenoic FA. *Cis* monoenoic FA affected differently *B. fibrisolvens* growth, 18:1 *cis*-9 led to a 2 h lag phase while 18:1 *cis*-11 had no effect. Geometrical isomers of dienoic FA also exhibited different effects with the all *cis* configuration being more toxic than the all *trans* configuration. Lag phase with 18:2 *n*-6 lasted for 6 h whereas with 18:2 *trans*-9,*trans*-12 lasted for 4 h. Maximum growth of *B. fibrisolvens* was lower with *trans* FA than with *cis* FA, at 12 h incubation.

The effect of 18:1 *trans*-11 and 18:2 *n*-6 on the growth of *B. fibrisolvens* was determined (**Figure 2.5**). Incubations were carried out similarly to those previously described, except for non-esterified FA addition. *B. fibrisolvens* JW11 was inoculated in M2 medium with 50 μg mL^{-1} 18:2 *n*-6, 50 μg mL^{-1} 18:1 *trans*-11, or 50 μg mL^{-1} 18:2 *n*-6 plus 50 μg mL^{-1} 18:1 *trans*-11, added to M2 medium as an ethanol solution (5 mg mL^{-1}). No lag phase was observed in incubations with 18:1 *trans*-11, whereas 18:2 *n*-6 induced a 6 h lag phase. These results are similar to those presented in Table 1. When *B. fibrisolvens* was incubated with 18:2 *n*-6 plus 18:1 *trans*-11, the lag phase was of 4 h. Although FA concentration on 18:2 *n*-6 plus 18:1 *trans*-11 cultures was two times higher (100 μg mL^{-1} *vs*. 50 μg mL^{-1}) a 2 h lag phase reduction was observed, compared to cultures incubated with 18:2 *n*-6 alone.

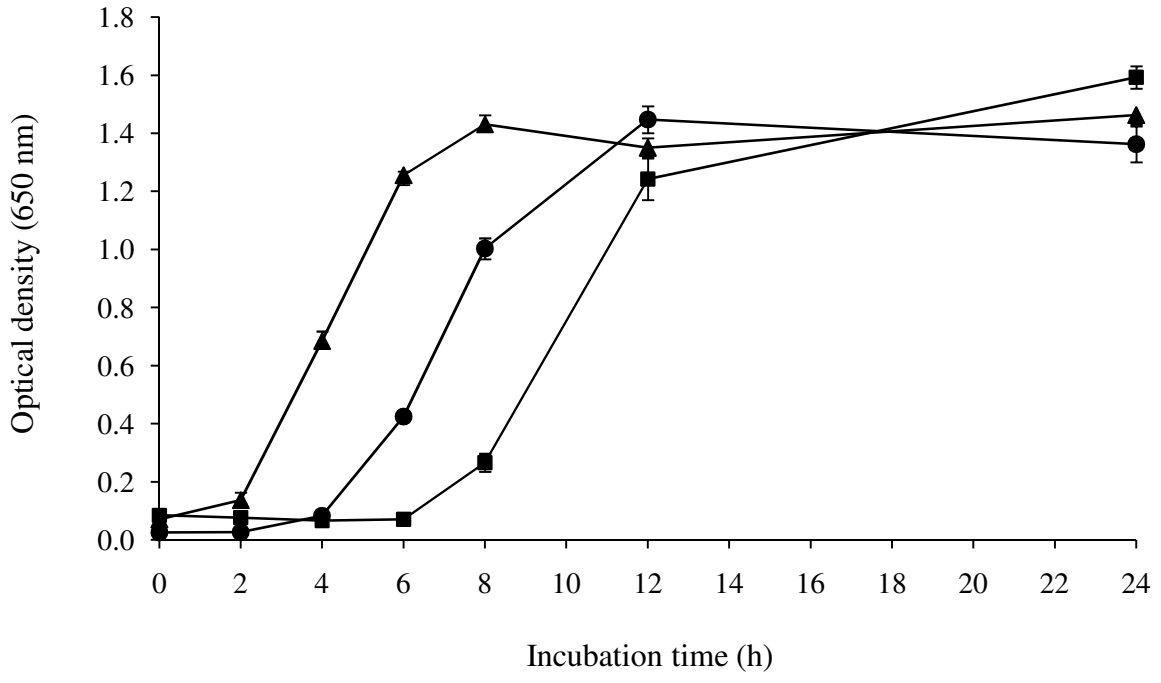

Figure 2.5. Influence of 50 μg mL^{-1} linoleic acid (18:2 *n*-6), 50 μg mL^{-1} vaccenic acid (18:1 *trans*-11) and 50 μg mL^{-1} linoleic acid plus 50 μg mL^{-1} vaccenic acid (18:2 *n*-6 plus 18:1 *trans*-11) on growth of *Butyrivibrio fibrisolvens* JW11. 18:2 *n*-6 (■); 18:2 *n*-6 plus 18:1 *trans*-11 (●); 18:1 *trans*-11 (▲). Results are mean values and standard deviations from three cultures.

2.3.4. Influence of fatty acids on bacterial cell integrity

2.3.4.1. Influence of linoleic acid on cell integrity of different species of ruminal bacteria

Fluorescence of PI was used to assess the effects of 18:2 *n*-6 on cell integrity of some of the most common species of ruminal bacteria (**Figure 2.6**).

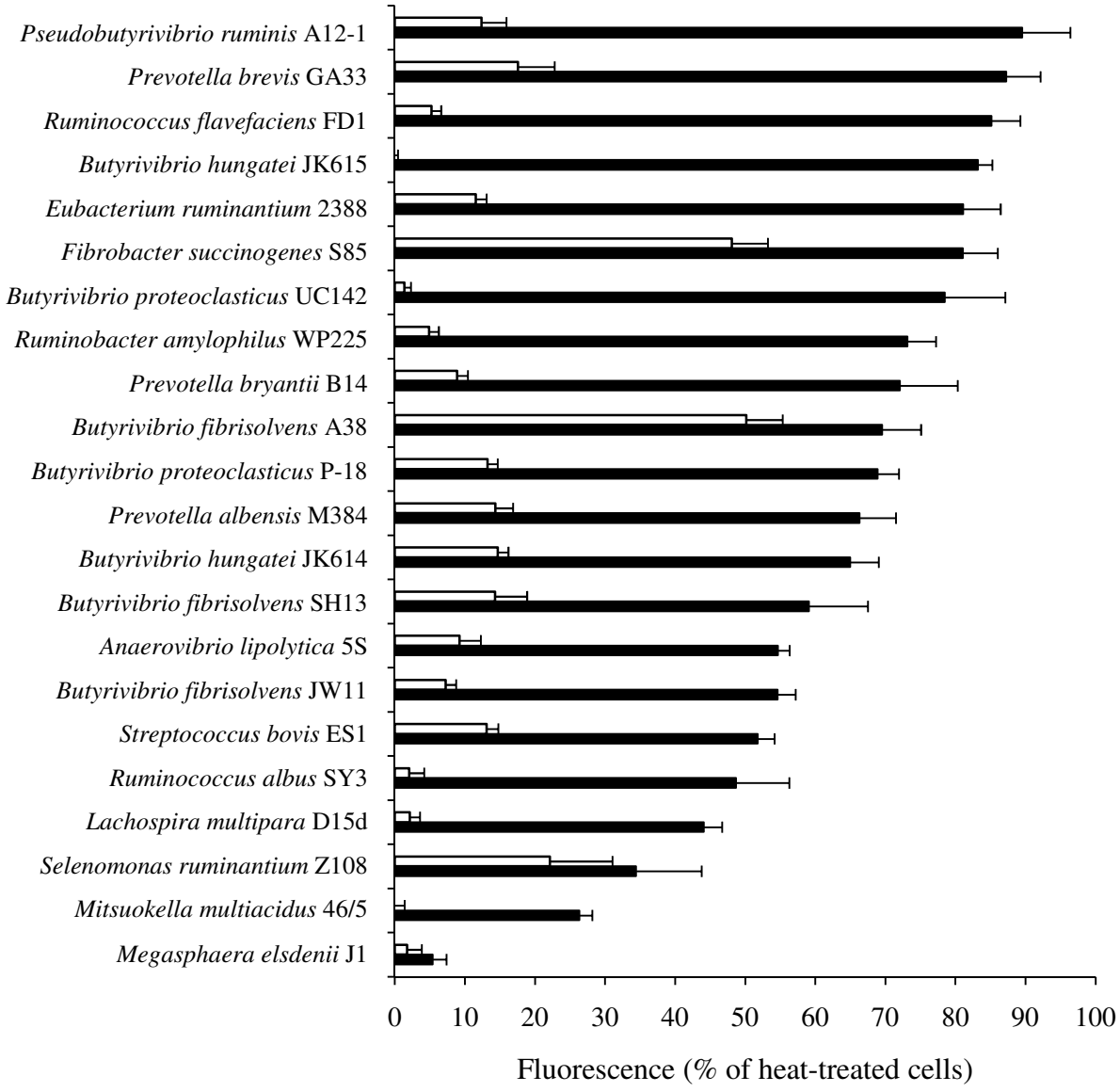

Figure 2.6. Influence of 50 μg mL^{-1} linoleic acid (18:2 *n*-6) on membrane integrity of different strains of ruminal bacteria. Loss of cell integrity was determined by fluorescence of propidium iodide. One hundred % loss of cell integrity was taken as the fluorescence of the same culture which had been incubated at 100 °C for 20 min. No addition (□), 18:2 *n*-6 (■). Results are means and standard deviations from 3 cultures, each of which was subject to 8 replicate measurements (n = 24).

Propidium iodide fluoresces when in contact with DNA, but due to its high molecular weight is normally impermeant across the cell membrane. Thus, only cells which have lost their cell membrane integrity fluoresce with PI. Fluorescence readings from treated cell suspensions with 50 μg mL^{-1} 18:2 *n*-6 were therefore compared with values obtained from the same culture untreated and heat-treated at 100 ºC for 20 min. *Fibrobacter succinogenes* S85 and *B. fibrisolvens* A38 showed the highest permeability to PI in the absence of 18:2 *n*-6, of nearly 50% of the values obtained with boiled cells while others gave 20% or less. Addition of 18:2 *n*-6 increased the PI fluorescence of all bacteria. Propidium iodide fluorescence increase differed between different species and even among the same species or closely related bacteria, such as the *Prevotella* spp. Sensitivity to 18:2 *n*-6 varied considerably, being *Megasphaera elsdenii* the least sensitive species.

2.3.4.2. Influence of C18 fatty acids on cell integrity of Butyrivibrio fibrisolvens

Propidium iodide fluorescence was used to determine the effects of different C18 FA, added as non-esterified and methyl esters, on cell integrity of *B. fibrisolvens* JW11 (**Figure 2.7**).

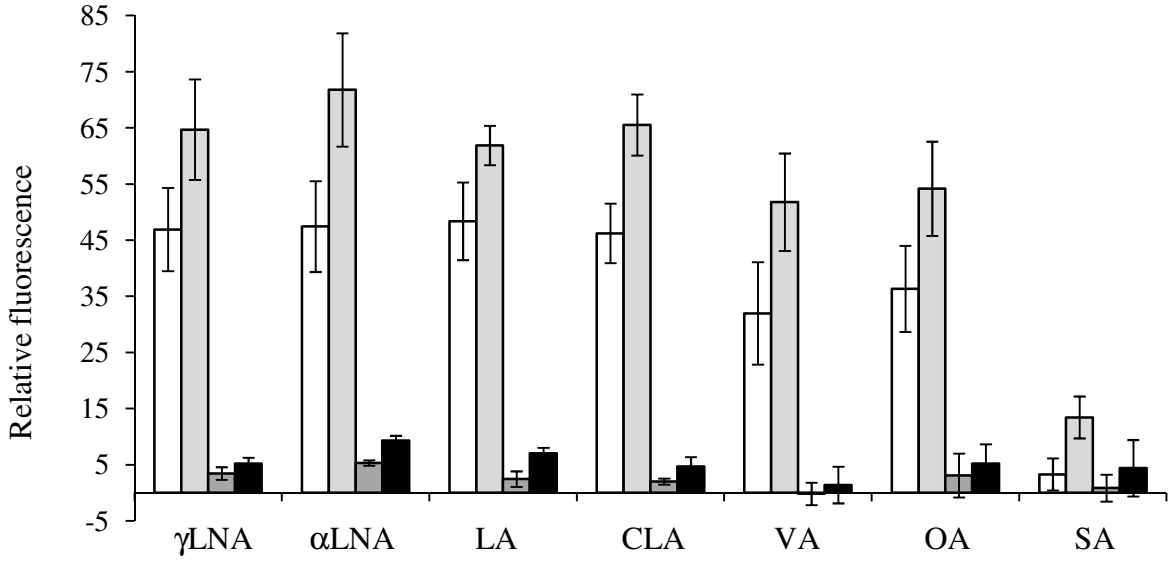

Figure 2.7. Influence of C18 non-esterified fatty acids and fatty acid methyl esters on cell integrity of *Butyrivibrio fibrisolvens* JW11. Loss of cell integrity was determined by fluorescence in the presence of propidium iodide. γLNA, 18:3 *n*-6; αLNA, 18:3 *n*-3; LA, 18:2 *n*-6; CLA, a mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12; VA, 18:1 *trans*-11; OA, 18:1 *cis*-9; SA, 18:0. In order of increasing shading density: 50 μg mL^{-1} non-esterified fatty acid, 200 μg mL^{-1} non-esterified fatty acid, 50 μg mL^{-1} fatty acid methyl ester, 200 μg mL^{-1} fatty acid methyl ester. Results are means and standard deviations from three determinations.

All unsaturated FA, including the monoenoic 18:1 *cis*-9 and 18:1 *trans*-11, had a similar effect. The addition of 200 μg mL^{-1} FA increased the fluorescence slightly compared to that of 50 μg mL^{-1}. Oppositely to unsaturated FA, 18:0 had a small effect on PI fluorescence. Addition of FA methyl esters caused only about one-tenth of the disruption caused by non-esterified FA.

Wallace et al. (unpublished results) found an increased lag phase of *B. fibrisolvens* JW11 in M2 medium with 50 mg mL^{-1} 18:2 *n*-6 in the presence of 70 mM sodium lactate. Therefore, the influence of 18:2 *n*-6 on PI fluorescence was determined in the presence and absence of sodium lactate (**Figure 2.8**). As observed before, 18:2 *n*-6 increased the fluorescence of PI, indicating that cell integrity had been disrupted. Sodium lactate did not alter the response significantly.

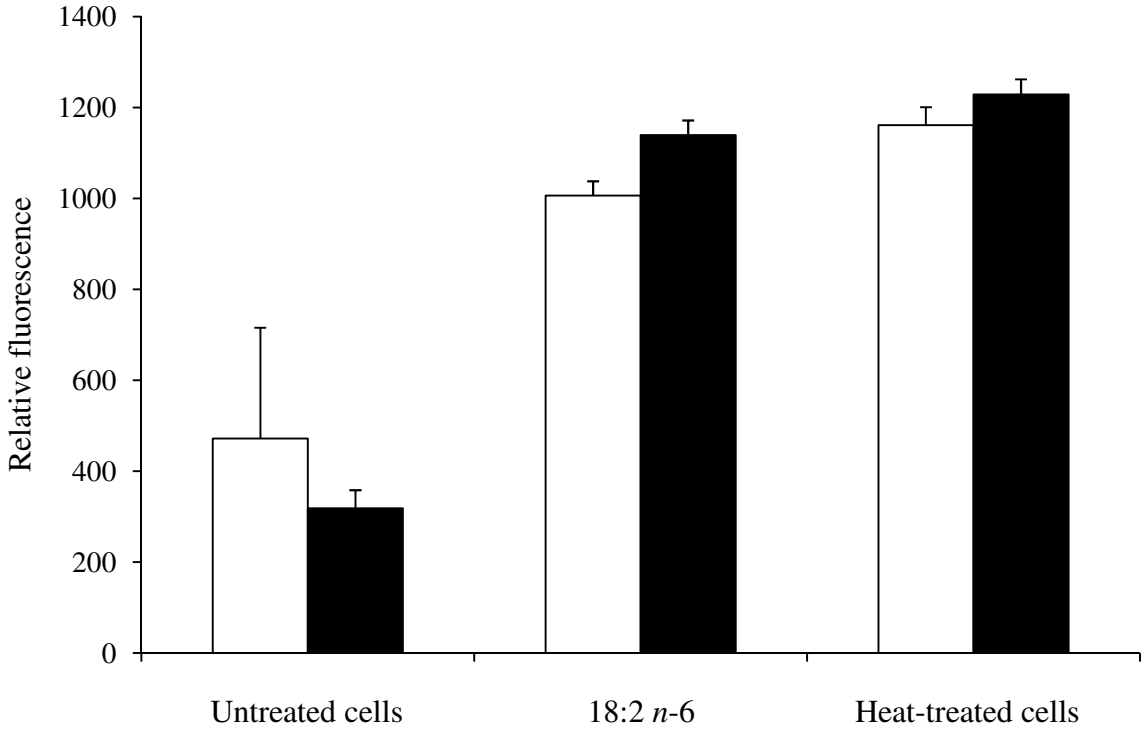

Figure 2.8. Influence of sodium lactate (70 mM) on the loss of cell integrity of *Butyrivibrio fibrisolvens* JW11 following incubation with 50 μg mL^{-1} linoleic acid (18:2 *n*-6). Loss of cell integrity was determined by fluorescence in the presence of propidium iodide. Sodium lactate plus 18:2 *n*-6 (□), 18:2 *n*-6 alone (■). Results are means and standard deviations from three cultures, each of which was subjected to 8 replicate measurements (n = 24).

2.3.5. Influence of fatty acids on membrane potential of Butyrivibrio fibrisolvens

Fluorescence of $DiOC_2(3)$ was used to determine the influence of C18 FA on membrane potential of *B. fibrisolvens* JW11 (**Figure 2.9**). The red to green fluorescence ratio is a measure of membrane potential. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler, depolarized the cells membrane potential causing an increase in the red to green fluorescence, whereas heat-treated cells, at 100 ºC for 20 min, decreased the fluorescence ratio. Addition of 18:2 *n*-6, CLA, a mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12 isomers, and 18:1 *trans*-11 had a similar effect to FCCP in reducing membrane potential. On the other hand, 18:3 *n*-3 tended to follow the pattern of heat-treated cells by reducing the fluorescence ratio. 18:0 had little or no effect as the results were similar to untreated cells.

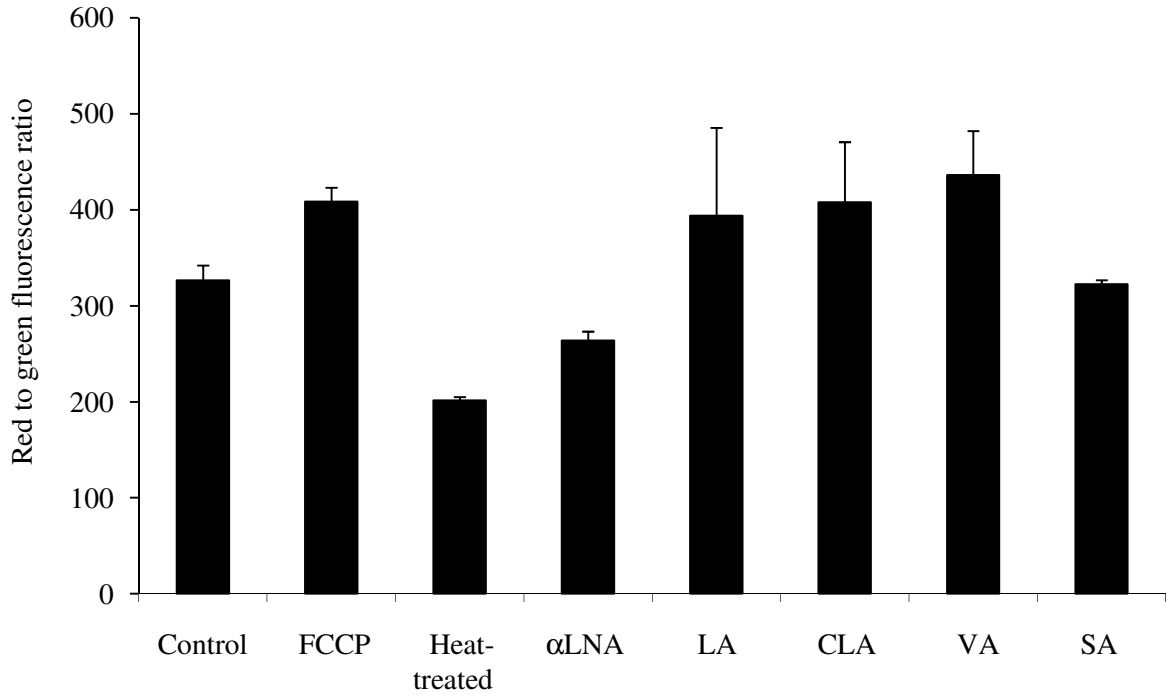

Figure 2.9. Influence of different fatty acids on red to green fluorescence of *Butyrivibrio fibrisolvens* JW11 in the presence of $DiOC_2(3)$ as measured by flow cytometry. FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; αLNA, 18:3 *n*-3; LA, 18:2 *n*-6; CLA, a mixture of 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12; VA, 18:1 *trans*-11; SA, 18:0. Results are means and standard deviations from three incubations, each of which was subject to three replicate measurements ($n = 9$).

2.4. Discussion

Over the last few years, ruminal biohydrogenation regained a new interest with the emergence of ruminant-derived products as functional foods. Most recent studies aimed to increase the healthiness of ruminant products by improving their FA profile, whether by enhancing or inhibiting biohydrogenation. Inhibition of dietary 18:2 *n*-6 and 18:3 *n*-3 biohydrogenation in the rumen would result in an increased deposition of these PUFA in the ruminant products. Nevertheless, promotion of a partial biohydrogenation would led to an increased concentration of PUFA biohydrogenation intermediates, such as 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11, which exhibit health-promotion effects. It has emerged that PUFA themselves might be useful to control biohydrogenation, as some members of the *Butyrivibrio* genus, the bacteria by far the most active in biohydrogenation and formation of 18:2 *cis*-9,*trans*-11, were extraordinarily sensitive to PUFA (Wallace et al., 2006). The present study evaluated the toxicity of PUFA to the ruminal biohydrogenating bacterium *B. fibrisolvens* and investigates some cellular mechanisms by which it may occur.

2.4.1. Toxicity of polyunsaturated fatty acids to Butyrivibrio fibrisolvens

Butyrivibrio fibrisolvens is a small, Gram-positive bacterium that is particularly prevalent in the rumen of grazing animals (Hazlewood, Orpin, Greenwood & Black, 1983), with high proteolytic (Strydom, Mackie & Woods, 1986) and fibrolytic (Stewart, Flint & Bryant, 1997) activities. The importance of *B. fibrisolvens* in ruminal biohydrogenation was first documented by Polan et al. (1964). Although other bacteria have been implicated (Harfoot & Hazlewood, 1997), biohydrogenating activity is high among all members of the *Butyrivibrio* phylogenetic tree (Paillard et al., 2007), many times higher than in other species (Maia, Chaudhary, Figueres & Wallace, 2007).

The pattern of 18:2 *n*-6 and 18:3 *n*-3 metabolism by *B. fibrisolvens* JW11 here observed, as well as the intermediates formed, followed the established biohydrogenation pathways (Kepler et al., 1966; White, Kemp & Dawson, 1970; Kemp et al., 1975; Hazlewood, Kemp, Lander & Dawson, 1976). The observations linking growth and 18:2 *n*-6 metabolism with *B. fibrisolvens* JW11 are consistent with those obtained with *B. fibrisolvens* A38 (Kim et al., 2000) and *B. fibrisolvens* TH1 (Fukuda, Furuya, Suzuki, Asanuma & Hino, 2005). The results obtained showing that growth of *B. fibrisolvens* only occurred after PUFA were

biohydrogenated to 18:1 *trans*-11, clearly demonstrate that biohydrogenation is a detoxification process necessary for *B. fibrisolvens* to escape from the bacteriostatic effects of PUFA. Moreover, they explain that the concentration-dependence of 18:2 *n*-6 toxicity and its apparently lower toxicity in growing cultures, observed by Kim et al. (2000), have to be considered in terms of time required by different biomass concentrations to hydrogenate, and thereby detoxify, different concentrations of FA.

More research emphasis has been placed on 18:2 *n*-6 biohydrogenation and its metabolism, as 18:2 *cis*-9,*trans*-11 exhibits health-promoting effects (Kritchevsky, 2000; Whigham et al., 2000). Nevertheless, it should be noted that biohydrogenation is probably more important for *B. fibrisolvens* to survive high 18:3 *n*-3 concentrations, as this PUFA is more toxic than 18:2 *n*-6 and is usually present at higher concentrations in forages. In temperate fresh grass, 18:3 *n*-3 may correspond up to 55 to 65% of total FA (Bauchart, Verite & Remond, 1984). The biohydrogenation intermediates formed by *B. fibrisolvens* in pure culture, were the same as those found when 18:3 *n*-3 was metabolized by the mixed ruminal microbiota *in vitro* (Wąsowska et al., 2006). Among these intermediates, the conjugated trienoic acid 18:3 *cis*-9,*trans*-11,*cis*-15 may have beneficial health effects, similarly to those found with CLA isomers. Indeed, different conjugated linolenic acid isomers have been shown to suppress the growth of human tumour cells (Suzuki et al., 2001; Kohno et al., 2004; Yasui, Hosokawa, Kohno, Tanaka & Miyashita, 2006a and 2006b; Coakley et al., 2009) and to be as effective as, or even more effective than, CLA in reducing body fat mass in rodents (Koba et al., 2002; Koba et al., 2007). Most interestingly, the 18:3 *cis*-9,*trans*-11,*cis*-13 isomer was shown to be converted by endogenous Δ13 saturation to 18:2 *cis*-9,*trans*-11 in the liver, intestine, kidney and mammary gland of rodents (Tsuzuki et al., 2004; Cao, Chen, Yang & Chen, 2009).

The bacteriocidal and bacteriostatic effects of FA are well known (Nieman, 1954). Henderson (1973) examined the effects of FA on ruminal bacteria. A *Butyrivibrio* sp. was generally most sensitive to FA, but only saturated and monoenoic FA were included in the study. 18:1 *cis*-9 was found to be more toxic than the saturated FA (Henderson, 1973; Maczulak, Dehority & Palmquist, 1981). Marounek et al. (2002) found that medium-chain FA, 8:0 and 10:0, were more toxic to ruminal and rabbit caecal bacteria than other chain lengths, but again the study was of saturated FA and 18:1 *cis*-9 only. In non-ruminal bacteria, 18:2 *n*-6 and 18:3 *n*-3 were more toxic than saturated or monoenoic acids (Galbraith, Miller, Paton & Thompson, 1971). In the present study, PUFA were found to be much more toxic than monoenoic and saturated FA. Additionally to the number of unsaturations, the position and configuration of double bounds seemed to have an effect on FA toxicity to *B. fibrisolvens*. Geometrical isomers

exhibit different toxicity, with *cis* FA being more toxic than *trans* FA, except for 18:1 *cis*-11. The *cis* or *trans* configuration of long-chain FA have been described to influence the degree of inhibition on pure cultures (Maczulak et al., 1981) and on mixed bacteria *in vitro* (Demeyer & Henderickx, 1967), with the *cis* isomers of C18 FA being more toxic. A proposed explanation for the relative inhibitory effects of different FA was their selectivity to complex with calcium and to form insoluble soaps (Maczulak et al., 1981). The order of completeness of soap formation measured in a mixed culture *in vitro* was 18:0 > 18:1 *trans*-11 > 16:0 > 18:1 *cis*-9 (Jenkins & Palmquist, 1982), which relates to the minimal effect of 18:0 and the maximum effect of 18:1 *cis*-9 (Henderson, 1973; Maczulak et al., 1981; Marounek et al., 2002). Although the effects of saturated and monoenoic FA could be partially explained, the inhibitory effects of PUFA here described are hardly explained by the formation of soaps. The two trienoic acids evaluated, 18:3 *n*-3 and 18:3 *n*-6, had completely different toxic effects on *B. fibrisolvens*, inhibiting growth for 35 h and over 72 h, respectively. This difference in toxicity seems difficult to be explained by strong differences in physicochemical properties of the trienoic FA. Moreover, a free carboxyl group was also necessary for toxicity and detoxification to occur. A specific effect of PUFA on the biohydrogenating bacterium *B. fibrisolvens* seems more likely.

Two main explanations for high biohydrogenating activity had been proposed. The first suggested that biohydrogenation was a means of disposing of reducing power (Lennarz, 1966), while the second proposed that biohydrogenation was in fact a detoxification mechanism hypothesis (Kemp & Lander, 1984; Kemp, Lander & Gunstone, 1984). The reducing power disposal hypothesis seems difficult to sustain. The disposal of hydrogen in an anaerobic ecosystem is vital to fermentation. However, the amount of hydrogen equivalents that would be removed by biohydrogenation of unsaturated FA is tiny in proportion to total metabolism. It might be argued that any additional advantage gained by one species, however slight, may be crucial in a highly competitive microbial ecosystem. Yet the reductase which converts 18:2 *cis*-9,*trans*-11 to 18:1 *trans*-11 in *B. fibrisolvens* comprises 0.5% of the total cell protein (Hughes, Hunter & Tove, 1982), which in itself represents a very significant expenditure of cellular resources. Even more convincing was the argument made by Harfoot and Hazlewood (1997) that *B. fibrisolvens* possesses a hydrogenase, which is a much more efficient method for removing excess reducing power than biohydrogenation of unsaturated FA. Therefore, and considering the toxicity and detoxification mechanism described here, the detoxification hypothesis seems to carry even more weight.

In non-ruminal bacteria, *trans* unsaturated FA have been described as an adaptation mechanism to stress stimuli (Guckert, Hood & White, 1986; Sikkema, de Bont & Poolman, 1995). Some bacteria species possess direct *cis-trans* isomerases which permits the inclusion of *trans* FA in the lipid bilayer membrane, and thereby regulate its fluidity, as a defence mechanism to extreme environmental changes (Keweloh & Heipieper, 1996). Bessa, Santos-Silva, Ribeiro and Portugal (2000) suggested that the accumulation of *trans* octadecenoates in the rumen might be beneficial to bacteria as response to environmental stress, such as low pH, presence of ionophores or high concentration of FA. Indeed, the growth of *B. fibrisolvens* only occurred after FA were biohydrogenated to 18:1 *trans*-11. Moreover, the addition of 18:1 *trans*-11 alleviated the inhibitory effects of 18:2 *n*-6, by decreasing the lag phase, compared to 18:2 *n*-6 alone, although the FA concentration in the medium was twice as higher the lag phase was reduced by one third. During 18:3 *n*-3 metabolism, 18:2 *trans*-11,*cis*-15 appeared to permit growth while other dienoic acids studied here did not. One explanation for the non-toxic effect of 18:2 *trans*-11,*cis*-15 may be explained by a protective effect of 18:1 *trans*-11. In fact, growth only occurred in the presence of 18:2 *trans*-11,*cis*-15 when considerable amounts of 18:1 *trans*-11 were also present. Thus, besides biohydrogenation being a detoxification mechanism, its end-product 18:1 *trans*-11 might play a protective role to the biohydrogenating bacterium *B. fibrisolvens*.

2.4.2. Mechanisms of toxicity of polyunsaturated fatty acids

Members of the *Butyrivibrio* genus seem to be particularly sensitive to bacteriostatic effects of PUFA, particularly the 18:0-producers *B. proteoclasticus* (Wallace et al., 2006). Its sensitivity to unsaturated FA may be due to its particularly thin cell wall structure (Wallace et al., 2006). *B. fibrisolvens*, as well as other member of the genus, is a Gram-positive bacterium that stains Gram-negative, due to its 12 to 18 nm cell wall, nearly one third to one half of the thickness of usual Gram-positive cell walls (Cheng & Costerton, 1977). Galbraith et al. (1971), in non-ruminal bacteria, found a relation between FA toxicity and cell wall structure, with growth of Gram-positive bacteria being sensitive to FA, while Gram-negative species were insensitive. The insensitivity of Gram-negative bacteria is attributed to the lipopolysaccharide layer of their outer membrane which prevents FA penetration (Nagaraja, Newbold, Van Nevel & Demeyer, 1997). Unsaturated FA are generally considered to be more toxic than saturated FA because double bonds alter the shape of the FA molecule, and kinked unsaturated FA would thereby disrupt the lipid bilayer structure (Keweloh & Heipieper, 1996). Indeed, the double

bond with *cis* configuration has a bend with an angle of 30º in the acyl chain, whereas *trans* configuration allows the chain to be extended, being more similar to saturated FA. However, it is not clear from the present study that the PUFA toxicity was a membrane effect. The free carboxyl group was necessary for growth inhibition to take place. Fatty acid methyl esters, which might be expected to be sufficiently hydrophobic to be incorporated into a membrane just as efficiently as a non-esterified FA, were non-toxic. They were metabolised in the same way as non-esterified FA, however, as they were hydrolysed by bacterial esterase activity. Furthermore, cell integrity, as measured by PI fluorescence, was poorly affected by the number of double bonds in the FA molecule and could not explain why trienoic acids were more toxic to growth than dienoic acids, and especially why 18:1 *trans*-11 was not toxic to growth. The free carboxyl group was also necessary for disruption of cell integrity, as measured by PI ingression. Wallace et al. (unpublished data) noted an enhanced toxicity of 18:2 *n*-6 in the presence of sodium lactate but, similarly to observed with *B. proteoclasticus* (Maia et al., 2007), no similar effect was found on cell integrity of *B. fibrisolvens*. Moreover, toxicity of 18:2 *n*-6 to different species of ruminal bacteria did not relate to changes in cell integrity disruption. Thus, the loss of cell integrity was most likely not the primary toxicity mechanism.

It might be argued that in the presence of FA, PI ingression may be facilitated into bacterial cell, as PI is a lipophilic molecule, which would explain the discrepancies between FA effects on growth and on cell integrity. Nevertheless, PUFA would not be expected to be any more effective than saturated FA in this respect. Neither would be non-esterified FA in relation to FA methyl esters.

An alternative possibility is that the ready diffusion of the non-esterified FA across the membrane causes chemiosmotic difficulties, perhaps uncoupling the proton-motive force (Mitchell, 1979), dissipating the membrane potential (Nichols, 1982) or decoupling intramembrane pathways (Rottenberg & Hashimoto, 1986; Rottenberg & Steiner-Mordoch, 1986). Here, the effects of different FA on membrane potential were determined using the carbocyanine dye, $DiOC_2(3)$, which when excited at 488 nm releases green (530 nm) and red (>600nm) fluorescence. According to Novo et al. (1999), the $DiOC_2(3)$ green fluorescence is related to particle size of an individual bacterium or clumps of bacteria, and largely independent of membrane potential. On the other hand, red fluorescence is a function of particle size as well as concentration. The red to green ratio largely eliminate the effect of size variation and depends primarily on dye concentration, which is sensitive to membrane potential and independent of cell size. The ratio was calculated by subtracting the log green

value from the log red value and by adding a constant (384) as proposed by Shapiro (1995). Membrane potential is reduced to zero either by heat-treament at 100 ºC or by ionophore uncouplers, such as FCCP. However, heat-treated cells are inappropriate to use as depolarized controls, due to changes in structure and permeability which consistently increased $DiOC_2(3)$ red to green ratio compared to the uncoupler carbonyl cyanide *n*-chlorophenylhydrazone (Novo et al., 1999). The results obtained indicate that, as with cell integrity, the effects of different FA on membrane potential did not correspond to their effects on growth. In particular, 18:1 *trans*-11, which had no effect on growth, had a similar effect on depolarizing the membrane potential to the growth-inhibitory dienoic acids. Moreover, 18:3 *n*-3 appeared to hyperpolarize *B. fibrisolvens* cells, a result difficult to explain biologically.

Polyunsaturated fatty acids toxicity to *B. fibrisolvens* was suggested to be a metabolic rather than a membrane effect (Maia et al., 2010). Measurements of CoA metabolic pools in *B. fibrisolvens* JW11 showed all acyl-CoAs, except acetoacetyl-CoA, to be diminished by over 96% when 18:2 *n*-6 was added to the medium. In contrast, the ATP pool remained at about one-third of the control values, presumably due to the contribution of glycolysis. Where FA might act is not clear, although their effects on metabolic pools suggest that there may be disruption of acyl-CoA metabolism. Acyl-CoA pool sizes were different for the different intermediates, with acetyl-CoA and butyryl-CoA present at highest concentration and the butyrate pathway intermediates at much lower concentrations.

A connection between PUFA toxicity and butyrate formation may exist. In fact, toxicity in different species of ruminal bacteria was related partly to whether the bacteria produced butyrate, with cellulolytic bacteria being the other most sensitive species (Maia et al., 2007). Within the *Butyrivibrio* phylogenetic group, the most sensitive species were those that formed butyrate *via* the butyrate kinase mechanism rather than butyryl-CoA:acetate-CoA transferase (Paillard et al., 2007). A metabonomic analysis (Nicholson, Lindon & Holmes, 1999) might help to identify precisely where the PUFA act and the possible connection to butyrate formation mechanism.

2.5. Conclusions

The present study contributed to clarify the purpose of biohydrogenation in ruminal bacteria, particularly in *B. fibrisolvens*. Biohydrogenation is a detoxifying mechanism to PUFA, however it was not possible to determine the cellular mechanisms by which FA are toxic to *B. fibrisolvens* JW11.

Growth only occurs after octadecatrienoic and octadecadienoic acids were hydrogenated to 18:1 *trans*-11. When PUFA were not biohydrogenated to the *trans* monoenoic acid, growth did not occur. The octadecenoic acid 18:1 *trans*-11 also appeared to have a protective effect on *B. fibrisolvens* metabolism. In the presence of 18:1 *trans*-11, 18:2 *n*-6 toxicity was reduced and no growth inhibition was observed with 18:2 *trans*-11,*cis*-15.

It is not clear that the toxicity of PUFA was a membrane effect. *Butyrivibrio fibrisolvens* membrane integrity decreased in the presence of C18 unsaturated FA. Also a decrease in membrane integrity was observed across most representative rumen bacteria in the presence of 18:2 *n*-6. However, membrane disruption results were not consistent with growth inhibition. Similarly, membrane potential results were not related to effects observed on growth of *B. fibrisolvens*. Membrane potential was altered by all C18 unsaturated FA, including 18:1 *trans*-11, which could not explain the differences in toxicity observed on growth between FA.

A free carboxyl acid is a requisite for PUFA toxicity, as FA methyl esters had no effect on membrane integrity or on growth. Nevertheless, FA methyl esters were biohydrogenated to similar end products as non-esterified FA, except for 18:3 *n*-6.

2.6. References

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Chapter 3

Effects of starch and pH on the biohydrogenation pattern of lucerne hay supplemented with soybean oil in a semi-continuous fermentation system (RUSITEC)

3.1. Introduction

Dietary lipids once in the rumen are rapidly and extensively hydrolysed by microbial lipases. The non-esterified fatty acids (FA) therefore released are adsorbed onto feed particles and biohydrogenated (Demeyer & Doreau, 1999). Ruminal biohydrogenation is a unique process by which dietary polyunsaturated fatty acids (PUFA), mostly C18 FA, are hydrogenated into more saturated FA by rumen microbial activity (Viviani, 1970). The main dietary PUFA, namely α-linolenic acid (18:3 *n*-3) and linoleic acid (18:2 *n*-6), are initially isomerized to *trans* conjugated FA and further reduced by hydrogenation of the double bonds (Harfoot & Hazlewood, 1997) until the formation of stearic acid (18:0). During this process a wide range of FA are formed as transient intermediates, such as trienoic, dienoic and monoenoic FA (Bickerstaffe, Annison & Noakes, 1972). Biohydrogenation results in the formation of the potential health-promoting FA conjugated linoleic acid (CLA) isomers and vaccenic acid (18:1 *trans*-11) as intermediates (Harfoot & Hazlewood, 1997). The main isomer present in ruminant-derived foods, corresponding to over 80% of total CLA isomers (Chin, Liu, Storkson, Ha & Pariza, 1992) is 18:2 *cis*-9,*trans*-11. In animal models, 18:2 *cis*-9,*trans*-11 has been demonstrated to have anticarcinogenic effects (Parodi, 1999, Park, 2009). Although CLA effects in human studies are less conclusive (Bhattacharya, Banu, Rahman, Causey &

Fernandes, 2006; Salas-Salvado, Marquez-Sandoval & Bullo, 2006; Park, 2009), 18:2 *cis*-9,*trans*-11 is considered to be the main health-promoting CLA isomer for humans (Pariza, 2004). Nevertheless, the other main CLA isomer with known biological properties, 18:2 *trans*-10,*cis*-12, has also been implied to have anticarcinogenic properties (Ip et al., 2002, Park, 2009), and to be involved in reduction of body weight and fat composition (Park, Storkson, Albright, Liu & Pariza, 1999; Park et al., 2000). Increased outflow of 18:1 *trans*-11 from the rumen is considered as beneficial as it is endogenously converted by Δ9-desaturase to 18:2 *cis*-9,*trans*-11 in animal tissues (Griinari et al., 2000; Corl, Barbano, Bauman & Ip, 2003; Kay, Mackle, Auldist, Thomson & Bauman, 2004).

Lipolysis and biohydrogenation pattern, i.e., the distribution of FA intermediates and end-products, are affected by several factors, such as ruminal pH (Van Nevel & Demeyer, 1996), basal diet, amount and type of dietary lipids, and ionophores (Bessa, Santos-Silva, Ribeiro & Portugal, 2000). In order to fulfil the energy requirements of high production animals, energy-rich concentrates may correspond to 50 or 60% of the total diet. Digestion of high fermentable non-structural carbohydrates leads to an accumulation of organic acids, with a consequent drop of the ruminal pH. High concentrate diets, and consequently low ruminal pH, are generally associated to a decrease of feed intake (Bradford & Allen, 2007), fiber digestion (Mould, Ørskov & Manns, 1983; Grant & Mertens, 1992), microbial yield (Strobel & Russell, 1986), milk fat content (Palmquist, Beaulieu & Bardano, 1993), and ruminal lipolysis by lowering the number of lipolytic bacteria (Latham, Storry & Sharpe, 1972; Grant & Mertens, 1992). A reduction of lipolysis in animals fed high concentrate diets has a negative effect on biohydrogenation, as a free carboxyl group is required for biohydrogenation to take place (Hawke & Silcock, 1970).

Products from ruminants fed pasture have highest amounts of 18:1 *trans*-11 and 18:2 *cis*-9,*trans*-11, while 18:1 *trans*-10 and 18:2 *trans*-10,*cis*-12 concentrations increase in animals fed high concentrate diets (Piperova et al., 2002). This biohydrogenation shift towards 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 formation was associated with milk fat depression (Griinari et al., 1998) when lactating dairy cows were fed low fiber diets.

It is well established that the 18:1 *trans*-11 to *trans*-10 shift in biohydrogenation pathways is associated to high concentrate diets, particularly when supplemented with C18 PUFA. However, as high starch content and low ruminal pH are strictly related, it is not clear if the *trans*-10 shift is caused by a direct effect of the starch supplementation on the ruminal ecosystem or its indirect effect on lowering the ruminal pH. Thus, the aim of this study was to determine the individual effects of starch and of pH, as well as their interaction, on PUFA

biohydrogenation pattern and intermediate FA profile in a semi-continuous fermentation system (RUSITEC), namely, on discriminating high starch from low pH effects on the *trans*-11 to *trans*-10 shift generally observed in animals fed high concentrate diets supplemented with vegetable oils.

3.2. Material and Methods

3.2.1. Donor animals

Three adult, castrated, Serra da Estrela rams with an average live weight of 50 (± 1.5) kg and fitted with permanent rumen cannula (6 cm internal diameter) were used as rumen content donors. Rams were kept in individual stalls with free access to water and mineral licking blocks (SC Sheep Rockies, Rockies, Winsford, Cheshire, UK) containing (per kg) 10,000 mg Ca, 10,000 mg P, 5,000 mg Mg, 150 mg Co, 250 mg I, 500 mg Mn, 1,000 mg Zn, 42 mg Se, and 380,000 mg Na. Animals were fed 1 kg (DM basis) of granulated dehydrated lucerne once a day at 09:00 h. Rumen contents were collected before feeding by vacuum to a preheated flask. Animal handling followed the EU directive number 86/609/EEC concerning animal care.

3.2.2. Dietary treatments and experimental design

Two experimental diets designed to radically differ on the starch content, low or high, were supplemented with 8% (wt/v) soybean oil. Low starch diet (LS) was of lucerne hay and high starch diet (HS) of lucerne hay plus starch (50:50, wt/wt). Soluble starch (BDH Prolabo, VWR International LLC., Pennsylvania, USA) and lucerne hay milled through a 3 mm sieve were weighted separately and carefully mixed. Soybean oil (Ibersoja®, Copaz, Lisboa, Portugal) was added daily into each feedbag to previously weighted feeds, immediately before incubation, and carefully homogenized. pH levels were maintained by continuous infusion of artificial saliva buffered at low (6.0) and high (7.0) pH. The experimental treatments thereby obtained were: i) low starch diet incubated at low pH (LSLpH); ii) low starch diet incubated at high pH (LSHpH); iii) high starch diet incubated at low pH (HSLpH); and iv) high starch diet incubated at high pH (HSHpH).

The experimental design used was a 2 x 2 factorial arrangement, with two experimental diets (low starch and high starch) being incubated at two pH levels (6.0 and 7.0). The experiment was replicated twice, to account for effects of the fermentation system and rumen contents. Each replica lasted for 15 days, and comprised a 7 days adaptation period and a 8 days experimental period.

3.2.3. Artificial saliva buffer solution

A macroelements solution (McDougall, 1948) and a microelements solution (Durand, Dumay, Beaumatin & Morel, 1988) were used to prepare the artificial saliva buffer solution. Daily, 10 mL of microelements solution (3.68 mg $FeSO_4.7H_2O$, 1.9 mg $MnSO_4.7H_2O$, 0.44 mg $ZnSO_4.7H_2O$, 0.12 mg $CaCl_2.6H_2O$, 0.098 mg $CuSO_4.5H_2O$, 0.0174 mg $Mo_7(NH_4)_6O_{24}.4H_2O$ L^{-1}) were added to 1000 mL of macroelements solution (9.80 g $NaHCO_3$, 9.3 g $Na_2HPO_4.12H_2O$, 0.57 g KCl, 0.47 g NaCl, 0.12 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2$ L^{-1}), and the buffer solution bubbled with O_2-free CO_2 for 15 min. Artificial saliva pH was read and adjusted to pH 6.0 (± 0.1) or pH 7.0 (± 0.1) with 1N HCl or 1N NaOH solutions.

3.2.4. Rumen Simulation Technique (RUSITEC)

The rumen simulation technique (RUSITEC) was developed at the Hannah Research Institute by Czerkawski and Breckenridge (1977) based on the concept of compartmentation in the rumen. Although the rumen is a very complex organ, it could be represented as a basic four compartment open system in which microbial population are divided into microorganisms free in the rumen fluid (compartment 1), loosely associated with food particles (compartment 2), strongly associated with food particles (compartment 3), and associated with the rumen wall (compartment 4; Cheng & Costerton, 1980; Czerkawski, 1980).

Czerkawski and Cheng (1988) described the RUSITEC system as “a rumen without a rumen wall”, as it is formed by three compartments in all similar to those of the rumen. Compartment 1 has a large volume and a low concentration of microorganisms in free suspension, while microbial population comprising compartment 3 are strongly adherent to the solid digesta, have high cellulolytic activity, and may account for 5 to 15% DM of the washed undigested feed residues. Similarly to the rumen, in RUSITEC most of the

micropopulation (70 to 80%) is associated with the solid digesta, but part of it is loosely attached and can be removed by washing. These loosely attached microorganisms comprise compartment 2, which has lower microbial concentration than compartment 3 but quite higher than compartment 1 (Czerkawski & Cheng, 1988).

Although similarities in terms of microbial compartmentation, the RUSITEC system differs from the rumen. Diets are milled and contained inside a nylon bag, which is forced to pass through the diluted and stationary liquid phase, the undigested particles only leave the system by manual removal after 48 hours, and the fermentation end-products are removed by overflow, it lacks rumination and the microorganisms associated with the rumen wall.

3.2.4.1. Semi-continuous fermentation system description

The semi-continuous fermentation system used was a copy of the RUSITEC apparatus designed by Czerkawski and Breckenridge (1977), with four independent fermentation vessels, artificial saliva buffer solution infusion, effluent and fermentation gas collection, controlled temperature and stirring systems (**Figure 3.1**).

3.2.4.1.1. Fermenters, effluents collection and feedbags

Each fermenter was a cylindrical vessel made from transparent acrylic resin (254 x 76 mm) with 1 L capacity. The vessel was fitted to the bottom of a water-bath maintained at 39 ºC. Inside, a perforated feed container held two feedbags and allowed the free circulation of the liquid phase. The container lid was connected to a driveshaft ensuring the vertical movement of the feedbags through the liquid phase of the fermentation vessel. The vessel was sealed by a lid with an O-ring rubber held by a screwed flange, which was passed by the driveshaft and had two holes: a sampling port and an effluent (both liquid and gas) outlet. The artificial saliva was infused through the bottom of the vessel by a peristaltic cassette pump (IPS-4, Ismatec SA, Glattbrugg, Switzerland) at a dilution rate of 42 mL h^{-1}. The buffer solutions were in individual containers under continuous magnetic stirring.

Effluents of each fermentation vessel were collected in 2 L collection bottles placed in a water-bath at 4 ºC under continuous magnetic stirring, to stop the fermentation process. Liquid and gas effluents outflowed together from the fermenters. Once in the effluent

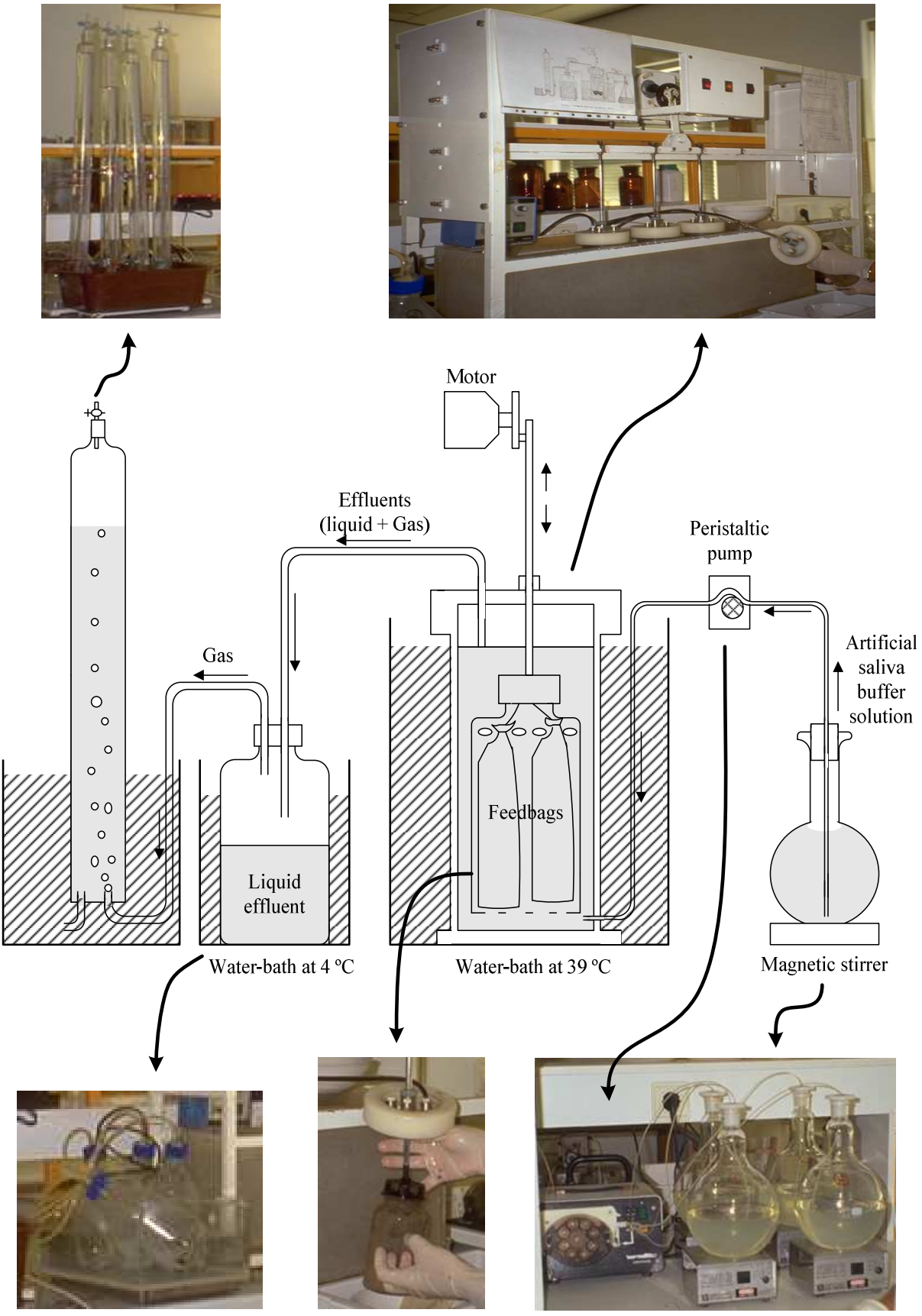

Figure 3.1. Schematic representation of the semi-continuous fermentation system (RUSITEC) used.

collection bottles, gas was released to a 2 L graduated glass column filled with water and placed in a water-bath. The pressure difference caused by the gas entrance in the column lead to the filling water to leave by a water fill outlet placed at the bottom of the column, next to the gas inlet.

Feedbags were in standard nylon (rumen *in situ* filter bags n.º 1020, Ankom, New York, USA) with a pore size of 50 (± 15) µm and dimensions of 150 x 100 mm. Lucerne hay was milled through a 3 mm sieve and 15 g of lucerne hay (LS diets) or 7.5 g of lucerne hay plus 7.5 g of starch (HS diets) were weighed to feedbags. Soybean oil (8% wt/v) was added daily to previously weighed feeds in each feedbag by pipeting, and the mixture homogenised.

3.2.4.2. Inoculation and daily manipulation

Rumen contents were sampled from three donor animals by vacuum pump before feeding, *ca.* 1,500 mL per animal, strained through four layers of linen cloth and both liquid and solid phases placed in thermos containers. Liquid and solid digesta were immediately taken to the laboratory, flushed with O_2-free CO_2 and incubated within 15 min. All materials and solutions used were pre-heated at 39 ºC to assure the maximum survival of the rumen micropopulation.

To each fermenter was added 500 mL of strained rumen fluid, 200 mL of buffer solution and 100 mL of distilled water. A feedbag with 80 g of solid digesta and a feedbag with 15 g of the experimental feed were placed in the perforated feed container of each fermentation vessel. Once the vessels were sealed with the lids, the RUSITEC system was started by turning on the motor, the peristaltic pump and the magnetic stirring systems.

Stirring and buffer solution infusion systems were stopped after 24 h of incubation. In each fermenter, the feedbag with the solid rumen content was replaced by a feedbag with the experimental feed. Daily, at each 24 h of incubation, the feedbag that had been in the fermenter for 48 h was replaced by a new one. The removed feedbag was washed twice with 40 mL of buffer solution at 39 ºC to detach and recover the microorganisms' loosely adherent to the solid phase. The washout liquid was added to the fermenter to maintain the biomass level in the vessel. The fermenters were immediately sealed and, when necessary, the volume made up to 1 L with buffer solution. The buffer solution infused and the liquid and gas effluents produced were measured daily. After the buffer solution containers refilled, effluent collection bottles emptied, and gas columns refilled with water, the RUSITEC system was re-started.

3.2.4.3. Sample collection

During the 8 day experimental period, at each 24 h of incubation, the system was stopped and measured the amount of artificial saliva buffer solution infused, and of liquid and gas effluents produced. Samples of the fermenter phase and effluent were collected before changing the feedbags. pH was immediately read and the samples kept at -20 ºC until further analysis. Feedbag residues removed after 48 h incubation were kept at -20 ºC. The residues from days 3, 5 and 7 of the experimental period were thawed, washed with deionised water for 15 min, under constant flow, and frozen. All feedbag residues were freeze-dried, milled through 1 mm sieve and kept at -20 ºC until further analysis.

3.2.5. Analytical procedures

Feed and feedbag samples ground at 1 mm were analysed for dry matter (DM; NP 875, 1983), and organic matter (OM; NP 872, 1983). Total nitrogen (N) was determined in feeds, washed feedbag residues and liquid effluents by macro-Kjedhall method (NP 2030, 1996). Crude protein (CP) was calculated as N x 6.25. Neutral detergent fibre (NDFom) and acid detergent fibre (ADFom) were determined in feeds and washed feedbag residues according to detergent procedure of Van Soest, Robertson and Lewis (1991) and Robertson and Van Soest (1981), and expressed without residual ash. During NDFom extraction α-amylase and sodium sulphite were not added. Ether extract (EE) was determined in feeds by extraction with petroleum ether (NP 876, 2000) using a Soxtec® system HT2 unit (Foss Tecator, Sweden). Starch content was determined by colorimetric method (DuBois, Gilles, Hamilton, Rebers & Smith, 1956). Feeds were analysed for DM digestibility and OM digestibility according to Tilley and Terry (1969) method. pH was determined in liquid effluents and fermenters liquid phase, immediately after sampling, by a 744 pH metter (Metrohm, Switzerland) with a pH combination electrode (Metrohm, Switzerland).

Non-ammonia nitrogen (NAN) was calculated in liquid effluents by subtracting the ammonia nitrogen ($N\text{-}NH_3$) fraction (OJEC, 1971) from the N fraction (NP 2030, 1996). Volatile fatty acids (VFA) were determined in 1.25 mL strained rumen fluid after addition of 0.25 mL ortophosphoric acid solution (25%) and centrifugation at 15,000 *g* for 10 min at 4 ºC. The supernatant was analysed by gas chromatography using a gas chromatograph HP6890 series

(Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and a semi-capillary column (MN 116; Permabond-FFAP, Macherey-Nagel GmbH & Co. KG, Düren, Germany) with 50 m, 0.25 mm internal diameter and 0.25 μm film thickness. Helium was the carrier gas and the split ratio was 50:1. The injector temperature was 230 ºC and the detector temperature was 280 ºC. Column initial temperature of 110 ºC was held for 1 min, increased to 170 ºC at a rate of 6 Cº/min and held for 1 min and then increased to 230 ºC at a rate of 15 Cº/min and held for 15 min. Volatile fatty acids were identified by comparison with retention times of known standards (Sigma-Aldrich Inc., St. Louis, MO, USA) and quantified by external standard calibration.

3.2.5.1. Fatty acid analysis

Fatty acids were extracted from feeds, non-washed feedbag residues, liquid effluents, and fermenters liquid phase as proposed by Folch, Lees and Stanley (1957) with some changes. Trichloromethane was replaced by dichloromethane and ultrasonic homogenization was used. Feedbag residues, liquid effluents, and fermenters liquid phase samples were acidified before extraction (Fellner, Sauer & Kramer, 1995) to recover non-esterified FA from soaps. A basic methylation followed by an acidic methylation was carried out according to Kramer et al. (1997) to prevent the isomerisation of conjugated dienes.

To 200 mg of dried samples were added 2 mL of ultra-pure water and 1 mL of 6 N hydrochloric acid. The mixture was vortexed for 1 min at low speed. Six millilitres of dichloromethane:methanol (2:1) solution was added, the mixture was vortexed for 30 s at medium speed, sonicated for 6 min at 25 ºC and centrifuged at 1,500 rpm for 5 min at room temperature. The dichloromethane phase was transferred to a new tube and 6 mL of dichloromethane:methanol (2:1) solution was added to the tube with the aqueous phase. The mixture was vortexed for 30 s at medium speed and centrifuged at 1,500 rpm for 5 min. The dichloromethane fractions were pooled and 3 mL of 0.8% potassium chloride solution added. After vortexing the mixture for 30 s at high speed, it was centrifuged at 1,500 rpm for 10 min. The aqueous phase was discarded and the dichloromethane fraction passed by a phase separator filter (PS1; Whatman International Ltd., Kent, UK) and took to dryness under N_2 flux at 37 ºC. To the dried lipid extracts were added 0.5 mL of 2 mg mL^{-1} tricosanoic acid (23:0, internal standard) in *n*-hexane and 2 mL of 2 N sodium methoxide in methanol. The mixture was vortexed at low speed for 30 s and placed in a water-bath at 50 ºC for 10 min.

Four millilitres of 3 N hydrochloric acid in methanol was added, the mixture vortexed at low speed for 30 s and taken to a water-bath at 80 ºC for 10 min. When solvent escaped, 2 mL hexane was added after cooling and tubes returned to the water-bath in order to ensure a complete methylation. When tubes were at room temperature, 1.5 mL ultra pure water and 2 mL hexane (3 mL for feed samples) were added, the mixture vortexed for 30 s at high speed and allowed to rest for 2 min. The hexane fraction containing the FA methyl esters was transferred to a gas chromatography vial, flushed with N_2 and immediately capped to prevent loss of highly volatile methyl esters, and kept at -20 ºC until analysis.

Fatty acid methyl esters were analysed using a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and using a fused silica capillary column (CP-Sil 88; Chrompack, Varian Inc., Walnut Creek, CA, USA) with 100 m, 0.25 mm internal diameter and 0.20 μm film thickness. Helium was used as carrier gas and the injector and detector temperatures were 280 and 260 ºC, respectively. The split ratio was 50:1 and the injection volume of 1 μl. Column initial temperature of 100 ºC was held for 15 min, increased to 150 ºC at a rate of 10 Cº/min and held for 5 min, then increased to 158 ºC at a rate of 1 Cº/min and held for 30 min, increased to at a rate of 1 Cº/min to 200 ºC and held for 15 min, and finally increased to 210 ºC at a rate of 10 Cº/min and held for 5 min. Fatty acids were identified by comparison with retention times of standards obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Matreya Inc. (Pleasant Gap, PA, USA). Structural analyses of some unknown peaks were conducted by gas chromatography-tandem mass spectrometry technique using a Varian Saturn 2000 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column.

The FA data presented in this chapter does not correspond entirely to the profiles obtained by gas chromatography analysis. In the elution region of conjugated trienoic FA, namely 18:3 *cis*-9,*trans*-11,*cis*-15, several peaks co-eluted together. Samples were analysed by covalent adduct chemical ionization tandem mass spectrometry, using acetonitrile as reagent of chemical ionization (Alves & Bessa, 2007), and putatively trienoic FA were identified in that region as well as dimethylacetals. Regardless the efforts that have been made to separate and clearly identify those FA no major breaks were achieved. Thus, we decided to exclude those putatively but not identified trienoic acids from the analysis.

3.2.6. Statistical analysis

Fermentative parameters (n = 64) and FA data of non-washed feedbag residues (n = 40), liquid effluents (n = 63) and fermenters (n = 64) were analysed using the MIXED procedure of SAS (2001) with day as repeated measurement. The model included the fixed effects pH level, starch inclusion level, pH level by starch inclusion level interaction, the random effect of replica, and the random residual error.

The most desirable covariance structure was determined according to the Akaike's information criterion and Schwarz's Bayesian criterion (Littell, Henry & Ammerman, 1998). First order autoregressive was the covariance structure chosen for all data. Least square means (LSM) were compared by least square difference test and differences declared significant at $P < 0.05$.

3.3. Results

3.3.1. Dietary treatments

Chemical composition and FA profile of dietary treatments are presented in **Table 3.1**.

The replacement of 50% lucerne hay by soluble starch in HS diets proportionally decreased CP and fibre contents. Ether extract contents were similar between feeds, although slightly higher in LS than HS.

Soybean oil supplementation to both diets led to similar FA profiles in LS and HS diets, with faintly higher proportions of 16:0 and 18:3 *n*-3 and lower proportions of 18:1 *cis*-9 and 18:2 *n*-6 in LS compared to HS diets.

3.3.2. Rumen fermentation pattern

Fermenters and effluents pH decreased in low pH (LpH) and HS treatments, whereas the volume of effluents produced, either gas or liquid, was not affected by incubation pH, inclusion of starch or their interaction (**Table 3.2**).

Table 3.1. Chemical composition and fatty acid profile of the experimental diets.

	Treatments[1]	
	LS	HS
DM (g kg^{-1})	924	911
Chemical composition (g kg^{-1} DM)		
OM	880	938
CP	228	124
EE	100	91.0
NDFom	285	131
ADFom	201	109
Starch	26.8	531
DM digestibility	695	748
OM digestibility	675	737
Fatty acid proportions (g 100 g^{-1} total FA)		
16:0	11.2	10.7
18:0	3.35	3.20
18:1 *cis*-9	20.0	20.7
18:2 *n*-6	52.8	54.6
18:3 *n*-3	8.36	7.03
Other FA[2]	4.29	3.77

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)].
[2]The remaining fatty acids, most of them unidentified.

Table 3.2. Effects of starch level and incubation pH on fermenters and effluents pH and on volumes of gas and liquid effluents produced daily.

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
pH								
Fermenters	6.03	5.81	6.84	6.68	0.059	<0.001	0.015	0.623
Effluents	5.99	5.88	6.76	6.57	0.050	<0.001	0.011	0.481
Effluents production (mL day^{-1})								
Gas	2798	2596	2615	2796	157	0.960	0.947	0.259
Liquid	1055	995	1032	1026	31.6	0.911	0.314	0.397

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.
[2]pH = pH level.
[3]S = starch content.

Most rumen fermentation parameters were not affected by pH level (**Table 3.3**). High pH (HpH) incubations increased N-NH_3 concentration, total VFA production, and molar proportion of acetate, whereas decreased molar proportion of butyrate. High starch diets decreased NAN and N-NH_3 concentrations. Additionally, HS diets promoted lower molar proportions of acetate and propionate, increasing butyrate. Interactions between pH incubation level and starch inclusion were observed for N-NH_3, total VFA, acetate and propionate.

Table 3.3. Effect of starch level and incubation pH on fermentation metabolites of liquid effluents.

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
N-NH_3 (mg L^{-1})	79.9^{b}	22.0^{a}	93.7^{c}	22.8^{a}	2.97	0.025	<0.001	0.043
NAN (mg L^{-1})	134	96	134	118	8.1	0.197	0.006	0.193
Volatile fatty acids (mM)								
Total	53.6^{a}	44.7^{a}	50.3^{a}	63.9^{b}	3.26	0.031	0.486	0.005
Molar proportion (mol 100 mol^{-1})								
Acetate	58.1^{bc}	47.1^{a}	59.9^{c}	55.6^{b}	1.02	0.001	<0.001	0.013
Propionate	21.8	14.5	18.3	15.9	1.19	0.436	0.015	0.109
Isobutyrate	0.230	0.390	0.369	0.396	0.0655	0.303	0.193	0.343
Butyrate	12.4^{a}	29.2^{c}	13.2^{a}	19.4^{b}	1.40	0.036	0.002	0.024
Isovalerate	1.28	2.53	1.47	3.27	0.749	0.580	0.135	0.742
Valerate	6.19	6.29	6.51	5.52	0.482	0.667	0.414	0.324
Acetate:Propionate	2.72	3.29	3.28	3.63	0.240	0.118	0.110	0.657

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

3.3.3. Feed residues, effluents and fermenters fatty acid composition

Fatty acid content and profiles, excluding C18 FA, of feed residues, effluents and fermenters are presented in **Tables 3.4**, **3.5** and **3.6**, respectively. On average, highest FA concentration was found in feed residues, which represented *ca.* 74% of total FA determined on all fractions. Fatty acid content of fermenters and effluents were more closely related, representing *ca.* 15% and 11% of total FA determined, respectively. Additionally, an

Table 3.4. Feed residues fatty acids profile (g 100 g^{-1} total FA) and content (mg g^{-1} DM).

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
10:0	0.134	0.124	0.101	0.189	0.0202	0.454	0.105	0.058
11:0	0.060	0.049	0.055	0.070	0.0072	0.321	0.784	0.106
12:0	0.762^{ab}	0.588^{ab}	0.518^{a}	0.818^{b}	0.0859	0.936	0.484	0.025
iso-13:0	0.092	0.103	0.887	0.094	0.0225	0.786	0.714	0.902
anteiso-13:0	0.052^{a}	0.046^{a}	0.060^{a}	0.089^{b}	0.0065	0.005	0.120	0.029
13:0	0.050^{a}	0.039^{a}	0.061^{a}	0.110^{b}	0.0080	<0.001	0.042	0.004
iso-14:0	0.050^{b}	0.017^{a}	0.057^{b}	0.065^{b}	0.0051	0.001	0.050	0.005
14:0	0.91	0.76	1.44	1.35	0.132	0.002	0.397	0.831
iso-15:0	0.349	0.278	0.388	0.489	0.0439	0.019	0.746	0.082
anteiso-15:0	0.385	0.139	0.562	0.160	0.0665	0.195	0.005	0.294
14:1 *cis*-9	0.121^{b}	0.103^{ab}	0.095^{a}	0.117^{ab}	0.0077	0.453	0.773	0.027
15:0	0.316^{b}	0.232^{a}	0.363^{b}	0.369^{b}	0.0183	<0.001	0.057	0.034
iso-16:0	0.036	0.051	0.044	0.049	0.0064	0.671	0.078	0.355
15:1 *cis*-10	0.417^{b}	0.254^{a}	0.300^{a}	0.275^{a}	0.0192	0.038	0.002	0.008
16:0	19.5	19.9	21.4	22.6	0.90	0.036	0.428	0.642
iso-17:0	0.077	0.095	0.166	0.148	0.0156	<0.001	0.997	0.275
16:1 *cis*-7	0.400	0.323	0.287	0.334	0.0399	0.248	0.731	0.181
16:1 *cis*-9	0.351^{b}	0.274^{a}	0.267^{a}	0.449^{c}	0.0229	0.069	0.039	<0.001
anteiso-17:0	0.055	0.058	0.098	0.087	0.0102	0.004	0.677	0.517
17:0	0.669	0.725	0.348	0.738	0.1118	0.195	0.071	0.163
C18 FA[4]	61.8	63.7	59.8	57.7	1.10	0.007	0.927	0.116
20:0	0.820	0.817	0.705	0.848	0.0338	0.241	0.067	0.057
20:1 *n*-9	0.271	0.275	0.224	0.295	0.0180	0.469	0.065	0.093
21:0	0.069	0.077	0.049	0.057	0.0097	0.034	0.340	0.947
22:0	0.883	0.732	0.840	0.886	0.0476	0.281	0.301	0.073
22:1 *n*-9	0.349	0.205	0.263	0.554	0.0655	0.201	0.452	0.058
20:4 *n*-6	0.088	0.154	0.107	0.173	0.0254	0.478	0.070	0.988
24:0	0.506	0.545	0.234	0.452	0.0823	0.184	0.260	0.433
Other FA[5]	11.0	9.9	11.5	11.3	0.47	0.084	0.222	0.390
OCFA[6]	1.04	1.00	0.77	1.21	0.118	0.843	0.112	0.065
BCFA[7]	1.06	0.740	1.46	1.14	0.0943	0.002	0.007	0.996
OBCFA[8]	2.10	1.73	2.23	2.36	0.170	0.047	0.496	0.173
BCFA:OCFA	1.19	0.91	2.03	1.13	0.156	0.005	0.003	0.075
Total FA (mg g^{-1} DM)	141	119	151	96.5	11.6	0.639	0.047	0.241

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

[4]Sum of octadecanoic acids.

[5]The remaining fatty acids, most of them unidentified.

[6]Odd-chain fatty acids.

[7]Branched-chain fatty acids.

[8]Odd- and branched-chain fatty acids.

Table 3.5. Effluents fatty acids profile (g 100 g^{-1} total FA) and content (mg g^{-1} DM).

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
10:0	0.120	0.150	0.171	0.177	0.0119	0.007	0.148	0.339
11:0	0.375	0.458	0.442	0.224	0.0787	0.335	0.427	0.114
12:0	0.524	0.486	0.677	0.642	0.0673	0.064	0.609	0.982
iso-13:0	0.110	0.115	0.118	0.091	0.0100	0.438	0.287	0.135
anteiso-13:0	0.270	0.192	0.283	0.174	0.0269	0.933	0.008	0.571
13:0	0.164	0.102	0.134	0.134	0.0335	0.987	0.381	0.399
iso-14:0	0.185	0.103	0.233	0.134	0.0237	0.040	<0.001	0.623
14:0	1.15	1.09	1.93	2.13	0.207	0.007	0.731	0.541
iso-15:0	0.357	0.292	0.307	0.258	0.0219	0.081	0.023	0.718
anteiso-15:0	1.86	1.01	1.57	1.01	0.098	0.177	<0.001	0.161
15:0	1.16	0.66	1.11	0.81	0.076	0.528	<0.001	0.232
iso-16:0	0.170	0.094	0.163	0.087	0.0131	0.618	0.001	0.993
15:1 *cis*-10	0.232	0.158	0.241	0.137	0.0174	0.739	<0.001	0.410
16:0	17.5	16.6	23.2	19.9	1.72	0.072	0.302	0.533
iso-17:0	0.103	0.110	0.134	0.133	0.0122	0.048	0.802	0.720
16:1 *cis*-7	0.288	0.217	0.271	0.214	0.0207	0.635	0.012	0.738
16:1 *cis*-9	0.277	0.259	0.234	0.245	0.0379	0.480	0.923	0.708
anteiso-17:0	0.248	0.220	0.226	0.179	0.0171	0.092	0.053	0.570
17:0	0.367	0.340	0.311	0.339	0.0227	0.229	0.999	0.244
C18 FA[1]	66.6	70.9	59.6	66.1	2.14	0.035	0.047	0.614
19:0	0.113	0.110	0.091	0.084	0.0107	0.037	0.613	0.818
20:0	0.491	0.465	0.504	0.523	0.0368	0.362	0.933	0.566
20:1 *n*-9	0.120	0.101	0.102	0.092	0.0130	0.286	0.267	0.722
22:0	0.499	0.547	0.450	0.478	0.0308	0.088	0.236	0.751
Other FA[2]	6.74	5.57	7.31	5.32	0.453	0.724	0.005	0.385
OCFA[3]	1.67	1.05	1.54	1.28	0.098	0.580	<0.001	0.094
BCFA[4]	3.30	2.12	3.03	2.07	0.165	0.360	<0.001	0.535
OBCFA[5]	4.96	3.17	4.58	3.35	0.257	0.689	<0.001	0.289
BCFA:OCFA	3.04	2.01	2.09	1.66	0.332	0.093	0.068	0.397
Total FA (mg g^{-1} DM)	21.5[a]	24.4[a]	21.8[a]	31.8[b]	1.58	0.028	0.001	0.041

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

[4]Sum of octadecanoic acids.

[5]The remaining fatty acids, most of them unidentified.

[6]Odd-chain fatty acids.

[7]Branched-chain fatty acids.

[8]Odd- and branched-chain fatty acids.

Table 3.6. Fermenters fatty acids profile (g 100 g^{-1} total FA) and content (mg g^{-1} DM).

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
10:0	0.117	0.133	0.177	0.204	0.0164	0.009	0.237	0.735
11:0	0.362[b]	0.106[a]	0.335[b]	0.180[a]	0.0189	0.206	<0.001	0.015
12:0	0.649	0.561	0.698	0.713	0.0607	0.145	0.569	0.423
iso-13:0	0.098	0.097	0.093	0.085	0.0087	0.344	0.576	0.714
13:0	0.133	0.103	0.150	0.254	0.0413	0.049	0.375	0.108
iso-14:0	0.323	0.462	0.313	0.381	0.0441	0.332	0.054	0.448
14:0	1.20	1.04	1.91	2.47	0.171	<0.001	0.276	0.064
iso-15:0	0.436	0.318	0.314	0.290	0.0323	0.052	0.062	0.186
anteiso-15:0	3.49	1.47	2.42	1.55	0.310	0.168	0.005	0.119
15:0	1.35[d]	0.66[a]	1.10[c]	0.86[b]	0.053	0.662	<0.001	0.004
iso-16:0	0.319	0.201	0.279	0.203	0.0227	0.399	<0.001	0.355
15:1 *cis*-10	0.156	0.126	0.097	0.114	0.0353	0.386	0.861	0.550
16:0	17.9	14.7	25.5	23.5	1.47	0.006	0.160	0.708
iso-17:0	0.086	0.062	0.066	0.079	0.0099	0.904	0.602	0.094
16:1 *cis*-7	0.264	0.180	0.228	0.259	0.0412	0.626	0.556	0.231
16:1 *cis*-9	0.233	0.266	0.262	0.284	0.0325	0.487	0.431	0.870
anteiso-17:0	0.082	0.079	0.054	0.061	0.0099	0.002	0.823	0.436
17:0	0.618[b]	0.395[a]	0.459[a]	0.490[a]	0.0294	0.314	0.012	0.003
C18 FA[1]	63.1	72.7	57.5	60.8	1.57	0.003	0.010	0.100
19:0	0.227	0.0483	0.362	0.153	0.0649	0.164	0.034	0.853
20:0	0.371	0.231	0.266	0.180	0.0538	0.199	0.080	0.645
22:0	0.446	0.446	0.399	0.381	0.0363	0.145	0.804	0.807
22:1 *n*-9	0.303	0.335	0.237	0.184	0.0560	0.047	0.844	0.419
20:4 *n*-6	0.374	0.337	0.376	0.319	0.0389	0.842	0.244	0.799
20:5 *n*-3	1.76	1.01	0.483	1.04	0.2249	0.071	0.700	0.064
Other FA[2]	5.98	4.50	5.14	4.70	0.447	0.492	0.060	0.275
OCFA[3]	2.10[c]	1.16[a]	1.71[b]	1.61[b]	0.085	0.758	<0.001	<0.001
BCFA[4]	4.75	2.65	3.47	2.62	0.375	0.131	0.008	0.146
OBCFA[5]	6.87	3.80	5.19	4.21	0.446	0.208	0.005	0.060
BCFA:OCFA	2.23	2.30	1.99	1.69	0.137	0.010	0.415	0.206
Total FA (mg g^{-1} DM)	15.1	22.0	17.7	20.6	2.73	0.826	0.148	0.508

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

[4]Sum of octadecanoic acids.

[5]The remaining fatty acids, most of them unidentified.

[6]Odd-chain fatty acids.

[7]Branched-chain fatty acids.

[8]Odd- and branched-chain fatty acids.

interaction was observed for total FA content in effluents. Total FA content increased with starch inclusion and pH level in effluents, decreased with starch in feed residues, and was not affected in fermenters.

Incubations at high pH negatively affected total C18 FA while increased or tended to increased 16:0 proportions in all fractions. Fatty acids in feed residues were mostly affected by pH incubation level, whereas starch inclusion mainly affected those in fermenters and effluents. Higher proportions of most feed residues odd-chain fatty acids (OCFA; 13:0, 15:0) and branched-chain fatty acids (BCFA; *anteiso*-13:0, *iso*-14:0, *iso*-15:0, *anteiso*-17:0) were promoted at HpH incubations in feed residues.

Inclusion of starch led to a decrease of most odd- and branched-chain fatty acids (OBCFA) in fermenters and effluents. Conversely, an increase of total C18 FA was promoted by HS diets.

No interactions between pH incubation level and starch inclusion were observed for most FA among the three fractions. However, the interactions were or tended to be significant for some OBCFA, particularly in feed residues.

3.3.4. Feedbag residues, effluents and fermenters C18 fatty acids

To focus on the effects of incubation pH level and starch inclusion on biohydrogenation, C18 FA were expressed as percentage of total C18 FA identified. C18 FA profile of feed residues, effluents and fermenters are presented in **Tables 3.7**, **3.8** and **3.9**, respectively.

Biohydrogenation of the dietary 18:2 *n*-6 and 18:3 *n*-3 FA was promoted both by high pH and starch inclusion, as these precursors decreased in feed residues and effluents fractions. Moreover, the sum of C18:3 and C18:2 FA decreased while C18:1 intermediates increased in feed residues of HS diets and HpH incubations. Although trienoic, dienoic, and monoenoic followed the same pattern in effluents, these were only affected by pH level. Among C18:1 FA, only 18:1 *cis*-9 proportion was negatively affected by pH level and only in effluents and fermenters. Higher proportions of the main monoenoic intermediate, 18:1 *trans*-11, were observed at HpH incubations in feed residues, effluents and fermenters. However, an increase of the biohydrogenation end-product, 18:0, at HpH only occurred in effluents fraction. 18:2 *cis*-9,*trans*-11 proportion was promoted by HpH in feed residues. Most C18 FA were not affected by pH level.

Starch inclusion decreased 18:1 *trans*-11 in all fractions while increased 18:1 *trans*-10 proportions. Additionally, a similar pattern was followed by 18:2 *cis*-9,*trans*-11 and 18:2

Table 3.7. Feed residues C18 fatty acids profile (g 100 g^{-1} total C18 FA).

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
18:0	9.2	10.7	9.4	13.3	1.00	0.222	0.031	0.260
18:1 *trans*-6,-7,-8	0.345	0.302	0.749	0.637	0.0761	<0.001	0.334	0.660
18:1 *trans*-9	0.386	0.321	0.506	0.635	0.0954	0.056	0.745	0.340
18:1 *trans*-10	1.92	5.23	2.02	6.50	0.935	0.486	0.005	0.548
18:1 *trans*-11	4.9[b]	1.2[a]	10.3[c]	3.6[b]	0.56	<0.001	<0.001	0.023
18:1 *trans*-12	0.196	0.217	0.509	0.431	0.0563	<0.001	0.626	0.400
18:1 *cis*-9	33.7	35.6	29.0	39.1	2.76	0.842	0.109	0.225
18:1 *cis*-11	2.62	2.75	2.41	3.19	0.209	0.608	0.097	0.198
18:1 *cis*-12	0.532	0.439	0.553	0.780	0.0866	0.074	0.464	0.106
18:1 *cis*-13	0.220	0.206	0.193	0.263	0.0213	0.509	0.230	0.091
18:1 *cis*-14+*trans*-16	0.122	0.126	0.168	0.162	0.0508	0.285	0.967	0.889
18:1 *cis*-15	0.375[ab]	0.242[a]	0.314[a]	0.768[b]	0.1288	0.101	0.241	0.046
18:2 *trans*-9,*trans*-12	0.158	0.173	0.168	0.241	0.0416	0.386	0.334	0.511
18:2 *trans*-11,*cis*-15	2.48	1.86	2.43	2.19	0.191	0.479	0.064	0.369
18:2 oi[4]	0.97	0.94	1.04	0.99	0.069	0.404	0.597	0.834
18:2 *n*-6	33.6[b]	31.1[b]	32.2[b]	19.8[a]	1.89	0.007	0.003	0.024
18:2 *n*-3	0.287	0.164	0.288	0.233	0.0217	0.143	0.002	0.148
18:3 *n*-6	0.255	0.195	0.303	0.197	0.0384	0.519	0.069	0.556
18:3 *n*-3	4.81	3.73	4.41	2.32	0.356	0.029	0.001	0.191
18:2 *cis*-9,*trans*-11	0.352	0.122	0.773	0.276	0.0660	0.004	0.001	0.088
18:2 *trans*-10,*cis*-12	0.229	0.408	0.210	0.550	0.0399	0.151	<.0001	0.071
CLA *trans*,*trans*	1.11[a]	1.55[a]	1.09[a]	2.60[b]	0.188	0.019	<0.001	0.017
C18:1[5]	45.3	47.4	46.5	55.3	1.65	0.021	0.009	0.074
C18:2[6]	40.4	37.8	39.4	29.1	1.83	0.027	0.007	0.063
C18:3[7]	5.07	3.92	4.71	2.50	0.364	0.034	<0.001	0.177
CLA[8]	2.94[a]	3.42[a]	3.28[a]	5.72[b]	0.313	0.001	<0.001	0.009

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

[4]Sum of octadecadienoic acids eluting between 18:1 *cis*-15 and 18:2 *trans*-11,*cis*-15;

[5]Sum of octadecenoic acids.

[6]Sum of octadecadienoic acids.

[7]Sum of octadecatrienoic acids.

[8]Sum of conjugated linoleic acid isomers.

Table 3.8. Effluents C18 fatty acids profile (g 100 g^{-1} total C18 FA).

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
18:0	23.8	19.0	31.7	30.6	3.17	0.028	0.395	0.581
18:1 *trans*-6,-7,-8	0.82	0.94	1.15	1.23	0.135	0.089	0.494	0.892
18:1 *trans*-9	0.631	0.653	0.843	0.862	0.0596	0.018	0.742	0.981
18:1 *trans*-10	3.5	10.2	2.9	8.1	1.22	0.343	0.013	0.589
18:1 *trans*-11	9.2[b]	3.0[a]	21.7[c]	11.5[b]	0.82	<0.001	<0.001	0.039
18:1 *trans*-12	1.22	1.28	1.82	1.67	0.163	0.040	0.818	0.541
18:1 *cis*-9	17.8	21.2	12.0	13.8	0.61	<0.001	0.002	0.216
18:1 *cis*-11	2.39	2.40	2.25	2.47	0.148	0.822	0.479	0.531
18:1 *cis*-12	1.00[a]	0.80[a]	0.97[a]	1.44[b]	0.099	0.018	0.230	0.012
18:1 *cis*-13	0.197	0.176	0.185	0.152	0.0123	0.175	0.047	0.630
18:1 *cis*-14+*trans*-16	0.675	0.517	0.746	0.613	0.1031	0.453	0.216	0.909
18:1 *cis*-15	0.262	0.320	0.302	0.380	0.0380	0.223	0.108	0.793
18:2 *trans*-9,*trans*-12	0.240	0.129	0.370	0.213	0.0492	0.017	0.004	0.559
18:2 *trans*-11,*cis*-15	0.99	1.15	1.64	1.80	0.144	0.003	0.308	0.981
18:2oi[4]	1.98	1.38	2.47	1.74	0.121	0.005	<0.001	0.609
18:2 *n*-6	23.8	29.4	8.8	16.1	3.96	0.019	0.167	0.837
18:2 *n*-3	0.211	0.267	0.207	0.177	0.0278	0.063	0.573	0.082
18:3 *n*-6	0.315	0.536	0.303	0.187	0.1728	0.365	0.776	0.394
18:3 *n*-3	3.68	3.15	2.86	2.20	0.342	0.049	0.143	0.854
18:2 *cis*-9,*trans*-11	2.01	1.37	1.69	1.29	0.261	0.467	0.082	0.668
18:2 *trans*-10,*cis*-12	0.601	0.613	0.473	0.891	0.1285	0.591	0.177	0.197
CLA *trans*,*trans*	3.99	1.95	4.51	2.57	0.303	0.083	<0.001	0.869
C18:1[5]	37.7	41.4	44.7	42.5	1.42	0.046	0.612	0.101
C18:2[6]	34.2	36.0	20.5	25.0	3.39	0.020	0.398	0.703
C18:3[7]	3.93	3.57	3.15	2.33	0.407	0.056	0.206	0.591
CLA[8]	6.80	4.03	7.09	4.95	0.567	0.312	0.002	0.591

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

[4]Sum of octadecadienoic acids eluting between 18:1 *cis*-15 and 18:2 *trans*-11,*cis*-15;

[5]Sum of octadecenoic acids.

[6]Sum of octadecadienoic acids.

[7]Sum of octadecatrienoic acids.

[8]Sum of conjugated linoleic acid isomers.

Table 3.9. Fermenters C18 fatty acids profile (g 100 g^{-1} total C18 FA).

	Treatments[1]				SEM	*P* value		
	LSLpH	HSLpH	LSHpH	HSHpH		pH[2]	S[3]	pH*S
18:0	43.6	27.6	43.9	39.5	5.78	0.349	0.153	0.374
18:1 *trans*-6,-7,-8	1.24	1.42	1.22	1.47	0.249	0.950	0.445	0.903
18:1 *trans*-9	0.792	0.895	0.856	0.807	0.1333	0.930	0.853	0.605
18:1 *trans*-10	4.7	13.1	2.9	8.5	1.70	0.141	0.020	0.461
18:1 *trans*-11	12.7	4.0	23.0	16.7	1.83	0.002	0.012	0.535
18:1 *trans*-12	1.30	1.39	1.51	1.74	0.177	0.158	0.390	0.695
18:1 *cis*-9	11.4	17.0	8.0	10.7	1.57	0.041	0.064	0.414
18:1 *cis*-11	2.31	2.54	1.83	2.76	0.253	0.635	0.083	0.236
18:1 *cis*-12	1.17^{ab}	0.97^{a}	0.84^{a}	1.48^{b}	0.130	0.532	0.161	0.030
18:1 *cis*-13	0.257	0.246	0.250	0.196	0.0154	0.078	0.045	0.188
18:1 *cis*-14+*trans*-16	1.31	0.90	1.78	1.40	0.108	0.001	0.004	0.870
18:1 *cis*-15	0.223	0.319	0.276	0.404	0.0529	0.167	0.046	0.732
18:2 *trans*-9,*trans*-12	0.387^{b}	0.308^{ab}	0.240^{a}	0.343^{ab}	0.0358	0.167	0.746	0.043
18:2 *trans*-11,*cis*-15	0.62	0.84	1.20	2.01	0.424	0.130	0.303	0.533
18:2oi[4]	0.83	0.98	1.12	1.01	0.194	0.423	0.914	0.531
18:2 *n*-6	9.0	21.5	3.6	5.0	5.10	0.121	0.265	0.356
18:2 *n*-3	0.519	0.451	0.198	0.143	0.1882	0.074	0.637	0.961
18:3 *n*-6	0.881	0.627	0.762	0.738	0.1035	0.969	0.267	0.344
18:3 *n*-3	2.64	2.41	2.11	0.85	0.613	0.185	0.307	0.457
18:2 *cis*-9,*trans*-11	1.24	1.32	0.86	0.97	0.292	0.235	0.749	0.974
18:2 *trans*-10,*cis*-12	0.468	0.853	0.230	0.929	0.2728	0.786	0.151	0.607
CLA *trans*,*trans*	3.24	0.37	3.63	1.74	0.315	0.017	<0.001	0.146
C18:1[5]	37.4	42.8	42.3	46.1	3.12	0.269	0.228	0.810
C18:2[6]	15.7	26.6	10.9	12.6	5.44	0.177	0.324	0.457
C18:3[7]	3.51	3.04	2.88	1.59	0.644	0.204	0.262	0.570
CLA[8]	4.88	2.77	5.03	3.66	0.688	0.468	0.033	0.602

[1]Diets are named according to the starch content [low starch (LS) or high starch (HS)] and pH incubation level (pH), respectively: LSLpH = low starch diet incubated at low pH; LSHpH = low starch diet incubated at high pH; HSLpH = high starch diet incubated at low pH; HSHpH = high starch diet incubated at high pH.

[2]pH = pH level.

[3]S = starch content.

[4]Sum of octadecadienoic acids eluting between 18:1 *cis*-15 and 18:2 *trans*-11,*cis*-15;

[5]Sum of octadecenoic acids.

[6]Sum of octadecadienoic acids.

[7]Sum of octadecatrienoic acids.

[8]Sum of conjugated linoleic acid isomers.

trans-10,*cis*-12 in feed residues. Inclusion of starch promoted a decrease of 18:1 *cis*-13 in effluents and fermenters, while increased 18:1 *cis*-9 in effluents and tended to increase it (P = 0.064) in fermenters. Higher 18:0 proportions were observed in HS feed residues.

Significant pH incubation level by starch inclusion interactions were observed for 18:1 *trans*-11 in feed residues and effluents, and for 18:1 *cis*-12 in effluents and fermenters. Additionally, a significant interaction for 18:2 *n*-6 was observed in feed residues.

3.4. Discussion

In vitro studies had been extensively used throughout the years to evaluate ruminal biohydrogenation. Most *in vitro* studies were carried out using *batch* technique, but more recently continuous-culture techniques had been used. Fievez, Vlaeminck, Jenkins, Enjalbert and Doreau (2007) reviewed the use of *in vivo*, *in vitro* and *in situ* techniques to access biohydrogenation and noted that, despite the low number of publications, the continuous-culture technique seemed to simulate *in vivo* biohydrogenation. Nevertheless, factors such as dilution and feed processing must be taken into consideration when using *in vitro* systems, as they may alter lipolysis and biohydrogenation rates (Wu & Palmquist, 1991; Fellner et al., 1995).

3.4.1. Rumen fermentation parameters

Fermentation pH was maintained at LpH or HpH values by continuous infusion of buffered artificial saliva. A constant fermentation pH does not reflect biologically what occurs in the rumen ecosystem, as diurnal variations are found *in vivo*. Nevertheless, as the main objective of this study was to differentiate pH incubation level from starch inclusion effects on ruminal biohydrogenation, fermentation pH should be kept as constant as possible (AbuGhazaleh, Riley, Thies & Jenkins, 2005). Buffered artificial saliva was continuously infused into the fermentation vessels; however, differences occurred in fermenters and outflow pH. Although infused saliva pH level had the most marked effect, pH decreased with incubation of HS diets both in fermenters (-0.19 units) and in effluents (-0.15 units).

It is well recognized that animals fed high-concentrate diets have lower ruminal pH, lower acetate and acetate to propionate ratio, and higher propionate than those fed high-forage diets.

Our results did not follow the expected pattern, as both propionate and acetate proportions decreased with HS diets. High starch diets promote the growth of amylolytic bacteria, which usually produce high amounts of propionate (Hungate, 1966). Nevertheless, Strobel and Russell (1986) found that mixed ruminal bacteria incubated *in vitro* with soluble starch decreased both acetate and propionate concentration at pH 6.0 compared to pH 6.7, while lactate concentration increased. Lactate is a strong acid that can be an intermediate from the conversion from starch to propionate (Hungate, 1966), and which accumulation can lead to low ruminal pH (Burrin & Britton, 1986). Large amounts of lactate can accumulate in the rumen when animals abruptly change from forage based diets to high-concentrate diets (Lana, Russell & Van Amburgh, 1998). In fact, incubation of HS diets with rumen fluid from exclusively forage fed animals, may have led to a shift in the ruminal micropopulation towards to an increase of the lactate-producing bacteria instead to an increased of the propionate-producing bacteria.

Ruminal bacteria respond to an increased availability of fermentable substrates, such as starch, by increasing growth rates and fermentative activities. A significant interaction was observed in VFA, HpH promoting a higher increase in HS diets reflecting the higher fermentability of these diets.

In the present experiment, an interaction was observed in N-NH_3 concentration, increasing in LS diets concentration but particularly at HpH incubation level. Lower ruminal concentrations of N-NH_3 were observed with increased fermentable carbohydrates levels *in vivo* (Overton, Cameron, Elliot, Clark & Nelson, 1995; Surber & Bowman, 1998) and in RUSITEC (Abel, Coenen & Immig, 1990). A decrease in N-NH_3 concentration may be explained by higher organic matter fermentation in the rumen, which led to more energy for the utilization of N-NH_3 in microbial protein synthesis (Overton et al., 1995). According to Satter and Slyter (1974), a minimum ruminal concentration of 50 mg N-NH_3 L^{-1} is required to maximize microbial protein synthesis. Although N-NH_3 was determined in effluents, its concentration from LS fermentation was above the recommended in the rumen, while that from HS diets was nearly half. Nevertheless, CP content of HS diets were not expected to limit microbial biohydrogenating activity. Gerson, John and Sinclair (1983) determined lipolysis and biohydrogenation rates of diets high in starch (30 to 50% DM basis) and containing different levels of N *in vivo*, and found the rates of lipolysis and biohydrogenation to increase in diets with dietary N concentrations between 1.2 and 2.5%. However, at higher N concentrations, between 2.5 and 3.7% N, lipolysis rate was not further increased and biohydrogenation rate

appeared to decrease. High starch and LS diets used in the present study contained 1.98% and 3.65% N, respectively.

3.4.2. Total fatty acids content

Total FA content of feed residues was much higher than that in effluents or fermenters. This difference may be explained by the high FA concentration of the experimental diets incubated in the feedbags. Moreover, bacterial population associated feed articles, i.e., solid associated bacteria (SAB), have higher FA content than liquid associated bacteria (LAB; Bauchart, Legay-Carmier, Doreau & Gaillard, 1990; Bessa et al., 2000), freely distributed across effluents and fermenters. Residues from HS diets presented lower FA content than LS ones, most probably reflecting the fat content differences in HS and LS substrates. In fact, starch was virtually fat free and replaced lucerne hay DM by 50% in HS feeds. Although the lower fat content of HS substrates and total FA concentration in HS feed residues compared to those of LS, total FA content of the liquid outflow was higher in HSHpH treatment, whereas no differences were observed among treatments in fermenters. Additionally, differences in FA content among feed residues, effluents and fermenters could be due to differences in bacterial composition (Vlaeminck, Fievez, Cabrita, Fonseca & Dewhurst, 2006). Higher proportion of Gram-negative bacteria would result in higher FA content, as bacterial FA are markedly associated with bacterial cell wall.

3.4.3. Fatty acid profile

Most worth noting effects of pH and starch on FA profile were on OBCFA. Vlaeminck et al. (2005) proposed the use of OBCFA as potential microbial markers in rumen contents, as these FA are mainly formed by ruminal microflora and virtually inexistent in feeds (Diedrich & Henschel, 1990; Kim, Sanderson, Dhanoa & Dewhurst, 2005). Although no attempt was made to isolate bacterial fractions, no OBCFA traces were found in the experimental diets, which strongly suggest that all OBCFA of residues, effluents, and fermenters had microbial origin.

Lower proportions of BCFA were observed in residues, effluents, and fermenters of HS diets, and of OCFA in effluents and fermenters. Differences in BCFA and OCFA proportions with

starch inclusion might be a reflection of cellulolytic and amylolytic population shifts. In fact, ruminal cellulolytic bacteria contain higher amounts of BCFA whereas amylolytic bacteria are richer in OCFA (Viviani, 1970; Minato, Ishibashi & Hamaoka, 1988; Vlaeminck, Fievez, Demeyer & Dewhurst, 2006). The replacement of 50% (DM basis) of lucerne hay by soluble starch in HS diets reduced the NDF content by nearly half and, most probably, decreased cellulolytic population (Weimer, Waghorn, Odt & Mertens, 1999). An increase of the amylolytic bacteria was also expected in HS diets (Weimer et al., 1999), however proportions of OCFA did not followed the expected, decreasing in effluents and fermenters, and not being affected in residues.

Odd- and branched-chain fatty acids profile in the rumen is mainly determined by the availability of precursors (Ifkovitz & Ragheb, 1968; Vlaeminck et al., 2006a). Branched-chain FA are formed using isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA as primers whereas OCFA are formed by using proponyl-CoA (Fulco, 1993; Kaneda, 1991). The lower proportions of BCFA observed in all fractions of HS incubations might be suggested to be related to the lesser availability of primers in these diets. In fact, HS diets had lower CP content of HS diets. However, molar proportions of isobutyrate and isovalerate were not affected by starch inclusion while those of acetate and propionate decreased.

Low ruminal pH levels are known to inhibit the growth of some bacteria, exhibiting stronger effects on the cellulolytic population which might cease growth at pH values below pH 6.0 (Russell & Dombrowski, 1980; Palmonari, Stevenson, Mertens, Cruywagen & Weimer, 2010). Fiber digestion requires bacterial adhesion to facilitate its hydrolysis prior to the fermentation of the carbohydrate oligomers (Weimer, 1996). Thus, reduced ability to adhere cellulose or inhibition of catabolism of hydrolytic products at low pH levels, can lead to lower cellulolytic populations. In the present study, a decrease of BCFA and of BCFA to OCFA ratio were observed in residues of LpH incubations, which may reflect a negative effect on cellulolytic bacterial population. An increased of the BCFA to OCFA ratio was observed in fermenters and tended (P = 0.093) to occur in effluents of LpH incubations. Differences between residues and fermenters OBCFA profiles suggest differences of the microbial population associated to those fractions. In fact, fermenters liquid are expected to reflect mostly soluble-substrate user bacteria while the population associated with residues is mostly cellulose-degrading bacteria (Legay-Carmier & Bauchart, 1989).

Additionally to changes of OBCFA profiles and ratio, proportion of 16:0 increased while C18 FA decreased in all fractions of HpH incubations, compared to LpH.

3.4.4. Biohydrogenation intermediates

Proportions of C18 FA biohydrogenation intermediates were mainly affected in residues and effluents by starch inclusion and pH level, respectively. Conversely, most C18 FAs of fermenters were not affected by any factor or their interaction.

Biohydrogenation of the main C18 FA precursors, 18:2 *n*-6 and 18:3 *n*-3, was promoted at HpH incubations as their proportions decreased in residues and effluents, not being affected in fermenters however. Additionally, C18:3 and C18:2 biohydrogenation intermediates decreased while C18:1 FA increased in residues and effluents. The main octadecaenoic intermediate, 18:1 *trans*-11 proportion increased in all fractions at HpH incubations, compared to diets incubated at LpH. Incubation pH level by starch inclusion interaction was significant in residues and effluents. Starch inclusion decreased 18:1 *trans*-11 proportion at HpH incubation more strongly than at LpH. Although biohydrogenation was promoted in incubations at HpH, the proportion of end-product 18:0 was not affected in residues and fermenters, while increased in effluents. The results from the present experiment suggest a higher biohydrogenation activity in incubations at HpH. Similarly, an inhibition of PUFA biohydrogenation by low ruminal pH had been reported in several studies (Latham et al., 1972; Van Nevel & Demeyer, 1996; Martin & Jenkins, 2002; Wang, Song, Son & Chang, 2002; Troegeler-Meynadier, Nicot, Bayourthe, Moncoulon & Enjalbert, 2003; Wang & Song, 2003). pH also influenced negatively lipolysis, in fact more markedly than biohydrogenation (Van Nevel & Demeyer, 1996). Lipolytic activity was significantly inhibited at pH 6.0 whereas biohydrogenation was preferentially inhibited below pH 5.2. Although lipolysis was not determined in the present experiment, at an average fermentation pH of 6.03 in LSLpH and of 5.81 in HSLpH, an inhibition of the lipolytic activity might have occurred and consequently inhibited biohydrogenation, as the presence of a free carboxyl group is an absolute requirement (Hawke & Silcock, 1970).

Unlike most monoenoic FA, 18:1 *cis*-9 proportion in effluents and fermenters decreased at HpH incubations No effect was observed in residues, however. Mosley, Powell, Riley and Jenkins (2002) showed *in vitro* that 18:1 *cis*-9 was biohydrogenated by mixed ruminal bacteria to multiple C18:1 FA, ranging from 18:1 *trans*-6 to 18:1 *trans*-16, and to 18:0. Inter-conversion among C18:1 *trans* FA were also shown to occur (Proell, Mosley, Powell & Jenkins, 2002). Nevertheless, AbuGhazaleh et al. (2005) found a decreased in the isomerization of 18:1 *cis*-9 to most C18:1 *trans* FA whereas an enrichment of 18:0 when

incubation pH was reduced from 6.5 to 5.5. A marked effect of pH was observed in 18:2 *cis*-9,*trans*-11 in residues, with HpH presenting higher proportions. However, no effects were observed in effluents and fermenters. Formation of 18:2 *cis*-9,*trans*-11 is the first step of 18:2 *n*-6 isomerization (Kepler, Tucker & Tove, 1967) by linoleate *cis*-12,*trans*-11 isomerase action (Kepler, Tucker & Tove, 1970). Besides biohydrogenation inhibition of C18 FA precursors, LpH may had a direct effect on isomerase activity, as this enzyme optimal pH ranged between 7.0 and 7.2 (Kepler & Tove, 1967). Although inhibition of biohydrogenation occurred at LpH incubations its pathway was not altered, being predominantly *via* formation of 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11.

Starch inclusion promoted the biohydrogenation of C18 FA precursors, as 18:2 *n*-6 and 18:3 *n*-3 proportions decreased in residues. Similarly, C18:3 and C18:2 biohydrogenation intermediates decreased while C18:1 FA increased in residues of HS diets. However, no effect was observed on effluents and fermenters. The end-product 18:0 only increased in residues of HS diets, compared to those of LS. Results suggest a promotion of C18 FA biohydrogenation in residues of HS diets, but not in liquid fractions. Differences among fractions may be related to differences of the microbial population associated. Solid associated bacteria comprise most bacterial population with biohydrogenating activity and are in close association with residues. On the other hand, LAB comprises most bacterial population of effluents and fermenters, and is considered to play a smaller role on C18 FA biohydrogenation, compared to SAB (Bauchart et al., 1990). A more complete biohydrogenation may be related to an increase of the microbial DM production (Loor, Hoover, Miller-Webster, Herbein & Polan, 2003) and the number of viable bacteria (Wang & Song, 2001) with starch supplementation.

In the present study, fermentations of HS diets changed the biohydrogenation pattern. The dienoic intermediate 18:2 *cis*-9,*trans*-11 decreased in residues and tended (P = 0.082) to decrease in effluents, while 18:2 *trans*-10,*cis*-12 increased in residues. Moreover, 18:1 *trans*-11 decreased in residues, effluents and fermenters of HS incubations, whereas 18:1 *trans*-10 increased in all fractions. In fact, 18:1 *trans*-10 was the main monoenoic intermediate found in HS residues and HSLpH fermenters. Concentrations of 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 are known to increase in products of animals fed high cereal diets supplemented with oils (Griinari et al., 1998; Piperova et al., 2000; Jurjanz, Monteils, Juaneda & Laurent, 2004). A pathway for 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 formation from 18:2 *n*-6 was proposed (Griinari & Bauman, 1999). Once having the same precursor, increased 18:2 *n*-6 isomerisation towards 18:2 *trans*-10,*cis*-12 will lead to less 18:2 *cis*-9,*trans*-11 formation. However, CLA isomers 18:2 *cis*-9,*trans*-11 and 18:2 *trans*-10,*cis*-12 isomers are synthesized

by two different mechanisms (Wallace, McKain, Shingfield & Devillard, 2007). Isomerisation of 18:2 *trans*-10,*cis*-12 occurs by an ionic reaction which does not involve an exchange with water, while 18:2 *cis*-9,*trans*-11 formation is based on a radical intermediate enzyme type and involves a hydrogen abstraction from water. A rapid hydrogenation of 18:2 *trans*-10,*cis*-12 to 18:1 *trans*-10 was proposed by Loor et al. (2003) to explain the high outputs of 18:1 *trans*-10 at low outputs of 18:2 *trans*-10,*cis*-12. An alternative explanation may be the direct isomerization of 18:1 *cis*-9 (Mosley et al., 2002) or 18:1 *trans*-11 (Mosley & McGuire, 2008) to 18:1 *trans*-10, and the conversion between *trans* C18:1 FA (Proell et al., 2002).

High starch diets decreased the proportion of 18:2 *n*-3 in residues, compared to LS residues. Harfoot (1978) proposed the occurrence of 18:2 *n*-3 as intermediate of 18:3 *n*-3 biohydrogenation based on findings of Kemp, White and Lander (1975) that 18:1 *cis*-15 was the major end-product of *Fusocillus* strain. Recently, Alves and Bessa (2007) identified this minor intermediate in fat from ruminants fed edible vegetable oils.

Starch inclusion almost had no effect on the extent of biohydrogenation but an extremely marked effect was observed on biohydrogenation pattern by increasing 18:1 *trans*-10. Outputs of 18:1 *trans*-10 and or 18:2 *trans*-10,*cis*-12 were previously reported to be sensitive to low pH (Loor et al., 2003; Choi et al., 2005). Jurjanz et al. (2004) reported an increased proportion of 18:1 *trans*-10 in milk fat when cows were fed a rapidly fermentable starch, compared to slowly fermentable starch, concluding that the higher drop of ruminal pH was the main cause for 18:1 *trans*-10 production. Similarly, Fuentes, Calsamiglia, Cardozo and Vlaeminck (2009) found that pH, but not forage to concentrate ratio, was the main cause for 18:1 *trans*-10 and 18:2 *trans*-10,*cis*-12 accumulation in effluents of a dual-flow continuous culture. The results from the present experiment suggest that the *trans*-10 shift which occurred during a 15 day adaptation period of the ruminal microbiota was caused by the presence of starch rather than incubation pH. This contrasts with the results of Choi et al. (2005), who found that lowering pH in a 6 h incubation caused the isomers of CLA to switch from 18:2 *cis*-9,*trans*-11 to 18:2 *trans*-10,*cis*-12. To our knowledge, this is the first report that clearly identifies the *trans*-10 shift to a direct effect of starch inclusion, independently of pH level.

Starch presumably enriches, over time, bacteria that form 18:2 *trans*-10,*cis*-12. In fact, shift in microbial populations have been reported, with severe reductions in *Butyrivibrio* population whereas increases in *Megasphaera elsdenii* (formerly *Peptostreptococcus elsdenii*) and *Lactobacillus* in animals fed high-concentrate diets compared to those fed low-concentrate diets (Latham et al, 1972; Wolstrup, Jensen & Jensen 1974). The adaptation of the ruminal microorganisms to a high-starch diet involves an increase of the bacterial lactate-utilizing

populations (Nagaraja & Titgemeyer, 2007), being *Megasphaera. elsdenii* one of the most important (Counotte, Prins, Janssen & Debie, 1981). Kim, Liu, Rychlik & Russell (2002) demonstrated, *in vitro*, that *M. elsdenii* YJ-4, a strain isolated from the rumen contents of cows fed a 90% corn diet, produced more 18:2 *trans*-10,*cis*-12 from 18:2 *n*-6 than the control, although other bacterium may be involved. In a recent study, Maia, Chaudhary, Figueres and Wallace (2007) found no formation of 18:2 *trans*-10,*cis*-12 when used the same strain of *M. elsdenii* as used by Kim et al. (2002), but observed the formation of 18:2 *trans*-10,*cis*-12 from 18:2 *n*-6 biohydrogenation by *Propioniobacterium acnes*. Although *P. acnes* and *Propionibacterium freudenreichii* produced 18:2 *trans*-10,*cis*-12 from 18:2 *n*-6 (McKain, Shingfield & Wallace, 2010), these species occur in low numbers in the rumen (Verhulst, Janssen, Parmentier & Eyssen, 1987; Jiang, Bjorck & Fonden, 1998).

Biohydrogenation has been mainly associated with rumen solids (Harfoot, Noble & Moore, 1973; Bauchart et al., 1990), particularly in oil supplemented diets as the preferential adhesion of lipids to the particulate matter may result in an uneven distribution of the biohydrogenating bacteria in the rumen (Ward, Scott & Dawson, 1964). Nevertheless, in the present experiment biohydrogenating activity was found in feed residues, fermenters and effluents. These results suggest that bacterial population associated with each fraction, namely SAB in residues and LAB in fermenters and effluents, exhibit biohydrogenation activity. Boeckaert et al. (2009) added equal amounts of FA to the LAB and SAB fractions and observed similar disappearances, yet differences in the biohydrogenation occurred with LAB fraction containing higher C18:1 FA and lower 18:0 than SAB. The authors suggested that bacteria involved in the last step of biohydrogenation were largely absent in the LAB fraction. Although no attempt was made to isolate bacterial fractions in the present experiment, a worth noting difference was observed among fractions which might be a reflex of distinct associated bacterial populations. The proportion of C18:1 intermediates from C18 FA biohydrogenation, i.e., all monoenoic acids except 18:1 *cis*-9 and 18:1 *cis*-11, is much higher in fermenters and effluents than in residues, being on average 28.0, 23,0 and 11.5, respectively. Additionally, proportions of 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 in fermenters and effluents was *ca.* two folds higher than in residues. Similarly, Kim et al. (2002) found higher production of 18:2 *trans*-10,*cis*-12 in LAB after incubation with 18:2 *n*-6, compared to SAB. Furthermore, Vlaeminck et al. (2006b) reported an increase of the 18:1 *trans*-10 to 18:1 *trans*-11 ratio in LAB and SAB fractions, being the increase 3.4 times higher in LAB than in SAB. These findings suggest that 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 producing bacteria may be preferentially in the LAB fraction than in SAB.

3.5. Conclusions

Biohydrogenation was promoted in incubations at HpH, with dietary precursors being extensively hydrogenated to 18:1 *trans*-11 in residues, effluents and fermenters, and to 18:0 in effluents. The inhibition of biohydrogenation at low pH might have been caused by an inhibition of lipolysis or a decrease of the cellulolytic population and/or activity, compared to HpH. In fact, *Butyrivibrio fibrisolvens*, a ruminal cellulolytic bacterium, is one of the major biohydrogenating bacteria in the rumen ecosystem. Although biohydrogenation was affected by pH level, the main pathway was not altered with intermediates being preferentially formed *via* 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11 production.

Starch inclusion promoted an increase of biohydrogenation activity in residues, but not on effluents and fermenters. Most marked effect of starch was on biohydrogenation pattern. High starch diets decreased 18:2 *cis*-9,*trans*-11 while 18:2 *trans*-10,*cis*-12 increased in residues. Moreover, 18:1 *trans*-11 proportion decreased whereas 18:1 *trans*-10 increased in all fractions of HS incubations, compared to LS. In fact, results suggest that biohydrogenation shift towards *trans*-10 was a direct effect of starch inclusion, independently of pH level. The *trans*-10 shift was most probably related to changes in the rumen microbial population. Starch presumably enriches, over time, bacteria that form 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10. Increased *Megasphaera elsdenii* and *Propionibacterium acnes* and decreased *Butyrivibrio* populations were observed in animals are fed high-concentrate diets, compared to those fed low-concentrate diets. fed high-starch diets and these bacteria were recently shown to produce 18:2 *trans*-10,*cis*-12. In fact, the lower proportions of 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11 with HS diets might be due to lower *B. fibrisolvens* numbers or activity, as fibre content was half of LS diets.

Biohydrogenation activity was detected in feed residues, fermenters and effluents. However, C18 FA profiles differed among fractions. Higher proportion of C18:1 intermediates were observed in fermenters and effluents. Moreover, 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 in fermenters and effluents nearly double the proportions in residues. Thus, 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 producing bacteria was suggested to be preferentially in the LAB fraction rather than in SAB.

3.6. References

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Chapter 4

Dietary nitrogen source and soybean oil supplementation effects on fatty acid profiles of rumen contents and bacterial fractions

4.1. Introduction

Dietary unsaturated fatty acids (FA) are extensively converted into more saturated FA in the rumen by biohydrogenation. α-Linolenic acid (18:3 *n*-3) and linoleic acid (18:2 *n*-6) are the main polyunsaturated FA (PUFA) present in ruminant feed lipids (Morand-Fehr & Tran, 2001). During biohydrogenation, 18:3 *n*-3 and 18:2 *n*-6 are metabolised to a range of trienoic and dienoic acid intermediates, mainly non-conjugated and conjugated linoleic acid (CLA) isomers, and *trans*-octadecenoates, particularly vaccenic acid (18:1 *trans*-11), being stearic acid (18:0) the end-product (Harfoot & Hazlewood, 1997). The CLA have been shown to exert health-promoting and disease preventing effects in animal and human studies (Bhattacharya, Banu, Rahman, Causey & Fernandes, 2006; Park, 2009), being the stronger effects observed in animal studies, however. Although *trans* FA have been associated with cardiovascular disease and cholesterol (Williams, 2000; Givens, 2005), 18:1 *trans*-11 was recognised as beneficial to human health. 18:1 *trans*-11 is endogenously converted by Δ9-desaturase in tissues to 18:2 *cis*-9,*trans*-11 (Corl, Barbano, Bauman & Ip, 2003; Kay, Mackle, Auldist, Thomson & Bauman, 2004), a CLA isomer with anticarcinogenic properties (Parodi, 1997). Rumenic acid has been proposed as the common name for 18:2 *cis*-9,*trans*-11 (Kramer et al., 1998), as it is the main CLA isomer present in ruminant meat and milk fat (Chin, Liu, Storkson, Ha & Pariza, 1992; Parodi, 1997).

Biohydrogenation is carried out by microbial activity, mostly bacterial, and its extent and pattern of FA intermediates formed are strongly determined by dietary factors. Indeed, modifications have been reported with pH (Van Nevel & Demeyer, 1996), ionophores (Fellner, Sauer & Kramer, 1997), carbohydrate type and level (Latham, Storry & Sharpe, 1972; Leat, 1977; Gerson, John & King, 1985), lipid supplementation (Harfoot & Hazlewood, 1997; Glasser, Ferlay & Chilliard, 2008; Jenkins; Wallace, Moate & Mosley, 2008), and nitrogen level (Gerson, John, Shelton & Sinclair, 1982; Gerson, John & Sinclair, 1983). Changes induced by diet alter the predominant ruminal biohydrogenation pathways resulting in different FA profiles of digesta. However, these modifications reflect not only changes in biohydrogenation extent and intermediate profiles, but also changes in bacterial FA synthesis and, ultimately, in modifications of microbial populations in the rumen. Microbial FA have been used in systematic (Moss, 1981; Kaneda, 1991) and ecological studies (Olsson, 1999). Recently, research has focused on the use of odd- and branched-chain fatty acids (OBCFA) as biomarkers in rumen contents and milk (Vlaeminck, Fievez, Cabrita, Fonseca & Dewhurst, 2006; Bessa et al., 2009).

Oil supplementation effects on ruminal fermentation and biohydrogenation in particular, have been widely studied. Additionally, indirect evidences have suggested the putative effect of nitrogen source, non-protein nitrogen or true protein, on microbial FA composition (Cabrita, Fonseca, Dewhurst & Gomes, 2003). As far as we know, no study reported the direct effects of nitrogen source, or the nitrogen source by oil supplementation interaction, on the pattern of biohydrogenation intermediates and on FA composition of bacterial fractions in the rumen, therefore the present experiment was designed to address these issues.

4.2. Materials and methods

4.2.1. Animals and diets

Animal handling followed the EU directive number 86/609/EEC concerning animal care. Four white Merino rams aged 30 (± 4.0) months old, with an average live weight of 49 (± 2.0) kg and fitted with rumen cannula (2.5 cm internal diameter) were used in a 4 x 4 Latin square with a 2 x 2 factorial arrangement. Each experimental period lasted for 31 days and included a 15 days adaptation period to diets, a 9 days period for digestibility trial, and a 7 days period for rumen fluid collection (**Figure 4.1**).

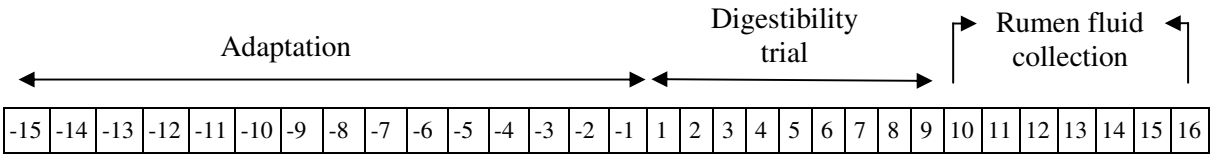

Figure 4.1. Schematic representation of each experimental period.

Rams were kept in individual stalls with free access to water and mineral licking blocks (SC Sheep Rockies, Rockies, Winsford, Cheshire, UK) containing (per kg) 10,000 mg Ca, 10,000 mg P, 5,000 mg Mg, 150 mg Co, 250 mg I, 500 mg Mn, 1,000 mg Zn, 42 mg Se, and 380,000 mg Na. Animals were fed 1 kg of a mixed diet comprised of 60% oat hay (32.4 g CP kg^{-1} and 736 g NDFom kg^{-1}; DM basis) and 40% of an experimental concentrate, in two equal meals at 09:30 and 16:30 h. To the morning feed were added 5 g of a multivitamin and mineral complex (Pecutrin, Bayer, Portugal) containing (per kg) 300,000 IU vitamin A, 50,000 IU vitamin D_3, 100 mg vitamin E, 196,000 mg P, 255,000 mg Ca, 12,000 mg Mg, 24,000 mg Na, 2,400 mg Zn, 1,000 mg Fe, 1,000 mg Mn, 600 mg Cu, 30 mg I, 30 mg Co, 10 mg Mo, and 10 mg Se.

Two experimental concentrates were formulated to be isonitrogenous, only differing in the main nitrogen source, urea or sunflower meal. The concentrates were prepared as a single batch with an experimental blender, split into two equal portions and 13% soybean oil added to one portion. The experimental diets thereby obtained were: i) oat hay plus urea concentrate (U); ii) oat hay plus urea concentrate with soybean oil (UO); iii) oat hay plus sunflower meal concentrate (SM); and iv) oat hay plus sunflower meal concentrate with soybean oil (SMO). The ingredient composition of the experimental diets is presented in **Table 4.1**.

Table 4.1. Ingredient composition of the experimental diets[1] (g kg^{-1}).

	U	UO	SM	SMO
Wheat	48	42	80	70
Wheat bran	252	219	-	-
Sunflower meal 28%	-	-	320	278
Oat hay	678	668	600	600
Urea	20	17	-	-
Sodium sulphate	2	2	-	-
Soybean oil[2]	-	52	-	52

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]Ibersoja®, Copaz, Lisboa, Portugal.

4.2.2. Sample collection

On the first day of the digestibility trial in each experimental period (**Figure 4.1**), sheep were moved to metabolic cages before morning feed. From day 1 to day 7 of the collection period, 10% samples of experimental diets were collected. At the end of the animal study, experimental diet samples were pooled per animal and per period, and ground in a hammer mill with a 1 mm sieve for further chemical analysis. Total faeces were quantified from day 3 to day 9 of the collection period. Faeces were collected daily in bags fixed to sheep by harness, weighted and 10% samples taken and kept at -20 ºC. At the end of the period all aliquots were pooled resulting in one sample per sheep per period. Faecal samples were freeze-dried and milled through 1 mm sieve for further analysis. After the end of the digestibility trial, sheep were moved from metabolic cages to individual stalls.

4.2.2.1. Total rumen contents collection and fractionation into bacterial pellets

Total rumen contents (TRC) were sampled from each ram by vacuum pump before morning feed on days 10 and 16 of the collection period. At each sampling *ca.* 1.5 L of TRC were collected from each sheep, pH was immediately recorded, and one aliquot was freeze-dried for further analysis. The remained TRC were further fractionated to obtain bacterial fractions (**Figure 4.2**), according to the procedure of Martín-Orué, Balcells, Zakraoui and Castrillo (1998), as described in detail below.

One litre of TRC was strained through eight layers of linen cloth and the solid residue obtained re-suspended in two times its weight of saline solution (NaCl 0.85%) at 39 ºC. The suspension was strained again through eight layers of linen cloth and the liquid phase from both filtrations pooled and centrifuged at 500 *g* for 5 min at 4 ºC. Liquid associated bacteria (LAB) were obtained by centrifugation of the supernatant at 20,000 *g* for 20 min at 4 ºC followed by two consecutive washes with saline solution and re-centrifugations. The filtered particles were re-suspended in three times its weight of saline solution with 1% methylcellulose at 39 ºC (Martín-Orué et al., 1998), vigorously hand-shaken for 2 min and incubated at 39 ºC for 15 min. The suspension was then kept at 4 ºC for 24 h and then homogenised six times for 30 s with a Waring blender (Waring Products Division, New Hartford, CT, USA). The suspension was strained through eight layers of linen cloth and the solid residue re-suspended in 300 mL of saline solution. The suspension was strained, the

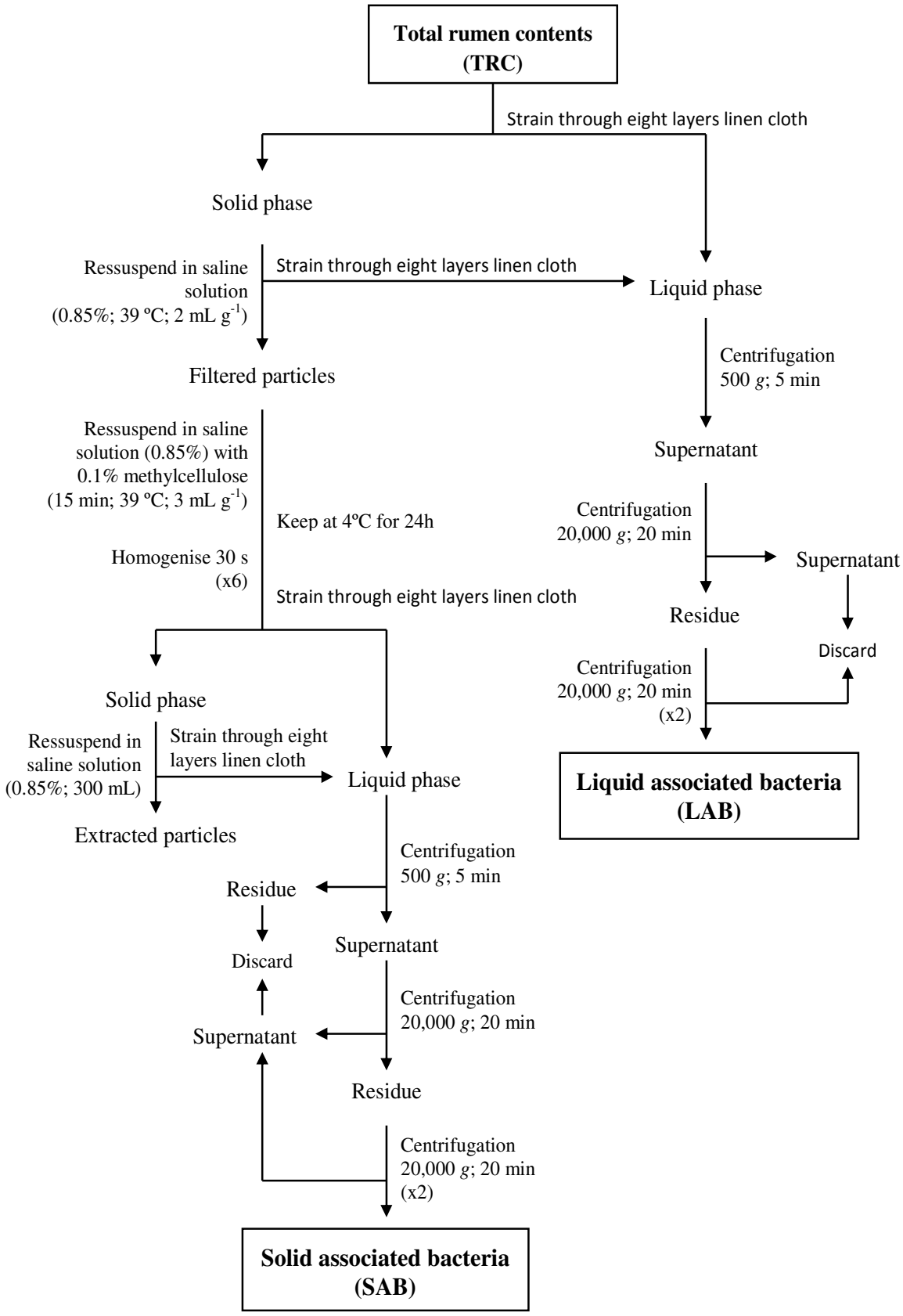

Figure 4.2. Schematic representation of the procedures used to fraction total rumen contents into liquid and solid associated bacterial fractions. Adapted from Martín-Orué et al. (1998).

liquid phase from both filtrations pooled, and the solid associated bacteria (SAB) obtained by differential centrifugation, as previously described for LAB. All samples were freeze-dried and kept at -20 ºC until subsequent analysis.

4.2.3. Chemical analysis

Composite samples of experimental diets and faeces were ground at 1 mm and chemically analysed. All samples were analysed for dry matter (DM; NP 875, 1993), organic matter (OM; NP 872, 1983), and neutral detergent fibre (NDF). Neutral detergent fibre was determined according to procedures of Van Soest, Robertson and Lewis (1991) and Robertson and Van Soest (1981), without addition of α-amylase or sodium sulphite, and expressed without residual ash (NDFom). Dietary samples were further analysed for total nitrogen (N) by the macro-Kjedhall method (NP 2030, 1996). Crude protein (CP) was calculated as N x 6.25. Acid detergent fibre and acid detergent lignin (ADL) were determined by detergent procedures (Robertson & Van Soest, 1981; Van Soest et al., 1991), and the acid detergent fibre expressed without residual ash (ADFom). Ether extract (EE) was determined by extraction with petroleum ether (NP 876, 2001) using a Soxtec® system HT2 unit (Foss Tecator, Sweden). Calcium and Mg were determined by atomic absorption after mineralization in an atomic absorption (NP EN ISO 6869, 2007) flame spectrophotometer model AA-680 (Shimadzu, Japan).

pH was determined in rumen contents immediately after collection by a 744 pH metter (Metrohm, Switzerland) with a pH combination electrode (Metrohm, Switzerland). Volatile fatty acids (VFA) were determined in 1.25 mL strained rumen fluid after addition of 0.25 mL ortophosphoric acid solution (25%) and centrifugation at 15,000 *g* for 10 min at 4 ºC. The supernatant was analysed by gas chromatography using a gas chromatograph HP6890 series (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and a semi-capillary column (MN 116; Permabond-FFAP, Macherey-Nagel GmbH & Co. KG, Düren, Germany) with 50 m, 0.25 mm internal diameter and 0.25 μm film thickness. Helium was the carrier gas and the split ratio was 50:1. The injector temperature was 230 ºC and the detector temperature was 280 ºC. Column initial temperature of 110 ºC was held for 1 min, increased to 170 ºC at a rate of 6 Cº/min and held for 1 min and then increased to 230 ºC at a rate of 15 Cº/min and held for 15 min. Volatile fatty acids were identified by comparison with retention times of known standards (Sigma-Aldrich Inc., St. Louis, MO, USA) and quantified by external standard calibration.

4.2.4. Fatty acid analysis

Fatty acids were extracted as proposed by Folch, Lees and Stanley (1957) with some changes. Trichloromethane was replaced by dichloromethane and ultrasonic homogenization was used. Total rumen contents and microbial fractions were acidified before extraction (Fellner, Sauer & Kramer, 1995) to recover non-esterified FA from soaps. A basic methylation followed by an acidic methylation was carried out according to Kramer et al. (1997) to prevent the isomerisation of conjugated dienes.

To 200 mg of dried samples were added 2 mL of ultra-pure water and 1 mL of 6 N hydrochloric acid. The mixture was vortexed for 1 min at low speed. Six millilitres of dichloromethane:methanol (2:1) solution was added, the mixture was vortexed for 30 s at medium speed, sonicated for 6 min at 25 ºC and centrifuged at 1,500 rpm for 5 min at room temperature. The dichloromethane phase was transferred to a new tube and 6 mL of dichloromethane:methanol (2:1) solution was added to the tube with the aqueous phase. The mixture was vortexed for 30 s at medium speed and centrifuged at 1,500 rpm for 5 min. The dichloromethane fractions were pooled and 3 mL of 0.8% potassium chloride solution added. After vortexing the mixture for 30 s at high speed, it was centrifuged at 1,500 rpm for 10 min. The aqueous phase was discarded and the dichloromethane fraction passed by a phase separator filter (PS1; Whatman International Ltd., Kent, UK) and took to dryness under N_2 flux at 37 ºC. To the dried lipid extracts were added 0.5 mL of 2 mg mL^{-1} tricosanoic acid (23:0, internal standard) in n-hexane and 2 mL of 2 N sodium methoxide in methanol. The mixture was vortexed at low speed for 30 s and placed in a water-bath at 50 ºC for 10 min. Four millilitres of 3 N hydrochloric acid in methanol was added, the mixture vortexed at low speed for 30 s and taken to a water-bath at 80 ºC for 10 min. When solvent escaped, 2 mL hexane was added after cooling and tubes returned to the water-bath in order to ensure a complete methylation. When tubes were at room temperature, 1.5 mL ultra pure water and 2 mL hexane (3 mL for feed samples) were added, the mixture vortexed for 30 s at high speed and allowed to rest for 2 min. The hexane fraction containing the FA methyl esters was transferred to a gas chromatography vial, flushed with N_2 and immediately capped to prevent loss of highly volatile methyl esters, and kept at -20 ºC until analysis.

Fatty acid methyl esters were analysed using a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector and using a fused silica capillary column (CP-Sil 88; Chrompack, Varian Inc., Walnut Creek, CA, USA) with 100 m, 0.25 mm internal diameter and 0.20 μm film thickness. Helium was used as carrier gas

and the injector and detector temperatures were 280 and 260 ºC, respectively. The split ratio was 50:1 and the injection volume of 1 µl. Column initial temperature of 100 ºC was held for 15 min, increased to 150 ºC at a rate of 10 Cº/min and held for 5 min, then increased to 158 ºC at a rate of 1 Cº/min and held for 30 min, increased to at a rate of 1 Cº/min to 200 ºC and held for 15 min, and finally increased to 210 ºC at a rate of 10 Cº/min and held for 5 min. Fatty acids were identified by comparison with retention times of standards obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA) and Matreya Inc. (Pleasant Gap, PA, USA). Structural analyses of some unknown peaks were conducted by gas chromatography-tandem mass spectrometry technique using a Varian Saturn 2000 system (Varian Inc., Walnut Creek, CA, USA) equipped with a CP-Sil 88 capillary column.

4.2.5. Statistical analysis

Dietary chemical composition and *in vivo* digestibility data were analysed by repeated measures analysis of variance using the MIXED procedure of SAS (2001) with period of sampling as repeated measurement. The model included the fixed effect of diet and the random residual error. Fermentative parameters, TRC, LAB, and SAB data were analysed by repeated measures analysis of variance using the MIXED procedure of SAS (2001) with day within animal as repeated measurement. The model included the fixed effects of period, nitrogen source, oil supplementation, the interaction between nitrogen source and oil supplementation, the random effect of animal and the random residual error.

The most desirable covariance structure was determined according to the Akaike's information criterion and Schwarz's Bayesian criterion (Littell, Henry & Ammerman, 1998). Compound symmetry was the covariance structure chosen for all data. Least square means (LSM) were compared by least square difference test and differences declared significant at $P < 0.05$.

4.3. Results

4.3.1. Dietary treatments

Chemical composition and FA profile of experimental dietary treatments are presented in **Table 4.2**.

Table 4.2. Chemical composition and fatty acid profile of experimental dietary treatments.

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
DM (g kg^{-1})	913	920	912	921	3.2	0.970	0.026	0.676
Chemical composition (g kg^{-1} DM)								
OM	925	932	929	931	3.7	0.671	0.238	0.624
CP	150	133	140	124	3.5	0.019	<0.001	0.889
EE	24.3	81.3	20.0	80.8	1.12	0.056	<0.001	0.120
NDFom	568	551	581	541	6.0	0.839	<0.001	0.086
ADFom	353	333	394	366	3.3	<0.001	<0.001	0.220
ADL	50.5	46.5	70.0	66.5	1.79	<0.001	0.058	0.891
Ca	3.70	3.53	4.50	4.40	0.632	0.210	0.832	0.954
Mg	1.33	1.30	1.80	1.43	0.106	0.015	0.083	0.123
Fatty acid profile (g 100 g^{-1} total FA)								
10:0	0.070	0.020	0.072	0.014	0.0049	0.690	<0.001	0.489
12:0	0.496	0.154	0.425	0.124	0.0312	0.129	<0.001	0.522
14:0	0.899	0.331	0.767	0.296	0.0472	0.103	<0.001	0.321
15:0	0.237^{d}	0.077^{b}	0.165^{c}	0.056^{a}	0.0066	<0.001	<0.001	0.002
16:0	20.4^{c}	12.7^{a}	15.6^{b}	11.9^{a}	0.30	<0.001	<0.001	<0.001
16:1 *cis*-7	0.100	0.036	0.090	0.027	0.0142	0.510	<0.001	0.993
16:1 *cis*-9	0.263	0.151	0.169	0.125	0.0239	0.029	0.007	0.180
18:0	2.58^{a}	3.09^{ab}	4.00^{c}	3.19^{b}	0.190	0.002	0.456	0.005
18:1 *cis*-9	19.5	19.5	20.7	20.6	0.77	0.159	0.948	0.901
18:1 *cis*-11	0.92^{b}	1.20^{c}	0.70^{a}	1.17^{c}	0.021	<0.001	<0.001	<0.001
18:2 *n*-6	36.7	49.3	41.6	51.1	1.24	0.019	<0.001	0.238
20:0	0.984	0.435	0.989	0.460	0.0505	0.775	<0.001	0.843
18:3 *n*-6	0.131	0.073	0.114	0.051	0.0152	0.228	0.002	0.901
18:3 *n*-3	4.98	6.22	3.32	5.81	0.353	0.013	<0.001	0.103
20:1 *n*-9	0.100	0.052	0.107	0.042	0.0161	0.921	0.004	0.591
21:0	0.086	0.036	0.088	0.037	0.0050	0.772	<0.001	0.923
20:2 *n*-6	0.299	0.167	0.198	0.121	0.0473	0.148	0.047	0.568
22:0	1.11^{b}	0.52^{a}	1.24^{c}	0.55^{a}	0.027	0.013	<0.001	0.070
24:0	1.04	0.22	0.92	0.18	0.060	0.215	<0.001	0.474
Other FA[4]	9.19	5.69	8.71	4.16	0.831	0.253	<0.001	0.540

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]The remaining fatty acids, most of them unidentified.

Although diets were formulated to be isonitrogenous, non-protein nitrogen diets (U and UO) had slightly higher CP content than true protein diets (SM and SMO). Higher ADFom and ADL content of true protein than non-protein nitrogen diets reflect differences of the ingredient composition used in formulation, sunflower meal and wheat bran, respectively.
Soybean oil supplementation increased EE content, proportionally reducing CP and fibre contents. Fatty acid profile of U diet had higher proportions of 16:0, 16:1 *cis*-9, 18:1 *cis*-11, and 18:3 *n*-3, and lower proportion of 18:2 *n*-6 compared to SM diet. All FA, with exception of 18:0 and 18:1 *cis*-9, were affected by oil supplementation. Major PUFA of soybean oil, 18:2 *n*-6 and 18:3 *n*-3, increased with oil supplementation, whereas most of the remaining FA decreased.

4.3.2. In vivo *digestibility*

Digestibility of dietary treatments determined *in vivo* is presented in **Table 4.3**. Oil supplementation decreased DM, OM and NDF digestibility, while nitrogen source had no effect. The interaction nitrogen source by oil supplementation tended to be significant for NDF digestibility.

Table 4.3. Effect of nitrogen source and oil supplementation on digestibility of diet components.

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
DM digestibility	59.1	54.0	57.9	55.7	1.23	0.788	0.004	0.131
OM digestibility	60.8	55.6	59.6	57.2	1.24	0.797	0.004	0.139
NDF digestibility	51.5	40.8	49.9	44.1	2.02	0.539	<0.001	0.086

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.

4.3.3. Rumen fermentation pattern

Dietary treatments affected rumen fermentation (**Table 4.4**). Rumen pH increased with oil supplementation, whereas total VFA production decreased. Additionally, oil supplementation decreased molar proportions of butyrate and caproate and increased propionate. True protein

diets promoted higher proportions of the branched-chain VFA isobutyrate and isovalerate, decreasing caproate.

Table 4.4. Effect of nitrogen source and oil supplementation on rumen metabolites

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
pH	6.68	6.91	6.70	6.86	0.028	0.499	<0.001	0.226
Volatile fatty acids (mM)								
Total	59.4	39.5	59.1	43.5	3.29	0.309	0.002	0.259
Molar proportion (mol 100 mol^{-1})								
Acetate	70.5	67.0	70.0	71.4	1.19	0.114	0.354	0.069
Propionate	16.0^{a}	24.2^{b}	16.8^{a}	17.8^{a}	0.72	0.021	0.003	0.010
Isobutyrate	0.70	0.73	1.26	1.30	0.050	<0.001	0.497	0.938
Butyrate	10.4	7.0	9.6	7.6	0.52	0.850	0.004	0.137
Isovalerate	1.14	1.13	1.83	1.67	0.195	0.039	0.686	0.719
Valerate	0.148	0.103	0.193	0.132	0.0694	0.449	0.303	0.863
Caproate	1.28	0.00	0.17	0.06	0.242	0.077	0.031	0.058

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.

4.3.4. Total rumen content and bacterial fatty acid composition

Fatty acid content and profiles, excluding C18 FA, of TRC, LAB, and SAB are presented in **Tables 4.5**, **4.6** and **4.7**, respectively. Total FA content increased with soybean oil supplementation in all ruminal fractions. However, marked differences were induced by oil supplementation. On average, FA content of LAB increased by nearly three folds in oil supplemented diets (UO and SMO) compared to unsupplemented diets (U and SM), whereas SAB FA content was nearly two folds higher. Most FA were negatively affected by oil supplementation compared to diets without oil. Conversely, total C18 FA increased in all rumen fractions with soybean oil supplementation, as well as 16:0 in LAB.

Nitrogen source had no effect on most of the FA proportions of the rumen fractions, although the effects observed on 16:0 and OBCFA are worth of being noted. True protein diets (SM and SMO), compared to non-protein nitrogen diets (U and UO), decreased 16:0 proportion, and induced, or tended to induce, higher proportions of most odd-chain fatty acids (OCFA; 13:0, 15:0, 17:0) and branched-chain fatty acids (BCFA; *iso*-13:0, *iso*-15:0, *iso*-17:0, *anteiso*-

Table 4.5. Total rumen content fatty acid profile (g 100 g^{-1} total FA) and content (mg g^{-1} DM).

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
10:1 *cis*-9	0.117	0.070	0.131	0.064	0.0103	0.719	0.001	0.360
11:0	0.052	0.035	0.086	0.031	0.0134	0.281	0.025	0.164
12:0	0.716	0.345	0.742	0.305	0.0471	0.882	<0.001	0.499
iso-13:0	0.216[b]	0.172[ab]	0.267[c]	0.130[a]	0.0162	0.766	<0.001	0.015
13:0	0.223	0.223	0.374	0.229	0.0402	0.046	0.059	0.052
iso-14:0	0.701	0.247	0.694	0.328	0.0363	0.330	<0.001	0.255
14:0	2.13	1.38	2.03	1.21	0.122	0.315	<0.001	0.791
iso-15:0	1.55	0.79	1.91	0.93	0.100	0.032	<0.001	0.305
anteiso-15:0	2.31[b]	1.38[a]	2.76[c]	1.19[a]	0.092	0.202	<0.001	0.008
15:0	2.14[b]	1.07[a]	2.88[c]	1.05[a]	0.152	0.053	<0.001	0.044
iso-16:0	1.30	0.36	1.34	0.55	0.068	0.110	<0.001	0.321
16:0	24.0	22.8	21.2	19.7	0.93	0.011	0.171	0.874
iso-17:0	0.413	0.170	0.494	0.197	0.0221	0.045	<0.001	0.247
16:1 *cis*-7	0.154	0.122	0.263	0.123	0.0282	0.082	0.014	0.088
16:1 *cis*-9	0.085	0.100	0.095	0.058	0.0112	0.201	0.342	0.059
anteiso-17:0	1.00	0.37	1.15	0.40	0.043	0.074	<0.001	0.214
Phytanic acid	0.236	0.217	0.211	0.136	0.0255	0.048	0.068	0.229
17:0	0.606[b]	0.340[a]	0.742[c]	0.316[a]	0.0271	0.039	<0.001	0.009
C18[4]	55.0	66.0	54.9	68.9	1.21	0.268	<0.001	0.250
19:0	0.067[bc]	0.058[ab]	0.078[c]	0.042[a]	0.0071	0.632	0.005	0.039
20:0	0.904	0.631	0.888	0.573	0.0220	0.136	<0.001	0.363
22:0	0.721	0.495	0.773	0.473	0.0188	0.384	<0.001	0.059
20:5 *n*-3	0.912	0.463	0.920	0.388	0.0808	0.587	<0.001	0.507
24:0	0.582	0.265	0.630	0.243	0.0377	0.720	<0.001	0.362
Other FA[5]	3.54	1.76	4.09	2.21	0.187	0.027	<0.001	0.792
OCFA[6]	2.97[b]	1.64[a]	3.99[c]	1.60[a]	0.203	0.048	<0.001	0.036
BCFA[7]	7.49	3.48	8.61	3.73	0.270	0.032	<0.001	0.137
OBCFA[8]	10.5	5.1	12.6	5.3	0.46	0.032	<0.001	0.065
Total FA (mg g^{-1} DM)	63[a]	101[b]	49[a]	119[c]	4.7	0.623	<0.001	0.010

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]Sum of octadecanoic acids.
[5]The remaining fatty acids, most of them unidentified.
[6]Odd-chain fatty acids.
[7]Branched-chain fatty acids.
[8]Odd- and branched-chain fatty acids.

Table 4.6. Liquid associated bacteria fatty acids profile (g 100 g^{-1} total FA) and content (mg g^{-1} DM).

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
10:0	0.163	0.057	0.129	0.066	0.0230	0.572	0.006	0.333
10:1 *cis*-9	0.159	0.046	0.138	0.052	0.0295	0.795	0.012	0.639
11:0	0.117	0.079	0.148	0.062	0.0162	0.695	0.004	0.179
12:0	0.881	0.512	0.861	0.528	0.0638	0.976	<0.001	0.781
iso-13:0	0.292	0.215	0.356	0.267	0.0173	0.008	0.001	0.732
anteiso-13:0	0.170	0.051	0.109	0.059	0.0184	0.145	0.002	0.073
13:0	0.72	0.60	1.68	0.81	0.322	0.104	0.163	0.278
iso-14:0	1.39[c]	0.44[a]	1.29[c]	0.79[b]	0.080	0.071	<0.001	0.007
14:0	4.21	2.79	4.70	2.63	0.507	0.718	0.007	0.484
iso-15:0	2.51	1.12	3.05	1.91	0.144	0.002	<0.001	0.391
anteiso-15:0	5.05	3.08	5.26	3.14	0.360	0.663	<0.001	0.814
14:1 *cis*-9	0.502	0.139	0.544	0.276	0.0714	0.256	0.004	0.525
15:0	3.52	1.97	4.78	2.15	0.283	0.031	<0.001	0.089
iso-16:0	1.95	0.47	2.06	1.15	0.179	0.053	<0.001	0.146
15:1 *cis*-10	0.421	0.255	0.685	0.275	0.0672	0.064	0.002	0.102
16:0	20.9	23.6	17.2	19.6	1.07	0.006	0.033	0.846
iso-17:0	0.489	0.221	0.557	0.276	0.0287	0.038	<0.001	0.771
16:1 *cis*-7	0.90	0.54	1.70	0.64	0.204	0.053	0.007	0.121
16:1 *cis*-9	0.272[a]	0.453[b]	0.296[ab]	0.225[a]	0.0531	0.089	0.325	0.042
anteiso-17:0	1.29	0.43	1.53	0.68	0.094	0.022	<0.001	0.972
Phytanic acid	0.375	0.170	0.256	0.190	0.0450	0.235	0.012	0.113
17:0	0.83[b]	0.53[a]	1.05[c]	0.46[a]	0.051	0.136	<0.001	0.020
C18[4]	44.3	57.7	41.5	58.7	1.63	0.582	<0.001	0.273
19:0	0.148	0.096	0.211	0.102	0.0269	0.227	0.015	0.309
20:0	0.717	0.532	0.654	0.519	0.0544	0.501	0.017	0.655
20:2 *n*-6	0.542	0.281	0.824	0.318	0.1892	0.420	0.073	0.534
22:0	0.443	0.341	0.543	0.363	0.0345	0.113	0.005	0.277
20:5 *n*-3	0.615	0.294	0.628	0.421	0.0729	0.362	0.006	0.460
24:0	0.356	0.172	0.354	0.196	0.0351	0.760	<0.001	0.720
Other FA[5]	5.80	2.79	6.6	3.14	0.694	0.304	<0.001	0.576
OCFA[6]	5.06	3.10	7.50	3.43	0.585	0.042	<0.001	0.105
BCFA[7]	13.1	6.0	14.2	8.3	0.67	0.035	<0.001	0.376
OBCFA[8]	18.2	9.1	21.7	11.7	1.10	0.021	<0.001	0.681
Total FA (mg g^{-1} DM)	90	251	75	231	26.5	0.523	<0.001	0.918

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]Sum of octadecanoic acids.
[5]The remaining fatty acids, most of them unidentified.
[6]Odd-chain fatty acids.
[7]Branched-chain fatty acids.
[8]Odd- and branched-chain fatty acids.

Table 4.7. Solid associated bacteria fatty acids profile (g 100 g^{-1} total FA) and content (mg g^{-1} DM).

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
10:0	0.045	0.027	0.047	0.022	0.0033	0.648	<0.001	0.225
11:0	0.051	0.039	0.045	0.025	0.0042	0.037	0.006	0.314
12:0	0.691	0.407	0.699	0.347	0.0383	0.512	<0.001	0.396
iso-13:0	0.163	0.192	0.199	0.181	0.0199	0.544	0.774	0.263
anteiso-13:0	0.130	0.045	0.086	0.033	0.0134	0.067	<0.001	0.257
13:0	0.220	0.154	0.246	0.147	0.0172	0.598	0.003	0.370
iso-14:0	0.954	0.413	0.954	0.566	0.0479	0.126	<0.001	0.126
14:0	2.52	1.67	2.30	1.40	0.158	0.128	<0.001	0.876
iso-15:0	1.62	1.25	2.16	1.33	0.152	0.071	0.006	0.158
anteiso-15:0	2.70[b]	2.19[a]	3.38[c]	1.96[a]	0.117	0.086	<0.001	0.004
14:1 *cis*-9	0.208[b]	0.127[a]	0.321[c]	0.128[a]	0.0185	0.016	<0.001	0.017
15:0	2.61	1.36	3.69	1.49	0.232	0.028	<0.001	0.073
iso-16:0	1.43	0.59	1.56	0.75	0.083	0.051	<0.001	0.862
15:1 *cis*-10	0.040[a]	0.042[a]	0.058[b]	0.035[a]	0.0045	0.266	0.046	0.021
16:0	23.0	22.8	20.3	19.7	0.74	0.003	0.643	0.794
iso-17:0	0.418	0.219	0.525	0.258	0.0257	0.029	<0.001	0.235
16:1 *cis*-7	0.106[a]	0.118[ab]	0.144[b]	0.101[a]	0.0111	0.358	0.200	0.036
16:1 *cis*-9	0.097[a]	0.137[b]	0.082[a]	0.067[a]	0.0116	0.007	0.295	0.046
anteiso-17:0	1.02[b]	0.50[a]	1.28[c]	0.58[a]	0.065	0.001	<0.001	0.022
Phytanic acid	0.215	0.236	0.166	0.180	0.0278	0.095	0.544	0.903
17:0	0.633[b]	0.360[a]	0.844[c]	0.361[a]	0.0423	0.044	<0.001	0.045
C18[4]	54.6	62.5	53.9	66.1	1.12	0.229	<0.001	0.084
19:0	0.088	0.075	0.113	0.087	0.0222	0.426	0.402	0.789
20:0	0.910	0.620	0.866	0.592	0.0435	0.308	<0.001	0.802
20:2 *n*-6	0.079	0.062	0.032	0.038	0.0156	0.057	0.757	0.504
22:0	0.675	0.462	0.692	0.460	0.0541	0.901	0.006	0.869
20:5 *n*-3	0.711	0.380	0.681	0.374	0.0398	0.660	<0.001	0.778
24:0	0.470	0.288	0.449	0.283	0.0284	0.667	<0.001	0.791
Other FA[5]	3.60	2.73	4.24	2.41	0.288	0.576	0.002	0.126
OCFA[6]	3.46	1.87	4.77	2.00	0.285	0.032	<0.001	0.067
BCFA[7]	8.4	5.4	10.1	5.7	0.34	0.024	<0.001	0.068
OBCFA[8]	11.9	7.3	14.9	7.7	0.59	0.024	<0.001	0.059
Total FA (mg g^{-1} DM)	232	393	194	396	25.5	0.516	<0.001	0.449

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]Sum of octadecanoic acids.
[5]The remaining fatty acids, most of them unidentified.
[6]Odd-chain fatty acids.
[7]Branched-chain fatty acids;
[8]Odd- and branched-chain fatty acids.

17:0) in TRC, LAB, and SAB fractions. No interactions between nitrogen source and oil supplementation were observed for most FA. However, the interaction was or tended to be significant in some OBCFA. Additionally, a significant interaction was observed for 16:1 *cis*-7 and 16:1 *cis*-9 in SAB, while in LAB it was only significant for 16:1 *cis*-9.

4.3.5. Total rumen content and bacterial C18 fatty acids

To focus on the effects of nitrogen source and oil supplementation on biohydrogenation, C18 FA were expressed as percentage of total C18 FA identified. C18 FA profile of TRC, LAB and SAB fractions are presented in **Tables 4.8**, **4.9** and **4.10**, respectively.

Soybean oil supplementation affected most C18 FA in all ruminal fractions. Biohydrogenation of the dietary 18:2 *n*-6 and 18:3 *n*-3 FA was promoted by soybean oil supplementation, as these precursors decreased in TRC, LAB and SAB fractions. Moreover, the sum of C18:3 and C18:2 FA decreased, whereas most monoenoic intermediates increased, particularly 18:1 *trans*-11. However, no concomitant increase of the biohydrogenating end-product, 18:0, was observed, although it tended ($P = 0.054$) to be higher in TRC.

Among C18:1 FA, only 18:1 *cis*-9 proportion decreased with oil supplementation in TRC, LAB and SAB fractions. Total CLA increased in TRC of soybean oil supplemented diets, while decreased in LAB fraction and was not affected in SAB fraction. Different effects were observed among fractions on the major CLA isomer. 18:2 *cis*-9,*trans*-11 increased in SAB, tended ($P = 0.073$) to increase in TRC with soybean oil supplementation, while no effect was observed in LAB.

Independently of the rumen fraction, nitrogen source had no effect on 18:0 and 18:1 *trans*-11 FA, neither did on their main precursor 18:2 *n*-6. However, a decrease of the 18:3 *n*-3 was observed in true protein diets, the interaction with oil being significant for LAB. Nitrogen source had only minor effects on C18 FA. The most relevant effect was the decrease of 18:1 *cis*-11 proportion in LAB and SAB with true protein diets. The interaction nitrogen source by oil supplementation was significant for both bacterial fractions. Additionally, a significant interaction for 18:2 *cis*-9,*trans*-13 was observed in all fractions, and for the CLA isomers 18:2 *cis*-9,*trans*-11, 18:2 *trans*-10,*cis*-12 and 18:2 *trans*-9,*trans*-11 in TRC.

Table 4.8. Total rumen content C18 fatty acids profile (g 100 g^{-1} C18 FA).

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
18:0	57.0	60.1	54.2	59.7	1.94	0.425	0.054	0.561
18:1 *trans*-4	0.098[a]	0.232[c]	0.097[a]	0.129[b]	0.0163	0.011	<0.001	0.012
18:1 *trans*-5	0.051	0.084	0.064	0.070	0.0094	0.974	0.075	0.202
18:1 *trans*-6,-7,-8	0.448	0.944	0.409	0.789	0.0794	0.253	<0.001	0.488
18:1 *trans*-9	0.392	0.706	0.328	0.616	0.0524	0.190	0.001	0.814
18:1 *trans*-10	0.485	1.04	0.453	0.977	0.0704	0.519	<0.001	0.837
18:1 *trans*-11	6.04	15.4	9.12	13.7	1.930	0.725	0.006	0.249
18:1 *trans*-12	0.749	1.36	0.590	1.11	0.0977	0.068	<0.001	0.650
18:1 *cis*-9	15.6	7.18	16.5	8.50	0.690	0.145	<0.001	0.758
18:1 *trans*-15	0.360	0.825	0.268	0.608	0.1074	0.184	0.005	0.577
18:1 *cis*-11	0.761	0.986	0.815	0.689	0.1011	0.206	0.582	0.086
18:1 *cis*-12	0.508	0.888	0.355	0.672	0.1257	0.183	0.030	0.808
18:1 *cis*-13	0.058	0.139	0.099	0.051	0.0441	0.604	0.722	0.176
18:1 *cis*-14+*trans*-16	0.611	1.08	0.462	0.760	0.1019	0.047	0.005	0.425
18:1 *cis*-15	0.057	0.114	0.051	0.090	0.0114	0.229	0.002	0.434
18:2 *trans*-9,*trans*-12	0.036	0.140	0.037	0.082	0.0194	0.181	0.004	0.164
18:2 *cis*-9,*trans*-13	0.361[b]	0.179[a]	0.758[c]	0.290[ab]	0.0441	<0.001	<0.001	0.012
18:2 *trans*-8,*cis*-12	0.121[b]	0.073[a]	0.049[a]	0.053[a]	0.0092	<0.001	0.048	0.022
18:2 *trans*-11,*cis*-15	0.151	0.238	0.170	0.266	0.0365	0.531	0.041	0.901
18:2 other isomers	0.344	0.325	0.592	0.292	0.1016	0.319	0.150	0.200
18:2 *n*-6	11.2	4.67	10.7	4.61	0.600	0.538	<0.001	0.644
18:3 *n*-3	1.69	0.792	1.37	0.574	0.0781	0.008	<0.001	0.549
18:2 *cis*-9,*trans*-11	1.64[a]	1.39[a]	1.73[a]	4.09[b]	0.511	0.029	0.073	0.037
18:2 *trans*-10,*cis*-12	0.152[ab]	0.001[a]	0.096[ab]	0.182[b]	0.0571	0.230	0.521	0.035
18:2 *cis*-9,*cis*-11	0.224	0.255	0.135	0.205	0.0663	0.235	0.376	0.721
18:2 *trans*-11,*trans*-13	0.289	0.336	0.350	0.188	0.1076	0.636	0.556	0.275
18:2 *trans*-9,*trans*-11	0.667[bc]	0.464[ab]	0.307[a]	0.729[c]	0.0774	0.536	0.180	0.005
18:3 *cis*-9,*trans*-11,*cis*-15	0.125	0.074	0.098	0.054	0.0197	0.234	0.037	0.816
C18:1[4]	26.2	30.9	29.6	28.7	2.31	0.803	0.427	0.258
C18:2[5]	15.0	8.09	14.7	11.0	0.847	0.109	<0.001	0.068
C18:3[6]	1.81	0.865	1.47	0.628	0.0803	0.006	<0.001	0.527
CLA[7]	2.81[a]	2.47[a]	2.41[a]	5.38[b]	0.527	0.037	0.032	0.013

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]Sum of octadecenoic acids.
[5]Sum of octadecadienoic acids.
[6]Sum of octadecatrienoic acids.
[7]Sum of conjugated linoleic acid isomers.

Table 4.9. Liquid associated bacteria C18 fatty acids profile (g 100 g^{-1} C18 FA).

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
18:0	65.4	60.0	59.2	66.8	3.58	0.928	0.762	0.111
18:1 *trans*-4	0.119	0.286	0.118	0.226	0.0307	0.303	0.002	0.316
18:1 *trans*-5	0.136	0.093	0.101	0.101	0.0351	0.716	0.554	0.554
18:1 *trans*-6,-7,-8	0.950	1.00	0.953	0.927	0.1840	0.848	0.946	0.838
18:1 *trans*-9	0.501	0.717	0.430	0.651	0.0682	0.313	0.013	0.972
18:1 *trans*-10	0.781	1.01	0.737	1.12	0.0876	0.722	0.007	0.409
18:1 *trans*-11	3.71	14.7	6.08	11.6	1.488	0.810	<0.001	0.099
18:1 *trans*-12	1.60	1.55	1.72	1.45	0.270	0.977	0.553	0.680
18:1 *cis*-9	7.97	5.88	9.12	5.54	0.689	0.500	0.002	0.233
18:1 *trans*-15	0.380	0.738	0.592	0.652	0.1709	0.723	0.252	0.405
18:1 *cis*-11	1.51^{a}	3.11^{b}	1.35^{a}	1.64^{a}	0.285	0.019	0.009	0.046
18:1 *cis*-12	1.05	1.27	0.568	1.00	0.156	0.035	0.056	0.470
18:1 *cis*-13	0.059	0.044	0.078	0.105	0.0201	0.085	0.774	0.328
18:1 *cis*-14+*trans*-16	0.622	1.13	0.499	0.983	0.1024	0.207	0.002	0.894
18:1 *cis*-15	0.236	0.165	0.136	0.159	0.0729	0.438	0.722	0.490
18:2 *trans*-9,*trans*-12	0.049	0.127	0.237	0.091	0.0817	0.382	0.694	0.210
18:2 *cis*-9,*trans*-13	1.10^{b}	0.304^{a}	2.62^{c}	0.808^{ab}	0.2219	0.001	<0.001	0.047
18:2 *trans*-11,*cis*-15	0.125	0.223	0.090	0.151	0.0273	0.060	0.014	0.447
18:2 other isomers	0.861	0.623	1.94	0.495	0.3643	0.223	0.046	0.131
18:2 *n*-6	9.36	5.46	10.4	4.11	0.975	0.870	0.002	0.266
18:2 *n*-3	0.361	0.293	0.363	0.274	0.0589	0.883	0.215	0.868
18:3 *n*-3	1.09^{c}	0.369^{a}	0.734^{b}	0.335^{a}	0.0451	0.002	<0.001	0.004
18:2 *cis*-9,*trans*-11	0.462	0.423	0.422	0.334	0.1058	0.552	0.558	0.820
18:2 *trans*-10,*cis*-12	0.497	0.038	0.279	0.051	0.1392	0.478	0.041	0.425
18:2 *trans*-9,*trans*-11	0.884	0.358	1.01	0.265	0.1699	0.915	0.005	0.529
18:3 *cis*-9,*trans*-11,*cis*-15	0.333	0.146	0.296	0.126	0.0806	0.736	0.070	0.917
C18:1[4]	19.6	31.7	22.5	26.1	2.13	0.542	0.005	0.079
C18:2[5]	13.6	7.84	17.3	6.58	1.877	0.639	0.005	0.234
C18:3[6]	1.38	0.515	1.03	0.461	0.0958	0.039	<0.001	0.102
CLA[7]	1.77	0.811	1.69	0.649	0.3024	0.663	0.009	0.876

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]Sum of octadecenoic acids.
[5]Sum of octadecadienoic acids.
[6]Sum of octadecatrienoic acids.
[7]Sum of conjugated linoleic acid isomers.

Table 4.10. Solid associated bacteria C18 fatty acids profile (g 100 g^{-1} C18 FA).

	Treatments[1]				SEM	*P* value		
	U	UO	SM	SMO		N[2]	O[3]	N*O
18:0	55.7	57.9	57.4	60.9	1.64	0.181	0.116	0.703
18:1 *trans*-4	0.072	0.176	0.062	0.108	0.0209	0.095	0.006	0.200
18:1 *trans*-5	0.045	0.131	0.045	0.077	0.0225	0.256	0.035	0.253
18:1 *trans*-6,-7,-8	0.377	0.908	0.419	0.749	0.0851	0.511	<0.001	0.266
18:1 *trans*-9	0.308	0.638	0.320	0.558	0.0505	0.518	<0.001	0.387
18:1 *trans*-10	0.396	0.898	0.444	0.810	0.0840	0.814	<0.001	0.440
18:1 *trans*-11	6.77	13.4	8.57	11.0	1.280	0.832	0.009	0.137
18:1 *trans*-12	0.530	1.16	0.507	1.01	0.0983	0.393	<0.001	0.526
18:1 *cis*-9	15.0	8.76	14.3	9.02	0.514	0.673	<0.001	0.376
18:1 *trans*-15	0.311	0.708	0.237	0.674	0.1026	0.611	0.003	0.850
18:1 *cis*-11	0.935[a]	1.38[b]	0.961[a]	0.874[a]	0.0631	0.007	0.024	0.004
18:1 *cis*-12	0.492	0.908	0.369	0.780	0.1374	0.385	0.015	0.987
18:1 *cis*-13	0.060	0.099	0.099	0.073	0.0468	0.895	0.891	0.505
18:1 *cis*-14+*trans*-16	0.562	0.984	0.435	0.844	0.1232	0.306	0.008	0.962
18:1 *cis*-15	0.099	0.155	0.077	0.172	0.0326	0.936	0.046	0.568
18:2 *trans*-9,*trans*-12	0.085	0.131	0.079	0.120	0.0173	0.643	0.032	0.886
18:2 *cis*-9,*trans*-13	0.381[a]	0.393[a]	1.06[b]	0.479[a]	0.0707	<0.001	0.003	0.002
18:2 *trans*-11,*cis*-15	0.152	0.287	0.140	0.225	0.0471	0.451	0.044	0.613
18:2 other isomers	0.383	0.521	0.511	0.391	0.1426	0.997	0.950	0.388
18:2 *n*-6	11.6	5.65	8.76	5.20	1.155	0.191	0.005	0.324
18:2 *n*-3	0.126	0.117	0.141	0.097	0.0207	0.890	0.170	0.348
18:3 *n*-3	1.48	0.870	1.21	0.674	0.0808	0.025	<0.001	0.666
18:2 *cis*-9,*trans*-11	1.64	2.15	1.55	3.22	0.328	0.169	0.009	0.112
18:2 *trans*-9,*cis*-11	0.055	0.055	0.033	0.067	0.0119	0.673	0.187	0.200
18:2 *trans*-10,*cis*-12	0.168	0.098	0.263	0.162	0.0305	0.014	0.010	0.528
18:2 *cis*-9,*cis*-11	0.119	0.094	0.060	0.133	0.0230	0.659	0.294	0.057
18:2 *cis*-10,*cis*-12	0.137	0.077	0.127	0.089	0.0115	0.925	0.003	0.381
18:2 *trans*-11,*trans*-13	0.650	0.150	0.871	0.133	0.1605	0.557	0.018	0.497
18:2 *trans*-9,*trans*-11	0.753	0.792	0.721	0.905	0.1243	0.752	0.392	0.574
18:3 *cis*-9,*trans*-11,*cis*-15	0.655	0.478	0.546	0.431	0.1756	0.667	0.428	0.864
C18:1[4]	26.0	30.3	26.8	26.8	1.63	0.451	0.236	0.226
C18:2[5]	16.2	10.5	14.0	11.2	1.23	0.553	0.007	0.261
C18:3[6]	2.14	1.35	1.76	1.11	0.170	0.102	0.002	0.706
CLA[7]	3.50	3.41	3.31	4.71	0.437	0.237	0.168	0.121

[1]Diets are named according to the nitrogen source [urea (U) or sunflower meal (SM)] and soybean oil supplementation (O), respectively: U = oat hay plus urea concentrate; UO = oat hay plus urea concentrate with soybean oil; SM = oat hay plus sunflower meal concentrate; SMO = oat hay plus sunflower meal concentrate with soybean oil.
[2]N = nitrogen source.
[3]O = soybean oil supplementation.
[4]Sum of octadecenoic acids.
[5]Sum of octadecadienoic acids.
[6]Sum of octadecatrienoic acids.
[7]Sum of conjugated linoleic acid isomers.

4.4. Discussion

4.4.1. Total fatty acid content

Fatty acid content (mg g^{-1} DM) differed among rumen fractions. Highest total FA content was found in SAB, followed by LAB and finally by TRC. Lower FA content of TRC may reflect a dilution of bacterial FA composition by ruminal digesta. Total rumen content FA concentration as well as profile might be influenced by other microbial populations, particularly by ciliate protozoa which are rich in unsaturated FA (Katz & Keeney, 1966; Harfoot & Hazlewood, 1997).

Differences between bacterial fractions might be related to differential distribution through rumen phases (Minato, Endo, Higuchi, Ootomo & Uemura, 1966), and distinct growth rates (Bates, Gillet, Barao & Bergen, 1985). The preferential adsorption site of dietary FA may facilitate the incorporation or adsorption onto bacterial cells and, thereby, promote a higher FA content in SAB (Harfoot, 1981), explaining the FA concentration found in this bacterial fraction. Moreover, SAB population has been reported to constitute most of bacterial cell mass present in the rumen (Forsberg & Lam, 1977; Legay-Carmier & Bauchart, 1989), and to have higher lipid content than LAB (Merry & McAllan, 1983).

Total FA content was not affected by nitrogen source whereas oil supplementation led to an increase in all fractions. Bacterial FA content determined at the present experiment was particularly high, ranging from 7.5 to 25% in LAB and from 19 to 40% in SAB of DM cell mass. Bacterial lipids normally constitute less than 10% of total cell mass (Harfoot & Hazlewood, 1997), although lipid inclusions have been observed in the cytosol of LAB and SAB after rapeseed and soybean oil supplementation (Legay-Carmier & Bauchart, 1989; Bauchart, Legay-Carmier, Doreau & Gaillard, 1990). These inclusions may alter the overall FA composition of bacterial cells once they can comprise a considerable part of the bacteria, as pointed out by Harfoot and Hazlewood (1997). Even though similar lipid inclusions might have occurred under our experimental conditions, FA content of bacterial fractions from animals fed diets supplemented with soybean oil are much higher than those described in literature (Legay-Carmier & Bauchart, 1989; Bauchart et al., 1990; O'Kelly & Spiers, 1991). The highest bacterial FA content reported was of 37% and 15% of DM cell mass in SAB and LAB, respectively, in sheep fed lucerne supplemented with 12% soybean oil (Bessa, 2001), twice the supplementation level used in our experiment. In the absence of oil supplementation

explanations are even more difficult, as no lipid inclusions were reported in these conditions (Bauchart et al., 1990). Differences in methodological procedures might have overestimated the FA contents of ruminal fractions, namely those of SAB. Fatty acids from TRC, LAB and SAB fractions were extracted by the dichloromethane and methanol procedure (Folch et al., 1957) after an initial acidification, as proposed by Fellner et al. (1995), to recover the saponified FA. Insoluble soaps are formed in the rumen by physical-chemical association of Ca and/or Mg ions to non-esterified FA released during lipolysis of dietary lipids. Soaps may constitute an important part of the FA pool in the rumen. *In vivo* studies suggested 60 to 80% of total ruminal FA content to be in the form of soaps (Palmquist, Jenkins & Joyner, 1986; Emanuelson, Murphy & Lindberg, 1991; Bock, Harmon, Brand & Schneider, 1991). Recovery and quantification of saponified FA might explain the higher FA content observed. Another possible explanation lays on the contamination of SAB fraction with fine particulate matter. This hypothesis might have been addressed and elucidated by determining the fibre content of the bacterial fraction, but the SAB pellet obtained after TRC fractionation was too small to proceed this approach. Therefore, reasons why bacteria incorporated or adsorbed FA to such an extent, nearly 40% in SAB with 6% soybean oil supplementation, are not clear or understandable from the results herein presented.

Although oil supplementation was reported to increase total FA of rumen contents and bacterial fractions (Bauchart et al., 1990; Bessa, Almeida, Ribeiro & Portugal, 1997; Sauvant & Bas, 2001), a differential FA enrichment of LAB and SAB was not promoted. However, in the present experiment, oil supplementation led to a preferential enrichment of the FA content in LAB. Indeed, the SAB to LAB FA ratio dropped from 2.6 to 1.6 with soybean oil inclusion. These results may reflect an increased availability of FA in the rumen liquid phase for LAB population or a modification of the bacterial fractions composition. Bacteria are not static microorganisms strictly confined into LAB or SAB fractions. Bacterial species or strains generally associated with particles, and therefore determined as SAB after TRC fractioning procedure, might be found loosely attached or free in liquid digesta during its life cycle. Rumen contents were collected from animals before morning feeding, i.e., 17 h after their previous meal. It is possible that, after such a long fast, part of bacteria from SAB fraction might have detached from digested particles in search for new feed particles for colonisation. Under such conditions, these bacteria with higher FA content, could have been isolated as LAB increasing the overall FA enrichment of this fraction, being the increased LAB FA content more evident in oil supplemented diets.

4.4.2. Fatty acid profile

The observed effects of oil supplementation on FA profile of TRC and bacterial fractions followed that described in literature as most FA proportions, either than C18 FA, decreased with soybean oil inclusion. Bacterial lipids may be originated from the uptake of dietary FA or by *de novo* synthesis, with FA proportions depending on the lipid content of the diet and bacterial species (Harfoot & Hazlewood, 1997). Lipid supplementation appears to enhance exogenous FA intake uptake by microorganisms (Jenkins, 1993). Indeed, most FA proportions decreased with lipid supplementation. The exception was C18 FA reflecting the uptake of dietary-derived FA.

Most marked effect of nitrogen source on FA profile was on OBCFA. The major source of OBCFA is of bacterial origin (Keeney, Katz & Allison, 1962), which compose their membranary lipids (Kaneda, 1991; Mackie, White & Bryant, 1991). Rumen microbial OBCFA profile is mainly determined by the FA synthetase of bacteria and the availability of precursors, and to a lesser extent to environmental and physiological conditions (Ifkovitz & Ragheb, 1968; Vlaeminck et al., 2006). Odd- and branched-chain FA have also been associated with bacterial membrane fluidity, namely *anteiso* FA which have a particularly low melting point (Annous, Bexker, Bayles, Labeda & Wilkinson, 1997). Bacterial OBCFA are formed by *de novo* synthesis by FA synthetases, the only difference between OCFA and BCFA being the primers used and respective products (Kaneda, 1991). Branched-chain FA are formed using isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA as primers whereas OCFA are formed by using proponyl-CoA (Fulco, 1993; Kaneda, 1991). Although synthesis of OCFA through α-oxidation were reported *in vitro* (Emmanuel, 1978), *in vivo* evidences of α-oxidation are still to be proven (Bauchart et al., 1990).

In the present experiment, non-protein nitrogen diets led to lower proportions of OBCFA in all rumen fractions studied. This effect is consistent with the lesser availability of primers for BCFA synthesis. Molar proportions of isobutyrate and isovalerate were higher in rumen fluid of animals fed true protein diets. Lower proportions of OBCFA promoted by non-protein nitrogen diets were accompanied by higher proportion of 16:0 in all rumen fractions. Additionally, 16:1 *cis*-9 tended (P = 0.089) to increase in LAB and increased in SAB, and 18:1 *cis*-11 proportion was higher in both fractions. These FA were present at higher proportions in non-protein nitrogen diets. Combined increases of 16:0 and of the unsaturated FA 16:1 *cis*-9 and 18:1 *cis*-11 when lower proportions of OBCFA are present, suggests a role on bacterial homeostasis, possibly on regulation of bacterial membrane fluidity.

Monounsaturated FA are synthesized by the anaerobic pathway (Fulco, 1983), constituting 15 to 20% of total bacterial FA (Jenkins, 1993). In the anaerobic pathway, the β-hydroxy 10:0 intermediate is dehydrate to 10:1 *cis*-3, and further elongated to 16:1 *cis*-9 and 18:1 *cis*-11 (Jenkins, 1993). In bacteria which do not synthesise OBCFA, such as *Escherichia coli*, 18:1 *cis*-11 is involved in adaptation to environmental temperatures (Gelmann & Cronan, 1972; Mendonza, Garwin & Cronan, 1982), growth rate (Arneborg, Salskoviversen & Mathiasen, 1993), and pressure resistance (Casadei, Manas, Niven, Needs & Mackey, 2002) to modify membrane lipids. Indeed, the ratio of 18:1 *cis*-11 to 16:0 has been used as an indicator of membrane fluidity in *E. coli* (Yuk & Marshall, 2006). Once low OBCFA are present in non-protein nitrogen diets, increases of 18:1 *cis*-11 might be related to bacterial membrane fluidity regulation. Additionally, an interaction was observed in 16:1 *cis*-9 both in LAB and SAB, increasing with oil supplementation in non-protein nitrogen diets and decreasing in diets with true protein. A tendency was observed for 18:1 *cis*-11 in LAB ($P = 0.077$) and a significant interaction in SAB, oil supplementation promoting a higher increase in non-protein nitrogen diets. Overall, these effects support the suggested role of 18:1 *cis*-11 on bacterial membrane fluidity.

4.4.3. Biohydrogenation intermediates

Proportions of biohydrogenation intermediates FA reported at the present experiment were mainly affected by soybean oil supplementation and only scarcely affected by dietary nitrogen source. Among biohydrogenation intermediates, non-protein nitrogen diets increased the proportion of 18:3 *n*-3 in TRC and SAB. In LAB, an interaction was observed with oil supplementation inducing a stronger decrease of 18:3 *n*-3 in U diet. These results reflect the lower 18:3 *n*-3 proportion in true protein diets as less forage was used in its ingredient composition. A strong effect of nitrogen source was observed in the non-conjugated dienoic acid, 18:2 *cis*-9,*trans*-13, in all rumen fractions, with true protein diets presenting higher proportions. Nitrogen source by soybean oil supplementation interaction was significant in TRC, LAB, and SAB. Oil supplementation decreased 18:2 *cis*-9,*trans*-13 proportion in SM diet more markedly than U diet, for TRC and LAB fractions, whereas in SAB no effect was seen on U diet. Destaillats, Trottier, Galvez & Angers (2005) proposed the occurrence of the non-conjugated dienoic isomer 18:2 *cis*-9,*trans*-13 as an intermediate of 18:3 *n*-3 biohydrogenation, based on the detection of 18:3 *cis*-9,*trans*-13,*cis*-15 isomer in milk fat.

Indeed, the soybean oil used in our experiment is a source of 18:3 *n*-3, which may explain the origin of this isomer.

Other worth noted effect of nitrogen source lays on CLA profile. Although nitrogen source played almost no effect on biohydrogenation precursors and end-product, one of the most important intermediates to human health was affected by its interaction with oil supplementation. Oil inclusion promoted a more marked increase of 18:2 *cis*-9,*trans*-11 in SM than U diets in TRC, and a similar tendency (P = 0.112), although not statistically significant, was observed for SAB fraction. Similarly, a nitrogen source by oil supplementation interaction was noted with other CLA isomers. Higher increases in the proportions of 18:2 *trans*-10,*cis*-12 and 18:2 *trans*-9,*trans*-11 in TRC and a tendency (P = 0.057) of 18:2 *cis*-9,*cis*-11 in SAB where observed in animals fed true protein diets supplemented with oil. CLA proportions of LAB were not affected. To our knowledge, this is the first report on the influence of nitrogen source on CLA proportions in ruminal digesta. The lack of effects of nitrogen source on LAB and SAB fractions suggest that the reported effects on TRC might be attributed to other ruminal microorganisms, namely to rumen ciliate protozoa.

Soybean oil supplementation provided an increment of FA available for rumen microbial population, namely 18:2 *n*-6 and 18:3 *n*-3, the main precursors of C18 FA biohydrogenation. Inclusion of soybean oil promoted the biohydrogenation of these precursors, as 18:2 *n*-6 and 18:3 *n*-3 proportions decreased in all rumen fractions. Additionally, C18:3 and C18:2 biohydrogenation intermediates decreased while the main octadecenoic intermediate, 18:1 *trans*-11, increased in TRC, LAB, and SAB. Although biohydrogenation was promoted with oil supplementation the proportion of 18:0, the end-product, was not affected in bacterial fractions and only tended (P = 0.054) to increase in TRC. These results may suggest that the last step of biohydrogenation, the saturation of 18:1 *trans*-11 to 18:0, was inhibited.

Hydrogenation of 18:1 *trans*-11 to 18:0 is carried out by members of the group B bacteria, while members of group A mostly hydrogenate 18:3 *n*-3 and 18:2 *n*-6 to 18:1 *trans*-11 (Kemp & Lander, 1984). Few are the bacteria known to have the ability to hydrogenate C18 FA to 18:0, among which are *Butyrivibrio hungatei* (van de Vossenberg & Joblin, 2003) and *B. proteoclasticus* (Wallace et al., 2006). Despite the classification of Kemp and Lander (1984) into two different groups, main biohydrogenating bacteria are phylogenetically closed, belonging to the *Butyrivibrio*/*Pseudobutyrivibrio* group (Paillard et al., 2007). Members of this group of bacteria are cellulolytic, although they can use a broad range of substrates (Stewart, Flint & Bryant, 1997). Overall metabolism of cellulolytic bacteria might have been

limited with oil supplementation as digestibility was negatively affected. However, an interaction tended ($P = 0.086$) to be significant for NDF digestibility, as negative effects of oil inclusion were more attenuated with true protein (SM) than U diet. Ruminal cellulolytic bacteria require branched-chain FA for growth (Dehority, Scott & Kowaluk, 1967; Slyter & Weaver, 1971). The limitation of branched-chain VFA in non-protein nitrogen diets might have conditioned cellulolytic bacterial growth and/or activity. Additionally, 18:0 producers are particularly sensitive to toxic effects of non-esterified unsaturated FA (Maia, Chaudhary, Figueres & Wallace, 2007; Paillard et al., 2007) hydrolysed from dietary lipids.

Biohydrogenation pattern and intermediates were similar across the ruminal fractions studied, with low proportions of most C18:2 and C18:3 intermediates and higher proportions of C18:1 intermediates. Unlike most monoenoic acids, 18:1 *cis*-9 proportion in TRC, LAB, and SAB decreased with oil supplementation, which is consistent with higher biohydrogenation activity. Mosley, Powell, Riley and Jenkins (2002) clearly demonstrated that 18:1 *cis*-9 was biohydrogenated by mixed ruminal bacteria to multiple C18:1 FA, ranging from 18:1 *trans*-6 to 18:1 *trans*-16, and to 18:0. Inter-conversion among C18:1 *trans* FA were also shown to occur (Proell, Mosley, Powell & Jenkins, 2002).

In animals fed diets supplemented with soybean oil (UO and SMO), the major C18:1 intermediate was 18:1 *trans*-11 in all rumen fractions studied. As rumen contents were collected before feeding, 17 h after the last meal, the FA profile found may reflect the end-point of the biohydrogenation activity, i.e., the equilibrium status for the specific diets offered to animals. The accumulation of 18:1 *trans*-11 in TRC, LAB, and SAB fractions after a prolonged fasting period might suggest a role of this particular FA in a possible adaptative response or mechanism of the rumen microbial ecosystem. Accumulation of *trans* octadecanoates in the rumen was suggested to be beneficial to bacterial population in face of environmental stress stimuli, as low pH, high concentration of FA and presence of ionophores (Bessa, Santos-Silva, Ribeiro & Portugal, 2000).

Lypolysis was not determined, nevertheless it is a crucial step as the presence of a free carboxyl group is an absolute requirement for biohydrogenation to occur (Hawke & Silcock, 1970; Hazlewood & Harfoot, 1997). Differences in ruminal lipolysis with different experimental diets or rumen fractions would reflect in limited or differentiated biohydrogenation, as the concentration of precursors available for biohydrogenation would, most probably, differ.

4.5. Conclusions

Nitrogen source had no influence on biohydrogenation extent or intermediates profile. The availability of true protein or non-protein nitrogen had a major effect on proportions of OBCFA, but another relevant effect was reported in bacterial fractions. The proportion of 18:1 *cis*-11 increased with limited availability of pre-formed amino acids. This suggests a compensatory mechanism with a shift from BCFA to monounsaturated FA *de novo* synthesis in ruminal bacteria, possibly as a way to maintain membrane fluidity.

Soybean oil supplementation, at 6% level, promoted an increase in the biohydrogenation activity. Dietary precursors were extensively hydrogenated to 18:1 *trans*-11 but not to 18:0. Most C18:2 and C18:1 FA intermediates increased with oil supplementation, the effects being more marked in SAB than LAB fraction. Bacteria responsible for hydrogenation of 18:1 trans-11 to 18:0, namely *B. proteoclasticus*, might have been inhibited by soybean oil supplementation. Conjugated linoleic acid proportions, mainly 18:2 *cis*-9,*trans*-11, increased more markedly in TRC of animal fed true protein diet supplemented with oil. A similar trend was observed in SAB but not in LAB. The lack of effects of nitrogen source and oil supplementation on bacterial fractions suggests the involvement of other microorganisms, as the rumen ciliate protozoa.

Total FA content increased in all ruminal fractions with oil supplementation, with LAB increasing more steeply than SAB. Nevertheless, SAB FA content was much higher than that of LAB. Differences may rest in higher proportion of cellulolytic bacteria, typically solid associated, found in free rumen fluid in search of new plant material for colonisation after a long fasting period.

4.6. References

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Chapter 5

General discussion

5.1. Discussion

Metabolism of polyunsaturated fatty acids (PUFA) in the rumen by the main biohydrogenating bacterium, *Butyrivibrio fibrisolvens*, and dietary factors affecting lipid metabolism were investigated during this PhD research. The main focus was on biohydrogenation intermediate profiles and mechanisms underlying the biohydrogenation process, in order to provide new insights for manipulating the rumen lipid metabolism by diet, and ultimately to optimise ruminant milk and meat fatty acids (FA) profile for human consumption.

As pointed out in **Chapter 1**, increased dietary fat consumption, namely the intake of saturated fat, has been associated with higher incidence of chronic diseases (Hu et al., 1997; Nugent, 2004). Ruminant edible products, milk and meat, are known to have a high saturated FA content (Banks & Hilditch, 1931). Nutritional recommendations are, thereby, to decrease consumption of these products and to increase vegetables and fish products intake, which are rich in PUFA, as well as to decrease the overall fat intake (Elmadfa & Kornsteiner, 2009). However, fat is an important nutrient to meet human energetic requirements and essential lipid-soluble vitamins uptake. Additionally, milk, dairy products and red meat have other beneficial nutrients to human health, as vitamins, minerals, FA, among others (Shingfield, Chilliard, Toivonen, Kairenius & Givens, 2008). Saturation of ruminant fats has two origins, the *de novo* synthesis of saturated FA and biohydrogenation, the latter being the most important. Dietary lipids entering the rumen are rapidly and extensively hydrolysed to non-

esterified FA and the PUFA, mainly α-linolenic (18:3 *n*-3) and linoleic (18:2 *n*-6) acids, are hydrogenated to stearic acid (18:0; Harfoot & Hazlewood, 1997; Jenkins, Wallace, Moate & Mosley, 2008). During this process, i.e. biohydrogenation, several biohydrogenation intermediates are formed, among which conjugated linoleic acids (CLA) and vaccenic acid (18:1 *trans*-11) are of particular interest. Several health-promoting or disease preventing properties have been described for CLA, including anticarcinogenic, antiatherosclerosis, and fat reduction effects (Park, Storkson, Albright, Liu & Pariza, 1999; Parodi, 1999; Belury, 2002; Pariza, 2004; Lee, Lee, Cho & Kim, 2005; Tricon, Burdge, Williams, Calder & Yaqoob, 2005; Bhattacharya et al., 2006; Salas-Salvado, Marquez-Sandoval & Bullo, 2006; Kelley, Hubbard & Erickson, 2007; Park, 2009). 18:1 *trans*-11 also exhibits beneficial health effects on its own or by its endogenously Δ9-desaturation to 18:2 *cis*-9,*trans*-11, a CLA isomer, in tissues (Banni et al., 2001; Voorrips et al., 2002; Corl, Barbano, Bauman & Ip, 2003; Rissanen, Knekt, Jarvinen, Salminen & Hakulinen, 2003; Lock, Corl, Barbano, Bauman & Ip, 2004; Kay, Mackle, Auldist, Thomson & Bauman, 2004). Indeed, 18:2 *cis*-9,*trans*-11 is the main CLA isomer found in ruminant products, thereby named rumenic acid (Kramer et al., 1998).

Ruminant milk, meat and dairy products are a considerably part of most diets in developed and developing countries, particularly in Western diets. Thus, FA profile of these products has a strong impact in humans' diet, and possibly on human health. Improving FA profile to meet nutritional recommendations is possible and, in fact, appears to be the best strategy to improve human FA intake without consumers' effort or altering their consumption habits.

Milk and meat FA profile are closely related, and dependent of lipid metabolism in the rumen, namely of biohydrogenation (Harfoot & Hazlewood, 1997). Many are the factors affecting biohydrogenation in the rumen (Bessa, Santos-Silva, Ribeiro & Portugal, 2000; Palmquist, Lock, Shingfield & Bauman, 2005), such as pH (Van Nevel & Demeyer, 1996; Kalscheur, Teter, Piperova & Erdman, 1997), forage to concentrate ratio (Latham, Storry & Sharpe, 1972; Leat, 1977; Gerson, John & King, 1985; French et al., 2000), oil supplementation (Harfoot, Noble & Moore, 1973; Kennelly, 1996; Bu, Wang, Dhiman & Liu, 2007; Glasser, Ferlay & Chilliard, 2008), and ionophores (Fellner, Sauer & Kramer, 1997). Manipulation of animal diet composition appears to be a good strategy to enhance modifications on biohydrogenation. However, in order to alter biohydrogenation and promote more health beneficial FA outflow from the rumen, more research is needed. The investigations presented in this thesis were designed to address specific issues and provide new insight in this area of research.

The experiment in **Chapter 2**, contributed to clarify the purpose of biohydrogenation in ruminal bacteria, particularly in *B. fibrisolvens*. Biohydrogenation was shown to be a detoxifying mechanism to PUFA. Growth only occurred after PUFA were hydrogenated to 18:1 *trans*-11, *B. fibrisolvens* biohydrogenation end-product. When PUFA were not biohydrogenated to the *trans* monoenoic FA, growth did not occur. Underlying mechanisms of this toxicity were not possible to determine. Membrane integrity, as measured by propidium iodide uptake, and membrane potential were not associated with toxic effects of PUFA on *B. fibrisolvens* JW11 growth, however. A metabolic effect rather than a membranary effect may be on the basis of the PUFA toxicity. 18:1 *trans*-11 appeared to have a protective effect on *B. fibrisolvens* metabolism, as it attenuated toxic effects of 18:2 *n*-6 on growth. A free carboxyl group was a requisite for PUFA toxicity, as FA methyl esters had no effect on membrane integrity or growth.

When animals are fed high-concentrate diets supplemented with oil, biohydrogenation pathway of 18:2 *n*-6 is altered towards the formation of 18:2 *trans*-10,*cis*-12 and 18:1 *trans*-10 FA, instead of the commonly 18:2 *cis*-9,*trans*-11 and 18:1 *trans*-11 (Griinari & Bauman, 1999; Offer, Marsden & Phipps, 2001; Daniel, Wynn, Salter & Buttery, 2004). This modification is usually referred as the "*trans*-10 shift", and has been associated with milk fat depression (Bauman & Griinari, 2001; Peterson, Matitashvili & Bauman, 2003; Shingfield et al., 2006; Shingfield & Griinari, 2007). Two factors have been identified in promoting the *trans*-10 shift; high starch content and low fermentation pH. As these factors are interdependent and their effects hard to distinguish *in vivo*, an *in vitro* approach in a long-term fermentation system (RUSITEC) was used to identify the main factor, or factors, involved in the *trans*-10 shift, i.e., pH, starch and/or their interaction (**Chapter 3**). Low pH inhibited C18 FA biohydrogenation, which may have been caused by an inhibition of lipolysis or a decrease in cellulolytic bacteria numbers. Indeed, *B. fibrisolvens*, one of the major biohydrogenating-bacteria in the rumen, is a cellulolytic bacterium. High starch inclusion (50%) promoted a more extensive biohydrogenation and altered the biohydrogenation pathway and intermediates formed. A shift on C18 FA biohydrogenation from *trans*-11 towards *trans*-10 formation was only observed in high starch diets, regardless of fermentation pH. This shift was suggested to be caused by changes in ruminal micropopulation, as the 18:2 *trans*-10,*cis*-12 producers *Megasphaera elsdenii* (Kim, Liu, Rychlik & Russell, 2002) and *Propionibacterium acnes* (Wallace et al., 2006; Wallace, McKain, Shingfield & Devillard, 2007) populations were shown to increase in animals fed high-concentrate diets (Stewart et al., 1997). To our

knowledge, this is the first report showing the specific effect of starch inclusion on biohydrogenation shift towards 18:1 *trans*-10 production.

Dietary nitrogen source was found to indirectly affect the overall rumen metabolism (Cabrita, Fonseca, Dewhurst & Gomes, 2003). Level of dietary nitrogen were reported in middle 80's not to affect biohydrogenation (Gerson, John, Shelton & Sinclair, 1982; Gerson, John & Sinclair, 1983) but information on nitrogen source is lacking. The *in vivo* experiment in **Chapter 4** was designed to address the effects of nitrogen source (non-protein nitrogen *vs.* true protein) and oil supplementation on FA composition and biohydrogenation profiles of rumen contents and bacterial fraction. Nitrogen source had no influence on biohydrogenation extent or intermediates profiles, but affected odd- and branched-chain fatty acids (OBCFA), 16:1 *cis*-9 and 18:1 *cis*-11 proportions. The increased proportion of 18:1 *cis*-11 in the absence of pre-formed amino acids suggested a compensatory mechanism involving a shift from OBCFA to monounsaturated FA *de novo* synthesis in order to maintain membrane fluidity in ruminal bacteria. Soybean oil promoted biohydrogenation, with FA precursors being extensively hydrogenated to 18:1 *trans*-11 but not to 18:0. Results suggest a possible inhibition of the activity or population of bacteria responsible for hydrogenation of 18:1 *trans*-11 to 18:0, namely *Butyrivibrio proteoclasticus* (Wallace et al., 2006). Biohydrogenation profile did not differ between liquid associated bacteria (LAB) and solid associated bacteria (SAB), and total FA increased with oil supplementation, more markedly in LAB. However, total FA content of SAB was much higher than that of LAB. Accumulation of 18:1 *trans*-11 was observed after a long fasting period in bacterial fractions of animals fed oil supplemented diets, suggesting a possible role in the rumen ecosystem. Indeed, as observed in **Chapter 2,** 18:1 *trans*-11 attenuated the inhibitory effects of 18:2 *n*-6 on growth of *B. fibrisolvens*. A similar protective role may be suggested by the accumulation of 18:1 *trans*-11 after 17 h of fast in bacterial fractions. The accumulation of *trans* octadecenoates in the rumen was proposed to be beneficial to bacteria as response to environmental stress, such as low pH, presence of ionophores or high concentration of FA (Bessa et al., 2000).

Changes in *trans* octadecenoates profile were associated with modifications in bacterial population, namely within the *Butyrivibrio* group, after algae supplementation (Boeckaert et al., 2008). However, the concentration of the main 18:0-producer, *B. proteoclasticus*, did not relate to the drop of 18:0 in the rumen (Boeckaert et al., 2008). Similarly, no correlation was found between *B. proteoclasticus* concentration in strained rumen fluid and duodenal 18:0 flow (Kim et al., 2008; Huws et al., 2010), or between DNA concentration of *Butyrivibrio* 18:0-producing group and 18:0 concentration of rumen planktonic and biofilm samples

(Huws et al., 2008). These findings suggest that other and yet uncultivated microbial species might be involved in 18:0 production and/or play a more important role in the final step of the biohydrogenation process. It is presently accepted that most commonly studied ruminal bacterial species represent only a small fraction of the total bacterial population that inhibit the rumen microbial ecosystem (Stevenson & Weimer, 2007; Firkins, Karnati & Yu, 2008; Wallace, 2008). Therefore, there is a strong possibility that 18:0 production is associated to yet uncultivated bacteria. Despite efforts on isolation of new 18:0-producers from the rumen, few achievements have been made. Isolation of new biohydrogenating bacteria is a laborious process, as bacteria have to be grown in a medium with PUFA, which are by themselves toxic to growth of bacteria with biohydrogenating activity (Wallace et al., 2006; Maia et al., 2007). Although bacterial isolation and pure culture studies have provided extremely important informations on the genus and strains involved in rumen biohydrogenation, their relative importance in *in vivo* rumen lipid metabolism is yet to be clarified (Palmquist et al., 2005). To present knowledge, biohydrogenation in the rumen ecosystem is a synergistic process which involves a consortium of bacteria, each playing a role in the transformation of dietary unsaturated FA into more saturated ones (Harfoot & Hazlewood, 1997).

5.2. Future work

The present PhD research provided new data and insights on ruminal biohydrogenation, and factors affecting it pattern and intermediate profile. Nevertheless, more than major breakthroughs and conclusions, several were the suggestions, hypothesis and questions that emerged along the way, namely on microbial population profiles and effects on ruminant product. Although not being a new or hot topic, the *trans*-10 shift is, in our opinion, of high interest to animal nutrition and management but also to beneficial FA profile of ruminant products for human consumption.

The *trans*-10 shift observed in animals fed high-concentrate diets supplemented with oils has been associated with a marked decrease on CLA content (Griinari et al., 1998; Piperova et al., 2002), namely 18:2 *cis*-9,*trans*-11, and milk fat depression (Bauman & Griinari, 2003). Although content of 18:2 *cis*-9,*trans*-11 in ruminant products is a result of ruminal biohydrogenation (Harfoot & Hazlewood, 1997) and Δ9-desaturation in tissues (Corl et al., 2003; Kay et al., 2004), 18:2 *trans*-10,*cis*-12 is thought to arise only from biohydrogenating activity (Griinari et al., 2000). Additionally, CLA isomers have different physiological

effects, though anticarcinogenic properties have been implied to both isomers (Ip et al., 2002, Park, 2009). The isomer 18:2 *cis*-9,*trans*-11 was involved in the inhibition of TNF-α, growth promotion in rodents, and improvement of lipoprotein profiles (Cook, Miller, Park & Pariza, 1993; Yang & Cook, 2003; Valeille et al., 2004), whereas 18:2 *trans*-10,*cis*-12 was involved in body weight, fat composition, and inhibition of Δ9-desaturase activity, protein and/or mRNA (Park et al., 1999; Park et al., 2000).

The biohydrogenation shift towards *trans*-10 production would thereby provide less beneficial FA for human consumption. However, induction of milk fat depression may play an important role in the transition period and early lactation. Dairy cows in early lactation are in negative energy balance due to the rapid increase in nutrient demands for milk synthesis (Bell, 1995). Thus, the *trans*-10 shift might be used as a nutritional management of early lactation cows by inducing milk fat depression while simultaneously maintaining feed intake and yield of other milk components, and preventing metabolic diseases (Griinari & Bauman, 2006). However, in order to use it as dietary management, the identification of dietary factors and microorganisms involved in *trans*-10 shift is crucial.

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Chapter 6

Concluding remarks

Overall data herein presented suggest biohydrogenation as a detoxification mechanism to the bacteriostatic effects of dietary polyunsaturated fatty acids (PUFA), namely α-linolenic acid (18:3 *n*-3) and linoleic acid (18:2 *n*-6), on the rumen microbial ecosystem. Although bacterial membranes could be easily accepted as the primary site for PUFA toxicity, a metabolic effect seems to carry more weight, as *Butyrivibrio fibrisolvens* growth inhibition was not related to changes in membrane integrity or membrane potential. Additional suggestion of a possible metabolic effect of PUFA on rumen microbial population emerged from *de novo* FA synthesis modifications in bacterial fractions (liquid and solid associated bacteria). This alteration suggested a possible adaptative mechanism in order to regulate bacterial membrane fluidity when branched amino acids were less available in diets for odd- and branched-chain fatty acids synthesis.

Biohydrogenation of non-esterified dietary PUFA involves an initial isomerisation to *trans* fatty acids (FA) intermediates which are further hydrogenated until the formation of 18:0. *Trans* unsaturated FA thus formed could also be used to regulate membrane fluidity, as these have lower melting points than *cis* isomers. Another possible adaptative mechanism to the rumen microbial ecosystem involving biohydrogenation is the suggested protective effect of 18:1 *trans*-11 to *B. fibrisolvens*. Not only biohydrogenation is a detoxification mechanism to one of the major biohydrogenating bacterium in the rumen, *B. fibrisolvens*, as its end-product may attenuate toxic effects of 18:2 *n*-6 on bacterial growth.

Biohydrogenation extension and intermediates profile are affected by dietary factors. High starch but not low pH promoted a shift in biohydrogenation pathway towards *trans*-10

production instead of the typical *trans*-11, whereas nitrogen source had no effect on FA intermediates formation. Additionally to changes in biohydrogenation, changes on the overall rumen metabolism, possibly involving modifications of the microbial population composition or profile, may also occur.

Diet manipulation may indeed be the most effective strategy to improve FA profile of ruminants-derived products profile for human consumption without altering consumers' habits. However, in order to achieve ruminant products with healthier FA profiles, more research is needed to better understand and predict modifications caused by ruminal biohydrogenation. Major achievements have been made on identification of factors affecting milk and meat fat and FA composition, over the last years. However, the approach used to study the rumen lipid metabolism has been mostly as a black box. Ruminal biohydrogenation is indeed an important metabolic process to the rumen microbial ecosystem with direct consequences on the fat composition and FA profile of ruminant products as milk, dairy products and meat, and ultimately to human health.

Annex

List of publications

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