UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

STRATEGIES FOR THE PROTECTION OF EICOSAPENTAENOIC ACID (EPA) FROM THE OVINE RUMEN MICROBIOTA USING *NANNOCHLOROPSIS OCEANICA*

ANA CRISTINA MARTINS DE MIRANDA VÍTOR

Orientador(es): Professor Doutor Rui José Branquinho de Bessa
Professora Doutora Susana Paula Almeida Alves
Professora Doutora Sharon Ann Huws

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

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It's surely our responsibility to do everything within our power to create a planet that provides a home not just for us, but for all life on Earth.

David Attenborough

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Resumo

Estratégias para a proteção do ácido eicosapentaenóico (EPA) da microbiota ruminal de ovina utilizando *Nannochloropsis oceanica*.

Os óleos marinhos derivados de microalgas são uma fonte rica em ácidos gordos polinsaturados de cadeia longa ómega-3 para ruminantes, com várias vantagens ambientais e de produtividade em relação ao óleo de peixe. A *Nannochloropsis oceanica* é uma microalga marinha unicelular particularmente rica em ácido eicosapentanóico (EPA). A biomassa da *N. oceanica* pode ser seca por diferentes métodos, tendo diferentes impactos na capacidade de preservar o EPA no seu interior. O objetivo desta tese foi explorar o grau de proteção ruminal do EPA *in vivo* e a sua deposição nos tecidos de borregos alimentados com dietas suplementadas com diferentes biomassas desidratadas de *N. oceanica*. A microscopia eletrónica de varredura confirmou que as células de *N. oceanica* na biomassa liofilizada foram mais bem preservadas do que na biomassa seca por atomização. No rúmen, as estimativas da extensão da biohidrogenação (desaparecimento) do EPA também confirmaram que a *N. oceanica* liofilizada estava mais protegida da biohidrogenação. Um menor rácio *t*10/*t*11-18:1 foi observado em animais alimentados com *N. oceanica* em comparação com animais controlo. No geral, não foram encontradas diferenças nos parâmetros da fermentação nem nos indicadores de paraqueratose ruminal. A análise do microbioma revelou que o *t*10-18:1 poderá ser um melhor indicador, quando comparado com o *t*11-18:1, para determinar perfis específicos de bacterioma ruminal. A incorporação de *N. oceanica* nas dietas resultou em um maior teor de ácidos gordos polinsaturados de cadeia longa ómega-3 na carne e no tecido adiposo subcutâneo dos borregos suplementados, sendo o EPA o mais significativo. Também foi possível concluir que o conteúdo de EPA no cérebro não respondeu à suplementação dietética. No entanto, a retina demonstrou uma resposta significativa em borregos suplementados com *N. oceanica*. Um novo método de encapsulamento de ácidos gordos polinsaturados de cadeia longa ómega-3 resistente à metabolização ruminal, usando algenano extraído da *N. oceanica,* foi desenvolvido e testado *in vitro* com resultados ainda inconclusivos em relação à proteção do EPA.

Palavras-chave: *Nannochloropsis oceanica*, biohidrogenação, ácidos gordos polinsaturados de cadeia longa ómega-3, ruminantes.

Abstract

Strategies for the protection of eicosapentaenoic acid (EPA) from the ovine rumen microbiota using *Nannochloropsis oceanica*.

Marine oils derived from microalgae are a rich source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) for ruminants, with several productivity and environmental advantages over fish oil. *Nannochloropsis oceanica* is a single-celled marine microalga particularly rich in eicosapentaenoic acid (EPA). *N. oceanica* slurry biomass can be dried using different methods, having different impacts in the ability to preserve EPA in its interior. The aim of this thesis was to explore the *in vivo* EPA ruminal protection degree and tissues deposition in lambs that were fed diets supplemented with different *N. oceanica* dried biomasses. Scanning electron microscopy confirmed that *N. oceanica* cells in freeze-dried biomass were better preserved than in spray-dried biomass. In the rumen, the estimates of biohydrogenation extent (disappearance) of EPA also confirmed that freeze dried *N. oceanica* was more protected from biohydrogenation. A lower *t*10/*t*11-18:1 ratio was observed in animals fed *N. oceanica* compared to animals fed the control diet. Overall, no disturbances were found in fermentable parameters nor indicators of ruminal parakeratosis. Microbiome analysis revealed that *t*10-18:1 may be a more suitable indicator compared to *t*11-18:1, for determining specific bacteriome profiles in the rumen. Incorporating *N. oceanica* into the diets resulted in a higher content of n-3 LC-PUFA in the meat and subcutaneous adipose tissue of the supplemented lambs, with EPA being the most significant contributor. It was also possible to conclude that brain EPA content was not responsive to dietary supplementation. However, the retina showed a significant response in lambs supplemented with *N. oceanica*. A novel ruminal-resistant n-3 LC-PUFA encapsulation method using *N. oceanica* extracted algaenan was developed and tested *in vitro* with yet inconclusive results regarding the protection of EPA.

Keywords: *Nannochloropsis oceanica*, biohydrogenation, long-chain n-3 fatty acids, ruminants.

Resumo alargado

Estratégias para a proteção do ácido eicosapentaenóico (EPA) da microbiota ruminal de ovina utilizando *Nannochloropsis oceanica*.

O crescimento da população mundial, aliado à crescente necessidade de produção de proteína animal tem aumentado a necessidade de procurar novos recursos para alimentação animal que permitam manter um compromisso entre a manutenção da produtividade e a sustentabilidade do sistema de produção.

No que concerne à produção de ruminantes, acresce a necessidade da procura de estratégias para a melhoria do perfil nutricional de ácidos gordos dos produtos edíveis, com vista à contribuição para a melhoria da saúde do consumidor. O aumento do teor de ácidos-gordos polinsaturadas n-3 nos produtos edíveis de origem animal é desejável, no sentido em que o seu consumo está associado a um menor risco de ocorrência e severidade de doenças cardiovasculares, neurodegenerativas e inflamatórias generalizadas.

As principais fontes de ácidos gordos polinsaturados de cadeia longa n-3 para a alimentação de ruminantes, como o ácido eicosapentaenóico (EPA) e o ácido docosahexaenóico (DHA), têm origem em produtos de origem marinha, como o óleo de peixe e as microalgas. Tradicionalmente, o óleo de peixe tem sido amplamente utilizado em ensaios *in vivo* para avaliação da deposição de EPA e DHA em produtos edíveis de ruminantes. No entanto, preocupações crescentes com a sustentabilidade do uso de óleo de peixe para este propósito têm colocado em questão a utilização de óleo de peixe para suplementação de dietas. A utilização do óleo de peixe tem, então, sido crescentemente substituída pela utilização de microalgas como suplemento naturalmente rico em ácidos gordos polinsaturados n-3. As microalgas apresentam a vantagem de não requererem terra arável para a sua produção, têm a possibilidade de ser cultivadas com recursos hídricos não potáveis corretamente tratados, terem uma elevada taxa de crescimento e geração de biomassa, aleando todos estes benefícios com uma composição nutricional e em compostos bioativos de extremo valor, de entre os quais se destacam os ácidos gordos polinsaturados n-3.

A *Nannochloropsis oceanica* é uma microalga marinha unicelular particularmente rica em EPA. Por forma a ser incorporada na dieta de ruminantes, a biomassa da *N. oceanica* pode ser seca por diferentes métodos, tendo diferentes impactos na capacidade de preservar o EPA no seu interior. A necessidade de preservação do EPA no interior da célula da microalga prende-se com a necessidade de evitar a

biohidrogenação ruminal do EPA, levada a cargo pelo microbioma naturalmente presente no rúmen, e consecutiva perda de funções biológicas. Os principais métodos de secagem alvo de estudo na presente tese são a secagem por atomização (*spray-drying*) e a liofilização (*freeze-drying*).

Num trabalho preliminar *in vitro* anteriormente efetuado, foi possível concluir que a microalga *Nannochloropsis oceanica*, por via das suas características morfológicas, especialmente relacionadas com sua parede celular, apresentava um forte potencial como fonte natural de ácido eicosapentaenóico protegido. Assim sendo, surgiu a necessidade de confirmação dos resultados de eficiência de proteção *in vivo*.

O objetivo desta tese foi explorar o grau de proteção ruminal do EPA *in vivo* e a sua deposição nos tecidos de borregos alimentados com dietas suplementadas com diferentes biomassas desidratadas de *N. oceanica*. A hipótese previamente desenvolvida será de que a microalga seca por atomização apresentará uma menor capacidade para proteger o EPA no interior quando comparada com a microalga seca por liofilização, uma vez que o processo de atomização, por utilizar um binómio de elevada temperatura/ elevada pressão para a secagem da biomassa, irá comprometer em maior grau a integridade da parede celular da microalga. A microscopia eletrónica de varrimento das duas biomassas confirmou que a integridade da parede celular células de *N. oceanica* na biomassa liofilizada foi mais bem preservada do que na biomassa seca por atomização. No rúmen, as estimativas da extensão da biohidrogenação (desaparecimento) do EPA também confirmaram que a *N. oceanica* liofilizada garantiu uma maior proteção do EPA contra a biohidrogenação. Assim sendo, a *N. oceanica* liofilizada pode constituir uma melhor fonte de EPA ruminal protegido em comparação com *N. oceanica* seca por atomização, uma vez que níveis mais elevados de EPA foram encontrados no rúmen e abomaso de animais alimentados com a primeira biomassa, indicando um menor desaparecimento do EPA ou uma menor completude da sua biohidrogenação ruminal. No entanto, elevados teores de EPA foram ainda encontrados no conteúdo do ceco e nas fezes, sugerindo que a sua absorção no intestino delgado não foi totalmente eficiente. Quando dietas com baixo teor de forragem são suplementadas com elevados níveis de ácidos gordos polinsaturados e/ou amido, o pH ruminal é reduzido e ocorrem alterações na composição do microbioma, envolvendo principalmente uma mudança de uma população bacteriana predominantemente celulolítica, produtora de *t*11-18:1, para uma população mais população fermentadora de açúcar que preferencialmente produz *t*10-18:1. O fenómeno é designado por *t*10-shift (*t*10-18:1/ *t*11-18:1 > 1). O *t*10-shift e, por conseguinte, um maior teor de *t*10-18:1 nos produtos edíveis está associado a efeitos

prejudiciais à saúde do consumidor. Um menor rácio *t*10/*t*11-18:1 foi observado em animais alimentados com *N. oceanica* em comparação com animais controlo. No geral, não foram encontradas diferenças nos parâmetros da fermentação nem nos indicadores de paraqueratose ruminal. Os resultados demonstram que a suplementação de EPA em borregos através da adição de *N. oceanica* às dietas pode aumentar o conteúdo de EPA no trato gastrointestinal dos mesmos, sem comprometer a abundância das bactérias ruminais e a sua diversidade. Não foram encontradas correlações entre o teor de EPA e géneros bacterianos específicos, nem correlações significativas entre o teor de EPA e as vias metabólicas inferidos. A análise do microbioma revelou que o *t*10-18:1 poderá ser um melhor indicador, quando comparado com o *t*11-18:1, para determinar perfis específicos de bacterioma ruminal. À semelhança da tendência verificada nos conteúdos gastrointestinais, a incorporação de *N. oceanica* nas dietas resultou em um maior teor de ácidos gordos polinsaturados de cadeia longa n-3 na carne e no tecido adiposo subcutâneo dos borregos suplementados, sendo o EPA o mais significativo. No que diz respeito às recomendações diárias de ingestão de EPA + DHA para humanos, a carne dos borregos alimentados com a dieta com *N. oceanica* liofilizada registou um teor de 25 mg de EPA + DHA por cada 100 g de músculo, sendo aproximadamente o dobro do teor registado na carne de borregos alimentados com a dieta controlo (sem EPA). Consequentemente, 100 g de carne de borrego alimentado com a biomassa liofilizada atingiram aproximadamente 10% da dose diária mínima recomendada de EPA + DHA para humanos (250 mg). O enriquecimento dos produtos edíveis em ácidos gordos n-3 foi realizado sem penalização da performance produtiva dos animais e sem penalização das qualidades organoléticas da carne. A suplementação de níveis supranutricionais de vitamina E nas dietas suplementadas com fontes exógenas de EPA poderá ter contribuído positivamente para a redução da suscetibilidade da carne à lipoxidação. Também foi possível concluir que o conteúdo de EPA no cérebro não respondeu à suplementação dietética. No entanto, a retina demonstrou uma resposta significativa em borregos suplementados com *N. oceanica,* sendo apenas a magnitude de resposta mais elevada na gordura subcutânea e plasma. Um novo método de encapsulamento de ácidos gordos polinsaturados de cadeia longa n-3 resistente à metabolização ruminal, usando algenano extraído da *N. oceanica,* foi desenvolvido e testado *in vitro* com resultados ainda inconclusivos em relação à proteção do EPA. O alto custo de produção das microalgas continua a ser um desafio significativo para a sua utilização em produção comercial de rações, decorrente principalmente das tecnologias dispendiosas envolvidas na cultura, processamento/extração e secagem. Assim sendo, explorar e implementar produtos ou métodos alternativos para

impulsionar a utilização de microalgas na produção de ruminantes poderá representar um contributo para o aumento do valor da sua utilização. Assim sendo, assumimos o desafio de isolar e propor uma aplicação biotecnológica de componentes da parede celular da *N. oceanica*, neste caso algenanos, na conceção de um método de encapsulamento ruminal que necessita, contudo, de aperfeiçoamentos.

List of publications

This thesis was based on the following publications:

Vítor, A.C.M., Francisco, A.E., Silva, J. *et al.* Freeze-dried *Nannochloropsis oceanica* biomass protects eicosapentaenoic acid (EPA) from metabolization in the rumen of lambs. *Scientific Reports* **11,** 21878 (2021). https://doi.org/10.1038/s41598-021-01255-w

Vítor A.C.M., Godoy Santos, F., Creevey, C. *et al*. The ruminal bacteriome of lambs supplemented with *Nannochloropsis* sp. microalga and its relationship with biohydrogenation intermediates. (In advanced preparation to be submitted to Frontiers in Microbiology).

Vítor, A.C.M., Godinho, M., Francisco, A.E. *et al*. *Nannochloropsis oceanica* microalga feeding increases long chain n-3 polyunsaturated fatty acids in lamb meat. *Meat Science* **197,** 109053 (2023). https://doi.org/10.1016/j.meatsci.2022.109053

Vítor A.C.M., Correia J.J., Alves S.P. *et al*. Enrichment of brain n-3 Docosapentaenoic acid (n-3 DPA) and retinal Eicosapentaenoic acid (EPA) in lambs fed *Nannochloropsis oceanica* microalga. *Animals* **13**, 828 (2023). https://doi.org/10.3390/ani13050828

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%	Percentage
°C	Degree Celsius
µg	microgram
µL	microlitre
µm	micrometre
$\alpha*$	Redness (CIELAB colour dimension)
AA	Arachidonic acid (20:4n-6)
ACSL	Long-chain fatty acid CoA synthase
ADF	Acid Detergent Fiber
ADG	Average daily gain
ADL	Acid Detergent Lignin
ALA	α-Linolenic acid (18:3n-3)
atm	Atmospheres
ATP	Adenosine triphosphate
$b*$	Yellowness (CIELAB colour dimension)
BCFA	Branched-chain fatty acids
BH	Biohydrogenation
BHI	Biohydrogenation intermediates
BHT	Butylated hydroxytoluene
BI	Biohydrogenation intermediates
BW	Body weight
c	cis
$C*$	Chroma – colour saturation
CH_4	Methane
CLA	Conjugated linoleic acid
cm	Centimetre
CO_2	Carbon dioxide
CoA	Coenzyme A
CP	Crude protein
CVD	Cardiovascular disease
DGLA	Dihomo-γ-linolenic acid (20:3n-6)
DHA	Docosahexaenoic acid
DM	Dry matter
DMA	Dimethylacetals

DMI	Dry matter intake
DPA	Docosapentaenoic acid (22:5n-3)
DTG	Derivative thermogravimetry
DW	Dry weight
DWG	Daily weight gain
E	Energy
EE	Ethyl ester
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FAS	Fatty acid synthase
FCR	Feed conversion ratio
FDR	False discovery rate
FI	Feed intake
FE	Feed efficiency
FFA	Free fatty acid
FTIR	Fourier-transform infrared spectroscopy
g	gram
g	Relative centrifuge force
G	Gauge
GC	Gas chromatography
GC-FID	Gas chromatography with flame ionization
GIT	Gastrointestinal tract
h	Hour
*H**	Hue angle
HDL	High density lipoproteins
ItaMF	Intramuscular fat
IteMF	Intermuscular fat
keV	Kiloelectron volt
kg	kilogram
kJ	Kilojoule
L	litre
*L**	Lightness
LA	Linoleic acid (18:2n-6)

LC-PUFA	Long-chain polyunsaturated fatty acids - ≥20 carbons
LDL	Low density lipoproteins
LL	*Longissimus lumborum*
LT	*Longissimus thoracis*
lysoPC	Lysophosphatidylcholines
m	metre
M	Molar
mg	miligram
min	Minute
mL	millilitre
mm	millimetre
mM	millimolar
MUFA	Monounsaturated fatty acids
N	Normality
n.d.	Not detected
n-3 PUFA	N-3 polyunsaturated fatty acids
n3 LC-PUFA	N-3 long-chain polyunsaturated fatty acids
n-6 PUFA	N-6 polyunsaturated fatty acids
n-6 LC-PUFA	N-6 long-chain polyunsaturated fatty acids
NA	Not available
NADPH	Nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acid
NH_3	Ammonia
NL	Neutral lipids
NMR	Nuclear magnetic resonance
OA	Oleic acid (*c*9-18:1)
OBCFA	Odd and branched-chain fatty acids
PBR	Photobioreactor
PC	Phosphatidylcholine
PCA	Principal Component Analysis
pH	Potential of hydrogen
PL	Phospholipid
PRF	Perirenal fat
PUFA	Polyunsaturated fatty acids
py-GC-MS	Pyrolysis–gas chromatography–mass spectrometry

RBH	Ruminal biohydrogenation
ROSs	Rod outer segments
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
SAS	Statistical analysis system (Software package)
SCD	Stearoyl-CoA desaturase
SCF	Subcutaneous fat
SDA	Stearidonic acid (18:4n-3)
SEM	Standard error of the mean
SFA	Saturated fatty acids
sPLSDA	sparse Partial Least Squares Discriminant Analysis
t	trans
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TFA	Total fatty acids
TGA	Thermogravimetric analysis
TLS	Trilaminar sheat
TMR	Total mixed ration
UFA	Unsaturated fatty acid
v	Volume
VFA	Volatile fatty acids
VLCFA	Very long-chain fatty acid
VLDL	Very-low-density lipoprotein
w	Weight
WHO	World Health Organization

Introduction

Evidence suggests that n-3 polyunsaturated fatty acids (n-3 PUFA) play a central role in cell membrane function and the optimization of their intake appears to confer many health benefits (Ruxton, 2004; Punia et al., 2019), including reduced risk of heart disease (Elagizi et al., 2018), mental health disorders (Lange, 2020), and inflammatory conditions (Gioxari et al., 2018). The most usual beneficial biological effects are frequently associated with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Loftsson et al., 2016; Zhang et al., 2019b). Due to a low endogenous conversion of α-linolenic acid into EPA and DHA, in animals, (Russell & Bürgin-Maunder, 2012) together with α-linolenic acid, these n-3 long-chain-PUFA (n-3 LC-PUFA) are termed essential and, therefore, need to be provided through the diet. The main dietary sources of EPA and DHA are derived from aquatic organisms, like seafood and microalgae (Hixson et al., 2015). Due to consumption habits, however, in most western countries, EPA and DHA consumption lies below the recommendations (Meyer et al., 2003; FAO/WHO, 2008; Howe et al., 2006). Because meat represents a more substantial source of n-3 LC-PUFA in some western diets than marine resources, the consumption of EPA and/ or DHA in enriched foods (e.g., enriched meat) can constitute a strategy to improve n-3 LC-PUFA consumption in humans. However, it is essential to maintain a balanced approach to meat consumption in light of the potential increased intake of saturated fatty acids and *trans*-fatty acids, which can have adverse effects on the consumer health.

In general, ruminants have a low PUFA content in the edible tissues (Wood et al., 2004), therefore, lipid supplements are commonly used to improve the fatty acid composition of ruminant meat, including those rich in EPA and DHA (e.g., algae and fish oil). Conflicting results regarding the effects of microalgae supplementation in lamb production traits have been published, depending on the microalga used, the feed incorporation rate and supplementation duration (Madeira et al., 2017). However, low incorporations can have no impact on feed intake (FI) and even increase body weight (BW) and average daily gain (ADG) (Sucu et al., 2017). Increasing n-3 LC-PUFA in ruminant meat can sometimes have detrimental effects in terms of meat oxidation status (Nute et al., 2007; De la Fuente-Vázquez et al., 2014), however, it can be counteracted with antioxidants as vitamin E (Ponnampalam et al., 2016). Regarding the sensory properties of the meat (Nguyen et al., 2018a), once again literature results might not always be conclusive (Álvarez-Rodríguez et al., 2022) but higher incorporation can easily result on odour, flavour and modified colour (Urrutia et al., 2016).

In addition to the challenges in the productive impact and sensory quality of the meat, the efficiency of the supplementation of ruminants with marine oils strongly relies on the degree of n-3 LC-PUFA ruminal biohydrogenation (RBH). The metabolization of ingested fatty acids (FA) is responsible for the fact that the profile of ingested lipids does not necessarily translate the lipid profile of ruminant tissues, reducing the supplementation efficiency (Jenkins et al., 2008). As a result of ruminal microbiome action on the ingested lipids (Jenkins et al., 2008), lipolysis and biohydrogenation processes convert dietary unsaturated FA (UFA) into saturated FA (SFA) (Jenkins et al., 2008). Therefore, RBH-protection technologies have been tentatively developed to reduce the ruminal metabolization of dietary FA, aiming to increase the amount of UFA bypassing the rumen, and consecutively the ones deposited in the peripheral tissues (Gadeyne et al., 2017). The strong resistance of microalgae cell wall structure can constitute a natural source of protected lipids (Alves et al., 2018), therefore we proposed to further investigate the ability of some microalgae to innately protect lipids from RBH.

This thesis aims to increase the tissue content of n-3 LC-PUFA, and namely EPA, by supplementing lambs with an EPA-rich microalga – *Nannochloropsis oceanica*, without compromising animal's production traits and meat quality. Therefore we aim to decrease RBH as much as possible to maximize EPA absorption, without a negative impact in ruminal bacteriome. We also propose to further characterize *N. oceanica* cell wall components and try to develop a novel ruminal-protection technology based on those components. Globally, the present work intends to contribute to a better understanding of the physiology of n-3 LC-PUFA-rich microalgal supplementation in lambs.

This thesis is structured in 7 chapters. In Chapter 1, "Scientific background and objectives", n-3 LC-PUFA consumption pattern and health benefits will be addressed. After that, the content of n-3 LC-PUFA in ruminant tissues will be described and sources of n-3 LC-PUFA supplementation for ruminants will be focused on fish oil and microalgae. Since microalgae are the focus in this thesis, their production and nutritional composition will be further explored. A special attention will be given to *N. oceanica* since it was the microalga specie selected for this work. Various results of the impact of feeding microalgae to ruminants will also be presented and an overview of the commercial application of microalgae in ruminant production will be addressed in a few comments. A general overview of the lipid metabolism in ruminants will be given. In the final part of the first chapter, a brief mention about lipid protection technologies will be made. After this overview, the specific objectives of the present work will be described. Chapters 2 to 5 are based on scientific manuscripts already published (3) or in advanced preparation for submission to publication (1). Previous work from our group showed that *N. oceanica* can be considered as a novel natural source of rumen-protected EPA for ruminants, *in vitro* (Alves et al., 2018). However, studies with dietary interventions using *N. oceanica* in ruminant feed are inexistent. Therefore, we aimed to confirm, *in vivo*, our previous *in vitro* results with a more complete characterization of the microalga drying methods (focusing on freeze-drying and spray-drying) and their impact in the cell wall structure and EPA protection efficiency (presented in Chapter 2). The better cell wall integrity of the freeze-dried microalga played an important role in the results. The concentration of EPA in the rumen was 50% higher in the freeze-dried microalga fed lambs compared with the ones fed the spray-dried one. An important find was that *N. oceanica* could mitigate the shift from the *t*10 biohydrogenation pathways to the *t*11 pathways compared to the control diet.

The impact on the ruminal bacteriome is presented in the following chapter (Chapter 3). No major impact was observed in the ruminal bacterial abundances and no changes in bacterial genera diversity occurred in *N. oceanica* supplemented lambs. However, because the *t*10/*t*11–18:1 ratio was mitigated by the microalgal biomass supplementation, correlations between microbial abundances and the abundance of C18 RBH intermediates were explored. It is still not fully known which microbial agents are responsible for the *t*10-shift altered BH pattern, therefore, in Chapter 3, considerations about the correlation between ruminal bacterial genera abundances and the presence of *t*10-18:1 were made.

Having confirmed that the freeze-drying method better conserved the ability to protect EPA inside the microalgal cell, we wanted to further explore the success of intestinal EPA absorption by evaluating EPA deposition in lambs' subcutaneous adipose tissue and muscle (Chapter 4), according to the different microalgal drying methods. Both dried microalgal biomasses (freeze-dried and spray dried) efficiently increased EPA in the tissues, without a negative impact on animal productive performance and meat sensory traits. Once again, the *t*10/*t*11–18:1 ratio decreased with all *Nannochloropsis*-containing diets, reinforcing the findings in the rumen.

Because n-3 LC-PUFA are known to represent a high proportion of n-3 PUFA in the brain and retina (Svennerholm, 1968; Querques et al., 2011), we proposed to evaluate their deposition in the brain and retina of lambs (Chapter 5). No microalgal supplementation studies have addressed the FA composition of brain and retina of lambs before. Our results demonstrate that, overall, brain FA composition was not responsive to the dietary intervention, but docosapentaenoic acid (DPA, 22:5n-3) enhancement in both the hippocampus and prefrontal cortex was observed in *N. oceanica* fed lambs. Retinal tissues were particularly responsive to the dietary intervention, with a 4.5-fold enhancement of EPA in the freeze-dried-fed lambs compared with the control.

We could conclude that *N. oceanica* can be a good supplementation feed to increase beneficial n-3 LC-PUFA in lambs' tissues but the commercial applicability of microalgae inclusion in ruminant diets is still lacking due to their high costs. Having gone into more detail about the microalgal cell wall, we developed the concept of utilizing *N. oceanica* algaenans in a novel rumen-protection technology (Chapter 6). Algaenan from freeze-dried *N. oceanica* was isolated, characterized and used in capsules developed to protect an n-3 LC-PUFA-rich oil, *in vitro*. Apparently, the amount of n-3 LC-PUFA in the produced capsules would have been adequate, in some cases, to produce a response in terms of EPA and DHA metabolization but the overall *in vitro* results were not conclusive.

The final chapter (Chapter 7) intends to summarize and discuss the main results obtained and, in each part (Chapter) of this thesis, integrating them in order to meet the research aims. The main conclusions and future perspectives are also pointed in this chapter.

1. CHAPTER 1 | Scientific background and objectives

1. N-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA).

1.1. Definition and relevance.

N-3 polyunsaturated fatty acids (n-3 PUFA) possess all *cis* methylene interrupted double bonds, being the last methylene interrupted double bond in n-3 PUFA, located on the third carbon atom, counting from the methyl end of the fatty acid (FA) chain. Hence, these FA are named n-3. The n-3 PUFA include, among others, α-linolenic acid (ALA, 18:3n-3), stearidonic acid (SDA; 18:4n-3), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and docosahexaenoic acid (DHA, 22:6n-3).

In humans, as in vertebrate animals, FA can be desaturated endogenously only up to the Δ9 position due to the lack of certain enzymes, such as Δ12- and Δ15-desaturases, and a low Δ-6 desaturase activity contributes to a poor conversion ratio of ALA to EPA and DHA (Russell & Bürgin-Maunder, 2012). For this reason, linoleic acid (LA, 18:2n-6) and ALA are termed essential, and EPA and DHA conditionally essential depending on essential FA availability (Tvrzicka et al., 2011), therefore needing to be taken from the diet.

In contrast to humans and vertebrate animals, terrestrial plants produce and contain fairly high amounts of ALA (Hixson et al., 2015) but are unable to produce LC-PUFA (Rizzo et al., 2023). N-3 LC-PUFA are, however, largely produced in marine ecosystems (Gladyshev et al., 2013) and fish and other seafood are good dietary sources of EPA and DHA.

The importance of the intake of N-3 LC-PUFA is related with their role in cell membrane function and development of neural tissues as in the brain and eyes, possessing several benefits (Ruxton, 2004; Punia et al., 2019), including reduced risk of heart disease (Elagizi et al., 2018), mental health disorders (Lange, 2020), and inflammatory conditions (Gioxari et al., 2018). An overview of the major health benefits of N-3 PUFA will be presented in the next topic.

1.2. Health benefits and recommendations.

1.2.1. Health benefits.

In 1971, Bang et al., 1986 reported low rates of cardiovascular diseases (CVD) in an Inuit population that consumed a fatty diet almost exclusively composed of fish and seal meat, establishing at that time a paradox with the association between dietary fat and CVD.

The interest in the health effects of marine lipids rich in n-3 FA, particularly EPA and DHA, increasingly gained economic interest and evolved in a way scientists and physicians started to approach them as medication with established recommended daily intake dosages. However, lack of efficacy of n-3 LC-PUFA in various health outcomes have been systematically reviewed (Kwak et al., 2012; Rizos et al., 2012) and conflicting results still arise, which may derive from variability in the marine oil compositions, namely n-3 PUFA content and EPA and DHA ratio (Albert et al., 2016).

Not much is known about the physiologic role of DPA or its nutritional health potential because most evidence for health benefits of n-3 LC-PUFA originates from intervention trials with fish oil supplementation, which is more rich in EPA and DHA (Howe et al., 2006). Therefore, the commonly observed beneficial biological effects are frequently associated with EPA and DHA (Loftsson et al., 2016) and are mostly related to positive outcomes of various diseases (Zhang et al., 2019b). However, the recognized DPA ability to increase EPA and DHA tissue status and its specific or shared biological effects, principally implicated in the pro-resolution of the inflammation (Drouin et al., 2019) have been contributing to a growing interest in its biological properties.

N-3 and n-6 PUFA are components of phospholipids (PL), which are constituents of cell membranes. Therefore, the increase in the intake of, for instance, n-3 PUFA starts with an alteration of the membrane FA composition (Figure 1.1). Due to the catalytic action of phospholipase A2, these FA are released from the membrane and become precursors of eicosanoids, such as prostaglandins, thromboxanes, leukotrienes or lipoxins, with a plethora of biological effects (Golanski et al., 2021).

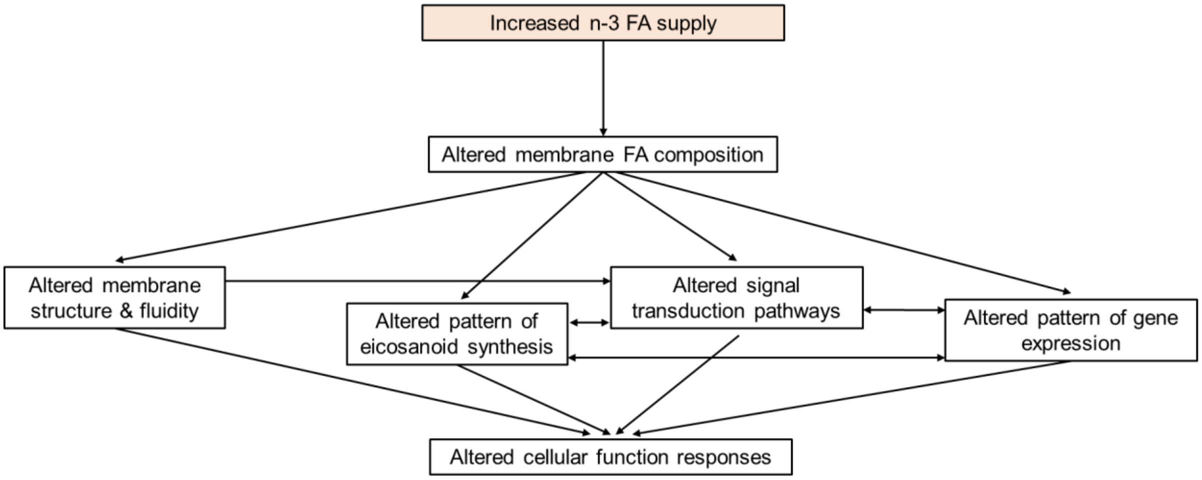

Figure 1.1- General scheme of the interacting mechanisms whereby long chain n-3 FA might influence cell function.

Adapted from Calder & Yaqoob, (2009a).

Several health benefits attributed to EPA and DHA (Russell & Bürgin-Maunder, 2012), relate to differences in regulation of transcription factors, different receptor-mediated effects, different incorporation of EPA and DHA into PL (the source of n-3 LC-PUFA may affect the extent to which EPA and DHA are incorporated), and different n-3 LC-PUFA metabolites (Russell & Bürgin-Maunder, 2012).

Cardiovascular diseases (CVD).

EPA and DHA have both shared and complementary benefits (Zhang et al., 2019b). Positive cardiovascular-related outcomes have been attributed to improvements in blood pressure control (Zhang et al., 2022), platelet reactivity and thrombosis (Dinicolantonio & Okeefe, 2019), plasma triacylglycerol (TAG) concentrations (Bornfeldt, 2021), vascular function (Colussi et al., 2017; Innes & Calder, 2020), cardiac arrhythmias (Dinicolantonio & Okeefe, 2020), and inflammation (Ishihara et al., 2019).

Most likely, EPA and DHA cardioprotective effects are linked to the beneficial modulation of a number of known risk factors for CVD, such as blood lipids, blood pressure, heart rate and heart rate variability, platelet aggregation, endothelial function and inflammation (Innes & Calder, 2020).

In general, n-3 PUFA possess anti-inflammatory, antiarrhythmic and antithrombotic properties (Jain et al., 2015; Golanski et al., 2021). They can stabilize the electrical activity of cardiac myocytes by inhibiting sarcolemmal ion channels, resulting in a prolonged relative refractory period (Jain et al., 2015). Both EPA and DHA lower blood concentrations of the acute phase protein, C-reactive protein and the pro-inflammatory cytokines, tumour necrosis factor and interleukin-6. They also have been reported to decrease plasma or serum concentrations of pro-inflammatory eicosanoids like thromboxane B2 and leukotriene B4 (Li et al., 2014; Jiang et al., 2016).

In a recent meta-analysis, n-3 FA intake was associated to reduced cardiovascular mortality, myocardial injuries, coronary heart diseases, major cardiovascular events and revascularization and the cardiovascular risk reduction was more prominent with EPA monotherapy, when compared to EPA + DHA (Khan et al., 2021). EPA has been shown to inhibit the synthesis of thromboxane A2, an eicosanoid that causes platelet aggregation and vasoconstriction. Ingestion of EPA has also been shown to reduce platelet adhesion and reactivity (Simopoulos, 1999), reductions in fibrinogen and increases in tissue plasminogen activator (Jain et al., 2015). EPA has also been shown to reduce the risk of myocardial infarction, stroke, hospitalization for unstable angina, or coronary revascularization in at-risk patients with elevated blood TAG (Mason et al., 2020).

Combined EPA + DHA or DPA + DHA are associated with lower risk of fatal cardiac events and DHA with lower risk of atrial fibrillation, suggesting a direct effect of DHA for cardiac arrhythmias (Mozaffarian & Wu, 2012). DHA appears to be higher related to the increase of high density lipoproteins (HDL) and low density lipoproteins (LDL) particle size, more efficient in decreasing blood pressure, heart rate and platelet aggregation when compared to EPA (Cottin et al., 2011).

Individual positive effects associated with dietary n-3 DPA and vascular health have also been found, such as a high endothelial cell migration ability, important in wound-healing processes (Kaur et al., 2011). Inverse associations between dietary intake of n-3 LC-PUFA and arterial intima/media thickness assed by ultrasonography was greatest for DPA (Hino et al., 2004). Also DPA has been shown to be a more potent inhibitor of platelet aggregation than EPA or DHA (Akiba et al., 2000).

Brain and visual system function.

Diet, in what concerns to its lipid imbalance (low intake of n-3 LC-PUFA) and the impact in intracellular biochemical processes and neuronal cell membranes, may lead to changes in brain functioning, causing or aggravating neuropsychiatric disorders (depression, schizophrenia, attention deficit hyperactivity disorder, and others) (Reimers & Ljung, 2019). The PL of the brain's gray matter and retina contain high concentrations of n-3 LC-PUFA, responsible for the membrane fluidity, plasticity and other essential physicochemical properties needed for the correct function of the nervous system (Stillwell & Wassall, 2003). Once neurodegenerative diseases might be associated with neuroinflammation, n-3 PUFA involvement in the synthesis of anti-inflammatory mediators might also represent a valuable contribution to the prevention of such diseases (Avallone et al., 2019). Membrane PL are specially enriched in DHA and arachidonic acid (AA) (Reimers & Ljung, 2019). DHA accounts for 10-15% of brain FA, participating in the formation of the lipid bilayer, signal transduction, neurotransmitter transmission, synapse formation and proliferation and differentiation of neurons, therefore helping to maintain normal brain development and function (Innis, 2008). Adequate DHA supplementation enhances neural networks and sensory normal functions, contributing to learning and memory ability (Heinrichs, 2010). Also, studies have found that an insufficient n-3 PUFA intake during pregnancy and lactation can reduce DHA levels in offspring brain tissues, affecting the normal development of dendritic spine density, synaptic plasticity and myelinisation in neurons, increasing the risk of various neurological diseases (Brenna, 2011).

Rapid urbanization and an overall transition from traditional lifestyles have been linked to increases in both physiological and mental illness, and nutrition is also a critical factor for the increasing prevalence and incidence in psychiatric disorders (Su et al., 2015). N-3 LC-PUFA have recognized positive outcomes of various neurological conditions (Dyall & Michael-Titus, 2008), namely depression, schizophrenia, attention deficit hyperactivity disorder, post-traumatic stress disorder, dementia and Parkinson's disease (Su et al., 2015; Reimers & Ljung, 2019). It has been observed that countries with a high consumption of fish diet appear to have a lower prevalence of major depressive disorder and bipolar disorders, implying a preventive effect of n-3 PUFA on mood disorders. Deficits in n-3 PUFA levels have been reported in other populations with mood disorders, including lower DHA and total n-3 PUFA in *postpartum* depression and lower DHA and EPA in social anxiety disorder (Su et al., 2015).

Neuroprotective effects have also been associated with n-3 DPA intake (Kaur et al., 2011), being them linked to better mental health and cognitive function (Byelashov et al., 2015). DPA levels in patients with depression and schizophrenia were lower than in healthy people (Hamazaki et al., 2013; Taha et al., 2013). Also, DPA supplementation of aged rats improved brain function, as measured by long-term potentiation and performance in spatial learning tasks, and reduced age-related oxidative damage of the brain (Kelly et al., 2011).

Another way in which n-3 PUFA may positively affect the central nervous system functions, such as cognition and behaviour is by affecting the gut's microbiota composition, its

FA composition, and by reducing intestinal inflammation, which in summary affects the gut–brain axis (Reimers & Ljung, 2019).

N-3 PUFA are also essential nutrients for the visual system (Zhang et al., 2019b). They are involved in beneficial clinical effects in age-related macular degeneration (Buschini et al., 2015) and epidemiological studies suggest that n-3 PUFA could have a protective role in reducing the onset and progression of retinal diseases (Querques et al., 2011). Diets rich in PUFA improve the retinal cellular response to ischemic, oxidative, and inflammatory damage in animal models of age-related macular degeneration (Simõn et al., 2016).

In vertebrates, although DHA represents a small percentage of the FA in most tissues (1–5%), it accounts for approximately 50–60% of the total FA content within rod outer segments of photoreceptors (ROSs) (Querques et al., 2011). DHA dietary deprivation leads to changes in retinal function in various animal species (Querques et al., 2011).

The majority of Australian and New Zealand optometrists recommends n-3-rich foods or supplements for dry eye disease, since they seem to modulate ocular surface inflammation and improving tear-lipid profiles (Zhang et al., 2020a). In addition to its anti-inflammatory effects, DHA is implicated in maintaining the structural and functional properties of the retina (van Leeuwen et al., 2018).

Other health conditions – cancer and lipid metabolism.

N-3 PUFA can contribute to other several beneficial outcomes for health disorders like cancer (Fabian et al., 2015), and chronic inflammatory diseases such as arthritis (Proudman et al., 2015), colitis (Hokari et al., 2013) and pancreatitis (Lei et al., 2015), some of which are related to the anti-inflammatory effects of the n-3 LC-PUFA (Ellulu et al., 2015; Ishihara et al., 2019).

Potential mechanisms for PUFA in cancer aetiology include their being precursors to lipid mediators regulating metabolic pathways and inflammatory responses, oxidative stress, and changes in membrane composition that could affect cell signalling pathways (Hanson et al., 2020). Epidemiological studies and animal experiments indicate that n-3 FA exert protective effects against cancers of the breast, colon and prostate (Simopoulos, 2003), being that PL rich in DHA/EPA can inhibit the proliferation of some tumour cells (Zhang et al., 2019a). EPA and DHA have shown to possess antiangiogenic activity (Tsushima et al., 2012) and it has also been accepted that n-3 PUFA can induce tumour cell apoptosis by damaging mitochondrial membrane, by promoting intracellular reactive oxygen species production, by activating caspase proteases and by regulating apoptosis-related gene expression (Zhang et al., 2019a). DPA contribution might be related to its role in resolution of chronic inflammation (Byelashov et al., 2015). However, evidence for effects of PUFA on risk of cancer is conflicting (Hanson et al., 2020). Nevertheless, even with uncertain direct effect on cancer onset and progression, n-3 PUFA can be of importance in cancer patients undergoing complications, such as anorexia-cachexia syndrome, pain, depression, and paraneoplastic syndromes (Freitas & Campos, 2019).

Various studies have reported that DHA/EPA-enriched PL can significantly improve glucose and lipid metabolism disorders (Zhang et al., 2019a). The regulation of several genes by n-3 PUFA can contribute to the improvement of lipid metabolism (Zhang et al., 2019a). Particularly EPA can inhibit the synthesis of endogenous TAG and cholesterol and decrease the levels of atherosclerotic factors, reducing arterial thickness (Berge et al., 2015; Zhang et al., 2017b). Despite a relatively low accumulation in adipocytes, n-3 LC-PUFA have beneficial effects on adipose tissue in obesity, as indicated by reduced body fat mass and stimulated lipid oxidation (Couet et al., 1997), improvement of body weight (BW) and satiety regulation (Abete et al., 2010), amelioration of cytokines profile, including leptin and adiponectin (Abete et al., 2010), and reduction of inflammation (Molendi-coste et al., 2011).

1.2.2. Consumption pattern and recommendations.

The main dietary sources of n-3 LC-PUFA are seafood and the by-products generated from its processing (Pateiro et al., 2021), however it is important to take into consideration that meat is by far a more substantial source of n-3 LC-PUFA in the typical western diet than marine resources. An analysis of Australian consumers revealed that meat, poultry, and game account for 47% of n-3 LC-PUFA intake. Hence, meat sources supply almost half of the n-3 LC-PUFA intake, even though fish and other seafood can have 5-15 times a higher n-3 LC-PUFA content than meat or poultry (Howe et al., 2006).

In the western diet, dietary n-6/ n-3 ratio varies between 10:1 and 20:1 (Molendi-coste et al., 2011) and it has been stated that the optimal n-6/ n-3 ratio should be around 1-4:1 (Simopoulos, 2016). However recent publications continue to point towards the need of a lower n-6/n-3 ratio (Patel et al., 2022), previously summarized FAO/WHO orientations point to a lack of scientific evidences and conceptual limitations to the standardization of recommendations for ratio of n-6 to n-3 FA (FAO/WHO, 2008).

The minimum intake levels for essential FA to prevent deficiency symptoms are estimated to be 2.5% of energy (%E) LA plus 0.5 %E ALA (FAO/WHO, 2008). Further elongation and desaturation of these FA to produce LC-PUFA, including EPA, DHA and AA (20:4n-6), is possible but not very efficient in humans, especially in the case of DHA (i.e., only 3–4% or as low as 0.05% is converted) (Burdge & Calder, 2005; Reimers & Ljung, 2019).

The total n-3 FA intake can range between 0.5 – 2%E. The higher value of 2 %E can be part of a healthy diet, with an acceptable macronutrient distribution range of 0.250 g – 2.0 g. For adult males and non-pregnant/non-lactating adult females, 0.250 g/day of EPA plus DHA is recommended. For adult pregnant and lactating females, the minimum intake for optimal adult health and foetal and infant development is 0.3 g/day EPA+DHA, of which at least 0.2 g/day should be DHA (FAO/WHO, 2008). However in most western countries it is generally considered that in non-fatty fish eaters, the intake of EPA + DHA is <0.2 g/day (Meyer et al., 2003; Howe et al., 2006)

Usually it is recommended that for chronic diseases prevention, the daily intake of EPA+DHA should be up to 0.5 g and to treat those chronic diseases, such as coronary heart disease and elevated TGA levels, up to 1 g and 1.2-4 g daily, respectively (Russell & Bürgin-Maunder, 2012). Several governments (France, Belgium, UK, The Netherlands, New Zealand, and Australia) and health organizations (FAO/WHO, American Dietetic Association and American Heart Association) recommend dietary intakes for total n-3 PUFA of 1.4 to 2.5 g/day, with EPA and DHA ranging from 140-600 mg/day depending on the guideline. Compared with the average recommendation of around 500 mg/day, FAO/WHO make a relatively low recommendation of 250 mg/day. To meat average recommendations, it would represent a minimum of 2 servings of fish per week (30-40 g/day) including one of oily fish (salmon, tuna, mackerel, and sardine). Probably the minimal EPA+DHA supplied for healthy adults should reach 0.5-1 g/day (2-4 servings per week of fish, half of oily fish) (Harris, 2007; FAO/WHO, 2008; Molendi-coste et al., 2011).

European Union’s EFSA Panel on Dietetic Products Nutrition and Allergies concluded that supplemental intake of EPA and DHA at combined doses up to 5 g per day, and supplemental intakes of EPA alone up to 1.8 g per day, do not raise safety concerns for adults (EFSA, 2012).

Despite the recognized importance of an adequate intake of n-3 LC-PUFA, it has been estimated that less than 20% of the world’s human population consume ≥ 250 mg/day of n-3 LC-PUFA from seafood-origin (Van Dael, 2021). Therefore, food enrichment is emerging as perhaps the best long-term solution to the chronically low intake of n-3 PUFA (Molendi-coste et al., 2011). For many individuals, the suggestion to consume more oily fish as a means of increasing their intake of EPA and DHA may not be a feasible option, thereby making it difficult to attain the recommended dietary intake of these nutrients. As a result, changing dietary habits may not be a viable solution for such individuals (Calder & Yaqoob, 2009b). Therefore, there are two main strategies to enhance the consumption of EPA and DHA that do not require dietary changes: a) intake of fish oil capsules or b) consumption of EPA and/ or DHA enriched foods. Various supplements, including fish oils, cod liver oil, krill oil and some algal oils, contain EPA and DHA (Innes & Calder, 2020). One should take into consideration that n-3 LC-PUFA supplements available are usually in the TAG, non-esterified FA (NEFA) or ethyl ester (EE) form, however, the PL forms have been shown to exhibit higher efficacy in efficiently enhancing EPA and DHA (Zhang et al., 2019b).

To attain the option of consumption of EPA and/ or DHA enriched foods, two routes to such enrichment of foods have been developed. The first is the addition of fish oil or similar oils to products such as spreads, yoghurts, or milk (i.e. food fortification). The second is feeding of farm animals with n-3 LC PUFA sources, enriching meat, milk, or eggs. One limitation of these methods relates to the degree of enrichment that can be attained. This may be constrained by factors such as metabolic processes in animals (in the case of the animal feeding approach), as well as various considerations related to food technology, processing, and storage (Calder & Yaqoob, 2009b).

1.3. N-3 LC-PUFA in ruminant tissues.

1.3.1. Meat and adipose tissue.

Lean meat.

The intramuscular fat (ItaMF) is composed by two main lipid classes: neutral lipids or TAG, with storage role, and polar PL (mainly glycerophospholipids), with structural and metabolic role. TAG are the main lipid component (>90%) of adipose tissue in mature animals (visible fat), whereas polar lipids are a constituent of cell membranes, contributing between 10% and 40% for the total FA in muscle (Wood et al., 2007). Apart from structural PL, also cholesterol is present in the cellular and intracellular muscular membranes (Bessa et al., 2015; Wood et al., 2008). TAG are deposited mostly in adipocytes (Raes et al., 2004) and in cytoplasmic droplets within myofibrils (Hocquette et al., 2010). The ItaMF deposit increase is achieved by increasing TAG's content, while structural PL content remains constant (Wood et al., 2008).

ItaMF composition in PL and TAG is regulated distinctly, being that different FA are preferentially deposited in PL or TAG (Bessa et al., 2015).

About 40-50% of the FA in ruminant meat are *cis*-monounsaturated FA (*c*-MUFA), predominantly oleic acid (OA, *c*9-18:1). This is the major FA in meat and is more predominant in the neutral lipid fraction (Wood et al., 2008). Saturated fatty acids (SFA) represent 30-40% of total FA, being stearic acid (18:0) the most abundant (30–40%) (Vahmani et al., 2020). The sn-1 position of the common glycerophospholipids is occupied mostly by a saturated carbon chain or less often by unsaturated chains, explaining the fact that FA composition of PL comprises a fairly constant and high content of SFA (Bessa et al., 2015). All SFA, mainly 14:0, 16:0, 18:0, and also *t*11-18:1 can be desaturated by the stearoyl-coenzyme A desaturase (SCD) to produce *c*9-14:1, *c*9-16:1, *c*9-18:1 and *c*9,*t*11-18:2 (Drackley, 2000; Berthelot & Gruffat, 2018). The medium chain FA are synthesised *de novo* from plasma acetate and β-hydroxybutyrate to generate 12:0, 14:0 and 16:0, whereas long chain FA (16:0, 18:0, 18:1, 18:2 and 18:3) arise from modified dietary lipids via plasma lipoproteins and are taken up through the action of the lipoprotein lipase (Drackley, 2000; Berthelot & Gruffat, 2018).

When compared to nonruminants, the FA composition of ruminant tissues contains higher proportions of *trans*-FA, FA with an odd number of carbon atoms (arising from rumen-derived propionic acid rather than acetate as a precursor for FA synthesis, e.g., 15:0 and 17:0), FA with branched chains (derived from the amino acids, leucine, valine, and isoleucine, i.e., 4-methyl octanoic acid, 8:0 and 4-methyl nonanoic acid, 9:0) and FA with conjugated double bonds (i.e., the bonds are on adjacent carbon atoms rather than being separated by a CH_2 group) (Berthelot & Gruffat, 2018). Most biohydrogenation intermediates, particularly those containing *trans* double bonds, including *c*9,*t*11-CLA, are found in TAG (Jerónimo et al., 2011).

The PL fraction has a much higher PUFA content in order to perform its function as a constituent of cellular membranes, where PUFA play a metabolic, functional and physiological status maintenance role (Wood et al., 2008; Ponnampalam et al., 2021). Because n-3 LC-PUFA are mainly incorporated into the membrane PL and are deposited in significant amounts in intramuscular tissue (Alvarenga et al., 2015), small amounts of n-3 LC-PUFA are incorporated into TAG in adipocytes. Thus, lean meat, per gram of tissue, is richer in n-3 LC-PUFA compared to fattier meat and adipose tissues (Nguyen et al., 2018a). Both LA and ALA are present in higher proportions in PL than neutral lipids (Wood et al., 2008). These FA are elongated and desaturated to longer n-6 and n-3 PUFA: AA and 22:4 for the n-6 PUFA and EPA, DPA and DHA for the n-3 PUFA (Berthelot & Gruffat, 2018).

Regarding LC-PUFA content, meat is particularly rich in AA (Givens et al., 2006). In conventional ruminant meat, n-3 LC-PUFA content ranges from 2.5 mg/ 100 g meat up to 40 mg/ 100 g depending if considering fatter meat from concentrate fed animals (Leheska et al., 2008) or lean meat from animals finished in lush pastures (Rosa et al., 2014), respectively. Green leafy materials usually contain more ALA in the chloroplasts, whereas the grain-based or cereal-based diets often contain more LA and MUFA (Ponnampalam et al., 2021). In general, more intensified ruminant production systems tend to yield animals with lower concentrations of n-3 PUFA and higher concentrations of n-6 PUFA, resulting in higher n-6/n-3 ratios in meat compared to animals that graze in range lands (Ponnampalam et al., 2021). On the other hand, pasture feeding systems or fodder feeds containing silage, produce meat with more n-3 FA (Nuernberg et al., 2005). Furthermore, pasture and forages possess secondary metabolites that can exert a protective effect on PUFA from the ruminal biohydrogenation (RBH), facilitating their absorption and deposition (Lourenço et al., 2008).

Apart from the basal diet, supplementation of various feeds, including oilseeds or plant and marine oils, has been investigated to improve the FA profile of ruminant meat. These supplements are commonly rich in ALA (e.g., linseed, chia seed, camelina oil), LA (e.g., corn oil, sunflower and safflower), EPA and DHA (e.g., marine algae and fish oil) or OA (e.g., canola/rapeseed and olive oil) (Ponnampalam et al., 2015; Chikwanha et al., 2018). In terms of individual n-3 LC-PUFA abundance in meat, DPA is the one that presents a higher content and a lower variability through feeding systems, whereas EPA seems to be the most responsive to dietary changes, and DHA is usually present in much lower concentrations (Bessa et al., 2015).

In general, despite ruminants have a low LC-PUFA content in the edible tissues (Wood et al., 2004), ruminant meat is an important part in global food and nutrition security, being a source of as much as 20% n-3 LC-PUFA for populations with low fish consumption (Russo, 2009).

It should be taken into consideration that, in ruminants, the extent of success in which meat is enriched with n-3 LC-PUFA is limited by (1) the extensive RBH of the dietary n-3 PUFA; (2) the low endogenous conversion of ALA into n-3 LC-PUFA; and (3) the capacity of muscle to incorporate and store the n-3 LC-PUFA (Bessa et al., 2015).

As previously stated, ruminants preferentially incorporate n-3 LC-PUFA into PL as opposed to TAG (Ashes et al., 1992; Bessa et al., 2015), being the maximum n-3 LC-PUFA enrichment in bovine muscle PL reported to be about 20%, achieved only with abomasal infusion of fish oil (Fortin et al., 2010) or high dietary incorporation of casein-formaldehyde protected fish oil (Ashes et al., 1992).

Muscle membranes accumulate more EPA than DHA, even when dietary marine oil sources are richer in DHA in comparison to EPA (Bessa et al., 2015), being that the DHA:EPA ratio in muscle PL remains close to 1 (Cooper et al., 2004). Therefore, the deposition of n-3 LC-PUFA in muscle neutral lipids and adipose tissue, although less extensive, can reflect more closely the dietary DHA:EPA ratio (Bessa et al., 2015). The evidence strongly indicates that the lower levels of DHA in muscle tissue are not due to insufficient activity of elongase and desaturase enzymes, but rather represent a programmed characteristic of membrane PL in muscle (Bessa et al., 2015).

Sheep meat in particular, when compared to beef, contains a higher proportion of n-3 PUFA (Enser et al., 1996; Chikwanha et al., 2018) which can be associated to a lower RBH (Prache et al., 2022). Also, in the work by Hopkins et al. (2014), it is stated that the very high n-3 LC-PUFA accumulation in lamb meat was only possible due to n-3 LC-PUFA accumulation in muscle TAG. In this case, the DHA supplementation provided by a microalgal source resulted in a n-3 LCPUFA deposition in TAG that contributed to 45% of total n-3 LC-PUFA content in the muscle. This data seems to illustrate that in small ruminants, contrary to what is described for bovines, it is possible to surpass the apparent blockage to n-3 LC-PUFA present in muscle neutral lipids. Lamb meat also presents a lower n-6/n-3 ratio when compared to other meats such as pork and chicken (Woods & Fearon, 2009) and possesses a high content of branched-chain FA (BCFA), also health-beneficial (Chikwanha et al., 2018).

Australian sheep meat provides > 60 mg of EPA and DHA per serving (135 g) (Williams, 2007) and Komprda et al. (2012) postulated that a 100 g portion of meat from grass-fattened lambs can provide > 30% of the daily recommended dietary allowance of EPA+ DHA, if considering it in the lower range of around 200 mg per day. Whereas, for DPA, lean trimmed meat can average 20 and 30 mg DPA/100 g for beef and lamb respectively (Byelashov et al., 2015).

Adipose tissue.

There are three main fat depots to consider, namely the subcutaneous fat (SCF), the intermuscular fat (IteMF) and the perirenal fat (PRF). Whereas cattle have faster SCF deposition than IteMF, and internal fat has an intermediate rate of fat deposition, sheep have a similar rate of SCF and internal fat deposition, with IteMF deposited at a slower rate (Kempster, 1981). Besides nutritional quality, animal fat deposition has a major impact on the lean meat yield from individual carcasses as well the perceived eating quality for consumers (Schumacher et al., 2022). Edible adipose tissues derived from ruminants have a low content of n-3 PUFA (Bessa et al., 2015) and however they have a much higher FA content than muscle, the FA composition of the two tissues (adipose and muscular) is broadly similar (Wood et al., 2008). PUFA composition of the adipose tissue varies between fat deposit, and within each deposit varies according to different factors such as age, muscle type, gender or breed, with diet being one of the main factors affecting it (Nürnberg et al., 1998; Kouba & Mourot, 2011; Wood et al., 2008).

Differences in FA composition of different fat depots have been described (Chilliard, 1993; Osorio et al., 2007; Mapiye et al., 2015; Peng et al., 2016), being that, for example, external depots (e.g., SCF) and internal depots (e.g. PRF) have the greatest propensity to: accumulate rumenic (*c*9,*t*11-18:2) and vaccenic (*t*11-18:1) acids, respectively (Jiang et al., 2013; Mapiye et al., 2014).

1.3.2. Other tissues.

Brain and retina.

The brain is particularly rich in lipids, containing around 24% PL and 22% cholesterol by dry matter (DM) (Svennerholm et al., 1997). However, brain's capacity to synthesize lipids is low, which rises the necessity for their supply from the peripheral blood circulation (Igarashi et al., 2007a; Lacombe et al., 2018).

FA are the organic compounds that are most abundantly present in the brain and more than 90% of PUFA in the mammalian brain are composed of LC-PUFA such as AA and DHA (Svennerholm, 1968). PUFA comprise 80% of total membrane PL and they influence brain functions by altering the biophysical properties of cell membranes (Fenton et al., 2000). DHA is the predominant PUFA in the brain, representing upwards of 40% of total brain PUFA (Lacombe et al., 2018). Together, AA and DHA, make up 20% of the human brain dry weight (DW), being the first concentrated in the neurons outer membrane and in the myelin sheath (Tallima & El Ridi, 2018).

High proportions of DHA (up to 35% in brain ethanolamine plasmalogen and phosphatidylserine in synaptic membranes) are characteristic of the mammalian brain, even in herbivores that consume no DHA and have low plasma and liver DHA (Innis, 2008).

Approximately 30% of the lipid fraction of the grey matter in the adult brain is made up of DHA (Svennerholm, 1968). N-3 DPA assumes also an important role, as it is the most abundant n-3 LC-PUFA in the brain after DHA and it can be specifically beneficial for elderly neuroprotection, and early-life development (Drouin et al., 2019). Despite being the second most abundant n-3 LC-PUFA, in relation to DHA, DPA cerebral concentration is about 70 times lower.

The brain has a low synthesis rate of DHA and AA and levels of enzymes involved in the synthesis of AA and DHA seem to be static, not being regulated in response to dietary supply. Collectively, these observations suggest that the brain relies on a constant supply of AA and DHA from the blood (Bazinet & Layé, 2014; Pifferi et al., 2021). There is evidence that these FA are maintained because they are mobilized from liver storages. In rats, liver but not heart or brain synthesis was upregulated (higher expression of DHA-synthesizing enzymes) when dietary n-3 PUFA content was reduced (Igarashi et al., 2008; Rapoport et al., 2010). On the other hand, SFA and MUFA can be synthesized *de novo* within the brain (Bazinet & Layé, 2014).

Lipoproteins containing PUFA, including low-density lipoproteins (LDL) (Edmond, 2001), very-low density lipoproteins (VLDL) and high density lipoproteins (HDL) (Polozova et al., 2006), along with lysophospohtidylcholine (lysoPC) (Thiès et al., 1992) and UFA (Robinson et al., 1992; Rapoport et al., 2001; Hamilton & Brunaldi, 2007) have been proposed as plasma pools for brain uptake. In 2014, Mfsd2a, member of the major facilitator superfamily, was identified as the major transporter for DHA uptake into the brain (Nguyen et al., 2014). Mfsd2a is expressed in the endothelium of the brain and transports DHA in phosphatidylcholine (PC) or lysoPC (Lacombe et al., 2018).

Upon entry into the brain, most PUFA — especially DHA and AA — are activated by a LC-FA coenzyme A synthase and then esterified to PL membranes. Other PUFA, such as LA, ALA and EPA, are β-oxidized (Bazinet & Layé, 2014).

Traditionally, the FA composition of sheep brain lipids has been reported by PL class (Palmer et al., 1985). The lipids of sheep brain, and associated nervous tissue, have been less well characterized than those of cattle but, in general, they do not appear to differ markedly from the latter (Christie, 1981).

In ruminants, essential FA intake is restricted by hydrogenation of PUFA in the rumen and this can also be reflected in the essential FA levels available for brain development as a foetus and neonate, being that the mechanism by which ruminants accommodate to this low essential FA supply is not fully understood. However, sheep, and probably ruminants in general, have adapted to conserve their essential FA supply for structurally important functions (Palmer et al., 1985), independently from dietary interventions (Alfaia et al., 2017). Among brain FA, DHA content appears to be the most resistant to dietary change (Rule et al., 2022).

The retina is a thin, highly organized neural tissue lining the posterior aspect of the eye and is responsible for initiating vision by transducing light into neural signals (Smith et al., 2009). Around 20% of retina DW is constituted by lipids, and membrane PL have the highest level of LC-PUFA of any tissue (~33% in humans) (Fliesler & Anderson, 1983; Acar et al., 2012; Albouery et al., 2020). The most abundant n-6 PUFA in the retina is AA (Acar et al., 2012; Albouery et al., 2020). But, as previously pointed, the retina is a tissue with a natural high content of n-3, particularly DHA. In this organ, DHA plays an important role in optimizing the fluidity of photoreceptors' membranes, retinal integrity and visual function (Querques et al., 2011). In the outer segments of photoreceptors, DHA accounts for around 50-60% (Stinson et al., 1991) of total FA content (Stillwell & Wassall, 2003), being mainly, but not exclusively located in photoreceptor cells, where it is esterified into PL. It also plays a role in the synthesis of disk membranes, renewal of outer segments, photoreceptor differentiation (Querques et al., 2011) and in the regeneration of rhodopsin (Bush et al., 1994). DHA has a protective role in the retina, participating with anti-inflammatory activity, antiangiogenesis, antiapoptosis, protection from neurotoxicity, enhancement of retinal pigment epithelium, and other (Querques et al., 2011).

Important knowledge on the physiological roles of DHA arose from studies on bovine retina ROSs, in which DHA is also high in PC. This is notable since cows consume a diet lacking DHA (Louie et al., 1991). In bovine photoreceptor outer segments, LC-PC species are significant components of lipid membranes since the C28-C36 LC-PUFA represent 10 mol % of total FA in PC (Aveldaño, 1987). Retinal C28-C36 LC-PUFA belong to the n-3 and n-6 families (Aveldaño & Sprecher, 1987).

Similar to what is pointed for the brain, analysis of sheep retina PL did not show any marked differences from those of cattle (Anderson, 1970). The individual simple lipids (Aveldaño & Bazán, 1974) and PL (Anderson, 1970) of retina characteristically contain appreciable amounts of DHA and, in the diglyceride and phosphatidylinositol fractions in particular, large amounts of AA (Anderson et al., 1970).

The PC are composed primarily of palmitic, stearic, and oleic acids, and contain small amounts of PUFA. Other three glycerolipid classes are composed of large amounts of PUFA, being that phosphatidylinositol contains predominantly AA and phosphatidylserine and phosphatidylethanolamine contain high levels of DHA (Anderson, 1970).

Interestingly, and consistent with the observations that the retina is derived from grey matter, the FA composition of each lipid class is similar to that of the same class from the grey matter of the brain. Furthermore, as also observed in the brain, retina lipids seem to have a constant profile across species, regardless the different environmental and nutritional differences between, with a remarkably similar PL class and FA compositions (Anderson, 1970).

Liver.

The edible internal organs of ruminants (i.e. heart, liver and kidney) are rich sources of essential FA (Le et al., 2019). They are widely consumed in many countries in traditional foods (Toldrá et al., 2012), therefore, there is also an interest in enhancing the FA profiles of lamb edible internal organs in order to increase the value-addition of edible meat by-products and generate extra income for lamb producers and slaughterhouses (Le et al., 2019).

Ruminant liver is a good source of LC-PUFA, with a desirable n-6:n-3 ratio for the human diet, particularly when derived from grassfed animals. Total PUFA content of lamb's liver resembles that of beef liver in terms of FA composition (Enser et al., 1998).

Although liver is a minor part of the edible carcass, this organ has a high content of PUFA (Enser et al., 1998) that might reveal differences in FA metabolism more clearly than the other tissues when n-3 PUFA supplementations are performed in the diets (Demirel et al., 2004; Alfaia et al., 2017). Furthermore, the liver is the first organ to receive and metabolise FA after lipid digestion and absorption. In general, lipids from ruminant liver appear to contain more 18:0, less 16:0 and more LC-PUFA than from ruminant muscle (Enser et al., 1998).

Contrary to other organs such as the brain, the liver has a very small amount of plasmalogens (Christie, 1978). However, it is suggested that the liver plays a role in plasmalogens biosynthesis by supplying plasmalogens to tissues via plasmalogen-containing lipoproteins (Nagan & Zoeller, 2001).

For both beef and lamb, liver is the richest source of DPA, containing approximately 140 mg DPA/ 100 g of edible portion (Byelashov et al., 2015). In the work by Le et al. (2019), liver EPA and DHA contents of PUFA fed lambs ranged from 162 to 414 mg/100 g, similar to EPA and DHA contents of wild Australian seafoods such as fish (~233 mg/100 g), shellfish (~150 mg/100 g) (Nichols et al., 2010).

Several dietary interventions have revealed success in increasing ruminants' liver PUFA content. Barrosã bulls fed an high silage diet had the highest percentages of 15:0, ALA and DPA in liver whereas those that a low silage diet had the percentages of DMA-18:1, *t*9-18:1, *t*10-18:1 and *c*13-18:1 (Alfaia et al., 2017). In a study by Demirel et al. (2004), significant increases in the n-3 PUFA content of the lipids of liver were obtained by feeding formaldehyde-treated linseed and a combination of this with fish oil. Supplementing lambs' diets with 5% flaxseed or canola oil pellets increased n-3 LC-PUFA in the liver (Nguyen et al., 2017a; Nguyen et al., 2017b) and medium-to-strong positive correlations were found between liver and plasma concentrations of total n-3 PUFA in rumen partially protected fish oil supplemented beef heifers (Hennessy et al., 2021). In these animals, the supplementation of 140 g of n-3 LC-PUFA per animal/ day allowed that, in a 100 g serving of uncooked liver, approximately 0.7 g of EPA and 0.5 g of DHA would be provided, relative to heifers on the control diet whose liver contained only 0.1 g and 0.2 g of the respective n-3 LC-PUFA (Hennessy et al., 2021). Also, six-month-old Tattykeel Australian White lambs (30 kg LW) fed n-3 oil fortified grain pellets registered a significantly higher proportion of EPA + DHA + DPA in the liver, when compared to the control lambs, being the rise equivalent to three-folds of what was obtained in the kidney and heart (Pewan et al., 2021), proving once again a good response to dietary interventions.

1.4. Sources of n-3 LC-PUFA for ruminants.

1.4.1. Fish oil.

Lipid supplements commonly used to improve n-3 LC-PUFA composition of ruminant meat include those rich in EPA and DHA (e.g., algae and fish oil). Most of the beneficial effects of fish oils have been attributed to DHA and then to EPA, with less references to DPA because, among other, it represents less than one-third of each EPA and DHA (Drouin et al., 2019). The fish oil used is normally extracted from tuna, sardine, herring, mackerel and salmon (Chikwanha et al., 2018). Fish oil is one of the primary natural n-3 LC-PUFA source of EPA and DHA and accounts for about 2 % of world consumption of fats and oils (Ivanovs & Blumberga, 2017). Traditionally, it is obtained as a by-product of the fish meal industry or from raw material such as smaller fish with a relatively high fat content – anchovies, sardines, herring, eels (Ivanovs & Blumberga, 2017). Fish oil is mainly used in food and pharmaceutical industry, agriculture and aquaculture as a feed additive (Ivanovs & Blumberga, 2017).

In the year of 2014, around the world, from 25−30 million tons of healthy fish and fish cuttings, approximately 1.1 million tons of fish oil were produced (Rizliya & Mendis, 2014). While only 5 % of it is used to extract the n-3 FA, the remaining is used in the aquaculture industry (Lembke, 2013). In 2020, approximately 23% of all captured fish was destined for non-food uses, mainly for fish and livestock feeds (FAO, 2020). Although the current situation with fish oil production is stable, future projections show that available wild marine resources are becoming increasingly limited while the demand for PUFA continues to rise (Šimat et al., 2019).

Fish-oil supplements typically contain 30% to 50% n-3 FA (Russo, 2009). Although the composition can vary widely, fish oil contains around 25.8% 18:1, 3.6% 18:2, 1.3% 18:3, 8.3% EPA, and 17.6% DHA (Najafi et al., 2012; Alvarenga et al., 2015).

The oil source in lamb' diets affects the FA in the PL fraction of the muscle, being that the inclusion of linseed oil increases the LA and ALA levels and decreases the EPA and DHA levels when compared with fish oil (Cooper et al., 2004). Rumen-protected fish oil supplements can be used to increase EPA and DHA concentrations several-fold and lower the n-6:n-3 ratio of lamb and beef (Gulati et al., 2005; Dunne et al., 2011), although with yet inconsistent results (Gadeyne et al., 2017).

Depending on the source and amount of fish oil supplement, concentrations of EPA and DHA in ruminant muscle can be increased by up to 2.33 and 2.55 g/100 g FA, respectively (Scollan et al., 2006; Sinclair, 2007; Noci et al., 2007). Also, studies have shown that inclusion of fish oil in the diet alone or in combination with plant oils increases both EPA and DHA contents in milk (Shingfield et al., 2003; Shingfield et al., 2006; Toral et al., 2012).

On the downside, fish oil has been found to suppress feed intake (FI) in many studies when fed at > 1% of dietary DM (Donovan et al., 2000; AbuGhazaleh et al., 2002; Whitlock et al., 2002; Kook et al., 2002), but this effect is not consistent (Table 1.1). Fish oil has a unique odour that may reduce FI when incorporated into the diet at high rates (Moallem, 2018). Findings of Parvar et al. (2017) showed that the inclusion of fish oil reduced average daily gain (ADG) of animals compared to a control. In contrast with the results of this study, Ferreira et al. (2014) reported that adding fish oil did not affect ADG. Most studies investigating fish oil supplementation have reported decreased FI and growth rate in lambs (Wachira et al., 2002; Annett et al., 2011; De la Fuente-Vázquez et al., 2014) and FI in steers (Wistuba et al., 2007) with up to 3.6% fish oil inclusion. These results could be caused by reduced palatability of diets (Annett et al., 2011). However, an increase in carcass fat scores was reported by Wachira et al. (2002) in lambs fed 3.6% fish oil, and by Demirel et al. (2004) in lambs fed 1.5% linseed and 1.5% fish oil.

Table 1.1- Fish oil supplementation and consequences in ruminant meat.

Species of origin	Ruminant species	Dosage (% diet DM)	Consequence of fish oil supplementation		Reference
			Positive	Negative	
Encapsulated MaxEPA® (Scherer Pty Ltd, Melbourne, Australia)	Bovine	20	The proportion of EPA and DHA increased 3-4-fold in the total muscle PL of sheep and cattle		(Ashes et al., 1992)
	Ovine	30			
na	Bovine	2.20	Doubled the proportion of EPA and DHA in muscle. No significant differences in FI, growth rate, cold carcass weight or carcass fatness. Higher carcass conformation score.	Tendency for FI of the fish oil diet to be lower.	(Scollan et al., 2001a)
na (linseed in basal ration)	Bovine	1.10			
na	Bovine	2.20	Higher CLA in the muscle		(Enser et al., 1999)
na (linseed in basal ration)	Bovine	1.10	No effect on FI		
na	Bovine	3.00	Increased n-3 LC-PUFA levels in muscle and fat Higher *t*11-18:1 in fat	Increased SFA in muscle and fat	(Wistuba et al., 2007)
Mackerel/herring oil (sunflower oil in basal ration)	Bovine (neutral lipids)	1,2,4	Increased CLA isomers, EPA, and DHA in the muscle and decreased n6/n3 ratio. Level of inclusion did not affect ADG or pre-slaughter weight of the steers. With wilted silage, carcass weight increased when animals were fed the 2 and 4% fish oil	Carcass weight decreased for 2% fish oil and unwilted silage fed animals. Higher muscular *trans*-FA	(Noci et al., 2007)
Anchovy	Bovine	0.70	No effect on slaughter weight, back fat thickness rib-eye area or dressing percent.	Decreased FI and ADG.	(Song et al., 2010)
na	Bovine (neutral lipids)	1.50	Increased EPA, DHA, and n-3 LC-FA in the muscle	Lower percentage of IM fat.	(Ponnampalam et al., 2001)
na	Ovine	3.00	Increased EPA, DPA and DHA in the muscle	Decreased ADG.	(Parvar et al., 2017)
na (canola in basal ration)		1.50			
na (sunflower in basal ration)		1.50			
Blend (soybean in basal ration)	Ovine	0.25, 0.50, 0.75	No effect on DMI, ADG, FE and final BW. No effect on carcass characteristics. Higher PUFA in the meat		(Ferreira et al., 2014)
Mackerel/herring-supplement Mackerel/herring-concentrate	Ovine	3.50	Increased levels of DPA and DHA in the muscle. No significant differences on DMI, animal performance or lamb carcass characteristics.		(Annett et al., 2011)
Herring	Ovine	3.60	Increased the muscle proportion EPA and DHA.	Decreased DMI and lamb live-weight gain	(Wachira et al., 2002)
Herring (linseed in basal ration)	Ovine (neutral lipids)	1.50	No significant differences on FI, growth rate or feed conversion ratio. Increased EPA and DHA in the muscle.		(Demirel et al., 2004)

Abbreviations: EPA – eicosapentaenoic acid; DHA – docosahexaenoic acid; PL – phospholipids; n-3 LC-PUFA – n-3 long-chain polyunsaturated fatty acids; CLA – conjugated linoleic acid; IM – intramuscular; na – non-available data; ADG- average daily gain; DMI – dry matter intake; FI- Feed intake FE – feed efficiency; BW – body weight. Adapted from Toral et al. (2018a).

Because fish is a restricted resource, there is a growing interest in exploiting alternative sources of n-3 LC-PUFA. Krill oil, extracted from Antarctic krill (*Euphausia superba*), is a rich source of n-3 LC-PUFA, being attractive for commercial harvest because it is by far the most dominant member of the Antarctic zooplankton community in terms of biomass. Another potential advantage relates to the fact that, even though both fish oil and krill oil contain a high proportion of EPA and DHA, in contrast to fish oil, krill oil contains a major part (30-65%) of these FA in the form of PL (mainly PC)(Tou et al., 2007). Because PL comprise the structure of cell membranes, n-3 LC-PUFA in the form of PL, in some cases, might facilitate the passage of FA through the intestinal wall and increase the bioavailability of these FA in krill oil, compared to when they are consumed from fish oil (Ulven & Holven, 2015). However, in the case of ruminants, this apparent advantage is lost due to the occurrence of extensive lipolysis in the rumen (Doreau et al., 2011). Furthermore, composition of krill oil resembles that of fish oil, but the EPA content is frequently higher (Tou et al., 2007).

Microalgae constitute another potential alternative to fish oil and when evaluating the efficacy of EPA and DHA-rich sources, marine algae are more effective at increasing n-3 LC-PUFA because they can have a lower rate of RBH compared to fish oil (Bessa et al., 2015). Supplementation of the diet with 3% fish oil increased the proportion of DHA in lamb from 0.22 to 2.65% (De la Fuente-Vázquez et al., 2014), while 2% of microalgae increased it from 0.25 to 3.35% (Díaz et al., 2017).

1.4.2. Microalgae.

As previously mentioned, marine oils derived either from fish or algae are a rich source of EPA and DHA (Chikwanha et al., 2018). However, the depletion of marine resource and the environmental pollution have seriously limited the use of n-3 PUFA from fish (Sehl et al., 2022). Furthermore, the ocean fishery has the problem of being highly season-dependent, resulting in price fluctuations of PUFA (Lu et al., 2021). On the other hand, microalgae are present as a good alternative, as they have the ability of growing in both freshwater system and marine system, being the major PUFA producers on the planet (Han et al., 2019). Microalgae cultivation is much more efficient, cost-saving and simple, confirming the advantages of microalgae over marine fish in the aspect of PUFA production (Lu et al., 2021; Sehl et al., 2022).

1.4.2.1. Microalgae production.

For a microalgae-based process to be competitive with fish-based processes and meet a growing market demand, it requires a high overall productivity and a certain degree of scalability (Sehl et al., 2022), which can be reached due to a large ability of microalgae to adapt to different environments. Microalgae can naturally grow fast under a variety of autotrophic, mixotrophic and heterotrophic culture conditions (Adarme-Vega et al., 2012). They can be cultivated in batch, fed-batch or continuously in open- or closed-systems (Tang et al., 2020). Currently, the two major types of mass-cultivation systems are photobioreactors (PBRs) and raceway ponds. Closed PBRs are chosen to produce a higher quality product in terms of less contamination and better physiochemical conditions, once they are a closed system with the ability to have a closer maintenance (Figure 1.2)(Enzing et al., 2014).

In terms of culture productivity, algae have the advantage over conventional plants in that they have much faster growth rates and they are grown from inocula of live cells that immediately can commence growth and biomass production. The productivity of algae in terms of biomass, as well as oil, is also much higher than that of higher plants (Ratledge & Cohen, 2008). Furthermore, they do not require arable land to grow on and non-potable water can be used (Peltomaa et al., 2017).

There is a marked difference in biomass processing relative to each use; however, three main steps are necessary after microalgal production, being them biomass recovery, which is usually done by flocculation, followed by sedimentation, centrifugation or filtration; biomass pre-treatment, to enhance digestibility, or the access to molecules of interest in the next processing step; and product isolation and purification (De Carvalho et al., 2020).

Once the microalgae reaches its mature stage, harvesting separates microalgae biomass from the culture medium, allowing the extraction and purification of specific compounds (Tang et al., 2020). After cultivation, the biomass is very diluted (0.5-5 g/L), therefore concentrating the cell suspensions is essential to reduce the processing volumes. Filtration, centrifugation, or sedimentation can be used as concentration methods, sometimes with the aid of flocculating agents. The resulted slurries or pastes reach concentrations of about 2-3% to 10% solids. Even vacuum- or pressure-filtered biomass cakes still have intracellular water, reaching at best 30% solids. The mineral content attached to the biomass can be high and depending on the use of the microalgae, the suspension can be washed one or more times during this process (De Carvalho et al., 2020).

Figure 1.2- Industrial tubular reactors at Allmicroalgae facilities – Pataias, Portugal.
Accessed in 01/03/2023 at https://www.allmicroalgae.com/pt-pt/o-nosso-processo-microalgas/.

The biomass concentrates can be stored refrigerated or frozen. Some pre-treatments such as hydrothermal liquefaction or wet extraction can handle wet biomass and others, especially for cell disruption, may require cells to be suspended in liquid. Sometimes biomass does not need to be dried initially, but rather conditioned-disrupted or modified. This step enhances digestibility and accessibility to biomolecules. The product of each extraction is a mixture of compounds that may require further purification, and, in some cases, the raw extract can be directly dried into a product. If a pre-treatment is applied, it can be one of four: 1) drying, 2) thermochemical processing, 3) cell disruption, and 4) targeted fractionation (De Carvalho et al., 2020).

After harvesting the biomass, drying it, or dewatering to an appropriately low water content may be necessary to obtain target products in an efficient manner. Although harvesting can result in a 50- to 200-fold concentration of algal biomass, the moisture percentage in the harvested slurry is still high enough to spoil the biomass after a few hours at room temperature. Therefore, a drying process (Table 1.2) is applied to convert the slurry into a stable, storable and easily transportable form (Chen et al., 2015b).

Depending on the requirements of the final products, i.e. if they can be somehow damaged or degraded by heat, light, or oxidation, drying processes can be chosen in order to overcome those technical problems and guarantee the final quality of the dried product. In that sense, solar drying, convective drying, spray-drying and freeze-drying can be applied differently, according to product specifications (Chen et al., 2015b). Spray-drying is usually the preferred method for drying high-value products producing microalgae, originating a powder (Chen et al., 2015b). Freeze-drying is also widely used in the food industry as well as in research because the cell constitutions are better preserved, and without rupturing the cell wall (Guldhe et al., 2014). Compared to freeze-drying, spray-drying originates a biomass that is less susceptible to lipolysis upon storage, but some molecules, like carotenoids, are more prone to oxidation (Chen et al., 2015b).

Table 1.2- Methods applicable for microalgae drying.

Drying method	Principle	Effect on biomass	Pros	Cons
Solar	Solar heating of the biomass	Compaction, some degradation during slow drying	Low energy cost	Slow, requires large areas, depends on the weather
Microwave drying	Microwave heating	Variable, compaction, expansion, or degradation can occur	Fast, highly controllable	High costs, higher safety concerns
Direct of convective. (Oven, tray, tunnel)	Warm air heats the biomass and carries the water	Compaction, degradation at higher temperatures	Practical	Can degrade biomass
Spray	Warm liquid and air carry the water	Enhanced permeation and some degradation can occur	Fast, generates power	Higher energy intensiveness, requires large air volumes
Fluidized bed, spout bed	Warm air heats and carries the water, in a mixed suspended solid medium	Enhanced permeation and some degradation can occur	Fast, generates power	Higher energy intensiveness, requires large air volumes
Conduction or contact. (Drum, tray)	Warm surfaces heat the biomass	Compaction, degradation likely	Fast	Degrades biomass
Lyophilization (freeze-drying)	Warm surfaces heat frozen biomass. Water sublimates	Low, can maintain cell viability, biomass cake very porous	High quality biomass	Slow, high costs

Adapted from De Carvalho et al. (2020)

Cell disruption pre-treatment encompasses several methods that permeate, disrupt, or dissolve cell walls or the cell membranes and techniques are divided into mechanical, physical, and chemical (Table 1.3). Chemical methods are generally scalable, while not all mechanical or physical methods are. Energy intensiveness is one of the keys to evaluate process suitability, because most of the energy applied in a mechanical disruption method is transformed in heat, and that limits the process application or throughput (De Carvalho et al., 2020).

Table 1.3- Cell disruption methods applicable for microalgal biomasses, and their primary targets.

Cell disruption method	Target	Principle	Reagents	Energy input, kW/kg DW	Note
Sonication	CW	Cavitation of microbubbles causes high shear in cell walls	-	6[a]	Severe heat generation, requires colling, harder to scale up
High pressure homogenization, impingement, and microfluidization	CW	High shear and impact, from pressure transformed in a steep velocity gradient in a homogenizer valve (HPH), impact chamber (impingement) or interaction chamber (fluid collision)	-	0.23[b] operating at 800 atm; not accounting for motor/ drive/ pump losses	Heat generation, roughly 0.024º/ atm of pressure applied. Feasible for most biomasses.
Microbead wet milling	Cw	High shear generated by the agitation of fluid with a high content of hard solids	Beads; diameter 100 to 200 µm; typical load 40-60% v/v	3.1 to 0.31[c]	Heat generation. Feasible for most biomasses.
Colloidal mills, rotor-starter homogenizers	CW	High shear by a high-speed mixer	-	Depends on efficiency. Generally lower energy per volume than HPH or microbead	Lower heat generation, inefficient for hardy biomasses
Grinding (microbead, ball or jet mills)	CW	Solid grinding; collision and attrition	Balls in some types	11.8[d]	Heat generation, slow process
Freeze-thawing	CW, CM	Ice crystals damage the cell wall and membrane	-	0.92[e]	Slow process; depends on freezing rate, gentle on the biomolecules
Autolysis	CW	Endoenzymatic lytic activity triggered by specific conditions	-	Very low	Slow process
Enzymatic hydrolysis	CW	Enzymes target structural components of the cell wall, such as cellulose, weakening, permeating, or dissolving it	Enzymes such as cellulase, xylanase, lysozyme	Very low	Enzymes are relatively expensive, but the process is gentle on non-target biomolecules
Alkaline treatment, energic	CW, CM	Very high pH catalyses the hydrolysis of CW and CM components	Base, KOH or NaOH	Low	Energic, can degrade cell components
Acid hydrolysis	CW	Low pH catalyses the hydrolysis of CW components	Acid, H2SO4 or another low-cost strong acid	Low	Energic, can degrade cell components
Alkaline treatment, mild	CM	High pH saponifies lipids. Milder temperature than the energic process	Base, KOH or NaOH	Low	-
Osmotic shock	CM	Immersion of cells in a hypotonic solution causes osmolysis	Buffers, sometimes enzymes to weaken the cell walls	Very low	Works only for cells with previously weakened or absent CW
Thermolysis	CM	Heating the suspension increases membrane fluidity, destabilizing it	-	Low	Depends on species, can denature enzymes
Detergent solubilization	CM	Detergents dissolve the cell membranes	Detergents such as SDS or bile salts	Very low	Can denature enzymes
Solvent solubilization	CM	Solvent destabilizes the cell membrane	Solvents such as toluene or xylene	Very low	Can denature enzymes
Pulsed electric fields	CM	Electroporation: high intensity, short durations electric fields (pulses) induce ion migration and membrane rearrangement, forming pores	-	0.17-1.47[f]	Generates heat

Abbreviations: CW (cell walls) or CM (cell membranes). Mechanical methods have high energy inputs, while chemical and physical methods use basically agitation but require specific reagents or heat exchange. All methods are described for wet pastes or suspensions, except grinding. [a]Industrial Sonomechanics ISP-3000 (EUA); [b]from Bernoullís equation with P converted to energy for water. Power input based on nominal power and throughput of various equipment: [c]Netzsch LME300 (Germany), [d]average for several microalgae,(Suarez Garcia et al., 2018); [e]phase change energy, based on water enthalpy of fusion, 6.02 J/mol; [f]Estimated from references (Eing et al., 2013; Grimi et al., 2014; Straessner et al., 2016). Adapted from De Carvalho et al. (2020).

Microalgae targeted for the production of EPA and DHA (Table 1.4) are often more resistant to the downstream processing conditions and are able to tolerate high extraction temperatures (60-105 ºC), being that they can be dewatered by centrifugation or membrane filtration, and dried by spray or convective drying, to decrease the operational cost (Chen et al., 2015b). Usually, a pre-treatment step may be needed to improve lipid extraction, through the disruption of the cellular integrity of oleaginous microorganisms, after which neutral lipids are separated from the rest of the cellular matrix and water, using techniques such as ultrasound-, microwave-, bead milling-, and detergent-assisted extractions (Barta et al., 2021).

Table 1.4- Screening of algal strains with the potential for PUFA production.

Algal strain	Source	Algae growth condition	Biomass yield or productivity	Percentage of PUFA	Reference
Arcocellulus cornucervis	Norwegian fjord waters	Marine and cold environment	8.67 mg/L/day	EPA: 1.36% if DW and 12.0% TFA DHA: 0.23% of DW and 2.02% of TFA	(Steinrücken et al., 2017)
Phaeodactylum tricornutum			60 mg/L/day	EPA: 3.14% of DW and 7.33% of TFA DHA: 0.25% of DW and 0.58% of TFA	
Attheya septentrionalis	Atlantic waters around Spitsbergen		22.22 mg/L/day	EPA: 4.58% of DW and 24.1% of TFA DHA: 0.60% of DW and 3.17% of TFA	
Thalassiosira hispida			12.86 mg/L/day	EPA: 4.10% of DW and 11.5% of TFA DHA: 0.47% of DW and 1.33% of TFA	
Scenedesmus dimorphus	Himachal Pradesh of India	Area with high altitude	14.0 mg/L/day	PUFA: 39.03% of TFA	(Gour et al., 2016)
Monodus subterraneus	NA	NA	0.45-1.42 g/L/day	GLA: 0.9-1.1% of DW and 20.2-27.5% of TFA	(Qiang et al., 1997)
Parietochloris incisa	NA		NA	DHGLA: 16.7-31.5% of TFA PUFA: 40.24% of TFA	(Iskandarov et al., 2011)
Ankistrodesmus fusiformis			0.24 g/L/day (lipid content: 20.66% of DW)	ALA: 26.28% of TFA LA: 12.23% of TFA	
Botryococcus terribilis	Eutrophic lagoon located at Salvador city, Bahia, Brazil		0.20 g/L/day (lipid content: 49.00% of DW)	PUFA: 12.56% of TFA ALA: 7.22% of TFA LA: 5.02% of TFA	
Chlorella vulgaris			0.73 g/L/day (lipid content: 28.07% of DW)	PUFA: 10.33% of TFA ALA 1.57% of TFA LA: 8.54% of TFA	(Nascimento et al., 2013)
Kirchneriella lunaris			0.14 g/L/day (lipid content: 17.30% of DW	PUFA: 44.83% of TFA ALA: 39.66% of TFA LA: 4.50% of TFA	
Attheya sptentrionalis	Arctic waters	Marine and cold environment	0.54-0.57 d-1	EPA: 7.1% of DW	(Steinrücken et al., 2018)
Botryococcus braunii			158.9 mg/L/day (lipid content: 41.98% of DW)	PUFA: 11.49% of TFA ALA: 8.41% of TFA	
Chlorella sp.			249.2 mg/L/day (lipid content: 21.54% of DW)	PUFA: 55.52% of TFA ALA:28.25% of TFA LA: 22.19% of TFA	
Chlorococcum humicola	Open drainages situated in Penang Island, Malaysia	Trophic regions with low latitude	198.3 mg/L/day (lipid content: 29.16% of DW)	PUFA: 14.07% of TFA LA: 10.45% of TFA	(Hena et al., 2015)
Chlamydomonas sp.			236.8 mg/L/day (lipid content: 21.92% of DW)	PUFA: 36.27% of TFA ALA: 14.16% of TFA LA: 19.35% of TFA	
Fistulifera sp.	The junction of the Sumiyo and Yakugachi Rivers, Japan	Marine diatom	NA	EPA: 17.1% of total FAME	(Liang et al., 2013)
Synedra fragilaroides			0.43 g/L (lipid content: 3.6% of DW)	PUFA: 36.8% of TFA EPA: 11.0% of TFA DHA: 2.1% of TFA	
Nitzschia Closterium	NA		0.10 g/L (lipid content: 13.5% of DW)	PUFA: 33.0% of TFA EPA: 19.5% of TFA DHA: 1.1% of TFA	(Ying et al., 2001)
Phaedactylum tricomutum	NA		0.22 g/L (lipid content: 21.3% of DW)	PUFA: 14.9% of TFA EPA: 8.8% of TFA DHA: 0.5% of TFA	

Individual fatty acids are a percentage of total fatty acids (TFA). Abbreviations: DW: Dry weight; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; PUFA: Polyunsaturated fatty acid; GLA: γ-linolenic acid; DHGLA: Dihomo-γ-linolenic acid; ALA: α-linolenic acid; LA: Linoleic acid. A “NA” refers to “Not available”. Adapted from Lu et al. (2021).

1.4.2.2. Microalgae nutritional composition.

Microalgae produce a range of biochemical molecules, like carbohydrates, proteins, lipids, and nucleic acids, as well as essential vitamins and minerals. The content of each fraction varies with microalgae strain and their physiological responses to biotic and abiotic factors (Barkia & Saari, 2019). Table 1.5 displays the average protein, lipids, ash and NDF content of microalgal species used as ruminant feed.

Table 1.5- Composition of microalgae species used for ruminants in their raw form (g/ kg DM).

Microalgae	Protein	Lipids	Ash	NDF	Ruminant species	References
Arthrospira platensis	460-744	20-150	47-257	35-87	Cattle	(Costa et al., 2016) (Lamminen et al., 2019) (Costa et al., 2020) (Panjaitan et al., 2010) (Panjaitan et al., 2015)
Chlorella pyrenoidosa	548-600	20-143	64-202	4	Cattle	(Costa et al., 2016) (Costa et al., 2020), (Becker, 2007)
Chlorella vulgaris	586	123	51	15	Cattle	(Lamminen et al., 2019)
Crypthecodinium cohnii	194	575	69	50	Sheep, Cattle	(Pickard et al., 2008) (Fievez et al., 2007) (Valente et al., 2021)
Dunaliella salina	62-570	60-281	90-787	0	Cattle	(Costa et al., 2016) (Costa et al., 2020) (Becker, 2007)
Nannochloropsis gaditana	385	192	158	2019	Cattle	(Lamminen et al., 2019)
Nannochloropsis oculata	289-292	197-292	81-89	69.5	Cattle, Goats	(Archibeque et al., 2009) (Kholif et al., 2020)
Prototheca moriformis	38-76	80-109	5-70	114	Cattle, Sheep	(Da Silva et al., 2016) (Stokes et al., 2015)
Schizocythrium sp.	130-208	38-577	74-139	163-369	Cattle, Sheep	(Boeckaert et al., 2006) (Meale et al., 2014) (Moate et al., 2013) (Till et al., 2019)

Adapted from Costa et al. (2022).

Proteins play a role in the structure of the cell and also in its metabolism, forming part of the membrane, light harvesting complex, and numerous enzymes (Barkia & Saari, 2019). The protein content of several species can be very high, varying between 50-70% of the microalga DM composition (Chew et al., 2017). *Arthrospira maxima* (60-70% DM), *Chlorella vulgaris* (51-58% DM), and *Scenedesmus obliquus* (50-56% DM) are among the main species used for protein production (Becker, 2007). Quality-wise, microalgal proteins contain essential amino acids that mammals require and the amino acid profiled is similar to high-quality protein sources such as lactoglobulin, egg albumin and soy (Becker, 2007; Williams & Laurens, 2010). In terms of animal feed, evaluations demonstrated the suitability of algae biomass as a valuable feed supplement or substitute for conventional protein sources (soybean meal, fish meal, rice bran, etc.) (Becker, 2007).

Microalgae typically have a high carbohydrate content which is about 50% of their DW (Chew et al., 2017), but it can vary from 25 to 60% (De Carvalho et al., 2020). Microalgal carbohydrates are mainly composed of glucose, starch, cellulose, and various kinds of polysaccharides. Carbohydrates including mono-, oligo-, and polysaccharides have both structural and metabolic functions. They can be found attached to proteins or lipids, and complex polysaccharides compose the major structural features of the cell wall (Arad & Levy-Ontman, 2010). Microalgal carbohydrates are able to modulate the immune system and inflammatory reactions, which makes them favourable sources of biologically active compounds that can be used for cosmetic additives, food ingredients and natural therapeutic agents (Chew et al., 2017).

Lipids are structural components of the cellular membrane and can also function as energy reservoirs, in the case of neutral lipids (Barkia & Saari, 2019). In microalgae, the lipid fraction is mainly composed of neutral lipids that include acylglycerols, free FA and carotenoids; and polar lipids, such as various PL and galactolipids. In the exponential phase of growth, most microalgae are rich in polar lipids, and they accumulate TAG under stress, typically during the stationary phase when nitrogen availability is lower (Ratledge & Cohen, 2008; Rodolfi et al., 2009; Chew et al., 2017). The accumulation of FA is linked to the cell growth stages, acting as an energy deposit during unfavorable conditions or cell division, having n-3 a high energy content and importance in cellular functions (Adarme-Vega et al., 2012). Marine microalgae can have oil contents ranging between 10-50% (w/w) and produce a high percentage of total lipids (up to 30-70% DW)(Ward & Singh, 2005). Most commonly microalgae n-3 FA consist of LC-FA, being the highest content associated with ALA, EPA and DHA (Perdana et al., 2021).

Microalgae are the initial EPA and DHA producers in the marine food chain (Adarme-Vega et al., 2012) with relative proportions frequently surpassing 12% of TFA (around 18% for EPA, 14% for DHA and 14% for DPA) (Perdana et al., 2021).

However, in specific cases, these contents can be much higher, such as in the case of *Dunaliella salina* MAScIR ALA content, reaching up to 45.3% of total FA content (Maadane et al., 2015). *Nannochloropsis* sp. can reach up approximately 40% of EPA (Adarme-Vega et al., 2012) and *Phaeodactyrum tricornutum* up to 52.6% of the total FA content (Suh et al., 2015). *Schizochytrium limacinum* can contain up to 30-40% DHA (Adarme-veja et al., 2012) and (Jang et al., 2017) reported levels of DHA content in *Paragymnodinium shiwhaense* up to 42.9% of total FA content. *Botryococcus braunii* has the highest lipid production capability, as, according to Tasić et al. (2016), the lipid content of *B. braunii* can reach up to 65% of its DW. However, the PUFA content is only 10.5% of the total lipid content.

Nannochloropsis genus is considered to be a model organism for lipid production (Simionato et al., 2013) and specially EPA, which content can average 4.3% on DW basis in *Nannochloropsis gaditana* (Mitra, Patidar, & Mishra, 2015; Ma et al., 2016; Janssen et al., 2019) and between 2.7 and 5.2% in *N. oceanica* (Ma et al., 2016). *Nannochloropsis* species are suggested to be among the most promising EPA and DHA producers for commercial applications (Adarme-Vega et al., 2012; Ma et al., 2016).

1.4.2.3. *Nannochloropsis* genus.

Hibberd (1981) described the genus *Nannochloropsis*, family *Monodopsidaceae*, order Eustigmatales, class Eustigmatophyceae (Hibberd, 1980). This genus is composed of non-motile spherical cells showing diameters ranging from 2 to 8 µm (Figure 1.3) (Zanella & Vianello, 2020). The systematics is extremely difficult due to its simple and little variable morphology. To date, seven "*Nannochloropsis* group" species have been formally described (Guiry & Guiry, 2020), comprising species now transferred to genus *Microchloropsis*. Due to their homogeneous morphological traits, genetic analysis is required for a reliable taxonomic diagnosis of these taxa. Furthermore, all these taxa show similar chemical composition and nutritional properties, at least in qualitative terms (Zanella & Vianello, 2020).

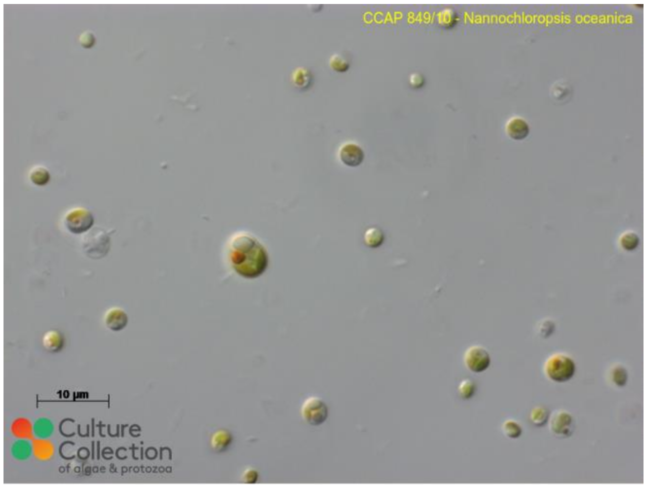

Figure 1.3- CCAP849/10 *Nannochloropsis oceanica*.
Accessed in 08/08/2022 in https://www.ccap.ac.uk/catalogue/strain-849-10.

Nannochloropsis is suitable for cultivation in PBRs, in which a light source (natural or artificial) is used for the autotrophic culture of this species. The culture medium usually consists of sterile sea water supplemented with mineral micronutrients. Culture densities can range from 0.74 to 0.90 g/L DW for *Nannochloropsis oceanica*, 1.2–2.2 g/L DW for *Nannochloropsis oculata* and 1.18–2.67 g/L DW for *Microchloropsis gaditana* (Ashour & El-Wahab, 2017).

The cell wall of *Nannochloropsis* spp. is made up of an inner cellulose-based layer and an outer algaenan-based layer (Scholz et al., 2014) (Figure 1.4) being the latter very resistant to enzymes and chemicals. Hence, *Nannochloropsis* cells cannot be easily ruptured in downstreaming processing.

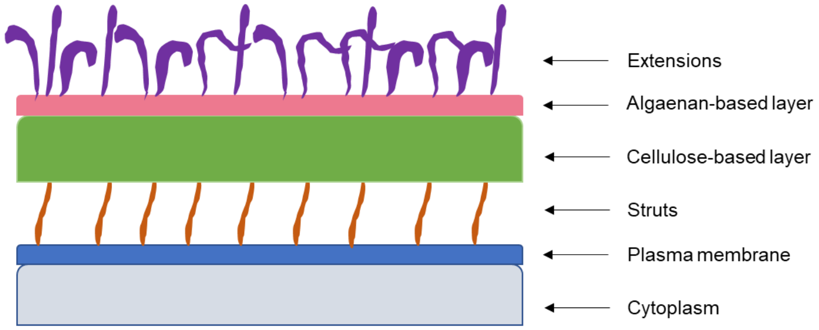

Figure 1.4- Model of the *Nannochloropsis* sp. cell wall.
Adapted from Scholz et al. (2014).

Apart from applications in aquaculture and development of biofuels, this microalga was later intensively exploited for its rich PUFA composition, favourable growth rate and high cell density under culture conditions (Scholz et al., 2014; Zanella & Vianello, 2020). PUFA production is more economically sustainable than biofuel production and a number of large scale producers are now focusing on nutritional PUFA production (Sayeda et al., 2015).

The chemical composition of *Nannochloropsis* biomass can show quantitative variations depending on the microalga strain and environmental conditions but overall, *Nannochloropsis* can be considered as a good source of proteins and fats, with a very desirable FA profile (Zanella & Vianello, 2020). *Nannochloropsis* tends to accumulate lipids in slow cell proliferation phases, when most energy is available for storing in reserve molecules (Rebolloso-Fuentes et al., 2001), and they include MUFA and PUFA of high nutritional value, among which an EPA is the predominant. Other dominant FA are 16:0, *c*9-16:0 and, with less regularity, *c*9-18:1 (Zanella & Vianello, 2020). EPA and other PUFA, cytoplasmic membrane components (Rebolloso-Fuentes et al., 2001), represent the most relevant elements of value for this microalga, enhanced by an important contribution of other antioxidant compounds with high biological activity, i.e. polyphenols, carotenoids, and vitamins. The intake of proteins is appreciable for quality and bioavailability, but given the high production costs, it would not be convenient to propose this microalga as a protein source (Zanella & Vianello, 2020). Members of this genus are characterized by the absence of chlorophyll b and the carotenoid composition is relatively simple, containing the major xanthophyll pigments β-carotene, violaxanthin, and a vaucheraxanthin-like pigment. Glucose is the dominant sugar in the polysaccharide composition and at protein level, amino acids aspartate, glutamate and proline predominate (Rebolloso-Fuentes et al., 2001). Despite a considerable variation in composition between *Nannochloropsis* strains (Table 1.6), PUFA range from 3 to 6% DW in most cases, mainly dependent on the contribution of EPA, which generally varies between 2.0 and 5.5% DW (Table 1.7) with an estimated average of 4% DW (Zanella & Vianello, 2020), and reaching as high as 44% of the total FA in the biomass (Safafar et al., 2016). Importantly, the EPA content is not strictly proportional to the amount of total lipids (Zanella & Vianello, 2020).

Table 1.6-Composition of *Nannochloropsis sp*. and other strains expressed as fraction dry weight (% DW) biomass.

Item	Strains										
	Nannochloropsis					***N. oceanica***	***N. oculata***	***N. granulata***	***N. limnetica***	***M. salina***	***M. gaditana***
Reference	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**	**(6-7)**	**(8)**	**(9)**	**(10-11)**	**(12)**	**(13-14)**
Compound	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**	**%**
Protein	28.8	30.29	26.7	43	28.4	14.5	22.6	45.8	37	18.1-36.2	47
Lipid	18.36	21.78	15.3	30		30.7	8.2	28.5	24	6.2-26.0	16.5
Carbohydrates	35.9	9.62	32.1	35		8.3	6.4	14.9	10	17.8-36.2	21.7
Fiber	2.41		17.7								4.0
Ash	9.44	11.32	8.31					7.8			10.1
Moisture	3.1	1.84									4.7
Nitrates	0.074										
Energy (KJ)	1760							2340			
SFA											
14:0	0.63				0.57	1.69	0.27		0.63		0.9-2.3
16:0	5.05		0.03		3.10	1.72	1.46		3.61		4.0-8.8
MUFA											
16:1n-7	4.72		2.6		4.0	1.82	2.18		3.71		4.4-11.9
18:1n-9	3.79		Nd		0.27	0.41	0.63		3.01		0.1-1.5
PUFA											
18:2n-6	0.36	0.411	Nd		0.34	0.97	0.24		0.38		1.6-3.5
18:3n-3		0.023				0.50			0.04		0.3-1.1
20:4n-6	0.69	0.745	Nd			0.37	0.58		0.53		0.4-3.4
20:5n-3	2.24	3.7	3.65		3.44	2.34	2.33		2.81		4.4-11

Whenever possible, values referred to standard cultures were chosen rather than data from experimental treatments. Abbreviations: SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. References: (1) (Rebolloso-Fuentes et al., 2001); (2) (Kent et al., 2015); (3) (Molino et al., 2018); (4) (Chua & Schenk, 2017); (5) (Bernaerts et al., 2020); (6) (Ashour & El-Wahab, 2017); (7) (Patil et al., 2007); (8) (Volkman et al., 1993); (9) (Tibbetts et al., 2015); (10) (Freire et al., 2016); (11) (Krienitz & Wirth, 2006); (12) (Safafar et al., 2016); (13) (Molino et al., 2019); (14) (Mitra et al., 2015). Adapted from Zanella & Vianello (2020).

Table 1.7- Minimum-maximum total contents (% DW) of lipid, PUFA and EPA in biomasses obtained from different *Nannochloropsis* strains and cultivation conditions.

Species	Total lipids	PUFA	EPA	References
Estimated average EPA (mg/g DW)			40	
N. oceanica NIVA-2/03	-	3.8	2.3	(Patil et al., 2007)
N. oceanica CY2			3.8-5.6	(Chen et al., 2015a)
N.oceanica IMET1	28-59		2.7-5.2	(Meng et al., 2015)
N. sp		5.1-5.9	3.6-4.3	(Chini Zittelli et al., 1999)
N. sp F&M-M24	28-60			(Rodolfi et al., 2009)
N. sp	-	-	2.3-5.7	(Zou et al., 2000)
N. sp	15.3	5.0	3.7	(Molino et al., 2018)
N. sp	21.8	4.5	3.7	(Kent et al., 2015)
N. sp	22-31	4.6-5.8	3.8-5.1	(Xu et al., 2004)
N. sp. F&M – M24 (N starved)	68.5	~9.3*	~4.1*	(Bondioli et al., 2012)
N. sp CCAP211/78		4.4-6.1	4.2-4.9	(Hulatt et al., 2017)
N. oculata ST-6 (wild type)		3.0	2.4	(Chaturvedi & Fujita, 2006)
N. limnetica SAG18.99		0.84-12.2	0.22-5.6	(Krienitz & Wirth, 2006)
M. salina		2.5-4.5	1.1-3.5	(Hoffmann et al., 2010)
M. gaditana	22.3-38.6	7.7-18.5	4.4-11	(Mitra et al., 2015)
M. gaditana	24-28	2.8-5.8	2.1-4.3	(Camacho-Rodríguez et al., 2014)
N. oceanica	12	3.9	2.9	(Alves et al., 2018)

Abbreviations: PUFA – polyunsaturated fatty acids; EPA – eicosapentaenoic acid. In order to show the maximum range of variation, each value has been reported as independent datum, without regard for the consistency of culture conditions, harvest timing, etc. The average content of EPA is estimated in the last row and used as representative of a generic biomass. *Approximate values estimated from graphs or variation ranges. Adapted from Zanella & Vianello (2020).

No toxins produced by *Nannochloropsis* are known (Enzing et al., 2014) and toxicological safety has been proved by its long term use as food for marine fish and shellfish larvae (Zanella & Vianello, 2020). All the available data information, i.e. use of *Nannochloropsis* as food for aquaculture species, on its composition and toxicological tests, supports the conclusion that this microalga shows no contraindication even for direct human nutrition (Zanella & Vianello, 2020).

In order to reduce production costs, cultivations in open systems might be proposed, if managed according to the related best practices in use for other microalgae intended for human diet (Zanella & Vianello, 2020) and dehydration by freeze-drying might be particularly suitable in order to preserve all nutritional and antioxidant properties of *Nannochloropsis*, since it is performed at low temperature and in the absence of oxygen, i.e. minimising oxidative reactions (Zanella & Vianello, 2020).

Although there are a lot of promising species and strains for EPA production (Gu et al., 2022), the development of scalable, cost-effective production processes is the factor limiting the photoautotrophic production of EPA using microalgae. Gu et al. (2022) explored the efficacy of industrial EPA production by nine microalgae strains, which included *Microchloropsis salina CS-190*, *Nannochloropsis oculata CS-179* and *Nannochloropsis oculata CS-192*. *Porphyridium pureum* and *Rodosorus* sp had a high fraction of EPA but low specific concentration due to low

FA content and *Pavlova lutheri* and *Rebecca salina* had high specific EPA concentration but low volumetric concentrations due to low biomass accumulation in flask cultures. The highest volumetric EPA concentrations were obtained with *Nannochloropsis oculata* strains (4-14 mg/L) (Gu et al., 2022).

Different strains of *Nannochloropsis* have been reported to have wide applications in aquaculture as live food and *Nannochloropsis oceanica* has been used as energy-rich food source for rotifers and fish larvae (Wan et al., 2013) and as one of the best strains for biodiesel production (Ma et al., 2014).

1.4.2.4. N-3 LC-PUFA-rich microalgae in lamb diets.

Increasing the content of n-3 PUFA in ruminant derived-foods is highly desirable due to their health promoting effects to humans (Scollan et al., 2006; Vahmani et al., 2020). There have been several attempts to implement strategies to improve lambs' tissues FA profile (Cooper et al., 2004). For that, various feed ingredients, including marine-derived oils (algae and fish) have been investigated (Ponnampalam et al., 2001). Moreover, as animal feed, microalgae can be a valuable source not only of n-3 LC-PUFA, but they might also have the ability to improve the levels of other FA intermediates (such as *t*11-18:1 and *c*9,*t*12-CLA), due to their impact in the extension of FA metabolization in the rumen (Halmemies-Beauchet-Filleau et al., 2018).

Impact on lamb production traits and carcass quality.

The addition of algae biomass or extracted oil to the lambs' diet on carcass composition and other aspects of meat quality are, however, largely unknown (Hopkins et al., 2014). Regarding the impact of microalgae supplementation on lamb production traits, conflicting results have been published (Madeira et al., 2017) mainly due to variations in supplemented species, incorporation percentages and duration of the supplementation (Table 1.8).

No major effects on lamb performance were found with a supplementation of *Dinophyceae* (155 g/kg diet) (Cooper et al., 2004) nor with *Aurantiochytrium limacinum* (2.5%) (Núñez-Sánchez et al., 2021). In general, no effects on ADG were observed with, *Schizochytrium* spp. (DHA-Gold) (1, 2, or 3% DM)(Meale et al., 2014; Hopkins et al., 2014; Fan et al., 2019) or with *Isochrysis sp*. supplementation (4%) (De la Fuente-Vázquez et al., 2014). Detrimental productive effects were observed with *Schizochytrium* spp. (DHA-Gold) up to

4%DM as in a work by Urrutia et al. (2016) or up to 6% as in the work by Valença et al. (2021). However, increased performance indicators such as improved final LW and ADG were observed with *Schizochytrium limacinum* (5 g/ day) (Sucu et al., 2017) supplementations.

No major effects on carcass characteristics were observed with *Schizochytrium* spp. (DHA-Gold) included as 2% DM as in the works by Hopkins et al. (2014) and Burnett et al. (2016), as 3% DM as described by Meale et al. (2014), as 4% DM as described by Urrutia et al. (2016), nor up to 6% as in the work by Valença et al. (2021). Regarding different genus, also no major carcass effects were reported with suplementtions of lambs with 4% *Isochrysis* sp. (De la Fuente-Vázquez et al., 2014) nor with a supplementation of 2.5% of *Aurantiochytrium limacinum* (2.5%) as in the work by Núñez-Sánchez et al. (2021).

Table 1.8 – Supplementation of n-3 PUFA in lamb diets: impact on productive performance and carcass.

N-3 PUFA source	Duration	Target animal	Impact on productive performance	Impact on carcass traits	Reference
Dinophyceae (155 g/kg diet)	Until half of their estimated mature live weight (40 kg)	Suffolk-crossbred wether lambs (LW of 29 ± 2.1 kg)	No effect on growth performance and FI		(Cooper et al., 2004)
Schizochytrium spp. (DHA-Gold) (1, 2, or 3% DM)	18 weeks	Lambs (LW of 22.7 kg)	No effect on daily DMI and ADG, with up to 3% DM.	No effect on carcass characteristics, except for body wall thickness	(Meale et al., 2014)
Isochrysis sp. supplementation (4%)	40.8 ± 0.7 days (slaughter weight of 26.2 ± 0.3 kg)	Weaned male Manchego lambs (LW of 14.7 kg)	No effect on ADG, average FI, FCR	No effect on carcass weight, carcass yield and backfat thickness	(De la Fuente-Vázquez et al., 2014)
Schizochytrium spp. (DHA-Gold) (2%)	6 weeks	3-month-old lambs (LW of 34.8 kg)	No effect on ADG, FCR	No effect on carcass weight	(Hopkins et al., 2014)
Schizochytrium spp. (DHA Gold) (1.8%)	3 months	Crossbreed lambs (LW of 32-34.5 kg)	Decreased DMI but no effect on growth rate	No effect on carcass weight	(Burnett et al., 2016)
Schizochytrium spp. (DHA-Gold) (4%)	Until commercial slaughter weight (24–28 kg LW)	55.1 days-old, weaned Navarra lambs with 16.3 kg LW (from 16.3 to 26.7 kg)	Decreased ADG and FE and increased slaughter age	No effect on carcass traits	(Urrutia et al., 2016)
Schizochytrium spp. (DHA-Gold) (2%)	Until target LW of 26 kg	7-8 weeks-old, weaned Manchego male lambs with 15.3 kg LW (from 15.3 to 26 kg)	Decreased ADG and FI, and increased fattening period, although not having effect on final LW		(Díaz et al., 2017)
Schizochytrium limacinum (5 g/ day)	7 weeks	5-months-old male Merino lambs (LW of 39.1 ± 1.28 kg)	Increased LW and ADG and no differences in average FI or FE.		(Sucu et al., 2017)
Schizochytrium spp. (3% DM)	60 days	80 ± 3 days old-Hu lambs (LW of 18.35 ± 1.39 kg)	No effect on final LW and ADG		(Fan et al., 2019)
Schizochytrium spp. (2,4 and 6% DM)	77 days	60 day-year-old Ile de France lambs (LW of 20.0 ± 0.2 kg)	Decreased DMI and ADG and increased FCR	No effect on carcass weight an yield	(Valença et al., 2021)
Aurantiochytrium limacinum (2.5%)	49 days	42 days-old Manchega-breed lambs (LW of 11.6 ± 1.67 kg)	No effect on growth performance	No effect on carcass characteristics	(Núñez-Sánchez et al., 2021)

Abbreviations: LW – live weight; FI – feed intake; ADG – average daily gain; FCR: feed conversion ratio; DM – dry matter; DMI – dry matter intake; FE – feed efficiency; BCS – body condition score.

Impact on lamb meat quality.

Table 1.9 resumes some results on the impact of supplementing lamb feed with microalgae and its impact on meat quality.

Increasing n-3 LC-PUFA in lamb tissues using marine lipid sources can have a detrimental effect in terms of meat oxidation status, making it less stable, with a reduced oxidative shelf-life and a higher content of thiobarbituric acid reactive substances (TBARS) (Nute et al., 2007; De la Fuente-Vázquez et al., 2014).

In a work by De la Fuente-Vázquez et al. (2014), the partial substitution of linseed with algae increased the meat lipid oxidation and the same was observed by Nute et al. (2007) when supplementing heavy lambs with algae. A stronger effect of marine lipids, when compared to other plant oils is pointed to be related to the fact that ALA is less susceptible to oxidation when compared to EPA and DHA (Kouba & Mourot, 2011). However, meat oxidation stability is strongly influenced by the presence of antioxidants as vitamin E (Ponnampalam et al., 2016). Ponnampalam et al. (2016) reported that a 2% *Schizochytrium* supplementation of lambs had no effect on colour but there was, however, an increase in meat TBARS. Also in a work by Valença et al. (2021), the inclusion of *Schizochytrium* in lambs' diets increased meat lipid oxidation.

In the same way, the colour of the meat is also a parameter that can be influenced by the presence of a higher PUFA content. The inclusion of EPA and DHA sources, like fish oil or algae sources, can result in lower colour stability during retail display (Nute et al., 2007; De la Fuente-Vázquez et al., 2014). Nute et al. (2007) reported that a less red coloured meat would be a result of higher metmyoglobin, caused by higher lipid oxidation. Contrasting with marine lipid sources, the dietary supplementation of several plant oils did not influence meat colour parameters in several studies (Atti et al., 2013; De la Fuente-Vázquez et al., 2014; Sekali et al., 2016; Buendía-Rodríguez et al., 2019).

The use of marine lipid sources in the diet can influence the sensory properties of the meat (Nguyen et al., 2018a), however, literature results might not always be conclusive (Álvarez-Rodríguez et al., 2022). Impact in meat tenderness and juiciness might not be affected by PUFA supplementation, as was verified by others (Nute et al., 2007; Urrutia et al., 2016; Díaz et al., 2017)

In general, microalgae supplementation can efficiently increase n-3 FA in both muscle and fat deposits in lambs, although with inconclusive impact on meat quality. A supplementation of *Schizochytrium* (2%) increased EPA between 1.2 (Ponnampalam et al., 2016) and 18 times

(Díaz et al., 2017) in muscle and fat tissues respectively, in relation to the control animals. DHA enhancement in muscle tissues varied between +3 and +29 times relatively to the control animals, with *Isochrysis* sp. (4%) (De la Fuente-Vázquez et al., 2014) and *Schizochytrium* spp. (DHA-Gold) (2%) (Urrutia et al., 2016) supplementations, respectively.

Table 1.9- Supplementation of n-3 PUFA in lamb diets: impact on meat quality.

N-3 PUFA source	**Duration**	**Target animal**	**Impact on meat quality**		**Reference**
			Oxidation and sensorial attributes	**n-3 FA (+ times increased compared to control)**	
Dinophyceae (155 g/kg diet)	Until half of their estimated mature live weight (40 kg)	Suffolk cross-wether lambs (LW of 29 kg)	Increased lipid oxidation, lower colour stability. No impact on tenderness and juiciness. Increased abnormal lamb flavours.		(Nute et al., 2007)
Schizochytrium spp. (DHA-Gold) (2%)	6 weeks	3-month-old lambs (LW of 34.8 kg)	No effect on meat colour and ph.	Increased EPA + DHA (+3) in *longissimus lumborum*	(Hopkins et al., 2014)
Schizochytrium spp. (DHA-Gold) (1, 2, or 3% DM)	18 weeks	Lambs (LW of 22.7 kg)		Increased EPA (+5), DPA (+3) and DHA (+17) in adipose tissue; Increased EPA (+4), DPA (+2) and DHA (+13) in the muscle.	(Meale et al., 2014)
Isochrysis sp. (4%)	40.8 ± 0.7 days (slaughter weight of 26.2 ± 0.3 kg)	Weaned male Manchego lambs (LW of 14.7 kg)	Increased meat lipid oxidation, lower colour stability. No effects on meat colour and ph.	Increased ALA (+5), EPA (+3), DPA (+2) and DHA (+3) in meat	(De la Fuente-Vázquez et al., 2014)
Schizochytrium (2%)	8 weeks	9-month-old cross ewe lambs (LW of 35.3 ± 3.1 kg)	No effect on colour and muscle fat. Decreased muscular vitamin E. Increase in meat TBARS	Increased EPA (+1.2) and DHA (+8)	(Ponnampalam et al., 2016)
Schizochytrium (2,4 and 6% DM)	77 days	60 day-year-old Ile de France lambs (LW of 20.0 ± 0.2 kg)	No effects on meat ph, colour, muscle cooking weight loss and shear force. Reduced cholesterol and increased meat lipid oxidation.		(Valença et al., 2021)
Schizochytrium spp. (DHA-Gold) (2%)	Until target LW of 26 kg	7-8 weeks-old, weaned Manchego male lambs with 15.3 kg LW (from 15.3 to 26 kg)	No effect on ph. Higher meat L*.	Increased EPA (+3/5), DPA (+2.4/1.4) and DHA (+29/20) in both subcutaneous and intermuscular fat	(Urrutia et al., 2016)
Schizochytrium spp. (DHA-Gold) (2%)	Until target LW of 26 kg	7-8 weeks-old, weaned Manchego male lambs with 15.3 kg LW (from 15.3 to 26 kg)	No effect on meat tenderness and juiciness.	Increased EPA (+8/+18), DHA (+13/+26), and total n-3 FA (+5) in both muscle and fat.	(Díaz et al., 2017)

Abbreviations: LW – live weight; TBARS- Thiobarbituric acid reactive substances; L*- lightness.

1.4.2.5. Commercial application of microalgae in ruminant production.

Environmental threats such as the prediction of continuous population growth, coupled with climate change phenomena and exploitation of natural ecosystems, with severe consequences in the availability of arable land and freshwater resources, can ultimately culminate in a condition of future global food insecurity (Kusmayadi et al., 2021).

Within ruminant-based food production, there are potential means to improve global food supply and to decrease its environmental footprint without compromising the quality of the edible products (Halmemies-Beauchet-Filleau et al., 2018). Even though ruminants possess a lower feed conversion efficiency when compared to monogastric, to produce the same amount of animal protein products, less human-edible feed is needed in ruminant systems when compared to monogastric systems (6 v. 16 kg of human-edible feed DM per kilogram of protein products (Mottet et al., 2017).

In order to improve the reduction of environmental footprint in ruminant production trough feedstock interventions, the feasibility of using alternative feeds for ruminants depends, among others, on the feed value of novel feeds, animal production responses and feed costs compared to the conventional feeds (Halmemies-Beauchet-Filleau et al., 2018). For that matter, microalgae cannot yet be regarded as an alternative to vegetables or cereals as protein source for the feed market due to high production costs mainly associated to technologies needed for cultivation, harvesting, and processing/extraction (Saadaoui et al., 2021). However, small-scale solutions can temporarily start being applied, as for example, a practical solution for extensive farming with the on-farm production of microalgae in ponds connected to animal drinking water system (Costa et al., 2022). This could lower the energy inputs of feed drying, preservation and transportation, for example (Panjaitan et al., 2010; Halmemies-Beauchet-Filleau et al., 2018). On-farm microalgae production (Figure 1.5) and delivery through the drinking systems (with desalinization when needed), could be an important protein source contribution for ruminants during the drier months of the year, being *Arthrospira*, *Chlorella* and *Nannochloropsis* the most suitable (Costa et al., 2022). Also, microalgae on-farm production could be linked to recovery and improvement in water quality of pits (Costa et al., 2022), and they could also be used as fertilizers (Baweja et al., 2019).

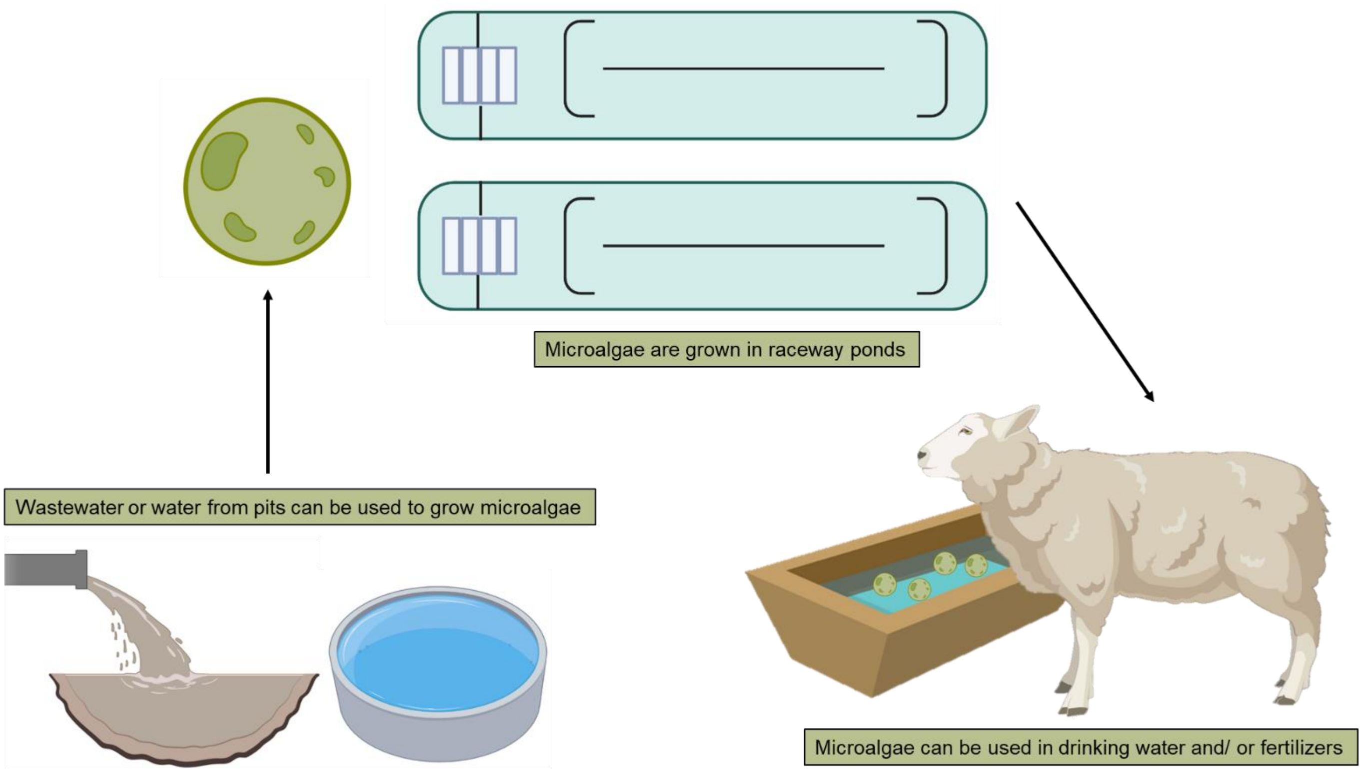

Figure 1.5 – On farm microalgae production and utilization.
Wastewater or water from pits can be used to grow microalgae on farm. Microalgae are grown in raceway ponds as a more affordable alternative. In case of a marine microalga, desalinization steps should be applied before the microalga is distributed in the drinking systems. Created with BioRender.com.

From a nutritional standpoint, microalgae offer a wide variety of compounds necessary for the improvement of animal productive performances, justifying their ability to add value in feed formulation in a medium/long-term. Moreover, the nutritional quality of animal products can be improved by several molecules provided by the ingested microalgae (i.e. carotenoids, antioxidants, iodine, n-3 LC-PUFA) which could have a positive impact on food safety (Bature et al., 2022).

Algae can be used in ruminant diets in their raw form if produced in excess, or as by-products from other industries (Costa et al., 2022). At the moment, the more realistic approach could be to incorporate microalgae as an enriching factor in early stages of animals development to improve the health and some productive traits (Vigani et al., 2015; Saadaoui et al., 2021), contributing to generate products of animal origin with added value. However, the differences in nutrient composition cause variable effects within the rumen and animal response (Costa et al., 2022) that require standardization for better consistency.

Furthermore, the possibility to grow microalgae in wastewater, their independence of arable land and weather conditions to grow, coupled with future technical developments, and policy interventions such as new incentives and carbon taxation will certainly contribute to the feasibility of including them in animal feedstocks.

In a recent review of the current status of the algae production industry in Europe, it is stated that upscaling of the production volumes and technological and market developments are key drivers to boost the growth of the sector in Europe (Araújo et al., 2021). Furthermore, goals for the EU to become climate neutral by 2050, the protection of biodiversity (EC, 2020c), the development of a circular economy (EC, 2020b) and the contribution to the farm to fork strategy for sustainable food (EC, 2020a) are important boosts for the development of the algae production sector.

Microalgae production for feed purposes is currently more practiced in Asia, and mainly China (Saadaoui et al., 2021), however the industry is also spreading to the United States and the United Kingdom. Although there is a long way to go before the production of microalgae-based feed sustainable and economically feasible, already around 30% of the world's microalgal biomass production is presently sold for animal feed applications (Saadaoui et al., 2021).

Another way of valuing microalgae in ruminant nutrition is their possible intervention in methane mitigation strategies. Their inhibitory effects can be attributed to their UFA content (Palangi et al., 2022). Microalgae species with potential as methane mitigators, such as *Schizochytrium* are high in PUFA which work as a H_2 sink, competing with pathways involved in methane formation (Boeckaert et al., 2006). However promising, evidence supporting meaningful enteric methane reductions *in vivo* using microalgae species is still lacking (Costa et al., 2022).

2. Lipid metabolism in ruminants.

2.1. Ruminal microbiome.

Ruminal microbiome is composed of bacteria (95%), archaea (2-4%), protozoa and fungi (1%), which corresponds to 10^{10} bacteria, 10^7 protozoa, 10^6 fungi and yeasts per mL of live liquor (Buccioni et al., 2012). Microbiome main function is to ferment and degrade plant fibres, converting plant lignocellulose material into digestible compounds (Brulc et al., 2009), and to convert non-protein nitrogen into microbial protein (Newbold & Ramos-Morales, 2020). The microbial fermentation of the ingested feedstuffs allows the production of energy-rich volatile fatty acids (VFA), that serve as the main energy source (Bergman, 1990), and microbial protein, the main protein source for ruminants (Kay, 1969). Therefore, microbiome activity supplies ruminants with most of their dietary requirements, supplanting up to 90% of their metabolic needs (Mizrahi & Jami, 2018).

Rumen harbours different types of bacteria, which are most actively involved in the plant fiber degradation. Bacteria associated with feed particles account for nearly 50-75% of the total microbial population. This adhesion to solid substances is an important factor for the success of the digestion of solid feed (Minatoi et al., 1966). Ruminal bacteria can be found free floating in the liquid phase, attached to the feed particles (firmly or loose) or in the rumen epithelium (Choudhury et al., 2015). They can be classified as fiber-degrading bacteria, lactic acid utilizers, acetogens, starch utilizers, etc (Choudhury et al., 2015). Usually fiber digester account for up to 25% of the rumen bacterial population (Choudhury et al., 2015). The rumen wall-associated bacteria scavenge oxygen and break down urea into CO_2 and NH_3, that enter the rumen via feed, saliva, and blood. The extramural bacteria (99%) can be either free floating or attached to protozoa (i.e. symbiotic methanogens) or feed particles. About 75% of the bacteria are associated with feed particles and from those, 80-90% are responsible for fiber digestion, 75% for protein digestion and 70% for starch digestion. Free-floating bacteria are about 30% (Choudhury et al., 2015). Despite several factors influencing the abundance and composition of ruminal microbiome, there is a shared core, composed of approximately 30 bacterial taxa, dominated by *Prevotella* (Bacteroidetes), *Butyrivibrio* and *Ruminococcus* (both Firmicutes), unclassified *Lachnospiraceae*, *Ruminococcaceae*, Clostridiales (all Firmicutes) and Bacteroidales (Bacteroidetes) (Henderson et al., 2015). *Ruminoccocus*, *Butyrivibrio* and *Fibrobacter* are the main cellulolytic genus and *Prevotella*, thought non-cellulolytic, also displays carbohydrate degradation capabilities (Accetto & Avguštin, 2015).

Archea make up 0.3-3% of the rumen microbiome (Janssen & Kirs, 2008). They are represented by methanogens, which are dominated by *Methanobrevibacter* (Henderson et al., 2015). This genus is hydrogenotrophic, producing methane from H_2, CO_2 and formate (Janssen & Kirs, 2008). Methanogens have the ability to use H_2 produced through bacteria fermentation, reducing CO_2 to methane (CH_4), serving as electron sink to decrease H_2 pressure in the rumen and avoid inhibition of bacterial activity (Morgavi et al., 2010). Other significant hydrogenotrophic genera include *Methanosphaera*, *Methanimicrococcus* and *Methanobacterium*. Less abundant methylotrophs, producing methane from methylamines and methanol are Methanosarcinales, *Methanosphaera* and *Methanomassiliicoccaceae*, and producing methane from acetate, aceticlastic archea like *Methanosarcinales* (Morgavi et al., 2010).

Eukaryotes are represented by ciliate protozoa and fungi (Brulc et al., 2009). Although smaller in richness compared with bacteria and archaea, they reach up to 50% of the ruminal biomass (Williams & Coleman, 1992) and are highly active (accounted for the % of ribosomal RNA (rRNA) expressed) (Comtet-Marre et al., 2017), playing an important role in fiber digestion and the modulation of the fermentation profiles, producing fermentation end products similar to those made by the bacteria, particularly acetate, butyrate, and H_2 (Choudhury et al., 2015). Protozoa are not crucial for animal survival once defaunation has been previously observed in apparently healthy animals. However, defaunation can have an influence in methane emissions physiology once protein supply decreases (Newbold et al., 2015) and some protozoa like *Holotrich* contain endosymbiotic methanogens (Belanche et al., 2015). Protozoa also play a role in RBH, however, RBH seems to only be slightly impacted after defaunation and the presence of protozoa appears not to be essential for it to occur (Dawson & Kemp, 1969). There is a suggestion that the participation of protozoa in RBH has to do with the activity of ingested or associated bacteria (Girard & Hawke, 1978), as the previously mentioned endosymbiotic methanogens. Most protozoa in the rumen are ciliates, with a few flagellate species (Newbold & Ramos-Morales, 2020).

Anaerobic fungi have been described under the order Neocallimastigales, class *Neocallimastigomycetes* in the phylum Neocallimastigomycota (Choudhury et al., 2015). Chitin measurements and rRNA transcript abundance indicate that anaerobic fungi represent 10-20% of the rumen microbiome (Huws et al., 2018) and they are thought to be crucial fiber degraders (Krause et al., 2013). Six genera are commonly recognized: the monocentric Neocallimastix, Caecomyces and Piromyces and the polycentric Anaeromyces, Orpinomyces and Cyllamyces (Newbold & Ramos-Morales, 2020).

The rumen virome remains poorly characterized. Lytic phages have been isolated from the rumen and studies on their diversity have been reported, including the evidence to suggest that energy intake may be a major driver of the rumen virome (Gilbert & Klieve, 2015). Bacteriophages are present typically at $>10^9$ particles per mL and are considered to be obligate pathogens for the bacteria, and probably by lysing the bacterial cells, the bacterial protein is easily made available to the animals as a source of amino acids (Choudhury et al., 2015).

The complex succession of microorganisms takes part in the cooperative catabolism of substrates in the rumen and the production of fermentative end products, along with BCFA, vitamins and other cofactors for other microorganisms (Choudhury et al., 2015). Microbiome modulation has been investigated and is still in various optimization steps, remaining a current challenge in the field of animal production. Attempts with transient microbiome modifications have involved introducing indigenous to the rumen (inoculations) and more long-term modification have been achieved with early interventions, namely at the time when the microbiome is developing, through dietary modulation (Mizrahi & Jami, 2018). Because diet has profound effects on the composition of the rumen microbiome, modulation of rumen microbial composition is an opportunity to modulate host metabolism (Zhang et al., 2021).

2.2. Microbial lipids.

Bacteria and protozoa are able to synthesise and incorporate LC-FA in their membrane lipids (Vlaeminck et al., 2006). The total lipid content of bacterial dry mass in the rumen ranges from 10 to 15%, being proportions lower in liquid-associated bacteria than in solid-associated bacteria (Bauchart et al., 1990). Bacterial lipids can be originated from exogenous sources, such as the uptake of dietary LC-FA, and endogenous sources, such as *de novo* synthesis. The contribution of each source depends on lipid content of the diet and bacterial species (Harfoot & Hazlewood, 1997).

2.2.1. Branched-chain fatty acids (BCFA).

Branched-chain fatty acids (BCFA) are a class of primarily SFA with an alkyl group branch, usually methyl, on the carbon chain (Vahmani et al., 2020). Rumen bacteria membrane lipids are rich in odd and branched-chain fatty acids (OBCFA), such as *iso* tetradecanoic acid (*iso*-14:0), pentadecanoic acid (15:0), 13-methyltetradecanoic acid (*iso*-15:0), 12-methyltetradecanoic acid (*anteiso*-15:0), *iso* hexadecanoic acid (*iso*-16:0), heptadecanoic acid (17:0), 15-methylhexadecanoic acid (*iso*-17:0), 14-methylhexadecanoic acid (*anteiso*-17:0) and small amounts of heptadecenoic acid (*cis* 9-17:1) (Kaneda, 1991; Fievez et al., 2003b). Odd chain FA 15:0 and 17:0 are formed through elongation of propionate or valerate, whereas precursors of BCFA (*iso* and *anteiso*) are branched-chain amino acids like valine, leucine and isoleucine and their corresponding branched short-chain carboxylic acids (isobutyric, isovaleric, and 2-methyl butyric) (Kaneda, 1991).

Because the major source of OBCFA in ruminant tissues, such as meat or milk, is from rumen bacterial origin rather than from endogenous synthesis, they have been suggested to be used as markers to quantify bacterial matter leaving the rumen (Keeney et al., 1962) and recent studies have associated them with specific bacterial groups (Conte et al., 2022). Also, the different profiles of their abundance can be related to differences in the animal feeding regimen and rumen fermentation patterns (Vlaeminck et al., 2005; Vlaeminck et al., 2006). Also, interactions between ruminant species, as for examples cows vs goats, and nutrition occur relative to differences in specific rumen OBCFA content (15:0, *anteiso*-15:0, 17:0 and *iso*-18:0), suggesting differences in the rumen microbiota between species (Toral et al., 2015).

2.2.2. Dimethyl acetals (DMA).

A high proportion of bacterial lipids are also composed of plasmalogen lipids, containing alk-l-enyl (vinyl) ether chains. Under methanolic hydrolysis conditions, the vinyl esters are liberated and transformed into dimethyl acetals (DMA) (Miyagawa, 1982). Bacteria might adjust their plasmalogen content and composition in response to different environmental stimuli (Alves et al., 2013) manly because they seem to be involved in the membrane fluidity regulation (Goldfine, 2010).

The DMA composition of rumen contents (i.e. DMA containing saturated, branched or monounsaturated carbon chains) is similar to the fatty acid methyl ester (FAME) composition (Alves et al., 2013) and dietary factors like basal diet, fat source and supplementation levels can modify DMA composition (Bas et al., 2003), being that bacteria can adjust individual DMA proportions but maintain their total content at the same level (Alves et al., 2013). Due to this fact, similarly to what is postulated for OBCFA, DMA composition can also be used as an internal microbial marker in the rumen ecosystem (Alves et al., 2013). Moreover, DMA potential can eventually surpass OBCFA' potential due to the fact that the first are not present in feedstuffs, in opposition to odd-chain FA that can occur in some silages (Alves et al., 2011).

2.3. Dietary lipid metabolism in the rumen.

Lipids incorporated in ruminant diets usually contain structural or polar lipids (glycolipids, PL), free fatty acids (FFA), TAG and sterol esters, being that forages and grains are particularly rich in structural lipids, whereas oil seeds and oils are rich in TAG (Sterk, 2011). The most abundant FA in traditionally used feedstuffs like forages, cereals, and oil seeds are 18-carbon PUFA, mainly LA and ALA, whereas some oil seeds are rich in MUFA (mainly OA) and marine products (fish oil, algae, etc.) are rich in LC-PUFA, mainly EPA and DHA (Chilliard et al., 2007) (Sterk, 2011).

However, the profile of ingested lipids does not necessarily translate the lipid profile of ruminant tissues, mainly due to ruminal metabolization of ingested FA (Jenkins et al., 2008). After ingested, dietary FA undergo rumen metabolization, a process that occurs with major transformations in relation to the amount of ingested UFA and SFA leaving the rumen to the following gastrointestinal (GIT) compartments (Jenkins et al., 2008). This FA transformations taking place in the rumen are responsibility of ruminal microbiome action on the ingested lipids, through lipolysis and biohydrogenation processes (Jenkins et al., 2008), in an overall phenomena designated as ruminal lipid metabolism. This process results not only in the production of stearic acid (18:0) but also a wide range of isomers of PUFA and MUFA, especially *trans* and conjugated FA (Chilliard et al., 2007). As a result, ruminant meat and milk present a low content of UFA, regardless the fact that they are abundant in grass and other ruminant feedstuffs (Jenkins et al., 2008), and a more saturated FA profile, when compared to products derived from non-ruminants (Jenkins et al., 2008).

2.4. Lipolysis.

Lipolysis occurs fast in the ruminal digesta (Dawson et al., 1977) and is carried out by microbial lipases, in a process comprised of extensive hydrolysis (85%) of the ester linkages in TAG, PL, and glycolipids. As a result, FFA and glycerol are released (Garton et al., 1961; Dawson et al., 1977). Under normal conditions, lipolysis is almost complete (Doreau et al., 2011). The resulting free carboxyl group of the FA is necessary for the successive hydrogenation step of the UFA (Ferlay et al., 2017).

In animals consuming concentrate diets, the lipids are mainly in the form of TAG and the hydrolysis mainly occurs by the action of microbial lipases (Dawson et al., 1977). On the other hand, in animals consuming forages or grazing, lipids are mainly in the form of galacto-, sulfo- and PL, and galacto- and phospholipases present in plant tissues contribute itself to ruminal lipolysis (Faruque et al., 1974). *In vitro* lipolysis rate seems to be enhanced by increasing the content of N and decreasing the content of fibre in detriment of starch (Dewhurst et al., 2006).

Among the various types of ruminal microorganisms, bacteria are the most active in lipolysis and some confirmed lipolytic bacteria have been identified such as *Anaerovibrio lipolytica* (Hobson & Mann, 1961), *Butyrivibrio*-like species (Hazlewood & Dawson, 1975), *Clostridium* and *Propionibacterium* (Ferlay et al., 2017). *A. lipolytica* is known to hydrolyse TAG and *Butyrivibrio* spp. is able to hydrolyse PL (Ferlay et al., 2017). Protozoa also seems to have lipase activity and it remains unknown whether fungi are or not involved in ruminal lipolysis (Jenkins et al., 2008; Lourenço et al., 2010).

2.5. Biohydrogenation.

Ruminal biohydrogenation follows lipolysis and converts UFA into SFA via isomerization to *trans* FA intermediates, and by hydrogenation of the double bonds (Harfoot & Hazlewood, 1997; Jenkins et al., 2008).

The putative aim of RBH is to protect bacteria from the toxicity of UFA (Maia et al., 2007; Maia et al., 2010). Bacteria play the central role in FA biohydrogenation (Jenkins et al., 2008), while protozoa role is negligible (Buccioni et al., 2012). This bacterial activity is mainly associated with bacteria attached to feed particles rather than with those in free liquid (Buccioni et al., 2012). Anaerobic fungi seem to have also a small contribution to RBH since it has been observed that they have 18:1 FA in their constitution, and are able to desaturate 18:0 to *c*9-18:1 and form CLA when incubated with LA and ALA (Ferlay et al., 2017)

The main biohydrogenation pathways of dietary LA and ALA initially involve an isomerization of the *cis*-12 double bond to a *trans*-11 configuration, resulting in a conjugated di- or trienoic FA, respectively, followed by a hydrogenation reaction with the conversion of an unsaturated double bond to a saturated single bond (Figure 1.6) (Ferlay et al., 2017). RBH culminates in the hydrogenation of the *trans*-11 double bond producing stearic acid (18:0) (Jenkins et al., 2008). A reduction in the ruminal BH results in lower production of 18:0 and a higher production of BH intermediates (BHI), like *cis*- and *trans*-18:1 and several CLA isomers (Chilliard et al., 2007), known to possess anticarcinogenic activity (Bessa et al., 2000). CLA isomers include both *cis-cis*, *cis-trans* and *trans-trans* geometry, with double bonds at 9 and 11, 10 and 12 or 11 and 13 positions (Bessa et al., 2000), being *c*9,*t*11 (rumenic acid) the predominant natural isomer in humans and animals (Lourenço et al., 2010). There are, however, times when the *t*10,*c*12 isomer (and also *t*rans-10 18:1) becomes a major intermediate, as in cases where the animal feeding has a high-starch content or is supplemented with fish or vegetable oil (Bauman & Griinari, 2001).

Ruminal biohydrogenation intermediates (BHI), formed between the ingested native forms and the final production of 18:0, vary with the diet composition and although some are directly absorbed in the small intestine and secreted into milk or deposited in the meat, some are further transformed in body tissues (Chilliard et al., 2007). The Δ9 SCD, present in the mammary gland tissue (Chilliard et al., 2007), muscle, adipose tissue (Gruffat et al., 2008) and liver (Xu et al.,

2015) has the ability to add a *c*9 double bond on different BHI (Palmquist et al., 2005), partially decreasing the saturation level.

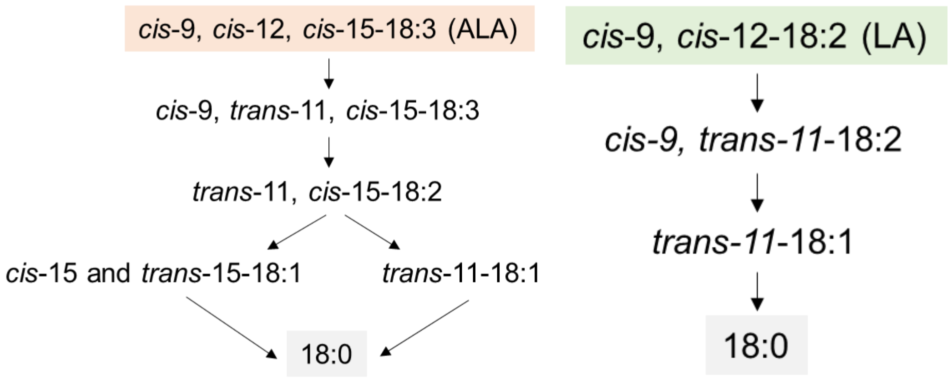

Figure 1.6- Biohydrogenation pathways of (A) α-linolenic and (B) linoleic acids.
The arrows point to the direction of the pathways. Adapted from (Harfoot & Hazlewood, 1997).

Usually, the main FA substrate for RBH in grazing or forage-fed animals is ALA, because it is the most abundant FA in plant glycolipids and PL. In animals supplemented with traditional dietary lipid supplements, usually LA in the form of TAG is the main RBH substrate (Lourenço et al., 2010). Overall RBH of LA and ALA ranges between 70 to 95% and 85 to 100%, respectively (Doreau & Ferlay, 1994; Doreau & Chilliard, 1997; Harfoot & Hazlewood, 1997; Chilliard et al., 2007). The biohydrogenation of OA varies from 58 to 87% (Shingfield et al., 2010a), being it rapidly converted to 18:0, with only a small amount of intermediary *trans*-18:1 formation (Jenkins et al., 2008). Overall, the extension of both the hydrolysis and biohydrogenation steps can be altered depending on the fat content in the diet, or on the ruminal pH (Beam et al., 2000; Van Nevel & Demeyer, 1996; Lock & Bauman, 2004). The amount of lipid in the diet and its composition influences the extent of RBH, however, to a lesser extent if the lipids are protected (Gadeyne et al., 2017), which can occur naturally by trapping in the vegetable cells. A low ruminal pH can also reduce the extent of RBH, and it is commonly associated to a high percentage of concentrate in the diet (> 70%) (Loor et al., 2004).

When marine oils are added to ruminants' diets, LC-PUFA are biohydrogenated via specific pathways (Ferlay et al., 2017). *In vivo* supplementation of ewes with fish oil inhibited the RBH of 16 and 18 carbon UFA, and resulted in the accumulation of *trans*-16:1, *trans*-18:1, and *trans*-18:2, 20, and 22 carbon metabolites (Toral et al., 2010). An *in vivo* study by Kairenius et al. (2011) with cows fed fish oil, suggested that the first committed steps of EPA, 21:5n-3 and DHA hydrogenation in the rumen involves the reduction and/or isomerisation of double bonds closest to the carboxyl group. In the same study, there was a detection of the formation of 20- and 22-carbon RBH products having at least one *trans* double bond, and *c*14-20:1, 20:2n-3, 21:4n-3 and 22:3n-6 FA, not contained in fish oil. However, the full comprehension of LC-PUFA RBH pathways remains unknown. Toral et al. (2018b) conducted a direct comparison of *in vitro* ruminal BH of EPA, DPA, and DHA and no conjugated 20:5, 22:5, and 22:6 isomers compatible with the first product of EPA, DPA, or DHA metabolism, respectively, were found. It was suggested that the main pathway explaining the *in vitro* BH of EPA and DPA seems to proceed via the reduction of the double bond closest to the carboxyl group (namely *cis* 5 in EPA and *cis* 7 in DPA) (Figure 1.7); a mechanism with much lower relevance in the DHA metabolism (Toral et al., 2018b).

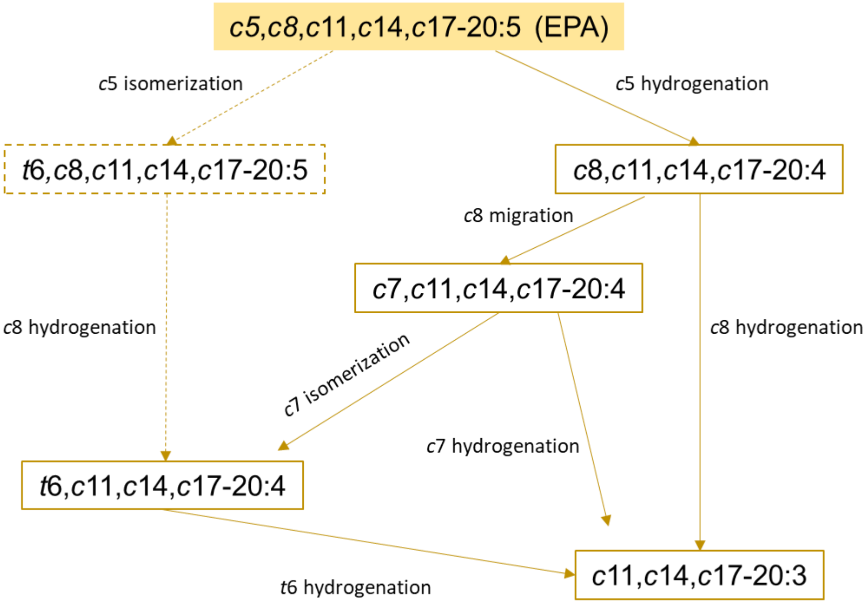

Figure 1.7 - Putative pathways describing the initial steps of EPA biohydrogenation.
Thick arrows highlight the potentially major pathways; dotted lines with arrows and text represent a hypothetical pathway involving the formation of a conjugated 20:5 intermediate. *c* stands for cis, and *t* stands for trans. Adapted from Toral et al. (2018b).

In a work by Aldai et al. (2018), after 6 hours of incubation, all DHA RBH products had a linear carbon chain of 22 carbons (Figure 1.8), indicating that there was no chain-shortening. This had been previously observed by others (Kairenius et al., 2011; Escobar et al., 2016; Jeyanathan et al., 2016). After 6 hours of incubation, 22:5 metabolites (presumable non-conjugated positional isomers of 22:5 with at least one *trans* double bond) were the most abundant (Aldai et al., 2018). Aldai et al. (2018) proposed the existence of two pathways in DHA RBH: (1) proceeding via *cis*-*trans* isomerization of DHA double bond to form mono-*trans* methylene-DHA interrupted isomer, which, following a *trans* double bond migration formed mono-conjugated-DHA isomers; (2) proceeding via an unidentified concerted isomerase via a '*t*14,*c*16' conjugated system.

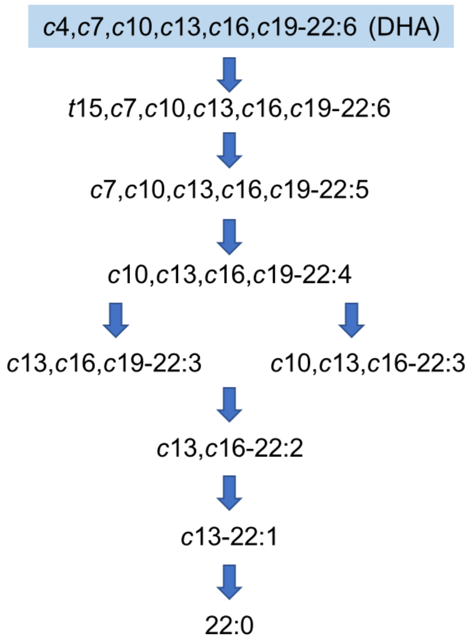

Figure 1.8- The putative biohydrogenation pathway of DHA in the rumen.
Adapted from (Huang et al., 2020). DHA – docosapentaenoic acid (c4,c7,c10,c13,c16,c19). The arrows represent possible major pathways. Neither all putative FA nor the numerous interconversions among C22:6 isomers are presented. Data is adapted from Jenkins et al. (2008), Shingfield et al. (2012), Jeyanathan et al. (2016), Kairenius et al. (2018), and Aldai et al. (2018).

In vitro studies have shown that, as a consequence of EPA and DHA impact on RBH completion extension, the content of *trans*-18:1 isomers, primarily *trans*-10 and *trans*-11, and *cis*-18:1 isomers is raised (Boeckaert et al., 2008b; Shingfield et al., 2010a; Toral et al., 2010). An *in vivo* postnatal supplementation of goat kids, from birth until 12 weeks old, with DHA-enriched microalgae, resulted in an increased ruminal proportion of *t*11-18:1 rather than *t*10 intermediates, which is suggested to be related to differences in microbiome profiles (Dewanckele et al., 2018). In the *in vitro* study by Carreño et al. (2019), ruminal incubations with the addition of EPA, DHA and DPA caused changes in the relative abundance of phyla, families and genera of bacteria, such as *Prevotella*, *Barnesiella*, *Ruminococcus*, *Butyrivibrio*, *Anaerovorax*, *Succinivibrio* or *Ruminobacter*, which might suggest a potential association with ruminal C18 RBH. Even though there is a putative microbiological toxicity associated with UFA (Maia et al., 2007; Maia et al., 2010), in a study by Toral et al. (2017) no direct relationship between *in vitro* disappearance of the incubated PUFA and effects on BH (in particular, inhibition of the last step) was found in either cows or ewes, calling into question a putative link between extent of disappearance and toxicity of these PUFA for microbiota.

2.5.1. *Trans*-10 shifted pathway.

When diets have low forage and are supplemented with high levels of PUFA and/ or starch, the ruminal pH is reduced and microbiome compositional changes occur, mainly involving a shift from a predominantly cellulolytic population, producer of *t*11-18:1, to a more sugar-fermenting population that putatively produces *t*10-18:1 (Loor et al., 2004; Scollan et al., 2017; Chikwanha et al., 2018). The change is accompanied by a reduction in both CLA isomers and atypical dienes at the expense of *c*9,*t*11-18:2 (Aldai et al., 2009; Bravo-Lamas et al., 2016).

The phenomena is called *trans*-10 shift (Figure 1.9) and defined as when *t*10-18:1/*t*11-18:1 ratio exceeds 1 (Bessa et al., 2015). The *trans*-10 shift has been associated with detrimental health effects for the consumer of ruminant products (Roy et al., 2007; Anadón et al., 2010), having cytotoxic, lipogenic and cholesterogenic properties (Vahmani et al., 2017). Furthermore, *t*10-18:1 cannot be desaturated in the tissues to *t*10,*c*12-18:2 (Alves et al., 2021).

Under the circumstances of occurrence of *trans*-10 shift, milk fat depression occurs, once *t*10 BHI seem to decrease mammary lipogenesis (Shingfield et al., 2009). Other consequences to the animal include lower intake and decreased fibre digestion (Bauman & Griinari, 2001).

A study comparing the ruminal metabolism in the cow and goat fed with similar diets supplemented or not with lipids, detected differences in the bacterial structure of the ruminal fluid of these two species. When starch-rich diets were fed, they induced a more pronounced shift in the *trans*-11 pathway to *trans*-10 in the cow when compared to the goat. This suggested that caprine RBH pathways are more stable in response to high-starch diets supplemented with lipids (Toral et al., 2016)

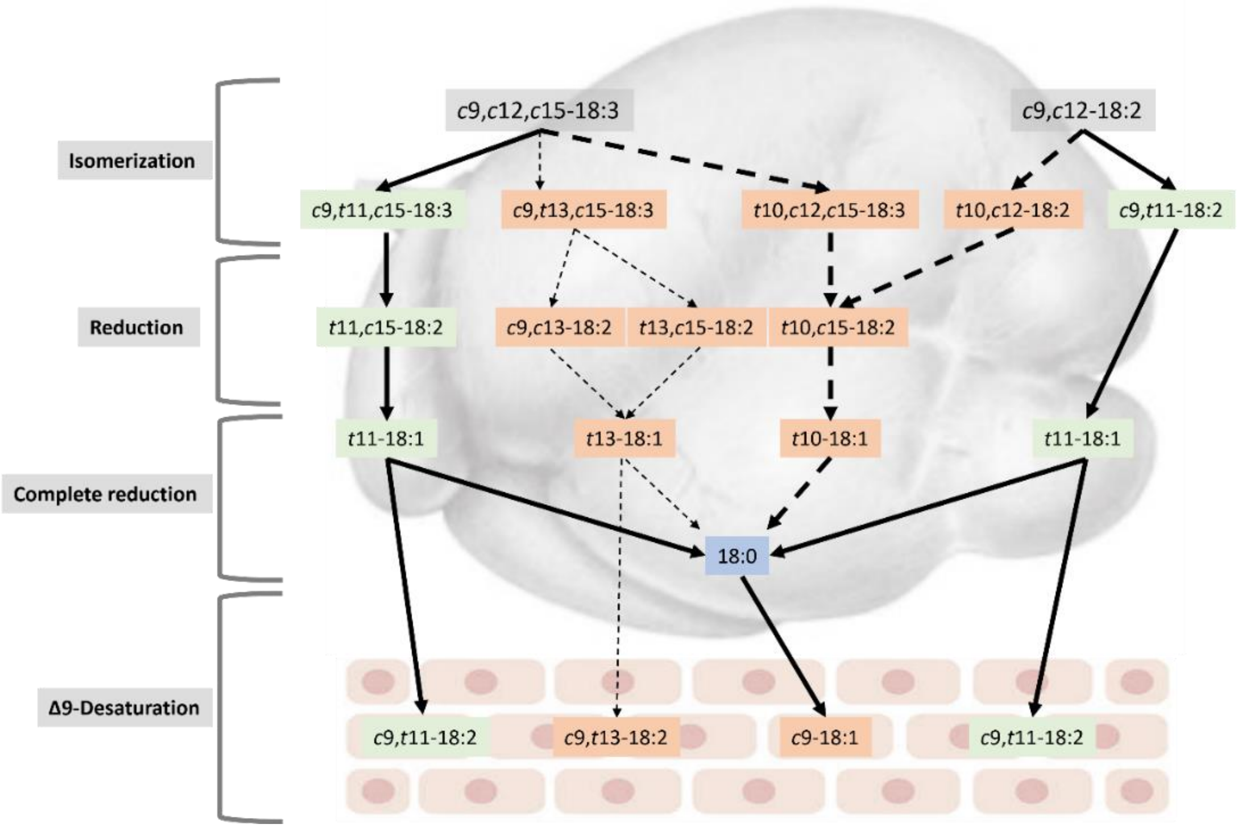

Figure 1.9- Normal and shifted ruminal biohydrogenation pathways of LA and ALA. Isomerization, reduction, and complete reduction steps occur in the rumen, whereas Δ9-desaturation occurs in the tissues. Adapted from Alves et al. (2021).

2.6. *De novo* lipid synthesis and deposition in tissues.

2.6.1. Lipid synthesis.

Three main lipogenic pathways are involved in TAG deposition in ruminants, being them: 1) *de novo* lipogenesis, 2) lipolysis and uptake of circulating FA and 3) esterification of FA and synthesis of TAG (Shingfield et al., 2013).

The handling and use of the FA absorbed from the intestine is done in the intestinal mucosal cells, whereas liver, adipose tissue and mammary gland are responsible for the *de novo* synthesis of FA (Laliotis et al., 2010).

FA are synthesized by an extramitochondrial system (Mayes & Botham, 2003), responsible for the synthesis of palmitate from acetyl-CoA in the cytosol (Laliotis et al., 2010). Contrary to what happens for non-ruminants, glucose is not the main contributor to FA synthesis in the tissues, being it associated to a low activity of adenosine triphosphate (ATP) citrate lyase and malate dehydrogenase enzymes (Laliotis et al., 2010). In the case of ruminants, ruminal-produced acetate and, in a less portion, propionate and butyrate, are the precursors for the initiation of lipogenesis in both adipose tissues and mammary gland (Vernon et al., 2001; Laliotis et al., 2010). After being produced, acetate is transformed into pyruvate and, within the mitochondria, via oxidation, is transformed into acetyl-CoA, which is the main building block of FA (Laliotis et al., 2010).

The contribution of liver is minimal and, instead, FA synthesis is particularly extensive in the adipose tissue (Ingle et al., 1972). Additionally, lipogenesis can take place in the intestinal mucosal cells. Contrarily to adipose tissue, in which the adipocytes use acetate as preferential lipogenic substrate, the intramuscular adipocytes use mostly glucose (Smith et al., 2009). This supports the concept that increasing the metabolic availability of glucose to ruminants seems to be the most effective dietary strategy for promoting intramuscular fat deposition (Pethick et al., 2004).

Synthesis *de novo* involves the activity of acetyl-CoA carboxylase and FA synthase (FAS) and the involvement of glucose-6-phosphate dehydrogenase or malic enzyme (Shingfield et al., 2013). *De novo* FA synthesis yields palmitic acid (16:0) as the final end product, which can serve as a substrate for further elongation or desaturation (Shingfield et al., 2013), taking place in the endoplasmic reticulum via the interaction of many catalytic enzymes (i.e. reductases, desaturases, elongases) (Laliotis et al., 2010).

Equation 1.1 displays the major biosynthetic steps involved in the formation of FA. The biosynthesis of palmitic acid is catalysed by the complex of FAS. The overall reaction can be summarized by the equation:

$$\text{Acetyl-CoA} + 7\text{Malonyl-CoA} + 14\text{NADPH} + 14\text{H}^{+} \rightarrow 16{:}0 + 14\text{NADPH} + 8\text{CoA} + 7\text{CO2}^{+} + 6\text{H}_{2}\text{0} \qquad \textbf{(1.1.)}$$

Equation 1.1- Major biosynthetic steps involved in the formation of FA.

Abbreviations: CoA- coenzyme A; NADPH- nicotinamide-adenine dinucleotide phosphate. Adapted from Laliotis et al. (2010).

It should be noted that in small ruminants, propionyl-CoA can be used in place of acetyl-CoA as the primer molecule for FA synthesis, giving rise to the odd-numbered FA (Vernon, 1981).

Apart from carbon substrate, also nicotinamide-adenine dinucleotide phosphate (NADPH) is required for the reduction of acetyl-CoA to FA (Laliotis et al., 2010). The principal enzymes that are responsible for the NADPH production in ruminants are: i) glucose 6-phosphate dehydrogenase (G6PD), ii) 6-phosphogluconate dehydrogenase (6PGD), iii) cytosolic NADP malate dehydrogenase and iv) cytosolic NADP isocitrate dehydrogenase.

Approximately 30 to 50% of the required NADPH comes from the catalytic action of G6PD and 6PGD (Belk et al., 1993; Smith, 1983). In cow and sheep this percentage can reach the 50-100% and 30-100%, respectively (Vernon, 1981).

Once palmitic acid is synthesized, other medium to LC SFA and MUFA are generated by a desaturation and elongation process. The essential FA LA and ALA are converted to their longer chain homologues by a combination of subsequent reactions in the microsomal fraction of the endoplasmic reticulum (Figure 1.10). Nevertheless, in ruminants as in other mammals, as previously pointed, this process is inefficient and most LC-PUFA in tissues derive from the diet (Urrutia et al., 2020; Ponnampalam et al., 2021).

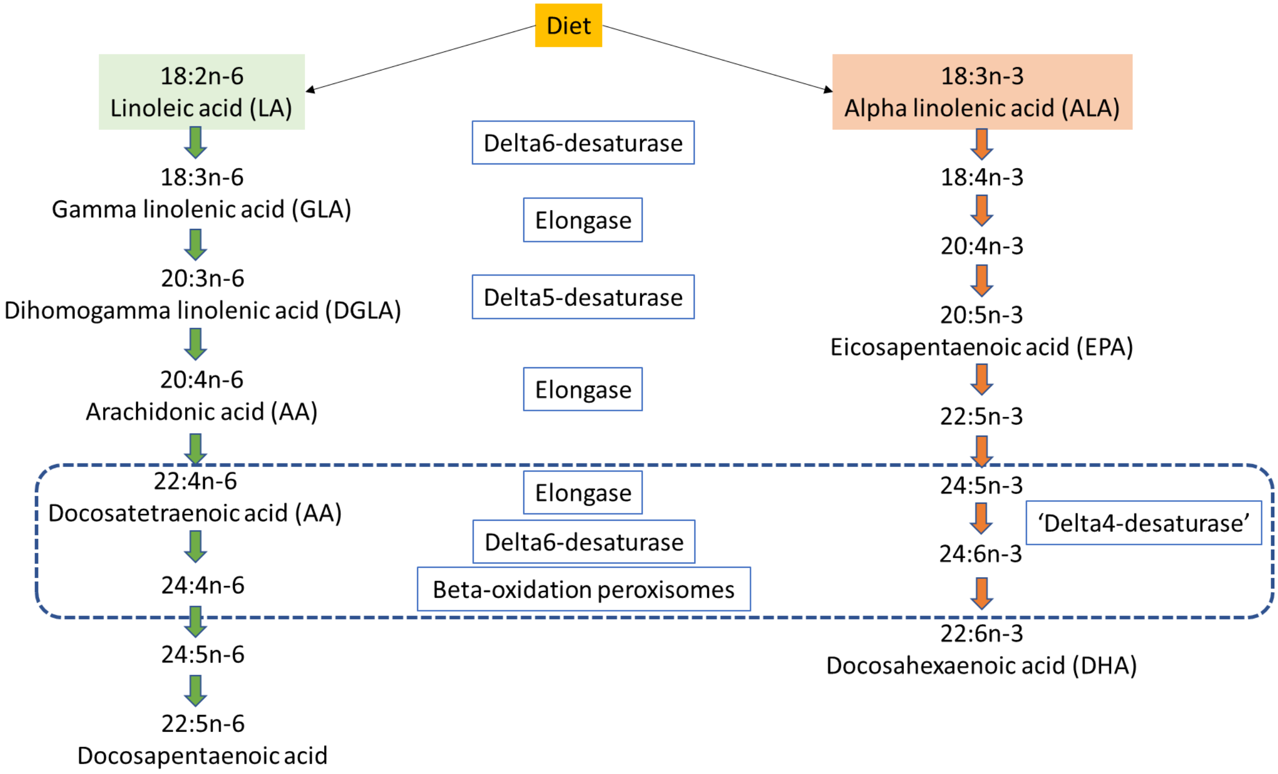

Figure 1.10-Elongation and desaturation of n-6 and n-3 polyunsaturated fatty acids.
Adapted from Simopoulos (2008).

2.6.2. Lipid deposition.

Several steps are involved in resynthesis and transport of lipids in ruminants from the enterocyte where FA are absorbed, until they reach the peripheral tissues such as adipose and muscle tissues. The FA, monoglycerides and diglycerides, reaching the jejunum from micelles, are absorbed into the epithelial cells of small intestine. These FA are esterified, and TAG and

PL are assembled into lipoprotein particles (chylomicrons, VLDL, etc.) in the enterocyte, which are then secreted into lymph vessels and enter the bloodstream (Ponnampalam et al., 2021).

Upon entry to the blood, chylomicrons and VLDL acquire apoproteins apo-C and apo-E provided by HDL. Apo-C inhibits liver removal of chylomicrons and VLDL, and this enhances the extent of diversion of these entities to other tissues. One of the apo-C components activates the lipoprotein lipase enzyme, which is situated primarily on the surface of the endothelium of skeletal muscle, adipose and mammary tissue sites. FA and partial glycerides are apportioned to TAG, PL and other lipids in the organs or oxidized for energy, according to the metabolic demands of the body either in skeletal muscles, adipose and/or mammary tissues (Ponnampalam et al., 2021).

The major lipid class in adipose tissue (>90%) is TAG or neutral lipid. In muscle, a significant proportion is PL, which has a much higher PUFA content in order to perform its function as a constituent of cellular membranes (Wood et al., 2008). When comparing cattle with sheep, it is often seen higher values for individual PL PUFA in the second (Wood et al., 2008).

Ruminants preferentially incorporate essential FA, with their important metabolic roles, into muscle rather than storing them in adipose tissue (Wood et al., 2008). N-3 and n-6 LC-PUFA are mainly found in PL but are detected in sheep muscle neutral lipids and adipose tissue (Cooper, 2002).

3. Protection of FA.

3.1. Lipid protection technologies.

Ruminal biohydrogenation-protection technologies have been developed to overcome the potentially negative effects of RBH, with the aim to increase the amount of UFA and other nutrients bypassing the rumen and resulting in a higher deposition in the peripheral tissues (Gadeyne et al., 2017). Labelling ruminant meat or milk as a "source of" or "rich in" n-3 PUFA can only be attributed if the enrichment is done in a natural way, i.e. through feeding (Gadeyne et al., 2017). Because unprotected n-3 PUFA sources usually fail to reach concentrations defined by the European Food Safety Authority (EFSA, 2009) to permit the labelling, there is a need to develop efficient protection technologies.

For the protection technology to be effective, it needs to confer a high protection in the rumen and a high post ruminal release of the bypass PUFA once it reaches the small intestine, in order to maximize absorption and consequent deposition in the peripheral tissues (Gadeyne et al., 2017). Despite a lot of protection technologies had already been developed (Gadeyne et al., 2017) (Table 1.10), many of these are harmful, non-cost-effective or lack efficiency consistency (Jenkins & Bridges, 2007).

In the work by Kandi et al. (2020), supplementation of ruminal protected calcium salt-linseed oil tended to increase ADG and increased blood glucose, cholesterol, and insulin concentrations in 12-day-old Farahani lambs. An experiment by Titi & Obeidat (2008) was conducted to evaluate the effect of a calcium salt supplement on lactating Awassi ewes and their lambs. Overall, milk production and fat yield increased with calcium salt level in the diet. Also, supplementation with calcium salts increased weaning weight of lambs and milk conversion ratio, as well as blood levels of cholesterol and TAG. Feeding lambs with LA and ALA encapsulated with formaldehyde-treated protein increased the levels of LA (247%) and ALA (57%), respectively relative to those fed unprotected lipids (Elmore et al., 2005). In another study, Noci et al. (2011) fed sodium hydroxide treated seeds (linseed & *Camelina sativa*) to lambs, resulting in greater ruminal protection of PUFA compared to amide-treated and their unprotected oils. In other studies, protection of LA- and ALA-rich diets with calcium salts (Majewska et al., 2016) bentonite and vermiculite clays (Oliveira et al., 2016) had no beneficial effects on FA composition of lamb.

Several commercially available protected fats and aminoacids are mentioned in the literature (Rotunno et al., 1998; Bittante et al., 2014; Pellattiero et al., 2015; Tsiplakou et al., 2017; Liu et al., 2021) but production specifications are often not mentioned, making it difficult to infer and compare the results on their protection efficiency.

More recent technologies to protect lipids and other valuable molecules, using encapsulation techniques have been applied, however, *in vivo* lamb and/or sheep trials are scarce or inexistent. Lipid nanoparticles carrying lysine successfully resisted ruminal digestion for up to 24h in a study by Albuquerque et al. (2020). Carvalho et al. (2019) studied the effect of slow release of urea microencapsulated in beeswax in the diet of adult sheep and verified that there was an improvement of fiber digestibility. Glycerol was encapsulated with sodium alginate and alginate-chitosan polymers, gaining greater protection in acidic pH *in vitro* (Gawad & Fellner, 2019). Flaxseed oil was encapsulated in chitosan nanoparticles and calcium alginate with a resulting reduced hydrogenation of rumen UFA (Besharati et al., 2022). Also, an EPA and DHA encapsulation with alginate resulted in increased *in vitro* intestinal availability of EPA and DHA (Olloqui et al., 2018).

Table 1.10-Protective mechanism and possible disadvantages for the most described or promising rumen lipid bypass technologies – ovine studies.

Principle	Protection technique	Description	Protective mechanism (+)	Disadvantages (-)	Protected product	Main results	Reference
Alteration of the fatty acid structure	**Calcium salts**	The insoluble character of the Ca salts permits an efficient bypass across the rumen. As dissociation constants (pKa) of Ca salts range between 4.5 and 6, salts dissociate again in the acid environment of the abomasum, which makes the FA available for absorption in the small intestine.	Blocking free FA carboxyl end	Protection impaired by dissociation if rumen pH < 6.3; Limited amount of protectable PUFA (at a given rumen pH, dissociation will occur with increasing concentrations of unsaturated FA); Free FA needed (as FA in most oils are present as TAG, hydrolysis is required before protection can be created).	Soya-bean meal	Total FA digestion increased. Nitrogen balance was improved.	(Sklan, 1989)
					Linoleic acid	No beneficial effects	(Fotouhi & Jenkins, 1992)
					Palm FA distillate (Megalac®)	No beneficial effects	(Horton et al., 1992)
					LC-FA (red palm oil)	Improvement of nutrient utilization without affecting the DMI	(Ramana Reddy et al., 2003)
					Ultralac$^{\text{TM}}$ 100-saturated fat	No beneficial effects	(Haddad & Younis, 2004)
					Dry fat	Improvement of milk production, growth rate of lambs, and production of higher quality milk	(Titi & Obeidat, 2008)
					LC-FA	Improvement of nutrients digestibility and increased serum concentrations of cholesterol and HDL	(Obeidat et al., 2012)
					FA (rice bran oil)	Improved body condition	(Bhatt et al., 2013)
					FA (rice bran oil)	improved gain, organic matter intake and digestibility during the post-weaning period	(Bhatt et al., 2015)
					FA (sunflower oil)	No beneficial effects	(Majewska et al., 2016)
					FA (rice bran oil)	Improved nutrient digestibility	(Bhatt & Sahoo, 2017)
					FA (palm oil)	Increased milk production and fat content	(Bianchi et al., 2018)
					EPA + DHA	Lipogenesis decreased in lambs born from EPA + DHA supplemented dams and supplemented with EPA + DHA during the finishing period.	(Coleman et al., 2019)
					FA (linseed oil)	Improved growth performance	(Kandi et al., 2020)
					FA	Improvement of sperm plasma membrane integrity and live sperm.	(Ahmad et al., 2021)
					FA (palm oil)	Decreased concentrations of free cholesterol,	(Behan et al., 2021)

						total cholesterol and esterified cholesterol in muscle	
	Fatty acyl amide	A FA is chemically linked through an amide bound to an amine. The microbial degradation of fatty acyl amides, resulting in a free carboxyl group, seems limited as the bulky fatty acyl and amine moieties provide steric hindrance around the amide bond impairing the access of microbial enzymes.	Blocking free FA carboxyl end	Free FA needed (as FA in most oils are present as TAG, hydrolysis is required before protection can be created).	Butylsoyamide (soybean oil and butylamine)	Increased plasma unsaturated fatty acids	(Jenkins, 1995)
					Hydroxyethylsoyamide (soybean oil and ethanolamine)	Increased plasma unsaturated fatty acids	(Jenkins & Thies, 1997)
Encapsulation in a microbe-resistant shell	**Formaldehyde/ other aldehydes**	Encapsulation within a protein aldehyde reaction product. Prior to aldehyde addition, lipids first need to be emulsified using proteins to ensure a homogeneous distribution of the lipid within the protein and can further be processed using spray-drying to obtain a coated particulate solid. The cross-linked protein matrix protects the lipid from degradation in the rumen but can be degraded in the abomasum.	Encapsulation within formaldehyde-protein matrix	Formaldehyde=toxic (possible residues in the final animal product); Untargeted reaction (hard to prevent formaldehyde from reacting with other nutrients); Expensive.	Safflower oil	Increased tissual linoleic acid. Higher plasma levels of urea, α-amino nitrogen, and glucose.	(Faichney et al., 1973)
					Linseed oil Coconut oil	Tendency for an increase in the efficiency of microbial protein synthesis with protected linseed oil.	(Sutton et al., 1983)
					Oil rich in 18:2n-6 and 18:3n-3	Higher levels of 18:2n-6 and 18:3n-3 in the meat.	(Elmore et al., 2005)
					Oil rich in EPA and DHA	Elevated milk n-3 LC-PUFA	(Nguyen et al., 2018b)
					Full-fat soybean	Increased depositions of 18:2n-6 and 18:3n-3 in muscle and adipose tissue.	(Lee et al., 2004)

					Sunflower oil gel	Higher PUFA in muscle and adipose tissue.	(Lee et al., 2007)
	Non-enzymatic browning	Encapsulation in protein capsules by cross-linking the proteins with reducing sugars. First, an aqueous emulsion of oil in a solution of protein and reducing sugars is made, second, emulsions are freeze dried to yield a dry powder, and third, the powder is browned in an oven to produce rumen protected granules.	Encapsulation within sugar-protein matrix	Potentially toxic Maillard compounds; Expensive (sugars and heating); Oxidation of PUFA during the heating step.			
	Lipid composite gels	Composite gels containing amino acids and lipids. The formation of composite gels, that is, the combination of gelled protein and lipid, basically consists of two steps: First, an emulsion is formed by mixing lipids with a high shear force in a matrix of dissolved and/or suspended proteins, and second, emulsions are heated at a temperature of 80–125°C to produce a composite gel.	Encapsulation within gelled-protein matrix	Contains large volumes of water (low shelf-life).			
	Encapsulation within lipid	Active compounds are protected in a microcapsule of lipids, formulated according to either one of the two basic concepts: Active compounds are either embedded in a lipid matrix or are formulated	Encapsulation within high melting point lipid matrix	Low payloads and low post-ruminal release (generally inversely related to the degree of rumen protection).	CLA	Higher CLA cis-9, trans-11 and trans-10, cis-12 intestinal flow. CLA isomers increased in the muscle.	(Wynn et al., 2006)
					FA	Enhanced EE and CF digestibility	(Behan et al., 2019)
					Acacia Tannin Extract	Reduced methane production and higher NDF digestibility	(Adejoro & Hassen, 2019)

		in small spheres, which then are coated with lipid. Generally, coatings are comprised of FA with a high melting point.			FA (palm oil)	Higher semen volume, improved plasma membrane integrity and live sperm.	(Ahmad et al., 2021)
					FA	Increased meat unsaturated fatty acids and decreased saturated fats	(Behan et al., 2021)
					Methionine (BY-MET^{TM})	No beneficial effects	(Baldwin et al., 1993)
	Other encapsulation techniques	Polymeric microspheres. Biocompatible polymer biodegradable by passage through the abomasum and intestine, but resistant to degradation in the rumen. Polymers are first dissolved in organic solvent together with unesterified FA and emulsified with a surfactant, resulting in the creation of microspheres.	Encapsulation within polymeric microspheres.	Low post-ruminal release	Betaine	Increased ADG	(Dong et al., 2020) (Yu et al., 2020)
		Polymeric coating. The functional ingredient is coated with multiple layers: First, an inner coating of enteric material, and second, an outer coating consisting of a delayed-release material.	Encapsulation within polymeric coating. Oil; Encapsulation-induced cross-linking	Low post-ruminal release			
		Hard gel beads. Oil beads are formed by injection of the emulsion in a solution containing a cross-linking agent to obtain hard gel beads.	Encapsulation within hard gel beads.	Low post-ruminal release	Urea	Increased NDF and ADF digestibility.	(Carvalho et al., 2019).

		Nano-encapsulation. Nano capsules are created by passing a pre-emulsion of targeted FA in water with lecithin and glycerol as emulsifier through a high-pressure homogenizer; organic solvent-free emulsification-sonication method	Encapsulation within nano capsules.				

Abbreviations: CLA – conjugated linoleic acid; DHA – docosahexaenoic acid; EPA – eicosapentaenoic acid; FA – fatty acids; LC-FA – long-chain FA. Adapted from Gadeyne et al. (2017).

3.2. Natural protection – microalgae cell wall.

The remarkably thick and resistant cell wall structure of certain species of microalgae (Figure 1.11) can be considered as a natural source of protected lipids. Frequently, high energy inputs or large quantities of chemicals are required to extract the compounds from within the cell due to recalcitrance, complexity, and diversity of microalgal cell walls (Alhattab et al., 2019). The composition is highly variable but generally contains carbohydrates, proteins, and lipids and all contribute to protect the algal cells from adverse environmental conditions (Scholz et al., 2014).

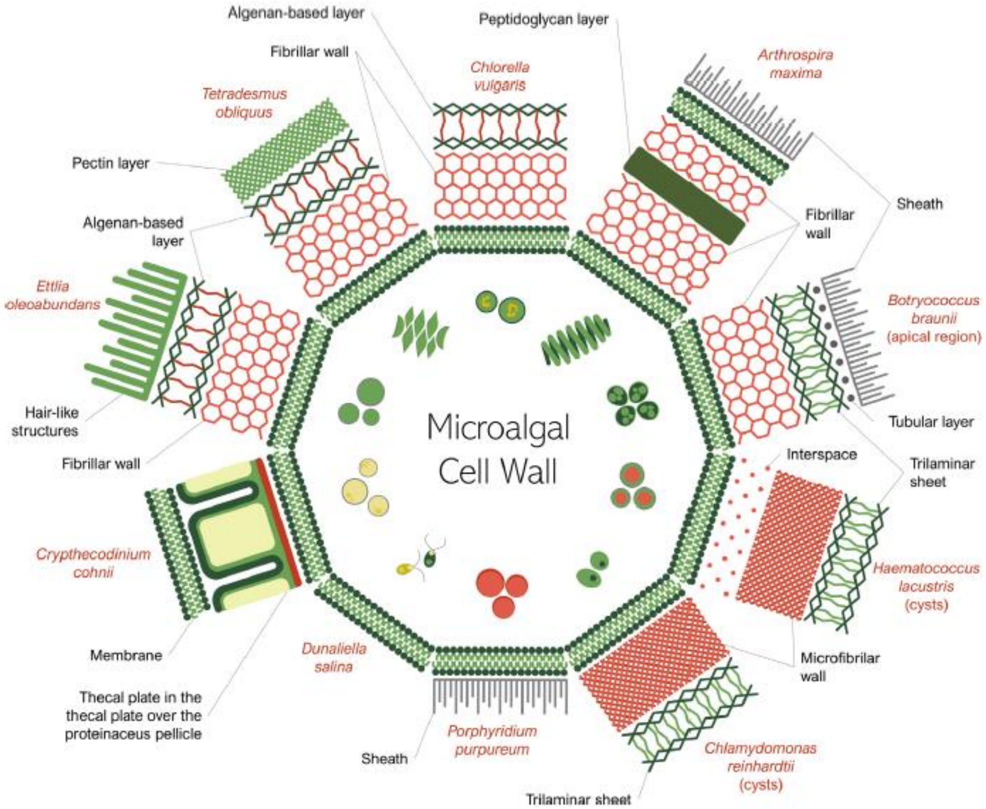

Figure 1.11-Cell wall structure of 10 important microalgae, illustrating the main components and the diversity of structure.
Layers are not to scale – the cell wall is 10 to 100 times thicker than the cell membrane. From de De Carvalho et al. (2020).

Between other, *Nannochloropsis* is among the genera possessing strong resistant cell walls (Scholz et al., 2014) and because these microalgae are naturally rich in n-3 LC-PUFA (Ma et al., 2016), they constitute potential candidates to be considered a natural source of protected lipids to ruminants.

The cell wall thickness in *Nannochloropsis* genus can vary from 63 to 119 nm depending on the specie and strain (Beacham et al., 2014) and among the most studied polymers of the algal cell wall are polysaccharides, including cellulose, chitin-/chitosan-like molecules, hemicelluloses, pectins, fucans, alginates, ulvans, carrageenans, and lichenins (Scholz et al., 2014). Brown (1991) reported that the polysaccharides of *Nannochloropsis oculata* contained ~68% glucose along with about 4 to 8% each rhamnose, mannose, ribose, xylose, fucose, and galactose. Later, Vieler et al. (2012) found that *Nannochloropsis oceanica* carbohydrates were 90% glucose, ~3% mannose, and the rest traces of rhamnose, fucose, arabinose, xylose, and galactose.

Previous studies shown that, as part of the outer cell wall of some microalgae, there is a type of aliphatic insoluble and chemically resistant biopolymer material termed algaenan (Tegelaar et al., 1989), that can be responsible for cell wall hardness (Zhang et al., 2019a). In *Nannochloropsis*, algaenans occur in the bilayer cell-wall, forming an algaenan-containing rigid trilaminar sheath (TLS), a narrow dark-light-dark domain at the wall periphery (Allard & Templier, 2000; Allard et al., 2002), as the external layer (Figure 1.12)(Arnold et al., 2015).

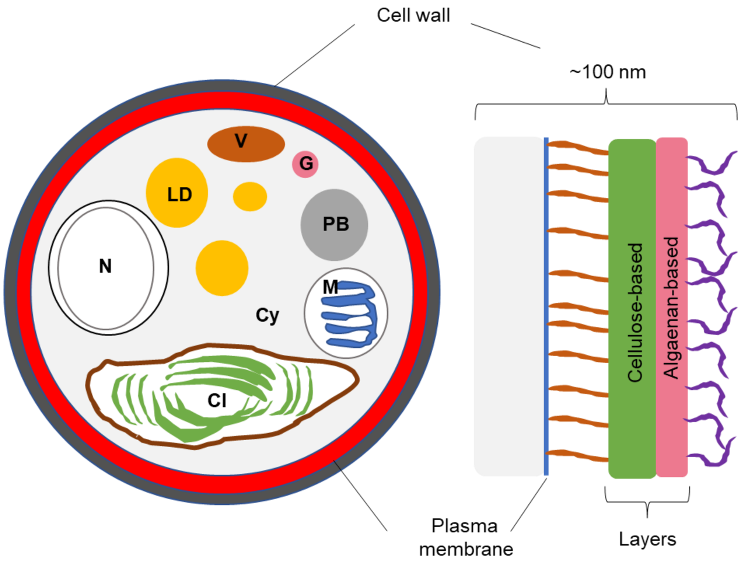

Figure 1.12- Schematic presentation of the structure of *Nannochloropsis gaditana* cell.
In the figure, (N): nucleus; (Cl): chloroplast; (Cy): cytoplasm; (LD): lipid droplet; (V): vacuole; (G): Golgi body; (PB): Pyrenoid-like body; (M): Mitochondrion. Adapted from Zhang et al. (2019a).

Algaenans are not only highly resistant to alkali/acid hydrolysis and aqueous/organic solubilization (Allard et al., 2002; Scholz et al., 2014), but also to many digestive enzymes and have been reported to help microalgae against microorganisms attack (Dunker & Wilhelm, 2018). This biopolymer is of great importance either due to its geochemical properties or economical value as a precursor of petroleum formation in the aquatic environment (Simpson et al., 2003). Algaenans are included in the selective preservation hypothesis for the kerogen formation (Biller et al., 2015) and are responsible for the microalgae wall's recalcitrance to breakage (Scholz et al., 2014). *Nannochloropsis* algaenan comprises long-chain aliphatic hydrocarbons that are subject to ether cross-linking reactions (Gelin et al., 1997b) (Figure 1.13). They are similar to cutan, a biopolymer that occurs in drought-resistant land plants, and contains long-chain (~C30) alkanes and alkenes joined by ether linkages (Boom et al., 2005). The elongation or condensation of a C18 FA with a similar FA molecule may produce C28~C34 algaenan constituents, which may be catalysed by the action of polyketide synthase (Jeong et al., 2017). Previous studies have unrevealed some parts of the structural information for algaenans using nuclear magnetic resonance (NMR) (Bertheas et al., 1998), size exclusion chromatography (Bertheas et al., 1998), tetramethylammonium hydroxide thermochemolysis (Allard and Templier, 2000; Zang et al., 2001) and pyrolysis techniques (Simpson et al., 2003). However, the biosynthetic pathway for algaenan production has not been elucidated (Jeong et al., 2017). Algaenans can be extracted, and it has been documented that upon drying, they become insoluble in organic solvents. Along with their extraction, other specimens can co-precipitate (Simpson et al., 2003) namely the so-called "botryals". These are not removed by pre-extraction with hexane and can be redissolved in chloroform. Two-dimensional NMR data supports the concept that the hexane insoluble botryals and the algaenan are similar in composition (Simpson et al., 2003).

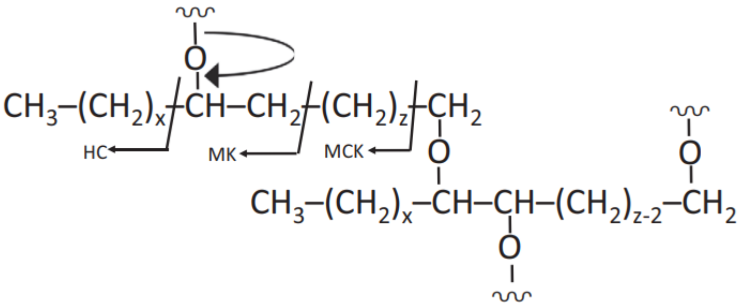

Figure 1.13- Proposed origins of the major products from pyrolysis of the structural units of algaenan.
Structural units of algaenan proposed by Gelin et al. (1997b). From Zhang & Volkman (2017).

Traditional algaenan isolation has depended on severe hydrolysis reactions at high temperature to degrade any reactive polysaccharides, lipids, and proteins (Gelin et al., 1997b;

Allard et al., 1998; Blokker et al., 1998), however, more recent approaches using sodium hydroxide at milder temperatures have been proposed as good alternatives (Obeid et al., 2015). Also, 24h digestion of pressed cell walls from *N. gaditana* at room temperature, first with cellulase and then protease, can leave preparations with algaenan-containing outer cells walls. This enzymatic approaches may allow the analysis of highly enriched algaenan preparations without exposure of the polymer to high temperature or strongly oxidizing/reducing conditions (Scholz et al., 2014).

4. Research objectives.

Dietary n-3 PUFA fed to ruminants are extensively hydrogenated through ruminal biohydrogenation, resulting in a very low efficiency of its transfer to tissues. The development or identification of novel strategies to protect PUFA from biohydrogenation, in order to increase the amount of PUFA bypassing the rumen, could possibly circumvent the problem. Preliminary results, based on *in vitro* batch incubations (Alves et al., 2018), showed that an EPA-rich microalgae could be a promising approach for PUFA bypassing the rumen, however much more research is needed. Thus, this PhD project aims to research effective strategies to protect PUFA from the rumen biohydrogenation by using naturally PUFA-protected sources such as microalgae biomass, and to develop novel PUFA-protection methods and evaluate their impact on rumen microbiome. The final goal is to enrich ruminant derived foods with health-promoting FA. To achieve this goal, two strategies will be investigated:

1. Elucidation if *N. oceanica* biomass can be a natural source of dietary rumen protected PUFA, increasing their availability and deposition in lamb tissues:

We will identify which microalgae dehydration method causes less alterations in the cell wall structure and integrity and evaluate cell integrity maintenance effects on EPA protection against rumen biohydrogenation *in vivo*. We will also evaluate if EPA from *Nannochloropsis* biomass is efficiently transferred to lamb tissues or if it promotes the formation of *trans*-FA by affecting the ruminal biohydrogenation of C18 PUFA in the rumen. The impact of this approach on rumen microbiome will also be assessed.

2. Alternative rumen lipid-bypass approaches based on algaenans.

We aim to develop a method to protect PUFA from the microbial RBH. The strategy involves the extraction of *N. oceanica* algaenan, its chemical characterization and its application in the development of a novel biopolymer-based technology for protecting lipids in the rumen.

2. CHAPTER 2 | Freeze-dried *Nannochloropsis oceanica* biomass protects eicosapentaenoic acid (EPA) from metabolization in the rumen of lambs.

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Abstract.

Eicosapentaenoic acid (EPA) from freeze-dried biomass of *Nannochloropsis oceanica* microalgae resists ruminal biohydrogenation *in vitro*, but *in vivo* demonstration is needed. Therefore, the present study was designed to test the rumen protective effects of *N. oceanica* in lambs. Twenty-eight lambs were assigned to one of four diets: Control (C); and C-based diets supplemented with: 1.2% *Nannochloropsis* sp. oil (O); 12.3% spray-dried *N. oceanica* (SD); or 9.2% *N. oceanica* (FD), to achieve 3 g EPA /kg dry matter. Lambs were slaughtered after 3 weeks, and digestive contents and ruminal wall samples were collected. EPA concentration in the rumen of lambs fed FD was about 50% higher than lambs fed SD or O diets. Nevertheless, the high levels of EPA in cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine. Furthermore, supplementation with EPA sources also affected the ruminal biohydrogenation of C18 fatty acids, mitigating the shift from the *t*10 biohydrogenation pathways to the *t*11 pathways compared to the Control diet. Overall, our results demonstrate that FD *N. oceanica* biomass is a natural rumen-protected source of EPA to ruminants.

2.1. Introduction.

The health benefits associated with the consumption of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are well known (Shahidi & Ambigaipalan, 2018; Zhang et al., 2019b). However, in ruminant edible fats their content is very low even when animals are supplemented with enriched n-3 LC-PUFA diets. The explanation for this finding relies on the ruminal microbiota intervention over the dietary lipids. Indeed, rumen microbes have the capacity to hydrolyse lipids and subsequently biohydrogenate the unsaturated fatty acids (FA). The biohydrogenation involves isomerization and hydrogenation of FA double bonds, forming a wide range of FA intermediates and saturated FA as end products, which will further alter the FA profile of ruminant edible products (Doreau et al., 2016).

Strategies to enhance the content of n-3 LC-PUFA in ruminant edible fat include the dietary supplementation with products derived from the marine food chain, as fish oil or microalgae, which are naturally enriched in EPA and DHA (Alvarenga et al., 2015; Scollan et al., 2017). However, the efficiency is rather low as it is well established that both EPA and DHA undergo extensive biohydrogenation in the rumen (Alvarenga et al., 2015; Doreau et al., 2016; Scollan et al., 2017). Thus, rumen-protected marine-derived supplements could be the most effective way to increase the concentration of EPA and DHA in ruminant derived foods.

Indeed, several rumen protection technologies have been proposed but often with low efficacy and with application difficulties (Gadeyne et al., 2017). Furthermore, the bypass must allow post ruminal release once it reaches the small intestine. Calcium salts have a disadvantage related to dissociation in lower pH, the limited amount of protectable polyunsaturated FA (PUFA) and the need of free FA to create an ionic bond between the free carboxyl group of the FA and Ca ions. Formaldehyde is a carcinogenic compound; the reaction is untargeted, and it is an expensive resource. Other methods such as fatty acyl amides, non-enzymatic browning, lipid composite gels, encapsulation within lipids and protein crosslinking, all have several disadvantages as many of these technologies use harmful products, are not cost-effective, or are lacking consistency regarding rumen protection efficiency (Gadeyne et al., 2017). Thus, the identification of PUFA sources naturally protected from rumen metabolism is highly relevant and promising.

Consequently, using *in vitro* batch incubations with rumen inoculum, our team was able to identify that biomass of *Nannochloropsis oceanica* microalgae was a partially protected source of EPA (Alves et al., 2018). The protection against rumen biohydrogenation was moderate with spray-dried (SD) biomass and exceptionally high when freeze-dried (FD) biomass was used. Microalgae are usually included in diet formulations after dehydration of the slurry biomass using industrial-scale SD technology. Dehydration by freeze-drying seems better in preserving the structural components and nutritional properties than SD (Lin, 1985; Zanella & Vianello, 2020). Thus, the higher rumen-protection of EPA observed *in vitro* with FD biomass was probably due to better preservation of *N. oceanica* cell walls than with SD biomass. However, the better preservation of cell walls might limit the *post*-ruminal release and availability for the EPA's absorption.

The objective of the present study was to evaluate FD *N. oceanica* as a natural rumen-protected source of EPA *in vivo*. We hypothesized that lambs fed a diet containing FD *N. oceanica* biomass would present a higher content of EPA through the main gastrointestinal tract compartments, comparing to those fed diets with SD biomass, or with *Nannochloropsis* sp. Oil (O).

2.2. Materials and methods.

2.2.1. Animals, diets, and management.

The experimentation involving live animals was conducted under strict compliance with international guidelines (Directive 2010/63/EU) regulating the use of production animals in animal experimentation, at the INIAV-Santarém facilities. The INIAV-Santarém facilities are certified by the competent veterinary authority (DGAV) to conduct animal experimentation (Ref: 04211000/000/2013). The experimental animal procedures were approved by the Ethical and Animal Well-Being Commission (CEBEA) of the Faculty of Veterinary Medicine, University of Lisbon, Portugal (Protocol FMV/CEBEA 007/2016). Animal management, handling, transport, and sacrifice were conducted replicating approved standard commercial practices regarding animal welfare, except that animals were individually housed. The study was also carried out in compliance with the ARRIVE guidelines.

Twenty-eight Merino Branco ram lambs were reared with dams on extensive grazing until weaning at approximately 60 days of age. After that, lambs were transported to INIAV—Santarém facilities, and randomly allocated to individual pens (1.52 m^2) with wood shaving beds, and with free access to clean water. Animals were allocated to one of 4 groups of 7 lambs each, and randomly allocated to diets, following a completely randomized design. Lamb's initial live weight averaged 21.8 ± 4.4 kg.

The C diet consisted of pellets containing dehydrated lucerne, barley and soybean meal (Table 2.1) and no added sources of EPA. The other diets maintained the same ingredients and proportion of C diet plus: SD diet—123 g/kg of spray-dried *N. oceanica* biomass; FD diet—92 g/kg freeze-dried *N. oceanica* biomass; O diet—12 g/kg of *Nannochloropsis* sp. free oil. The amounts of microalgae biomass or oil added to diets was determined to supply identical quantities of EPA (≈ 3 g/kg DM). The analysis of the final diets demonstrated that FD diet contained less EPA than the SD diet (Table 2.1), probably due to losses during handling, feed production, or differences among slurry batches. Diets containing *Nannochloropsis* were supplemented with 1.67 g/kg DM tocopheryl acetate (3a700 Vitamin E, 500 mg/g). Control diet was not supplemented because the premix already contained vitamin E in levels close to the NRC (NCR, 1985) requirements for growing-finishing lambs of 20–30 kg of body weight. Diets were not formulated to be isoproteic or isocaloric, but crude protein ranged from 191 to 211 g/kg DM.

The *N. oceanica* biomasses were produced at Allmicroalgae industrial plant located in Pataias, Portugal. Cultures were autotrophically grown in Guillard's F2 medium as previously described (Meehan et al., 2021). Around three months after inoculation, the microalgal biomass was harvested from the photobioreactors, concentrated in a membrane system, subjected to a short-term high temperature treatment, and dried in an industrial spray dryer to obtain the SD *N. oceanica*. The conditions were as follow: outlet temperature 85–90°C and inlet temperature 200–210°C. Frozen batches of slurry *N. oceanica* biomass were freeze-dried using a Scanvac Cool-safe Superior Touch freeze dryer (Scanvac, Denmark) with the following conditions: temperature, − 92°C; initial pressure 0.2 mbar; final pressure 0.07 mbar. The *Nannochloropsis* sp. oil was purchased from Qualitas Health (Houston, TX, USA). Feed ingredients were mixed and pelleted (3 mm diameter) at Instituto Superior de Agronomia, Universidade de Lisboa experimental feed mill.

During the adaptation period of 8 days, lambs were dewormed against gastrointestinal and pulmonary nematodes by dosing with Sinvermin (Lapsa—Portuguesa Pecuária Lda., Portugal) and coccidiosis by dosing with Vecoxan (Elanco GmbH—Germany) and vaccinated against enterotoxaemia with Miloxan (Merial Labs., Spain). After that, they went through a transition period of 6 days where they were given 1/3 of the pelleted experimental diet plus 2/3 of the basal ground feed ration, on the 1st and 2nd days and then the proportion of the experimental diet was regularly increased for the remaining days, until reaching 3/3. The experimental period started on the 14th day post arrival when the diet was exclusively composed by the experimental pellets and lasted for 3 weeks. During the first two weeks of the experiment, 1.2 kg of feed was offered once a day (0900 am) and thereafter animals were fed *ad libitum*. Feed intake was measured during the entire experiment and averaged 1.19 ± 0.13 kg of DM/day.

Table 2.1- Ingredients, chemical composition, and fatty acid profile of the experimental diets.

Item	Diets[1]			
	C	**O**	**SD**	**FD**
Ingredients (g/ kg DM)				
Barley	390	385	342	353
Soybean meal	170	168	149	154
Dehydrated alfalfa	400	395	351	363
Freeze dried *N. oceanica*	-	-	-	92
Spray dried *N. oceanica*	-	-	123	-
Nannochloropsis sp. oil	-	12	-	-
Calcium carbonate	13	13	11	12
Sodium bicarbonate	20	20	18	18
Marine salt	4	4	4	4
Premix[2]	3	3	3	3
Vitamin E	-	1.67	1.67	1.67
Chemical composition (g/kg DM)				
DM	904	907	902	907
Crude Protein	191	183	206	211
Ether Extract	13.3	32.5	35.2	33.9
NDF	267	267	232	290
ADF	173	174	150	167
ADL	30.3	28.1	26.8	31.2
Sugar	73.2	69.4	65.5	67.9
Crude energy (kJ/ 100g)	-	-	-	-
Ash	84.6	89.9	114.1	90.8
Total fatty acids (g/ kg DM)	13.7	20.6	19.5	20.2
FA profile (g/ kg DM)				
14:0	n.d.	0.26	0.47	0.46
16:0	3.49	4.74	4.66	5.27
*c*9-16:1	0.07	1.06	1.99	1.66
17:0	0.09	0.07	n.d.	n.d.
18:0	0.56	0.61	0.49	0.67
*c*9-18:1	2.55	2.99	2.30	2.77
*c*11-18:1	0.10	0.19	0.14	0.14
18:2n-6	5.62	6.49	5.50	5.88
18:3n-3	1.22	1.40	0.98	1.30
20:4n-6	n.d.	0.53	0.72	0.64
EPA	n.d.	2.14	2.20	1.39
22:0	n.d.	0.12	0.07	n.d.

[1] Diets (or treatments): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. [2] Premix composition: Vitamin A, 4,000,000 UI; Vitamin D3, 1,100,000 UI; Vitamin E, 7500 mg/kg; Vitamin B1, 250 mg/kg; Vitamin B2 250 mg/ kg; Zinc, 35,000 mg/kg; Iron, 12,500 mg/kg; Manganese, 17,500 mg/kg; Iodine, 200 mg/kg; Cobalt 250 mg/kg; Selenium, 100 mg/kg; Magnesium oxide (excipient) 40,000 mg/kg. n.d. – not detected.

2.2.2. Slaughter procedures and sampling.

At the end of the trial period, lambs were weighed at 0830 am at the housing facilities, without previous fasting, and immediately transported (circa 1 km) to the experimental abattoir of the INIAV—Santarém. Immediately after the slaughter, the whole rumen, abomasum, and lower intestine (cecum) digestive contents were collected from each lamb and frozen at − 80°C.

After that, they were freeze-dried, milled, and re-stored at −80°C until FA analysis. Faeces were collected directly from the rectum, frozen at −80°C, freeze-dried, milled and re-stored at −80°C. Samples of the ruminal wall (5 × 5 cm) were collected after washing the ruminal mucosa with tap water and fixed in 10% buffered formalin for histological examination. Ruminal content was straightened through 4 layers of cheesecloth and pH was measured in strained rumen fluid using a pH meter (Digital pH meter "pH-2005"; JP Selecta S.A, Barcelona, Spain). An aliquot of strained rumen fluid was immediately stored at −20 °C for volatile fatty acids (VFA) analyses and another aliquot preserved with 2 mL of 10% formalin solution and stored at 2°C until microscopic examination for rumen ciliate protozoa characterization.

2.2.3. Rumen mucosa evaluation.

Immediately after evisceration, the rumen was opened, emptied, and washed with tap water. Digital pictures (image size 16 mp) of macroscopic representative selected areas of the ruminal wall were taken with a Nikon D5100 Digital SLR camera (Nikon Europe BV, Badhoevedorp, The Netherlands) under constant lightning and photographic conditions. After, a 5 × 5 square from the ventral sac mucosa was collected for histopathological evaluation of parakeratotic lesions. Digital pictures were subjected to computational colorimetry tests on the mucosa colour (greyscale values) as an indicator of the degree of keratinization (Benavides et al., 2013), using image processing with a developed Python 3.8 script.

Samples from the ventral sac were fixed by immersion in 10% buffered formalin for at least 24 h. After fixation, samples were processed for paraffin embedding. Sections (3 μm thick) from each fragment were stained with haematoxylin and eosin for routine microscopic examination. From the 2 fragments corresponding to the ruminal wall of one animal, the 5 better preserved papillae were selected for histometry analysis, which was performed using a BX 511 microscope (Olympus, Tokyo, Japan) and the images were digitally captured using a DP 11 camera (Olympus, Tokyo, Japan) under a magnification of 20X. The measurements were made using DP-Soft (Olympus) and ImageJ 1.43 software (ImageJ, Health National Institute of Mental, Bethesda).

2.2.4. Rumen protozoa counting.

Protozoal densities were obtained individually by microscopic counting as previously described (Francisco et al., 2019b). Ciliate cell numbers were determined in duplicate for each sample and the identification at the genus level was made based on protozoa morphology, according to others (Ogimoto & Imai, 1981).

2.2.5. Chemical analysis.

Chemical analysis of diets was obtained as the average of the results of two pooled samples of each diet and analysed as previously described (Santos-Silva et al., 2016). Acid-insoluble ash of feed and faeces, mainly consisting in silica, was determined gravimetrically after drying, ashing, boiling of ash in hydrochloric acid (HCl), filtering and washing of the hot hydrolysate, and re-ashing (NP 2971, 1992). Ruminal volatile FA (VFA) were determined by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a Nukol (30 m × 0.25 mm, 0.20 μm film thickness, Supelco, Bellefonte, PA, USA) capillary column and quantification was made using calibration curves according to others (Oliveira et al., 2016).

Fatty acid methyl esters (FAME) of feed samples were prepared according to Sukhija and Palmquist (Sukhija & Palmquist, 1988). Freeze-dried rumen, abomasum and lower intestine contents, and faeces samples were prepared by direct transesterification by reaction with sodium methoxide (0.5 M) in methanol at 50°C for 15 min followed by addition of hydrogen chloride (1.25 M) in methanol at 80°C for 20 min. Methyl nonadecanoate (1 mg/mL) was added as internal standard. FAME and DMA were analysed by GC-FID using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with an SP-2560 (100 m × 0.25 mm, 0.20 μm film thickness, Supelco, Bellefonte, PA, USA) capillary column. The chromatographic conditions were as follow: injector and detector temperatures were set at 250°C and 280°C, respectively; helium was used as the carrier gas at 1 mL/min at a constant flow; the initial oven temperature of 50°C was held for 1 min, increased at 50°C/min to 150°C and held for 20 min, increased at 1 °C/min to 190°C and then increased at 2°C/min to 220°C and held for 40 min. Identification of FAME and DMA was achieved by comparison of fatty acid retention times with those of commercial standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA) and with published chromatograms (Alves et al., 2013). Additional confirmation of FAME and DMA was achieved by electron impact mass spectrometry using a Shimadzu GC–MS QP2010 Plus (Shimadzu, Kyoto, Japan). The chromatographic column and the GC conditions were like the ones in the GC-FID analysis.

2.2.6. Scanning electron microscopy (SEM) of *N. oceanica* biomass.

Spray-dried and FD microalgae samples were mounted on aluminium stubs with carbon tape and coated with an 8 nm thick palladium-gold film in a Quorum Q150T ES sputtering system. *N. oceanica* surface morphology was observed in a Carl Zeiss AURIGA Crossbeam SEM–FIB workstation, using an accelerating voltage of 5 keV with an aperture size of 30 microns.

2.2.7. Calculations and statistical analysis.

The biohydrogenation estimates (disappearance, %) in the rumen for linoleic acid (LA; 18:2n-6), linolenic acid (ALA; 18:3n-3), arachidonic acid (AA; 20:4n-6) and EPA, were obtained using the diminishing abundance of these FA, proportional to the sum of C18 FA or of C20 FA, between diet and rumen, assuming that no losses of FA occur in the gastric compartments as shown below in the Equation 2.1:

$$\text{Biohydrogenation UFA }(\%) = \frac{(|\text{UFA}-\text{D}|-|UFA-R|)}{|\text{UFA}-\text{D}|} * 100 \qquad \textbf{(2.1.)}$$

Equation 2.1 – Biohydrogenation UFA (%).

Where [UFA-D] and [UFA-R] are the proportions of each dietary unsaturated FA (UFA) expressed as % of total C18 FA or as % of total C20 FA, respectively for C18 UFA or C20 UFA. C18 biohydrogenation completeness (%) was estimated considering the maximum 18:0 in the rumen and abomasal digesta, assuming a complete biohydrogenation of the C18 FA from the diet (Alves et al., 2017). The calculations exemplified for rumen are presented in the Equation 2.2:

$$\text{C18 biohydrogenation completeness }(\%) = \frac{|SA-R|}{|Max\text{SA}-\text{R}|} * 100 \qquad \textbf{(2.2)}$$

Equation 2.2 - C18 biohydrogenation completeness (%).

The [SA-R] is the proportion of 18:0 in the rumen digesta and the [Max SA-R] is the maximum 18:0 proportion in the rumen, both expressed as % of total C18 FA, assuming that 100% of dietary unsaturated C18 FA biohydrogenated is converted to 18:0 and computed as shown in the Equation 2.3.

$$[Max\ SA-R]=([AO-D])-([AO-R])+([LA-D]-[LA-R])+([ALA-D]-[ALA-R])+[SA-D] \quad \textbf{(2.3)}$$

Equation 2.3 - Max SA-R.

Where: [SA-R], [AO-R], [LA-R], [ALA-R] are respectively the proportions of 18:0, *c*9-18:1, 18:2n-6 and 18:3n-3 in the rumen, and [SA-D], [AO-D], [LA-D], [ALA-D] the proportion of the same FA in the diets, expressed as % of total C18 FA. The whole tract apparent digestibility (WTAD) of EPA, which included both biohydrogenation and post ruminal digestion, was calculated using silica as the internal digestibility marker using marker and nutrients concentration ratios as described in Equation 2.4:

$$\text{WTAD (\%)} = 100-(100*(\frac{\text{[Marker]diet}}{\text{[Marker]faeces}}/\frac{[Nutrient]faeces}{[Nutrient]diet})) \quad \textbf{(2.4)}$$

Equation 2.4 - WTAD (%).

Post ruminal digestibility (PRD) of EPA was estimated using both WTAD and biohydrogenation values, by computing the balance between the proportion of EPA escaping rumen metabolization (EPA_{RE}) and the proportion of EPA escaping the digestive tract as faecal excretion (EPA_{FE}). PRD calculations are presented in the Equation 2.5:

$$\text{PRD (\%)} = \frac{(\text{EPA}_{\text{RE}})-(EPA_{FE})}{\text{EPA}_{\text{RE}}}*100 \quad \textbf{(2.5)}$$

Equation 2.5 - PRD (%).

Where: EPA_{RE} (%) = 100-% EPA biohydrogenation, and EPA_{FE} (%) = 100-% EPA WTAD.

Chemical analysis (FAME and DMA), histometric data, ruminal pH and ruminal mucosa greyscale evaluation and FA data were analysed as a completely randomized experimental design using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC), using diet as a fixed factor and the animal as the experimental unit. When needed, the group option of the repeated statement was included in the model to accommodate the variance heterogeneity. Least square means (LSM) and standard error of the mean (SEM) are reported, and main effects and their interactions were considered significant at $p < 0.05$ and trends toward significance at $0.05 < p < 0.10$. Due to an experimental incident the night before the slaughter, two animals receiving C diets accidentally had access to diets containing *Nannochloropsis* and consequently, those two animals were removed from the analysis.

2.3. Results.

2.3.1. Abundance of EPA in digestive tract and whole tract apparent digestibility.

The proportion of EPA in the rumen was higher ($p < 0.01$) in the FD treatment (Figure 2.1-a), reaching 3.5% of total FA and dimethyl acetals (DMA)(1.6 mg/g DM), compared to SD and O treatments, which reached 1.8% and 1.1% of total FA + DMA, respectively. In the abomasal digesta, the highest proportion ($p = 0.001$) of EPA (Figure 2.1-b) was found in FD treatment (3.4% of total FA + DMA) followed by O and SD treatments that averaged 2% of total FA + DMA. The EPA in cecum digesta and faeces (Figure 2.1-c, d, respectively) also differed ($p < 0.001$) among treatments, however both FD and SD treatments equally registered the higher content compared to O treatment.

The estimated whole tract EPA apparent digestibility was significantly different between diets ($p = 0.002$), being lowest in the FD diet (74.0% ± 2.31) fed lambs (Figure 2.2-a). The O treatment registered the highest EPA whole tract apparent digestibility (93.8% ± 0.38), and SD treatment presented an intermediate level (82.4% ± 1.02). The post ruminal apparent digestibility also differed among diets ($p = 0.012$) being highest in O diet (65.8% ± 4.34) and lowest in SD diet (33.4% ± 9.31) (Figure 2.2-b), while FD did not differ from both O and SD diets. The estimated biohydrogenation of EPA (Figure 2.2-c) differed among the *Nannochloropsis* treatments ($p < 0.001$), increasing from 44.7% with FD diets to 69.8% with SD and 80.7% with O diet, respectively.

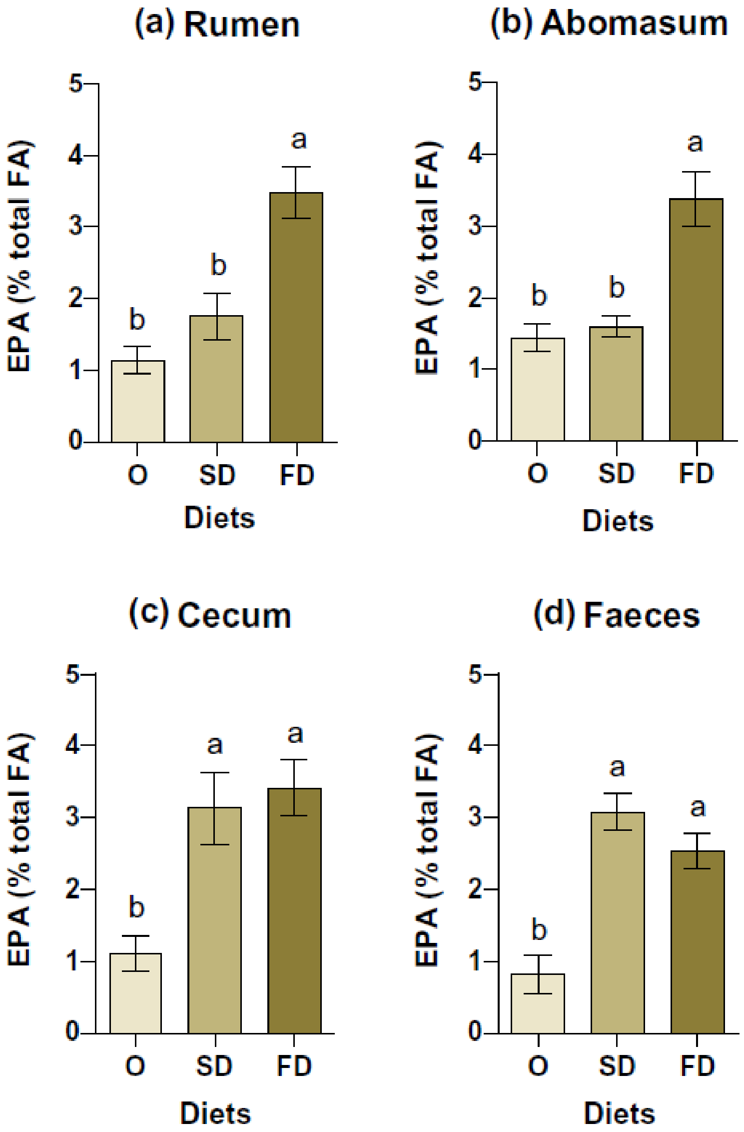

Figure 2.1- EPA content (% total fatty acids) in the (a) rumen content, (b) abomasal content, (c) cecal content, and (d) faeces.

Diets are represented as follow: O (*Nannochloropsis* sp. oil), SD (spray-dried *N. oceanica*) and FD (freeze-dried *N. oceanica*). Least square means are presented in each bar with respective standard error of the mean. Different letters (a–c) in each bar indicate significant differences ($p < 0.05$) among means.

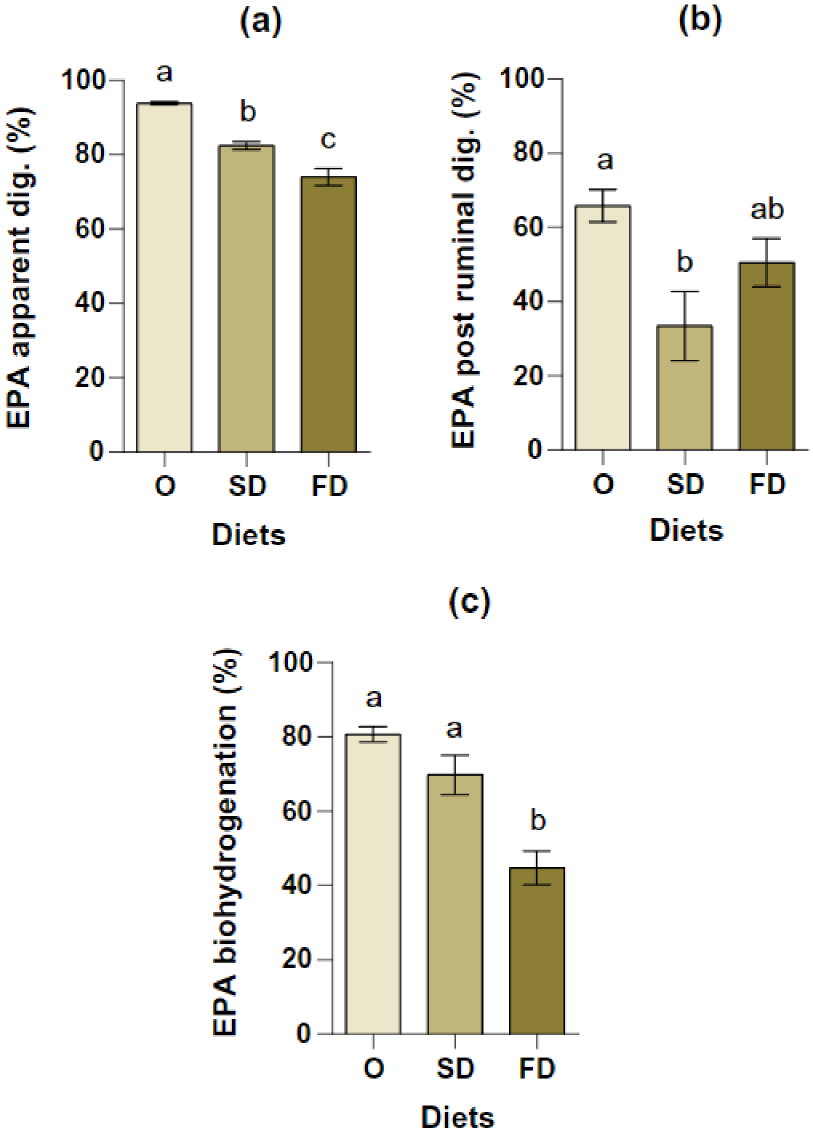

Figure 2.2- (a) EPA whole tract apparent digestibility (%), (b) EPA post ruminal digestibility (%) and (c) EPA biohydrogenation (%).

Diets are represented as follow: O (*Nannochloropsis* sp. oil), SD (spray-dried *N. oceanica*) and FD (freeze-dried *N. oceanica*). Least square means are presented in each bar with respective standard error of the mean. Different letters in each bar (a–c) indicate significant differences ($p < 0.05$) among means.

2.3.2. Scanning electron microscopy (SEM) of *N. oceanica* biomass.

The microscopic evaluation of the surfaces of SD and FD biomass incorporated in the diets revealed two different patterns (Figure 2.3-a to h). The FD biomass (Figure 2.3-a) consisted of a cluster of perfectly individualized cells (although organized in plate-like structures) that maintained an overall spherical shape. In contrast, SD biomass (Figure 2.3-b) consisted of a set of large amorphous granules consisting of clustered microalgae cells that loss the spherical structure, and thus they could not be easily individualized. Apparently, cell wall integrity was maintained in FD biomass but not in the SD form. Both FD and SD presented a wrinkled and slightly collapsed cell wall, although more severe alterations were found in the SD that presented fractured granules (Figure 2.3-g), and irregular shaped holes and cracks (Figure 2.3-h).

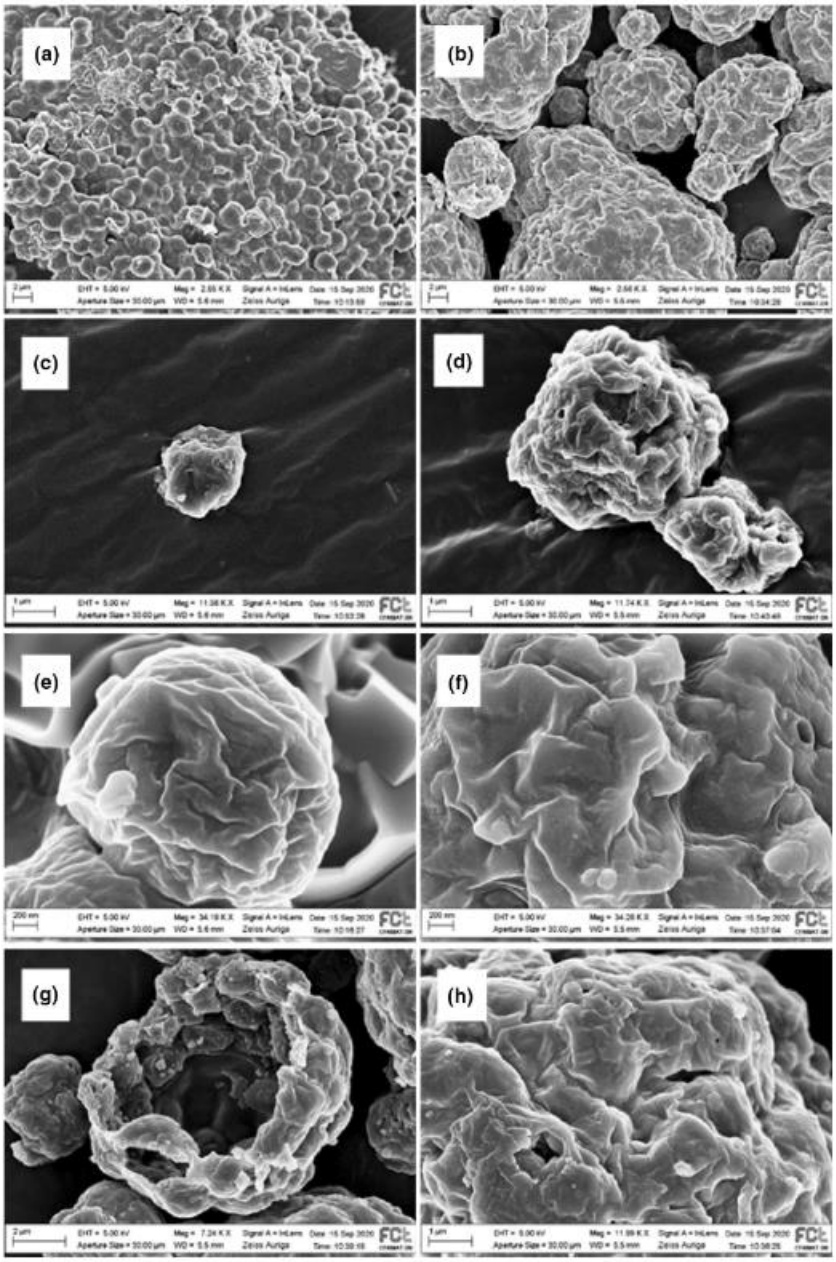

Figure 2.3- Scanning Electron Microscopy Focused Ion Beam (SEM–FIB) of freeze-dried (FD) and spray-dried (SD) *N. oceanica*.
Stabilization in carbon matrix and gold–palladium coating. (a) Granules correspond to individualized FD *N. oceanica* cells; (b) Granules correspond to clusters of more than one SD *N. oceanica* cell; (c) Individual FD *N. oceanica* cell; (d) SD *N. oceanica* cells cluster; (e) FD *N. oceanica* cell wall appearance detail; (f) SD *N. oceanica* cell walls appearance in detail; (g) SD *N. oceanica* fragmented granule; (h) SD *N. oceanica* granule surface in detail.

2.3.3. Fatty acid composition of digesta contents.

The total FA content, including DMA, and the partial FA sums in the rumen, abomasal and cecum digesta of lambs fed control (C) and *Nannochloropsis* supplemented diets are presented in Table 2.2. The complete FA and DMA profile are presented in Table 2.3, Table 2.4 and Table 2.5.

Table 2.2- Total FA and dimethyl acetals content (mg/g DM) and FA partial sums (% total FA + DMA) in the rumen, abomasum, and cecum.

Item	Diets[1]				*p*-value
	C	O	SD	FD	
		Rumen			
Total FA + DMA[2]	36.36^{b} ± 3.028	42.76^{ab} ± 4.461	38.80^{b} ± 1.545	45.79^{a} ± 1.143	0.004
SFA	49.28^{ab} ± 5.569	38.62^{b} ± 2.274	52.47^{a} ± 3.900	44.01^{a} ± 1.243	0.027
MUFA[3]	28.60 ± 4.662	30.40 ± 3.042	25.76 ± 2.648	30.35 ± 0.586	0.415
trans-MUFA	26.24 ± 4.830	26.20 ± 2.948	20.06 ± 2.434	21.90 ± 0.454	0.347
PUFA	12.18^{b} ± 1.279	17.75^{a} ± 1.569	12.66^{b} ± 1.657	17.70^{a} ± 1.387	0.012
DMA	4.87^{a} ± 0.328	3.96^{b} ± 0.171	3.82^{b} ± 0.284	3.44^{b} ± 0.307	0.030
C16:1 FA	0.23^{c} ± 0.030	1.78^{b} ± 0.177	2.75^{b} ± 0.648	5.93^{a} ± 0.436	< 0.001
BCFA	4.07^{a} ± 0.261	3.89^{a} ± 0.232	3.25^{b} ± 0.199	2.92^{b} ± 0.249	0.009
C18 FA[4]	66.62^{a} ± 0.601	52.15^{b} ± 3.302	47.82^{b} ± 0.397	43.81^{c} ± 0.1178	< 0.001
C18 BI[5]	29.52 ± 4.817	31.41 ± 3.182	24.18 ± 2.541	26.20 ± 0.556	0.316
Total C20 FA	1.35^{c} ± 0.127	7.04^{b} ± 0.791	7.67^{b} ± 0.223	9.08^{a} ± 0.269	< 0.001
C20:1 FA	0.06^{c} ± 0.034	0.58^{b} ± 0.088	1.06^{a} ± 0.162	0.64^{ab} ± 0.169	< 0.001
C20:2 FA	0.40^{c} ± 0.075	1.42^{a} ± 0.188	1.13^{ab} ± 0.132	0.77^{b} ± 0.126	< 0.001
C20:3 FA	0.12^{c} ± 0.021	1.59^{a} ± 0.259	0.72^{b} ± 0.171	0.99^{ab} ± 0.211	< 0.001
C20:4 FA	0.07^{c} ± 0.041	1.28^{ab} ± 0.372	1.15^{b} ± 0.212	2.07^{a} ± 0.190	< 0.001
		Abomasum			
Total FA + DMA[2]	42.43^{ab} ± 6.529	33.74^{b} ± 4.053	49.24^{a} ± 3.165	47.32^{a} ± 5.112	0.043
SFA	50.50 ± 6.970	41.20 ± 2.226	50.74 ± 3.463	44.14 ± 0.899	0.133
MUFA[3]	30.35 ± 5.149	33.81 ± 2.966	30.05 ± 2.819	33.34 ± 0.643	0.655
trans-MUFA	19.26 ± 4.351	23.01 ± 3.199	17.12 ± 2.526	16.81 ± 0.644	0.303
PUFA	11.44^{b} ± 1.672	16.75^{a} ± 1.592	11.82^{b} ± 0.586	16.08^{a} ± 1.090	0.003
DMA	2.68^{a} ± 0.191	2.32^{ab} ± 0.228	1.91^{b} ± 0.154	2.08^{ab} ± 0.285	0.036
BCFA	3.87^{a} ± 0.283	3.77^{ab} ± 0.404	3.15^{bc} ± 0.195	2.74^{c} ± 0.248	0.028
C16:1 FA	0.48^{c} ± 0.047	2.75^{b} ± 0.278	3.75^{b} ± 0.500	6.96^{a} ± 0.438	< 0.001
C18 FA[4]	68.22^{a} ± 0.387	54.63^{b} ± 2.041	48.59^{c} ± 0.547	43.58^{d} ± 0.500	< 0.001
C18 BI[5]	21.08 ± 4.310	25.60 ± 3.320	20.51 ± 2.528	19.24 ± 0.946	0.336
Total C20 FA	2.35^{c} ± 0.418	7.83^{b} ± 0.596	9.16^{ab} ± 0.376	9.80^{a} ± 0.343	< 0.001
C20:1 FA	1.27 ± 0.428	0.76 ± 0.212	1.52 ± 0.230	1.03 ± 0.229	0.136
C20:2 FA	0.33^{c} ± 0.078	2.04^{ab} ± 0.255	2.08^{a} ± 0.178	1.47^{b} ± 0.180	< 0.001

C20:3 FA	0.15^{c} ± 0.019	2.10^{a} ± 0.246	1.35^{b} ± 0.249	1.23^{b} ± 0.177	< 0.001
C20:4 FA	0.07^{d} ± 0.14	0.36^{c} ± 0.049	0.79^{b} ± 0.064	1.62^{a} ± 0.116	< 0.001
Cecum					
Total FA + DMA[2]	42.02 ± 8.230	34.88 ± 6.677	28.42 ± 5.836	27.50 ± 3.459	0.376
SFA	58.81^{ab} ± 5.640	48.39^{b} ± 2.335	56.41^{a} ± 2.962	55.62^{a} ± 1.083	0.055
MUFA[3]	22.38^{ab} ± 4.454	26.85^{a} ± 2.943	17.58^{b} ± 2.217	18.32^{b} ± 0.747	0.048
trans-MUFA	21.42^{ab} ± 4.207	23.26^{a} ± 2.816	13.13^{bc} ± 2.284	12.07^{c} ± 0.785	0.003
PUFA	6.14^{b} ± 0.874	12.71^{a} ± 1.079	12.23^{a} ± 1.121	14.18^{a} ± 0.830	< 0.001
DMA	4.26 ± 1.354	4.91 ± 1.413	5.25 ± 0.886	5.01 ± 0.608	0.943
BCFA	6.79 ± 1.013	6.34 ± 0.838	7.91 ± 1.369	6.31 ± 0.370	0.706
C16:1 FA	0.24^{d} ± 0.047	1.21^{c} ± 0.177	2.56^{b} ± 0.328	4.44^{a} ± 0.559	< 0.001
C18 FA[4]	57.37^{a} ± 2.984	47.98^{a} ± 3.806	34.06^{b} ± 4.010	30.86^{b} ± 1.361	< 0.001
C18 BI[5]	17.54^{ab} ± 3.860	21.70^{a} ± 2.650	11.33^{b} ± 2.303	11.59^{b} ± 0.817	0.008
Total C20 FA	1.65^{c} ± 0.363	5.69^{b} ± 0.461	8.56^{a} ± 0.520	8.79^{a} ± 0.354	< 0.001
C20:1 FA	0.05^{b} ± 0.027	0.45^{a} ± 0.127	0.25^{a} ± 0.055	0.21^{ab} ± 0.041	0.003
C20:2 FA	0.61 ± 0.579	1.13 ± 0.285	0.81 ± 0.249	0.60 ± 0.183	0.479
C20:3 FA	n.d	1.08 ± 0.230	0.61 ± 0.178	0.94 ± 0.101	0.212
C20:4 FA	n.d	0.33^{a} ± 0.068	0.12^{b} ± 0.016	0.23^{ab} ± 0.056	0.012

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($p < 0.05$). [1]Diets (or treatments): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. [2]The sum of total fatty acids includes also dimethyl acetals (DMA). [3]MUFA, includes fatty acids with undefined geometry.[4]C18 FA, fatty acids with a chain length of 18 carbon atoms. [5]C18 BI, C18 biohydrogenation intermediates, sum of total 18:1, 18:2, 18:3 and 18:0-oxo except the *c*9-18:1, *c*11-18:1, 18:2n-6 and 18:3n-3.

2.3.3.1. Ruminal digesta.

Individual FA are presented in the Table 2.3. Average FA and DMA content was highest in the FD (45.8 mg/g DM, $p = 0.001$), but it did not differ from O treatment (42.8 mg/g DM). The lowest total FA content (36.4 mg/g DM) was observed in the C treatment, but it did not differ from the O and SD treatments (Table 2.2). The proportion of saturated fatty acids (SFA) was affected by treatments ($p = 0.027$), being highest in the SD and lowest in the O treatment (52.5% and 38.6% of total FA + DMA, respectively). Neither monounsaturated FA (MUFA) nor *trans*-MUFA proportions in the rumen differed among treatments ($p = 0.415$ and $p = 0.347$, respectively), as there were no differences in total C18 biohydrogenation intermediates (BI). On the contrary, PUFA were higher in the O and FD treatments (averaging 17.7% of total FA + DMA) comparing to C and SD treatments (averaging 12.6% of total FA + DMA). The branched-chain fatty acids (BCFA) were higher in the rumen of lambs fed C and O and lower in those fed SD and FD diets. The highest proportion of the total C20 FA was found in the FD treatment

(9.1% of total FA + DMA), followed by SD and O treatments (7.7% and 7.1% of total FA + DMA, respectively), and the C showed the lowest proportion (1.4% of total FA + DMA) (Table 2.2).

Table 2.3- Fatty acid (FA) and dimethyl acetal (DMA) composition in the rumen (% of identified peaks).

Item	Diets^{1}				
	C	O	SD	FD	*p*-value
12:0	0.27±0.030	0.26±0.025	0.30±0.059	0.34±0.019	0.061
i-13:0	0.07^{c}±0.008	0.14^{b}±0.009	0.21^{a}±0.011	0.23^{a}±0.006	<0.001
13:0	0.07±0.016	0.07±0.010	0.08±0.013	0.08±0.014	0.970
i-14:0	0.45±0.042	0.42±0.056	0.51±0.047	0.66±0.068	0.064
14:0	0.97^{c}±0.273	1.20^{c}±0.110	1.78^{b}±0.092	2.53^{a}±0.117	<0.001
i-15:0	0.45^{ab}±0.059	0.48^{a}±0.050	0.58^{a}±0.075	0.34^{b}±0.031	0.017
a-15:0	2.00^{ab}±0.412	2.01^{a}±0.175	1.34^{b}±0.100	1.20^{b}±0.136	0.005
15:0	1.25±0.167	1.09±0.097	1.23±0.125	1.02±0.076	0.398
16:0	17.14^{c}±0.700	22.20^{b}±0.897	28.41^{a}±0.560	28.37^{a}±0.599	<0.001
i-16:0	0.60±0.141	0.35^{a}±0.057	0.27±0.029	0.33±0.111	0.137
c9-16:1	0.12^{c}±0.023	1.57^{b}±0.186	2.36^{b}±0.607	5.58^{a}±0.453	<0.001
t-16:1	0.11^{b}±0.024	0.22^{a}±0.099	0.38^{a}±0.058	035^{a}±0.058	<0.001
i-17:0	0.12±0.016	0.16±0.031	0.16±0.027	0.11±0.035	0.406
a-17:0	0.42^{a}±0.113	0.33^{ab}±0.074	0.18^{b}±0.009	0.21^{b}±0.015	0.034
17:0	0.65±0.084	0.64±0.062	0.65±0.068	0.56±0.042	0.538
17:0-cyclo^{2}	0.31^{a}±0.070	0.29^{a}±0.053	0.15^{b}±0.026	0.15^{b}±0.034	0.041
17:1	0.12±0.023	0.12±0.029	0.06±0.016	0.08±0.009	0.160
18:0	26.39^{a}±4.473	10.94^{bc}±0.876	16.91^{ab}±3.237	9.09^{c}±0.821	0.002
*t*6/*t*7/*t*8-18:1	0.88±0.207	1.18±0.194	0.96±0.171	1.04±0.077	0.745
*t*9-18:1	0.41±0.141	0.75±0.126	0.77±0.124	0.74±0.047	0.185
*t*10-18:1	14.0^{a}±4.236	10.50^{a}±3.246	3.22^{b}±1.022	5.49^{ab}±1.209	0.032
*t*11-18:1	2.31^{b}±0.715	6.24^{ab}±1.858	7.38^{a}±1.356	6.51^{a}±1.369	0.006
*t*12-18:1	0.75±0.079	0.66±0.138	1.04±0.105	0.92±0.137	0.104
*t*13/*t*14/*c*9-18:1	7.23±0.870	6.42±0.462	5.73±0.842	6.42±0.450	0.680
*t*15-18:1	0.56^{a}±0.066	0.24^{b}±0.086	0.59^{a}±0.086	0.43^{ab}±0.079	0.028
*c*11-18:1	0.987±0.156	1.06±0.134	0.94±0.080	1.15±0.108	0.345
*c*12-18:1	0.35±0.065	0.27±0.065	0.53±0.098	0.36±0.028	0.202
*c*13-18:1	0.05±0.010	0.05±0.008	0.07±0.008	0.06±0.011	0.315
*t*16/*c*14-18:1	0.34±0.062	0.15±0.074	0.38±0.077	0.23±0.064	0.118
*c*15-18:1	0.27^{ab}±0.075	0.29^{a}±0.061	0.12^{b}±0.020	0.25^{ab}±0.074	0.019
*c*16-18:1	0.08±0.009	0.11±0.013	0.09±0.017	0.11±0.016	0.404
t,*t*-18:2	0.09±0.023	0.17±0.048	0.05±0.009	0.06±0.012	0.059
*t*9,*c*12-18:2	0.07^{b}±0.012	0.16^{ab}±0.054	0.14^{ab}±0.051	0.21^{a}±0.049	0.037
*t*11,*c*15/*t*10,*c*15-18:2	0.45^{bc}±0.105	1.49^{a}±0.359	0.26^{c}±0.069	0.065^{b}±0.150	0.006

18:2n-6	9.027±1.148	7.99±0.680	4.94±1.147	6.95±0.863	0.083
*c*9,*t*11-CLA	n.d.	0.10±0.011	0.19±0.053	0.12±0.026	0.228
t,*t*-CLA	0.17^{a}±0.010	0.11^{b}±0.011	0.18^{ab}±0.033	0.32^{ab}±0.117	0.002
18:3n-3	1.69±0.148	1.81±0.127	1.78±0.056	1.57±0.072	0.138
oxo-18:0	0.64±0.239	1.58±0.395	1.55±0.275	1.21±0.290	0.077
20:0	0.60±0.048	0.50±0.038	1.49±0.481	0.61±0.117	0.104
20:2n-6	0.12±0.064	0.29±0.052	0.20±0.053	0.27±0.042	0.174
20:3n-6	n.d.	0.24±0.066	0.17±0.044	0.27±0.024	0.101
20:4n-6	0.07^{d}±0.041	0.29^{c}±0.057	0.76^{b}±0.106	1.62^{a}±0.122	<0.001
20:4n-3	n.d.	0.83±0.311	0.27±0.103	0.38±0.075	0.232
20:5n-3	n.d.	1.14^{b}±0.185	1.75^{b}±0.327	3.47^{a}±0.356	<0.001
21:0	0.06^{b}±0.004	0.14^{a}±0.025	0.17^{a}±0.037	0.15^{a}±0.034	0.001
22:0	0.56^{a}±0.025	0.54^{ab}±0.062	0.47^{b}±0.027	0.43^{b}±0.020	0.005
23:0	0.26±0.048	0.18±0.019	0.19±0.026	0.17±0.014	0.309
24:0	0.55^{a}±0.026	0.46^{ab}±0.041	0.41^{bc}±0.018	0.37^{c}±0.006	<0.001
24:1	0.09^{a}±0.008	0.07^{ab}±0.009	0.08^{ab}±0.015	0.06^{b}±0.007	0.024
26:0	0.27^{a}±0.024	0.23^{ab}±0.024	0.19^{b}±0.004	0.18^{b}±0.014	0.017
28:0	0.20^{a}±0.010	0.15^{b}±0.009	0.18^{ab}±0.014	0.11^{c}±0.007	<0.001
DMA					
i-14:0	0.11^{b}±0.028	0.18^{b}±0.045	0.30^{a}±0.033	0.20^{ab}±0.050	0.003
14:0	0.12^{c}±0.040	0.22^{bc}±0.048	0.45^{a}±0.063	0.34^{ab}±0.057	0.001
i-15:0	0.05±0.007	0.08±0.025	0.10±0.014	0.06±0.012	0.086
a-15:0	0.31^{b}±0.053	0.41^{ab}±0.056	0.56^{a}±0.046	0.33^{b}±0.069	0.009
15:0	0.31^{b}±0.060	0.22^{ab}±0.025	0.18^{bc}±0.018	0.14^{c}±0.017	0.016
i-16:0	0.71±0.256	0.37±0.089	0.38±0.083	0.49±0.083	0.498
16:0	2.35^{a}±0.224	2.05^{a}±0.086	1.36^{b}±0.104	1.50^{b}±0.172	<0.001
18:0	0.17^{a}±0.026	0.08^{b}±0.014	0.08^{b}±0.016	0.06^{b}±0.017	0.020
t-18:1	0.46^{a}±0.062	0.26^{b}±0.043	0.23^{b}±0.028	0.21^{b}±0.018	0.008
*c*9-18:1	0.109±0.005	0.14±0.045	0.19±0.047	0.15±0.035	0.080

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($p < 0.05$). [1]Diets (or treatments): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. [2]17:0-cyclo17, 11-cyclohexyl-11:0.

2.3.3.2. Abomasal digesta.

Individual FA are presented in the Table 2.4. Average FA and DMA content was lowest for animals fed O diet (33.7 mg/g DM), although not different from animals fed C diet (42.4 mg/g DM), while the highest content was observed in animal fed SD (49.2 mg/g DM), but it did not differ from those fed FD and C diets. Contrary to what was observed for the rumen contents, SFA did not differ among treatments ($p = 0.133$), but similarly, to what was observed in the rumen, the proportion of MUFA, *trans*-MUFA and C18 BI did not differ ($p > 0.05$) among treatments. The proportion of PUFA was higher (*p* = 0.003) in animals fed O and FD diets (averaging 16.4% of total FA + DMA) compared to C and SD diets, (averaging 11.6% of total FA + DMA). Similarly, to what was observed in the rumen, the total C18 FA differed among treatments (*P* < 0.001), being highest and lowest in the C (68.2%) and FD fed lambs (43.6%), respectively. The total DMA proportion was highest in C (2.7%) and lowest in SD treatments (1.9%) while O and FD presented intermediate proportions. The highest BCFA proportion was also observed in C diet (4.0% of total FA + DMA), but the lowest proportion was observed in FD diet (2.7% of total FA + DMA). The highest proportion of total C20 was observed in FD diet (9.8% of total FA), while in C diet it only reached 2.4% of total FA. There were also differences (*p* < 0.001) among treatments for the sums of C20:2, C20:3 and C20:4.

Table 2.4- Fatty acid and dimethyl acetal composition in the abomasum (% of identified peaks).

Item	**Diets[1]**				
	C	**O**	**SD**	**FD**	***p*-value**
12:0	0.25^{b}±0.025	0.34^{ab}±0.050	0.33^{ab}±0.074	0.38^{a}±0.032	0.028
13:0	0.13^{b}±0.014	0.19^{ab}±0.030	0.20^{ab}±0.034	0.24^{a}±0.021	0.002
i-14:0	0.12^{b}±0.016	0.21^{ab}±0.050	0.24^{a}±0.040	0.35^{a}±0.048	<0.001
14:0	0.82^{d}±0.216	1.35^{c}±0.094	1.68^{b}±0.091	2.52^{a}±0.103	<0.001
15:0	1.05±0.167	1.23±0.136	1.02±0.182	1.05±0.074	0.673
i-15:0	0.38^{b}±0.029	0.50^{ab}±0.052	0.58^{a}±0.039	0.39^{b}±0.043	0.004
a-15:0	2.219^{a}±0.128	1.98^{a}±0.229	1.38^{b}±0.090	1.20^{b}±0.148	<0.001
16:0	17.33^{c}±0.341	22.20^{b}±1.095	27.43^{a}±0.408	28.21^{a}±0.584	<0.001
i-16:0	0.57±0.119	0.42±0.084	0.29±0.038	0.38±0.105	0.117
*c*9-16:1	0.15^{c}±0.018	1.90^{b}±0.278	2.42^{b}±0.365	5.60^{a}±0.414	<0.001
16:0-Me_4	0.10^{b}±0.021	0.23^{ab}±0.091	0.42^{a}±0.058	0.25^{ab}±0.077	<0.001
17:0	0.877±0.061	0.84±0.082	0.74±0.050	0.70±0.063	0.456
i-17:0	0.18^{c}±0.027	0.29^{abc}±0.067	0.45^{a}±0.046	0.32^{b}±0.034	<0.001
a-17:0	0.43^{a}±0.105	0.38^{ab}±0.093	0.20^{b}±0.012	0.11^{c}±0.031	0.005
*c*9-17:1	0.16^{ab}±0.028	0.19^{a}±0.024	0.09^{b}±0.019	0.18^{a}±0.015	0.006
17:0-cyclo[2]	0.31^{ab}±0.088	0.36^{a}±0.072	0.20^{bc}±0.028	0.15^{c}±0.033	0.058
18:0	28.30^{a}±6.451	12.92^{b}±1.212	16.93^{b}±2.937	9.47^{c}±0.610	0.003
oxo-18:0	048^{b}±0.170	1.27^{a}±0.281	1.48^{a}±0.202	1.03a±0.282	0.007
*t*6/*t*7/*t8*-18:1	0.93±0.219	1.25±0.219	1.16±0.145	1.09±0.067	0.755
*t*9-18:1	0.656±0.135	0.74±0.165	0.89±0.116	0.78±0.056	0.347
*t*10-18:1	13.69^{ab}±3.855	13.08^{a}±3.307	3.69^{c}±1.084	5.56^{bc}±1.219	0.018
*t*11-18:1	2.31^{b}±0.734	7.43^{ab}±2.560	8.87^{a}±2.139	6.96^{a}±1.648	0.008
*t*12-18:1	0.78^{b}±0.090	0.88^{ab}±0.228	1.26^{a}±0.082	1.00^{ab}±0.156	0.006
*t*13/*t*14/*c*9-18:1	7.83^{a}±0.767	6.99^{a}±0.308	5.45^{b}±0.373	6.88^{a}±0.355	0.010
*t*15-18:1	0.55±1.110	0.46±0.185	0.69±0.092	0.39±0.083	0.159
*c*11-18:1	0.84^{b}±0.093	1.02^{a}b±0.201	1.02^{a}b±0.090	1.28^{a}±0.104	0.040

*c*12-18:1	0.41±0.059	0.38±0.067	0.72±0.136	0.44±0.026	0.176
*c*13-18:1	0.09±0.017	0.08±0.023	0.11±0.006	0.09±0.016	0.327
*t*16/*c*14-18:1	0.43±0.093	0.21±0.088	0.47±0.080	0.28±0.059	0.110
*c*15-18:1	0.30^{a}±0.054	0.38^{a}±0.053	0.19^{b}±0.018	0.27^{ab}±0.066	0.012
*c*16-18:1	0.11±0.018	0.15±0.013	0.16±0.016	0.15±0.021	0.304
*t*9,*c*12-18:2	0.08^{b}±0.012	0.22^{a}±0.041	0.22^{a}±0.035	0.25^{a}±0.038	<0.001
*t*11,*c*15/*t*10,*c*15-18:2	0.49^{bc}±0.131	1.89^{a}±0.430	0.33^{c}±0.064	0.76^{b}±0.182	0.005
18:2n-6	9.86^{a}±1.447	7.96^{ab}±1.020	3.90^{c}±0.424	6.66^{b}±0.702	<0.001
18:3n-3/20:1	0.51^{ab}±0.285	0.92^{ab}±0.313	1.08^{a}±0.114	0.27^{b}±0.078	0.003
*c*9,*t*11-CLA	n.d.	0.13b±0.030	0.26a±0.033	0.17ab±0.031	0.034
20:0	0.64±0.050	0.64±0.066	1.30±0.364	0.60±0.048	0.312
20:2n-6	0.14^{b}±0.052	0.49^{a}±0.090	0.52^{a}±0.107	0.47^{a}±0.101	0.002
20:3n-6	0.04^{b}±0.218	0.68^{a}±0.078	0.49^{ab}±0.092	0.45^{ab}±0.076	0.044
20:4n-6	0.07^{d}±0.014	0.36^{c}±0.049	0.79^{b}±0.064	1.62^{a}±0.116	<0.001
20:5n-3	n.d.	1.44b±0.192	1.60b±0.151	3.38a±0.390	0.001
21:0	0.02±0.071	0.12±0.031	0.15±0.025	0.14±0.030	0.426
22:0	0.56^{b}±0.037	0.80^{a}±0.048	0.54^{b}±0.045	0.53^{b}±0.030	0.001
24:0	0.61^{a}±0.035	0.56^{a}±0.036	0.43^{b}±0.011	0.38^{c}±0.016	<0.001
26:0	0.28^{a}±0.011	0.30^{a}±0.024	0.22^{b}±0.013	0.19^{b}±0.018	<0.001
DMA					
i-14:0	0.10^{b}±0.017	0.15^{ab}±0.027	0.24^{a}±0.050	0.19^{ab}±0.052	0.041
14:0	0.12^{c}±0.035	0.20^{bc}±0.038	0.35^{a}±0.044	0.32^{a}b±0.044	0.001
a-15:0	0.14±0.045	0.17±0.067	0.06±0.010	0.24±0.154	0.102
15:0	0.27^{a}±0.028	0.21^{ab}±0.024	0.16^{b}±0.022	0.16^{b}±0.037	0.021
16:0	1.63^{a}±0.183	1.31^{ab}±0.114	0.83^{c}±0.050	0.99^{bc}±0.134	<0.001
18:1	0.41±0.074	0.30±0.067	0.27±0.036	0.24±0.024	0.178

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($p < 0.05$). [1]Diets (or treatments): C, control; O, Control plus 1.2% Nannochloropsis sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. [2]17:0-cyclo17, 11-cyclohexyl-11:0.

2.3.3.3. Caecal digesta.

Individual FA are presented in the Table 2.5. Average FA + DMA content did not differ (p = 0.376) among treatments (Table 2.2). Following the same trend that was verified in the ruminal content, the proportion of SFA showed a tendency (p = 0.055) for being higher in the cecum of C, SD, and FD lambs (averaging 57.0% of total FA + DMA) than in the O fed lambs (48.4% total FA + DMA). Contrary to what was observed in both rumen and abomasum, the proportion of MUFA and *trans*-MUFA in cecum differed among treatments (p < 0.05), being the lowest proportions found in FD and SD fed animals. Moreover, total C18 FA and C18 BI differed among treatments (p < 0.001 and p = 0.008, respectively) being highest in the C and O treatment and lowest in the SD and FD treatments. In the cecum, neither total DMA nor BCFA differed among treatments ($p > 0.05$). PUFA presented higher proportions ($p < 0.001$) in *Nannochloropsis* fed lambs (averaging about 13% of total FA) compared to C fed animals that only showed 6.1% of total FA. The proportions of total C20 FA were higher ($p < 0.001$) for FD and SD treatments (8.8 and 8.6% of total FA + DMA, respectively) compared to O treatment (5.7% of total FA + DMA).

Table 2.5- Fatty acid and dimethyl acetal composition in the cecum (% of identified peaks).

Item	**Diets^{1}**				
	C	**O**	**SD**	**FD**	***p*-value**
12:0	0.42±0.079	0.43±0.120	0.60±0.116	0.58±0.038	0.210
13:0	0.11^{b}±0.016	0.18^{b}±0.046	0.37^{a}±0.051	0.54^{a}±0.123	<0.001
i-14:0	0.56±0.071	0.69±0.115	1.10±0.315	0.84±0.091	0.089
14:0	1.24^{c}±0.166	1.69^{c}±0.329	2.93^{b}±0.364	3.99^{a}±0.298	<0.001
i-15:0	0.53±0.075	0.82±0.227	0.89±0.123	0.70±0.121	0.104
a-15:0	2.577±0.489	2.69±0.394	2.31±0.358	2.24±0.121	0.656
15:0	1.88±0.184	1.74±0.211	2.43±0.280	2.10±0.143	0.222
i-16:0	1.06±0.231	0.60±0.057	0.86±0.175	0.81±0.154	0.126
16:0	20.26^{d}±0.274	23.79^{c}±0.866	28.75^{b}±0.334	31.44^{a}±0.559	<0.001
t-16:1	0.07^{b}±0.014	0.12^{ab}±0.035	0.21^{a}±0.027	0.21^{a}±0.037	0.001
*c*7-16:1	0.08^{b}±0.016	0.20^{a}±0.034	0.16^{a}±0.034	0.24^{a}±0.036	0.001
*c*9-16:1	0.09^{d}±0.026	0.88^{c}±0.115	2.24^{b}±0.320	3.99^{a}±0.514	<0.001
i-17:0	0.64^{c}±0.060	0.74^{bc}±0.126	1.38^{a}±0.205	0.92^{ab}±0.100	0.008
a-17:0	0.99±0.240	0.67±0.086	0.97±0.153	0.59±0.076	0.109
17:0	2.15±0.220	1.80±0.230	2.15±0.300	1.97±0.177	0.683
17:1	0.17±0.046	0.16±0.025	0.22±0.086	0.18±0.038	0.919
17:0-cyclo^{2}	0.44±0.119	0.48±0.123	0.31±0.086	0.35±0.096	0.658
18:0	28.92^{a}±5.692	14.37^{b}±2.678	13.77^{b}±3.483	10.77^{b}±1.071	0.026
*t*6/*t*7/*t*8-18:1	0.74±0.209	0.54±0.195	0.58±0.149	0.51±0.080	0.765
*t*9-18:1	0.37±0.098	0.63±0.173	0.40±0.109	0.36±0.040	0.515
*t*10-18:1	9.53^{a}±2.955	8.35^{ab}±3.041	1.92^{b}±0.881	2.41^{b}±0.803	0.042
*t*11-18:1	3.08±0.812	7.24±2.975	4.57±0.905	3.83±0.564	0.437
*t*12-18:1	0.69±0.078	0.52±0.038	0.70±0.132	0.49±0.063	0.148
*t*15-18:1	0.45^{a}±0.054	0.14^{c}±0.020	0.28^{b}±0.063	0.11^{c}±0.026	<0.001
*t*16,*c*14-18:1	0.31±0.074	0.11±0.038	0.28±0.067	0.14±0.031	0.054
*c*9/*t*13/*t*14-18:1	6.13^{ab}±0.869	5.63^{a}±0.491	4.27^{bc}±0.359	3.99^{c}±0.215	0.012
*c*11-18:1	0.76±0.101	0.99±0090	0.82±0.089	0.87±0.081	0.351
*c*12-18:1	0.57±0.130	0.68±0.190	0.70±0.137	0.54±0.061	0.671
*c*13-18:1	0.04±0.004	0.05±0.005	0.10±0.021	0.06±0.017	0.121
*c*15-18:1	0.22^{b}±0.048	0.44^{a}±0.079	0.13^{b}±0.021	0.24^{ab}±0.085	0.009
*c*16-18:1	0.13±0.017	0.14±0.032	0.08±0.012	0.08±0.017	0.062

t,*t*-18:2	0.08±0.013	0.13±0.030	0.10±0.012	0.06±0.007	0.053
t,*c*.18:2	0.07^{b}±0.017	0.13^{a}±0.015	0.14^{a}±0.026	0.11^{ab}±0.013	0.047
*t*11,*c*15/*t*10,*c*15-18:2	0.38^{b}±0.114	1.14^{a}±0.224	0.26^{b}±0.053	0.39^{b}±0.078	0.008
18:2n-6	3.28±0.598	4.13±0.531	2.81±0.372	2.80±0.300	0.179
18:3n-3/20:1	0.75^{b}±0.104	1.16^{a}±0.089	1.06^{ab}±0.149	0.85^{b}±0.052	0.018
*c*9,*t*11-CLA	0.311^{b}±0.008	0.18^{a}±0.015	0.30^{ab}±0.128	0.23^{a}±0.044	0.001
c,*t*-CLA	0.04±0.026	0.08±0.023	0.14±0.038	0.24±0.192	0.219
t,*t*-CLA	0.70±0.118	1.48±1.032	0.74±0.284	1.87±0.426	0.090
oxo-18:0	0.16±0.062	0.32±0.059	0.31±0.066	0.21±0.049	0.240
20:0	0.78±0.053	0.79±0.092	1.44±0.403	0.80±0.031	0.467
20:2n-6	0.39±0.175	0.33±0.061	0.52±0.165	0.53±0.104	0.386
20:3n-3	n.d.	0.67±0.113	0.44±0.103	0.60±0.075	0.317
20:3n-6	0.05^{b}±0.004	0.19^{a}±0.047	0.28^{a}±0.049	0.19^{a}±0.034	<0.001
20:4n-6	0.19^{b}±0.052	0.40^{b}±0.106	1.55^{a}±0.255	1.92^{a}±0.181	<0.001
20:5n-3	n.d.	1.11b±0.246	3.13a±0.498	3.41a±0.391	<0.001
21:0	0.08^{b}±0.006	0.12^{a}±0.012	0.18^{a}±0.035	0.14^{a}±0.017	0.002
22:0	0.79±0.067	0.87±0.079	0.85±0.088	0.73±0.029	0.231
23:0	0.31^{b}±0.026	0.39^{ab}±0.032	0.45^{a}±0.048	0.42^{a}±0.037	0.032
24:0	0.95±0.062	1.0±0.158	1.35±0.214	1.07±0.077	0.281
26:0	0.55±0.051	0.72±0.179	0.68±0.066	0.65±0.069	0.376
28:0	0.43±0.034	0.59±0.183	0.54±0.086	0.50±0.059	0.441
DMA					
i-14:0	0.12^{b}±0.021	0.24^{ab}±0.065	0.40^{a}±0.108	0.32^{a}±0.076	0.009
14:0	0.11^{b}±0.015	0.33^{ab}±0.140	0.72^{a}±0.161	0.66^{a}±0.127	<0.001
a-15:0	0.34^{b}±0.039	0.59^{ab}±0.164	0.63^{a}±0.065	0.53^{ab}±0.088	0.004
15:0	0.35±0.132	0.28±0.081	0.29±0.062	0.26±0.051	0.930
16:0	2.43±0.844	2.77±0.799	2.39±0.409	2.51±0.291	0.978
18:0	0.31±0.093	0.23±0.040	0.34±0.092	0.25±0.036	0.652
18:1	0.61±0.257	0.47±0.157	0.55±0.158	0.47±0.053	0.913

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($p < 0.05$). [1]Diets (or treatments): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. [2]17:0-cyclo17, 11-cyclohexyl-11:0.

2.3.4. Biohydrogenation of C18 PUFA in the rumen.

Diets were not formulated considering the C18 FA, nevertheless the content of the C18 FA (g/kg DM) was similar among diets. Also, to better evaluate the effect of *Nannochloropsis* supplementation on the biohydrogenation of C18 PUFA in the rumen, the profile of C18 FA was expressed as a percentage of total C18 FA (Table 2.6). Although the total C18 FA (mg/g DM) only tended to differ ($p = 0.080$) across treatments, several C18 BI differed statistically among treatments. The major BI were the *t*10-18:1 and *t*11-18:1. The *t*11-18:1 was higher with

Nannochloropsis supplemented diets (averaging 14.1% of total C18 FA) than with the C diet. On the contrary, the *t*10-18:1 tended (*p* = 0.057) to be highest in C and lowest in SD treatments. The ratio between *t*10-18:1 and *t*11-18:1 (*t*10/*t*11-18:1 ratio) was highest in the C treatment (7.6), although it did not differ from O treatment (6.5) due to one sample with a very high *t*10/*t*11-18:1 ratio (Figure 2.4), and it was lowest in the SD and FD treatments (0.47 and 1.69, respectively).

Other rumen C18 BI differed (*p* < 0.05) among treatments, namely *t*9-18:1, *c*16-18:1, and *t*9,*c*12-18:2 were highest in FD, and the *t*12-18:1, *c*12-18:1, *c*13-18:1 and oxo-18:0 were highest in SD, whereas the C treatment presented the lowest proportions of these BI (Table 2.6). No differences among treatments were observed for 18:2n-6, but the 18:3n-3 was 43% higher with *Nannochloropsis* supplemented diets than with C diet (*p* = 0.001). In contrast, the 18:0 was about two folds higher with C diet (40% of total C18 FA) than with FD diet (21%), and the O and SD diets presented intermediate values, i.e., 22% and 35% of total C18 FA, respectively (Table 2.6).

Consistent with these results, the estimated biohydrogenation of 18:2n-6 did not differ (*p* = 0.133) among treatments (Table 2.6), whereas the biohydrogenation of 18:3n-3 was highest in C and lowest in SD group (*p* < 0.001). Also, the estimated biohydrogenation completeness differed (*p* = 0.01) among treatments, reaching the highest value with C (55.0%) and the lowest with O and FD treatments (averaging 30.3%), while the biohydrogenation completeness in SD did not differ from the other treatments.

Table 2.6- C18 fatty acid content (mg/g DM) and composition (% of total C18) in the rumen and biohydrogenation (BH) indicators.

Item	Diets[1]				*p*-value
	C	O	SD	FD	
Total C18 FA[2]	24.26 ± 2.185	22.15 ± 2.41	18.57 ± 0.81	20.06 ± 0.49	0.080
18:0	39.86^{a} ± 7.145	21.86^{b} ± 2.751	35.28^{ab} ± 6.744	20.76^{b} ± 1.889	0.034
*t*6/*t*7/*t*8-18:1	1.32 ± 0.312	2.24 ± 0.305	2.00 ± 0.350	2.36 ± 0.171	0.056
*t*9-18:1	0.61^{b} ± 0.221	1.43^{a} ± 0.203	1.60^{a} ± 0.252	1.69^{a} ± 0.107	0.002
*t*10-18:1	20.80 ± 6.187	18.64 ± 5.655	6.66 ± 2.089	12.4 ± 2.718	0.057
*t*11-18:1	3.46^{b} ± 1.078	11.94^{a} ± 3.550	15.48^{a} ± 2.911	14.92^{a} ± 3.168	0.001
*t*12-18:1	1.12^{c} ± 0.113	1.29^{bc} ± 0.253	2.17^{a} ± 0.206	2.10^{ab} ± 0.315	0.001
*t*13/*t*14/*c*9-18:1	10.83 ± 1.259	12.54 ± 1.041	12.05 ± 1.880	14.66 ± 1.018	0.143
*t*15-18:1	0.84^{ab} ± 0.101	0.46^{b} ± 0.167	1.22^{a} ± 0.177	0.98^{ab} ± 0.183	0.036
*c*11-18:1	1.31^{c} ± 0.248	2.05^{ab} ± 0.239	1.97^{b} ± 0.169	2.64^{a} ± 0.247	0.010
*c*12-18:1	0.52^{b} ± 0.097	0.51^{b} ± 0.110	1.10^{a} ± 0.199	0.82^{a} ± 0.064	0.012
*c*13-18:1	0.08^{b} ± 0.015	0.09^{b} ± 0.015	0.14^{a} ± 0.016	0.13^{ab} ± 0.024	0.026
*t*16/*c*14-18:1	0.52 ± 0.099	0.29 ± 0.145	0.80 ± 0.161	0.53 ± 0.148	0.169
*c*15-18:1	0.41^{ab} ± 0.113	0.58^{a} ± 0.127	0.24^{b} ± 0.041	0.56^{ab} ± 0.168	0.038
*c*16-18:1	0.13^{b} ± 0.013	0.20^{a} ± 0.020	0.19^{ab} ± 0.034	0.25^{a} ± 0.037	0.003
t,*t*-18:2	0.13 ± 0.033	0.36 ± 0.138	0.10 ± 0.018	0.13 ± 0.027	0.224
*t*9,*c*12-18:2	0.11^{b} ± 0.018	0.29^{ab} ± 0.092	0.29^{ab} ± 0.105	0.47^{a} ± 0.112	0.006
*t*11,*c*15/*t*10,*c*15-18:2	0.66^{b} ± 0.155	2.97^{a} ± 0.742	0.55^{b} ± 0.147	1.48^{a} ± 0.339	0.007
18:2n-6	13.52 ± 1.681	15.56 ± 1.256	10.43 ± 2.532	15.84 ± 1.957	0.279
*c*9,*t*11-CLA	n.d	0.18 ± 0.022	0.40 ± 0.108	0.27 ± 0.061	0.102
t,*t*-CLA	0.26 ± 0.015	0.23 ± 0.036	0.37 ± 0.072	0.73 ± 0.267	0.119
18:3n-3	2.53^{b} ± 0.215	3.52^{a} ± 0.269	3.73^{a} ± 0.114	3.58^{a} ± 0.169	0.001
oxo-18:0	0.97^{b} ± 0.362	2.95^{a} ± 0.660	3.23^{a} ± 0.554	2.78^{a} ± 0.670	0.006
*t*10/*t*11-18:1 ratio	7.61^{a} ± 2.594	6.46^{ab} ± 4.859	0.47^{b} ± 0.124	1.69^{b} ± 0.725	0.022
			BH indicators		
BH-18:2n-6	75.8 ± 3.10	74.1 ± 2.01	82.1 ± 4.33	71. ± 3.58	0.270
BH-18:3n-3	79.1^{a} ± 1.77	73^{b} ± 1.60	64.^{c} ± 1.00	70.3^{b} ± 1.40	< 0.001
Completeness[3]	55^{a} ± 8.81	28.7^{c} ± 2.44	47^{ab} ± 7.78	31.9^{bc} ± 2.11	0.018

Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($p < 0.05$). [1]Diets (or treatments): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. [2]C18 FA, Fatty acids with 18 carbon chain length. [3]C18 biohydrogenation completeness (%) that was estimated considering the maximum 18:0 in the rumen and assuming a complete biohydrogenation of the C18 FA from the diet. n.d – not detected.

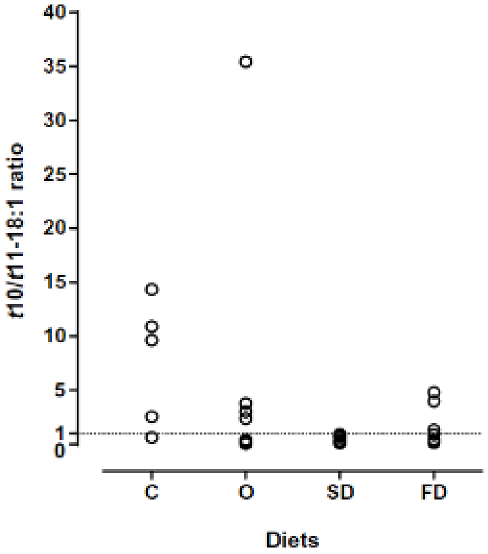

Figure 2.4- Dispersion of individual values of *t*10/*t*11-18:1 ratio in the rumen of lambs fed experimental diets.
Diets are represented as follow: C (Control), O (*Nannochloropsis* sp. oil), SD (spray-dried *N. oceanica*) and FD (freeze-dried *N. oceanica*). Least square means are presented in each point.

2.3.5. Fermentation parameters, protozoa counting, and rumen mucosa evaluation.

Rumen pH averaged approximately 6.0 ± 0.26 in animals consuming different diets and did not differ ($p = 0.782$) among them (Table 2.7). Total volatile fatty acids (VFA) concentration, which averaged 222 mmol/L, the molar proportions of linear chain VFA (2:0, 3:0, 4:0, 5:0 and 6:0) and the branched-chain VFA (iso-4:0 and iso-5:0) did not differ among treatments ($p > 0.05$).

Table 2.7- Rumen pH, volatile fatty acid (VFA) concentration and molar proportion, and mucosa variables of lambs fed experimental diets.

Item	Diets[1]				
	C	O	SD	FD	*p*-value
Rumen pH	5.7±0.27	6.0±0.23	6.0±0.26	5.8±0.26	0.782
Total VFA (mmol/ L)	199±31.6	246±45.9	233±63.8	211±31.2	0.847
VFA (mmol/ 100 mol)					
2:0	43.4±3.32	47.9±4.06	50.2±2.10	50.5±2.54	0.329
3:0	26.3±2.82	26.9±2.0	23.6±0.75	24.9±1.97	0.370
i-4:0	6.8±2.09	6.0±3.01	6.7±1.55	5.6±1.25	0.928
4:0	14.3±1.49	15.6±2.24	13.3±1.50	12.7±0.79	0.564
i-5:0	3.1±1.17	2.0±0.75	3.3±0.56	2.4±0.58	0.518
5:0	4.7±1.24	2.9±0.60	2.5±0.54	3.3±0.58	0.417
6:0	1.4±0.53	0.9±0.29	0.5±0.09	0.7±0.27	0.206
Rumen mucosa variables					
Papillae length (mm)	5.30±0.37	5.03±0.33	5.76±0.78	4.33±0.30	0.125
Epithelium thickness (µm)	195±20.3	173±20.2	182±16.8	167±7.5	0.558
Stratum corneum thickness (µm)	53.6±6.91	47.1±3.87	52.7±3.85	45.9±2.39	0.410
Grayscale value	59.3±4.86	61.8±3.56	61.7±6.15	56.1±6.20	0.867

Values are means ± standard error of the mean. [1]Diets: C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica.*

Most of the lambs were defaunated and only 3 presented countable rumen ciliate protozoa. Two lambs consuming SD diet had counts of 2.3 × 10^6 and 7.5 × 10^5 ciliate cells per mL and one animal consuming O diet had a count of 2.3 × 10^6 ciliate cells per mL. *Isotricha* genus was only found in one animal fed SD diet (1.0 × 10^5 cells per mL). *Epidinium* were only found in the lamb fed O diet (1.0 × 106 cells per mL). *Entodinium* sp. were found in three lambs, two from the SD group having 2.2 × 10^6 and 7.5 × 10^5 cells per mL and one from the O fed group having 1.3 × 10^6 cells per mL. Regarding the evaluation of rumen mucosa integrity in what to concerns to the presence of parakeratotic lesions, none of the histometric indicators (i.e., papillae length, epithelium thickness, and *stratum corneum* thickness) differed among treatments ($p > 0.120$) as shown in Table 2.7. Image greyscale values from ruminal mucosa digital photograph processing did not differ among treatments ($p = 0.867$) as well.

2.4. Discussion.

In a previous *in vitro* study (Alves et al., 2018), we found that EPA disappearance from *N. oceanica* dried biomass, after incubation with rumen fluid for 24h, was reduced compared with non-esterified EPA (unprotected EPA) and that the EPA disappearance was 25% lower when *N. oceanica* biomass was FD than SD. We hypothesised that protection against biohydrogenation was somehow related to *N. oceanica* cell walls' structure and that the freeze-drying would better preserve the integrity of cell walls (Alves et al., 2018). The present experiment was designed to confirm *in vivo* our previous findings related to the higher capacity of FD *N. oceanica* to decrease EPA disappearance on *in vitro* rumen batch incubations compared to SD *N. oceanica*. Thus, we designed the lamb diets to provide similar amounts of EPA, via free *Nannochloropsis* sp. oil or *N. oceanica* biomass, either SD or FD. However, after diet sampling, the final EPA content was slightly lower in FD diet compared to SD or O diets. This could be related with losses during handling, feed preparation or differences among slurry batches. Despite that, the results confirmed that the EPA from FD biomass was better protected from rumen biohydrogenation than SD biomass. The estimates of biohydrogenation extent (disappearance) of EPA in the rumen also confirmed that FD *N. oceanica* was more protected from biohydrogenation, as EPA biohydrogenation of FD was only 45% compared to high values found with SD (70%) and O (81%). Consistently the concentration of the EPA in the rumen of lambs fed FD was about 50% higher than lambs fed SD or O diets.

These results reinforce the hypothesis that the *N. oceanica* cell wall structure was differently affected by the slurry drying method allowing the lipids to be distinctly exposed to rumen microbes. Indeed, SEM images confirmed that *N. oceanica* cells in FD biomass were better preserved than SD biomass. Differential effects of spray-drying or freeze-drying on *Chlorella* and *Spirulina* microalgae biomass microstructure evaluated by SEM were previously reported (Lin, 1985). This author also observed that SD particles consisted of multicellular spheres with a void space in the middle. However, the *Chlorella* and *Spirulina* FD particles consisted in sheets of cells that were no longer spherical but adhered together in a linear fashion, which differed from what we presently found. The freeze-dried method can cause damage to cells, as intracellular water expands upon freezing (Lee et al., 2017), but this might be highly variable with the freezing conditions and with microalgae species. Nevertheless, the impact of the freeze-dried process on the cell wall structure seems to be less drastic compared with spray-dried as previously discussed (Alves et al., 2018). The changes on the surface of *N. oceanica* biomass morphology reported here evidenced that FD maintained a better overall cell structure (shape and integrity), while SD suffered more severe shape alterations, including the presence of cracks and holes. This integrity loss of SD *N. oceanica* biomass might have compromised the ability to keep the EPA inside the cell structures, exposing it to microbial metabolization. This could explain why rumen biohydrogenation of EPA with the SD treatment was much higher than FD's and similar to that found when free *Nannochloropsis* oil was used.

Ruminal biohydrogenation of EPA and DHA from several experiments with fish oil or marine microalgae averaged 80% as reviewed by Doreau and co-workers (Doreau et al., 2016). This value is similar to that found for the biohydrogenation of EPA from *Nannochloropsis* oil, which supports the evidence of the effective protection against microbial attack in the rumen offered by FD *N. oceanica* biomass. Considering that the *Nannochloropsis* sp. contains relevant amounts of *c*9-16:1 and 20:4n-6, both were also partially protected from ruminal metabolization with the FD *N. oceanica*. So, the ruminal biohydrogenation of 20:4n-6 was lowest in FD group and highest in lambs fed *Nannochloropsis* free oil. The highest proportion of 20:2 and 20:3 intermediates, likely to be formed from biohydrogenation of EPA and AA, was found in the O group. These results suggest that in oil, FA are more exposed to rumen microbes than in SD and FD *N. oceanica* biomass. Moreover, the formation of a large range of C20 intermediates from the ruminal biohydrogenation of unesterified EPA using deuterated (d5-20:5n-3) (Alves et al., 2018) and non-deuterated forms (Toral et al., 2017) was already demonstrated *in vitro*.

An efficient lipid-protection supplement needs to allow lipid release during abomasal passage and further digestion and absorption in the intestine (Gadeyne et al., 2017). We did not evaluate microalgae cell disruption in the abomasum, but the highest proportion of EPA in the abomasal digesta was found in FD *N. oceanica* fed animals, similar to what was observed in the ruminal digesta. Also, the proportions of total SFA, MUFA and PUFA in abomasal digesta were in the same range to those found in the rumen content. Indeed, the FA composition of abomasal contents has been shown to remain similar to that leaving the rumen (Alves et al., 2017) because medium- and long-chain FA are minimally absorbed or modified in the omasum or abomasum (Doreau & Ferlay, 1994). Additionally, the acid environment at abomasum should help disruption of *N. oceanica* cell walls and promote intracellular lipids to get released. In fact, low pH solution was reported to increased porosity of cell wall and help lipid extraction from several microalgae, including *Nannochloropsis* sp. (Melanie & Fithriani, 2019). This microalgae cell disintegration will allow the digestion and absorption of EPA and other nutrients in the intestine. However, the high levels of EPA in both cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine.

Compared to the literature, our estimates of post ruminal apparent digestibility of EPA are quite low for SD (33%) and FD (51%) treatments but similar to previous reported values for O treatment (66%) (Wachira et al., 2000). In sheep, the apparent intestinal digestibility of EPA supplied by fish oil ranged between 73 and 89% (Wachira et al., 2000). Moreover, Doreau et al. (2016), revising the literature, found an average apparent intestinal digestibility of EPA of 80%. Our figures are only crude estimates but clearly point to a large difference between EPA *post* ruminal apparent digestibility of *N. oceanica* biomass compared to free oil, which is consistent with the protection against EPA metabolization in the rumen. However, contrary to what could be expected the post ruminal apparent digestibility was lower with SD than with FD biomass. Thus, despite the EPA in the SD particles being more exposed to rumen metabolism than in FD particles, after rumen passage, the EPA remaining in the SD particles seems less available to intestinal digestion than that remaining on FD particles.

Dietary supplementation of EPA or DHA-enriched products, especially from marine origin, affects the ruminal biohydrogenation of both 18:2n-6 and 18:3n-3 by disturbing the rumen microbial population and inhibiting the final biohydrogenation reductive step, resulting in the accumulation of *trans*-18:1 isomers and reduction of 18:0 in the rumen (AbuGhazaleh & Jenkins, 2004b; Boeckaert et al., 2008b; Kim et al., 2008). Indeed, several studies reported that dietary supplementation of fish oil or microalgae lipid extracts decreased the proportion of 18:0 and increased the *trans*-18:1 in the rumen and in the duodenal flows (Kitessa et al., 2001; Kim et al., 2008; Lee et al., 2008; Toral et al., 2012). Consistently with the literature, the proportion of 18:0 in the rumen was highest in the C treatment and decreased in *Nannochloropsis* fed lambs (Table 2.3), even when expressed in percentage of total C18 FA. However, in SD fed lambs, neither the 18:0 nor C18 biohydrogenation completeness in the rumen differed from the C group. *In vitro* incubations with unesterified DHA or DHA-microalgae suggested that the DHA is the active component that promotes incomplete biohydrogenation of C18 PUFA (Boeckaert et al., 2007b) and this might also be extrapolated to EPA (Toral et al., 2017). Thus, inhibition of the completeness C18 biohydrogenation is expected to be directly related to EPA release in the rumen, being lowest in the more protected FD biomass, intermediate in SD biomass and highest in the free oil. However, our results do not fit this pattern, as this inhibition of the last reductive step of the biohydrogenation was highest for both O and FD and lowest for SD, suggesting that other factors besides the amount of EPA released in the rumen might modulate this effect. Factors like the type of metabolites formed, and the total PUFA concentration might have a role in disturbing the bacteria responsible for the last hydrogenation step to 18:0 (Kitessa et al., 2001).

Several individual C18 BI differed among treatments, with a particular interest in *t*11-18:1 and *t*10-18:1. The *t*11-18:1 is often the main *trans*-18:1 to accumulate in the rumen of animals fed forage-based diets but occasionally, a shift toward the formation of *t*10-18:1 at the expense of *t*11-18:1 (*t*10-shift, with *t*10/t11-18:1 ratio > 1) is observed, particularly in high-concentrate diets supplemented with vegetable oils and/or low rumen pH (Bessa et al., 2015). In the present experiment, the *t*10-shift was evident (i.e., *t*10/*t*11-18:1 ratio ≈ 7.61) in C treatment. As there were no differences in rumen pH (≈ 5.9) among treatments and the basal diet was the same of the C, it was expected that *t*10-shift observed in the C was also present in the *Nannochloropsis* fed animals. But surprisingly, lower *t*10/*t*11-18:1 ratio was observed in animals fed the *Nannochloropsis* dried biomasses compared to C diet, while *Nannochloropsis* sp. extracted oil did not differ among groups but showed a large individual variability. Large individual susceptibility of lambs and bulls to the *t*10-shift has been often observed, although not well understood (Rosa et al., 2014; Bessa et al., 2015). Lipid supplementation often favours the *t*10-shift, particularly when supplementing low-forage diets (Bessa et al., 2005; Alves et al., 2013). Marine lipids supplementations have been identified as one of the triggers of milk fat depression in dairy cows, which in great part could be explained by the rumen *t*10-shift occurrence (Dewanckele et al., 2020b). Nevertheless, the effect of marine lipids is often determined by the composition of typical dairy or finishing diets. Data on supplementation of high-forage, with marine lipids are more scarce and do support that marine lipids are not relevant as inducer of the *t*10-shift (Lee et al., 2008; Alves et al., 2019). The notable decrease in the *t*10/*t*11-18:1 ratio with the inclusion of *N. oceanica* biomass was due to a large increase of *t*11-18:1 accumulation and tended to decrease the accumulation of *t*10-18:1, indicating that *N. oceanica* biomass was effective in deviating the C18 biohydrogenation pathways toward the *t*11-18:1 production. A potential explanation for such positive effect of *N. oceanica* biomass in mitigating the *t*10-shift could be linked to the additional vitamin E added in the *Nannochloropsis* diets. Indeed, vitamin E has been suggested to inhibit the *t*10-shift (Juárez et al., 2010), but such preventive effect was not confirmed in beef cattle or lambs, as evaluated by *trans*-18:1 deposition in the tissues (Mapiye et al., 2012; Berthelot et al., 2014). Moreover, the O diet also included the same additional vitamin E content than SD and FD diets and did not offer such a clear mitigation of the *t*10-shift, suggesting a direct effect of *N. oceanica* biomass. The occurrence of the *t*10-shift in finishing ruminants is a major constraint of the strategies based on lipid supplementation designed to improve the nutritional value of meat (Bessa et al., 2015), and this promising effect of *N. oceanica* biomass might constitute a clue towards a novel approach to reduce *t*10-shift occurrence in animals fed high-concentrated diets.

Other major C18 BI were affected by treatments as the coeluted peak of *t*11,*c*15-/*t*10,*c*15-18:2 that was higher in lambs fed O and FD diets than in those fed C and SD diets. Both *t*11,*c*15-18:2 and *t*10,*c*15-18:2 are intermediates from the biohydrogenation of 18:3n-3 (Alves & Bessa, 2014), thus the low levels in the rumen of animals fed SD might be explained by the lower biohydrogenation estimated for 18:3n-3 compared with the other diets. The low levels in C group might be explained by the high C18 completeness, as previously discussed. Indeed, supplementation with microalgae has been reported to favour the accumulation of *t*11,*c*15-18:2 in rumen fluid of cows (Boeckaert et al., 2008b) and ewes (Toral et al., 2012). Other studies also reported that fish oil increases the ruminal outflow of *t*11,*c*15-18:2 (Lee et al., 2008; Shingfield et al., 2010b) due to incomplete biohydrogenation of 18:3n-3.

No differences among treatments were found for 18:2n-6 or for its estimated biohydrogenation. Thus, contrasting to what was observed for 18:3n-3, diets did not affect the initial steps of 18:2n-6 biohydrogenation. Similar results have been observed on the *in vitro* incubations with rumen fluid and SD or FD *N. oceanica* (Alves et al., 2018) Other authors also did not find effects of dietary microalgae inclusion on the concentration of 18:2n-6 in the rumen (Boeckaert et al., 2008b; Or-Rashid et al., 2008; Zhu et al., 2016).

Globally, our results are consistent with the evidence that supplementation with marine lipid sources rich in long-chain PUFA affects the biohydrogenation of C18 PUFA. However, the differences in C18 biohydrogenation intermediates among treatments indicate changes in the predominance of competing biohydrogenation pathways and eventually of the rumen microbiota. Although complete rumen microbiome analysis is not available, protozoal counts showed that many lambs were defaunated. However, because defaunation was observed in animals from both control and *Nannochloropsis* groups, any potential toxic effect of the microalgae was excluded, also the basal diet was a common diet as presented in Table 2.1. Rumen bacteria are known to play the main role in biohydrogenation; however, the relationship between rumen ciliate protozoa and biohydrogenation of C18 FA has been reported (Yáñez-Ruiz et al., 2007; Francisco et al., 2019b).

Incorporation of supplements containing very long-chain PUFA, as those supplied by fish oil or microalgae, can affect rumen fermentative activity often increasing propionic acid molar proportion (Fievez et al., 2003a). In our experiment, none of diets containing *Nannochloropsis* sources affected the rumen fermentation parameters, probably because the amount incorporated in the diet was not high (1.2% of oil or microalgae biomass supplying equivalent amount of EPA). Nevertheless, other authors reported increases on VFA and propionate in goats fed with dietary inclusions of *Nannochloropsis oculata* biomass as low as 0.5% (Kholif et al., 2020). The effects of *Nannochloropsis* microalgae on rumen fermentation might be dependent on the type of basal diet as reported for *Nannochloropsis salina*, that induced larger effects on rumen fermentation in continuous flow fermenters when fed with forage basal diet and minor effect when fed with a concentrate basal diet (Lodge-Ivey et al., 2014). Thus, differences in experimental conditions, including animal species, basal diets, or even in intrinsic differences in the nutrient composition and cell wall structure of each *Nannochloropsis* species might result in different effects on rumen fermentation.

In south-western Europe, light lambs are usually finished for few weeks after weaning with high-energy diets and slaughter with up to 25 kg of liveweight. In these conditions a high incidence of rumen mucosa lesions including ruminal parakeratosis are observed and thus we routinely evaluate the rumen mucosa lesions in our lamb experiments (Costa et al., 2017; Santos-silva et al., 2019; Francisco et al., 2020). Parakeratosis of rumen mucosa is characterized by an accumulation of layers of keratinized, nucleated squamous epithelial cells and excessive sloughing of the epithelium, increased thickness of the *stratum corneum* and consequent dark brown coloration of the mucosa (Tamate & Kikuchi, 1978; Steele et al., 2009; Benavides et al., 2013). In the present study, none of the histological parakeratosis indicators nor colour greyscale values differed among treatments, which indicates the absence of parakeratotic lesions. Despite neither the histometric parameters nor the pH differed among diets ($p > 0.05$), a negative Pearson correlation between *stratum corneum* thickness and ruminal pH was found ($r = -0.38$, $p = 0.043$). The inclusion of 35–40% of dehydrated alfalfa in lamb diets could have contributed to the lack of differences, although rumen lesions in lambs have been reported using similar diets (Santos-silva et al., 2019). These results indicate that dietary inclusion of *Nannochloropsis* biomass in lamb finishing diets does not influence the occurrence of ruminal wall lesions.

2.5. Conclusion.

We conclude that the drying method applied to *N. oceanica* strongly influences powder architecture and cell wall integrity, and consequently the degree of EPA protection against rumen microbes. Indeed, we confirmed that freeze-drying has an advantage over spray-drying in preserving *N. oceanica* cell wall. Thus, FD *N. oceanica* can constitute a better source of ruminal protected-EPA comparing to SD *N. oceanica*, once higher EPA levels were found in the rumen and abomasum, indicating a better escape against ruminal biohydrogenation. However, EPA was also found in cecum content and faeces, suggesting that its absorption at the small intestine was not totally efficient.

Moreover, the supplementation of high-concentrated diets with *Nannochloropsis* microalgae affected the biohydrogenation of C18 fatty acids. The most notable effect was the deviation from the *t*10 biohydrogenation pathways to the *t*11 pathways, resulting in the higher abundance of *t*11-18:1 over *t*10-18:1 in the rumen of lambs fed *N. oceanica*. Also, at this level of *Nannochloropsis* incorporation, no disturbances were found in fermentable parameters nor ruminal parakeratosis indicators. Further studies need to address if the ruminal microbiome was affected by the different treatments and if EPA was indeed successfully deposited in the lambs' meat and edible fats.

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Author contributions

S.P.A. and R.J.B.B. conceptualization and funding acquisition. A.C.M.V., R.J.B.B., S.P.A., S.A.H. and J.S.S. implemented the experiment. J.L.S. provided the microalgae. A.C.M.V., A.E.F., and J.S.S. conducted the animal experiment. A.C.M.V. and S.P.A. conducted laboratory analysis. M.P. monitored and assisted histopathological analyses. A.C.M.V., R.J.B.B., and S.P.A. performed data analysis. A.C.M.V., R.J.B.B., S.P.A. and S.A.H. interpretation of results. A.C.M.V. drafted the manuscript. S.P.A. and R.J.B.B. edited and revised the original draft. All authors revised and approved the final manuscript.

3. CHAPTER 3 | The ruminal bacteriome of lambs supplemented with *Nannochloropsis oceanica* microalga.

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Abstract.

Marine microalgae have been exploited with the aim of being a future source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) in the diet of ruminants. The enrichment of ruminant meat in health-beneficial eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) can constitute a positive contribution of red meat to meet human EPA and DHA dietary requirements. A few studies have evaluated the efficacy of n-3 LC-PUFA enhancement in lamb tissues after microalgae-supplemented diets but solid results on the associated changes in the ruminal bacteriome are largely absent from the literature, therefore this study focused on this aspect. Merino Branco lambs were fed four different pelleted diets, with one being the control diet (C – diet with no *Nannochloropsis* sp.), and three diets supplemented with *Nannochloropsis* sp. microalga adjusted to the same EPA content. Ruminal content was collected, and bacterial 16S rDNA was sequenced. No major differences were found between diets regarding genera abundances and diversity indexes. Although no correlations were found between EPA and bacterial genera, positive and negative correlations were found between bacterial genera and the main biohydrogenation intermediates (BI). Positive associations were also found between bacterial genera and branched-chain fatty acids and dimethylacetals. We concluded that EPA supplementation in lambs' feed can positively impact meat quality without compromising the overall ruminal bacterial abundances and diversity. Moreover, we found that, when considering the main BI, *t*10-18:1 is more clearly associated to a distinct bacteriome profile in the rumen when compared with *t*11-18:1.

Keywords: microbiome; n-3 fatty acids; rumen; eicosapentaenoic acid.

3.1. Introduction.

Marine microalgae are the primary producers of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) (Ma et al., 2016). These FA have been receiving growing interest, especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which are associated with numerous human health benefits e.g., prevention of heart disease, hypertension, inflammation, cancer, neurological disorders and other (Punia et al., 2019). In order to take advantage of the beneficial health effects of n-3 LC-PUFA from a consumer perspective, inclusion of microalgae in animal diets is considered as a means to enrich the human health properties in animal edible products (Madeira et al., 2017). A few have evaluated the efficacy of n-3 LC-PUFA enhancement in lamb tissues after the animals were fed microalgae-supplemented diets (De la Fuente-Vázquez et al., 2014; Meale et al., 2014; Ponnampalam et al., 2016; Urrutia et al., 2016; Díaz et al., 2017; Valença et al., 2021). Among the marine microalgae, *Nannochloropsis* is of particular interest due its content of PUFA (mainly EPA) (Zanella & Vianello, 2020). Supplementation of lambs with the microalga *Nannochloropsis oceanica* has been shown to increase n-3 LC-PUFA content across the gastrointestinal tract (Vítor et al., 2021) and in the meat and edible fat (Vítor et al., 2023).

The major constrains to the strategy of enhancing n-3 LC-PUFA in ruminant tissues is the occurrence of ruminal biohydrogenation (RBH) of unsaturated fatty acids (UFA). RBH involves extensive isomerization, hydration, or hydrogenation of non-esterified UFA, usually C18 FA, with the formation of partially saturated intermediates and fully saturated FA decreasing the availability and deposition of PUFA in the tissues (Bessa et al., 2015). RBH is a process under the control of rumen bacteria and therefore n-3 LC-PUFA can modulate RBH pathways and their FA products through changes in the associated populations of ruminal microorganisms (Kim et al., 2008; Dewanckele et al., 2018). Indeed, microbial toxicity associated to n-3 LC-PUFA can suppress the numbers of C18 UFA biohydrogenating ruminal bacteria (Maia et al., 2007), with EPA and DHA the having strongest toxicity (Abughazaleh & Jenkins, 2004a; Boeckaert et al., 2008a; Carreño et al., 2019). The impairment of the complete RBH process results in the accumulation of RBH intermediates like 18:1 isomers, particularly *t*11-18:1, but with an occasional shift towards the formation of *t*10 intermediates (*t*10-shift) (Bessa et al., 2015; Dewanckele et al., 2018). The *t*10-shift occurrence is primarily associated to animals with concentrate-based dietary regimens (Bessa et al., 2015) and the high content of *t*10 intermediates in the animals edible tissues might have potential detrimental effects for the human consumer (Ip et al., 2007; Tricon et al., 2004; Gebauer et al., 2011). The *t*10-shift is expected to be associated with a correspondent ruminal microbiome shift (Enjalbert et al., 2017; Dewanckele et al., 2020b), and there is a suggestion that this shift might result from a dysbiosis in favour of *t*10-producing bacteria at the expense of those producing *t*11 or a modification of bacterial activities (Zened et al., 2013b). However, the microbial etiology of the *t*10-shift is not well understood yet (Dewanckele et al., 2020b).

Rumen microbiota composition variations are currently evaluated by 16S DNA sequencing, but nevertheless certain microbial lipids such as plasmalogenic and odd- and branched-chain fatty acids (OBCFA) that are components of cell membranes can be used as microbial markers and also reflect changes in the microbiota associated to specific dietary regimens (Vlaeminck et al., 2006; Bessa et al., 2009; Alves et al., 2013).

In this study we investigated the effects of feeding Merino lambs *Nannochloropsis* sp. microalga on the gastrointestinal tract (GIT) microbiome using 16S rDNA metataxonomy.

Despite the well-established effects of n-3 LC-PUFA on RBH, we found only minor differences in C18 RBH, total BCFA and dimethyl acetals (DMA) when lambs were supplemented with *Nannochloropsis* oil, spray-dried and freeze-dried biomass, although the major differences between the major RBH intermediates (i.e. *t*10-18:1 and *t*11-18:1) were present (Vítor et al., 2021). Moreover, a high individual variability regarding the abundance of *t*10-18:1 and *t*11-18:1 (*t*10-shift) was observed, which should reflect differences in rumen microbiota. The purpose was to ensure that whilst feeding *Nannochloropsis* sp. has been shown to enhance the long-chain PUFA content in the lambs' GIT, that it does not cause any detrimental effects on the GIT microbiome, which may in turn cause health challenges. Thus, the present work aims to evaluate the impact of feeding lambs with *N. oceanica* supplemented diets on ruminal bacteria abundances, and to study possible correlations between ruminal bacteria and the ruminal profile of C18 biohydrogenation intermediates.

3.2. Material and Methods.

3.2.1. Animal management and diets.

Detailed information about ingredients, chemical composition, and fatty acid profile of the experimental diets is described in Vítor et al. (2021). The lambs were fed four different pelleted diets, being one the control diet (C - diet with no *Nannochloropsis* sp.), and three diets supplemented with *Nannochloropsis* sp. microalga adjusted to the same EPA content. These comprised the O diet (control diet plus 1.2% of *Nannochloropsis* sp. oil), SD diet (control diet plus 12% of spray dried *Nannochloropsis oceanica*), and FD diet (control diet plus 9.2% of freeze-dried *Nannochloropsis oceanica*). The current lamb trial was conducted in compliance with the ARRIVE and international guidelines, in certified facilities and was approved by an ethical and animal well-being commission, as fully detailed by Vítor et al. (2021). All animal handling and slaughter were conducted under the standard commercial practices regarding animal welfare, except the fact that animals were individually housed during the experiment. Briefly, twenty-eight Merino Branco ram lambs with approximately 60 days of age and weighting 21.8 ± 4.4 kg on average, were transported to INIAV – Santarém, Portugal facilities, and randomly allocated to individual pens with free access to clean water. The allocation of the animals to different groups followed the existence of 4 diet groups, composed of 7 lambs each, following a completely randomized design. The basal diet contained dehydrated lucerne, barley and soybean meal in a 60:40 concentrate:forage ratio and all 4 diets were isoproteic. The experimental period had a 3 week-duration.

3.2.2. Animal slaughter and sample collection and storage.

At the end of the trial period, lambs were weighed, without previous fasting, transported to the experimental abattoir of the INIAV - Santarém. Immediately after the slaughter, the whole rumen digestive content was collected from each lamb and frozen at -80°C. After that, samples were freeze-dried, and re-stored at -80°C for FA analysis and 16S rDNA sequencing analysis.

3.2.3. DNA extraction and 16S rDNA sequencing.

Lyophilized rumen content underwent DNA extraction using the FastDNA™ SPIN Kit for Soil (MPBio, France) followed by downstream microbiome diversity analysis using 16S rDNA metataxonomic sequencing. Samples of 50 mg of lyophilized ruminal liquid were resuspended in 500 µL of sterile PBS and placed in 2 mL tubes. DNA extractions were carried out using the FastDNA™ SPIN Kit for Soil following manufacturers' instructions, with slight adaptations. Sample's pellets were washed in PBS twice (ThermoScientific, microCL 21R) for 5 minutes at 21.1 g and 4ºC. Between each bead-beatings (FastPrep24, MPBio, 3*30 s, 6 m/s) the samples were placed on ice for 2 minutes. At the binding step, samples were homogenised manually and at the end, 100 µL of Dnase/Pyrogen-Free Water (DES) was added to the column to elute the DNA. Negative controls using phosphate-buffered saline (PBS) solution were obtained from each DNA kit box used. The DNA extraction efficiency was estimated using ZymoBIOMICS™ Microbial Community Standard D6300 (ZymoBiomics) as the positive control. At the end of the extraction, DNA concentration (ng/ µL) and purity (260/280 and 260/230 ratios) were determined using a NanoDrop spectrophotometer (ThermoFisher Scientific, USA). DNA samples were normalised to 5 ng/ µL using DES and 20 µL of each sample were transferred to a 96 well plate (Bio-Ras MLL9601). Plates were foil sealed (4ti-0550), and then delivered to Genomic Core Technology Unit (GCTU) at Queen's University Belfast, where DNA sequencing was performed following the protocol outlined by the Illumina MiSeq platform except for the PCR cycles used. Essentially, amplicons of V4 region from bacterial 16S rDNA were generated by PCR. PCR run conditions were the following: 95°C for 3 minutes, 20 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and then 72°C for 5 minutes. Illumina indexes (Nextera XT index kit v2) were attached to the 16S rDNA amplicons in a second PCR round with the following conditions: 95°C for 3 minutes, 8 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and then 72°C for 5 minutes (Mcgovern et al., 2018), using the primers described by Caporaso et al. (2011), with Illumina adapters (515F/806R - overhang adapter sequences).

3.2.4. Taxonomic assignment.

The paired-end Illumina raw sequences (2 × 250 bp) were imported and taxonomically assigned using QIIME 2 software (2021.8) (Bolyen et al., 2019). The sequences were paired, trimmed, and clustered by de novo operational taxonomic unit (OTU) picking using vsearch and an identity match of 99%. Taxonomic classification of 16S rDNA amplicon sequence data was performed using BLAST against the SILVA 16S rDNA reference database (2021.4).

3.2.5. CowPI software analysis.

After using QIIME2 for OTU picking and clustering, CowPI software was used to infer function. This involved the use of a fasta formatted file of consensus/representative sequences for each OTU from the combined Global Rumen Census (GRC)/Hungate 1000 16S rDNA sequences along with table of OTU abundances from the dataset to be analysed. These were used as input to mothur (Schloss et al., 2009) or usearch (Edgar, 2010), to classify the unknown 16S rDNA sequences against the 696,451 GRC sequences and a script in R was used to sum the values for OTU with common classifications. The resultant file was converted to biom format and submitted to standard PICRUSt steps for metagenome prediction using the KO frequencies calculated for the Hungate 1000 genomes (Wilkinson et al., 2018).

3.2.6. Fatty acid methyl esters (FAME) and dimethyl acetals (DMA) analysis.

Freeze dried rumen content samples were used for direct transesterification by reaction with 0.5 M sodium methoxide in methanol at 50°C for 15 min, followed by addition of 1.25 M hydrogen chloride in methanol at 80°C for 20 min, as described in a previous work (Vítor et al., 2021). Methyl nonadecanoate (1 mg/mL) was added as internal standard. Both FAME and DMA were analysed by GC-FID using a Shimadzu GC 2010- Plus (Shimadzu, Kyoto, Japan) equipped with an SP-2560 (100 m×0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA, USA) capillary column.

The chromatographic conditions were the same as the ones described in a previous work (Vítor et al., 2021). To identify fatty acid methyl esters (FAME) and dimethyl acetal (DMA) compounds, a comparison of the FA retention times was conducted using commercial standards (FAME mix consisting of 37 components from Supelco Inc., Bellefont, PA, USA) and published chromatograms (Alves et al., 2013). Additionally, confirmation of FAME and DMA was achieved using electron impact mass spectrometry via a Shimadzu GC-MS QP2010 Plus

(Shimadzu, Kyoto, Japan). The chromatographic column and GC conditions were consistent with those utilized in the GC-FID analysis.

3.2.7. Statistical analysis.

For the calculation of bacterial abundances and diversity indexes, OTU read counts were normalized to the minimum count. Diversity indexes were determined using Vegan package from R (Oksanen et al., 2022). Differences in indexes between treatments were determined using PROC MIXED by SAS. Unknown bacterial genera, uncultured and misidentified genera were discarded and bacterial genera with more than 22 zeros (more than 22 samples without presence of certain genera) were also discarded. Data were converted into proportions. Bacterial abundances, FA and CowPI pathways data were normalized and transformed using the function logratio.transfo from mixOmics package from R. Statistical differences between bacterial abundances and FA, regarding different treatments, were performed using PROC MIXED by SAS. *P*-values adjustments for multiple comparisons were made with P.adjust function from R considering the BH method (Benjamini & Hochberg, 1995). Correlations between ruminal bacterial genera and FA, as well as between DMA and BCFA and CowPI pathways were made using Spearman correlations using MetaboAnalyst 5.0. A Principal Component Analysis (PCA), a Sparse Partial Least Squares Discriminant Analysis (sPLSDA) and a Spearman correlation heatmap were also performed using MetaboAnalyst 5.0. Significant results registered a p-value < 0.05.

3.3. Results.

3.3.1. Phyla, families, and genera distribution in the rumen.

The rumen bacterial diversity of the 28 lambs was explored with a principal component analysis (PCA), using the 95% most abundant genera read counts, clustered by diet. As can be seen in the Figure 3.1, PC1 and PCA2 carry respectively 19.8% and 13.2% of the variance of the data and there was no major evidence of diet-based clusters, especially in the case of the C, O and FD diets, showing a common shared microbiome composition regardless of the treatment. The SD diet cluster was the one showing less overlapping with the remaining ones, illustrating less shared bacterial genera in its bacteriome.

Figure 3.1-Cluster PCA of the 95% most abundant genera in the rumen.
Cluster PCA figures illustrate the distribution of the 28 lambs according to the abundance of the 95% most abundant genera in the rumen, clustering by diet. The counts were normalized to the minimum. Analysis was made using res.pca and fviz_pca_ind by R. All 28 animals were included in the data. Different clusters are defined with different diets: C (control) – light blue, O (oil) - purple, SD (spray-dried *Nannochloropsis oceanica*) – light pink, FD (freeze-dried *Nannochloropsis oceanica*) - red.

In the rumen, 95% of the sequences had a phred score between 25 and 35, the other 5% ranged between 20 and 35. Before normalization, average hits by sample was 55500. In the ruminal fluid samples, a total of 17 phyla were identified (Figure 3.2). The most abundant phyla were Firmicutes (59%), followed by Bacteroidetes (30%), Proteobacteria (5%) and Actinobacteria (4%). A total of 103 families were identified (Figure 3.3). The most abundant families were *Prevotellaceae* (26%), followed by *Lachnospiraceae* (26%), *Ruminococcaceae* (7%), *Succinivibrionaceae* (5%) and *Erysipelatoclostridiaceae* (4%). A total of 209 bacterial genera were identified and 50 of them represented the 95% most abundant ones (Figure 3.4). The least abundant of the 95% most abundant genera was *Erysipelotrichaceae_UCG-007* with 0.2% abundance. Fourteen of the 50 genera presented an individual abundance > 2%. *Prevotella* was the most abundant genus (24%), followed by *Lachnospiraceae_NK3A20_group* (10%), *Ruminococcus* (6%), *[Ruminococcus]_gauvreauii_group* (5%), *Erysipelotrichaceae_UCG-002* (4%), *Acetitomaculum* (3%), *Succinivibrio* (3%) and *Olsenella* (3%). *Prevotella* and *Lachnospiraceae_NK3A20_group* were the most abundant genera in all 4 diets.

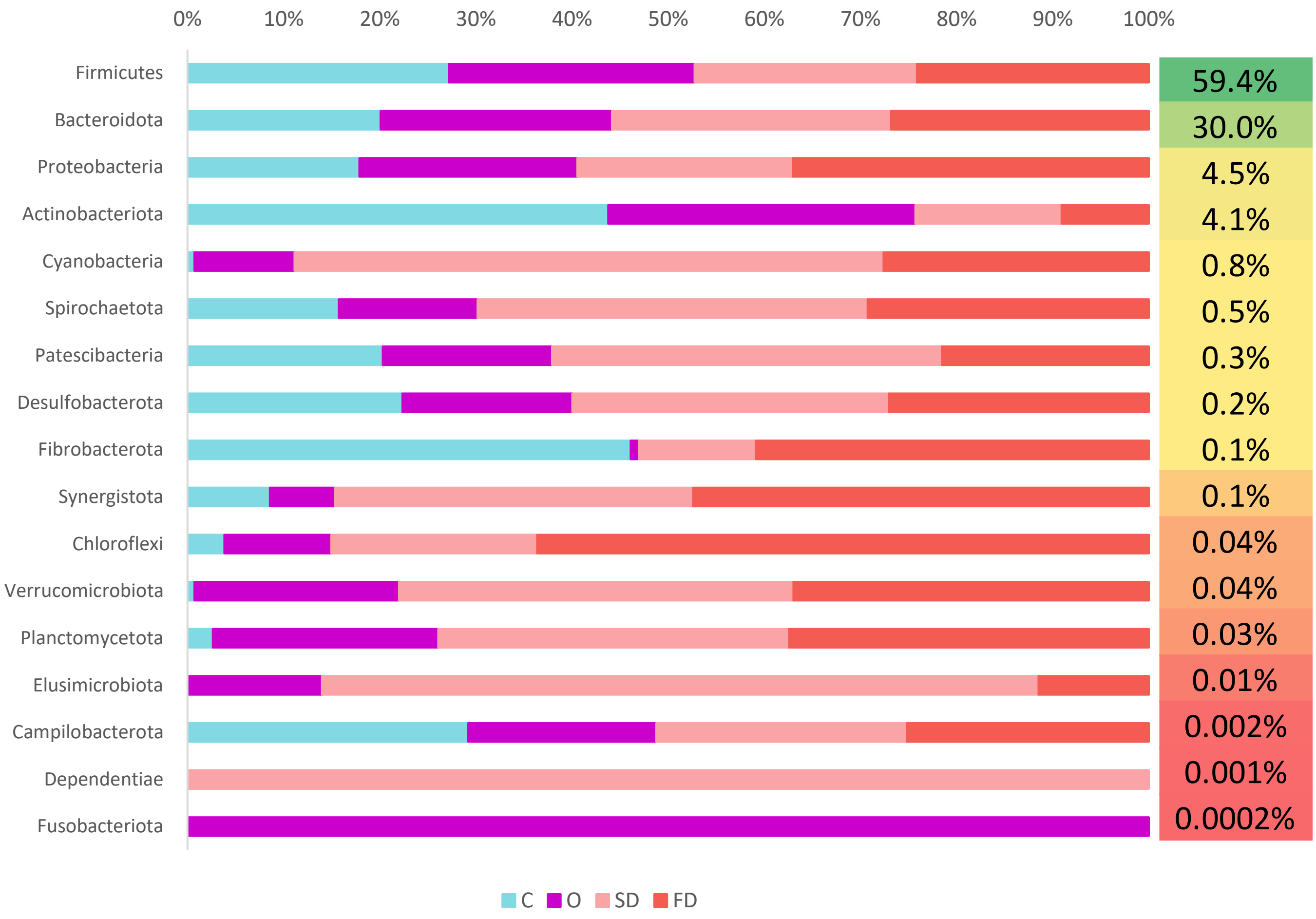

Figure 3.2- Phyla abundances in the rumen.

The read counts were normalized to the minimum. Unknown phyla were removed from the data. All 28 animals were included in the data. Diets are the following: C (control) – light blue, O (oil) - purple, SD (spray-dried *Nannochloropsis oceanica*) – light pink, FD (freeze-dried *Nannochloropsis oceanica*) - red. The most abundant phylum starts from the top of the figure. A colour-scaled bar is displayed on the left side of the graph indicating the relative abundance of each phylum.

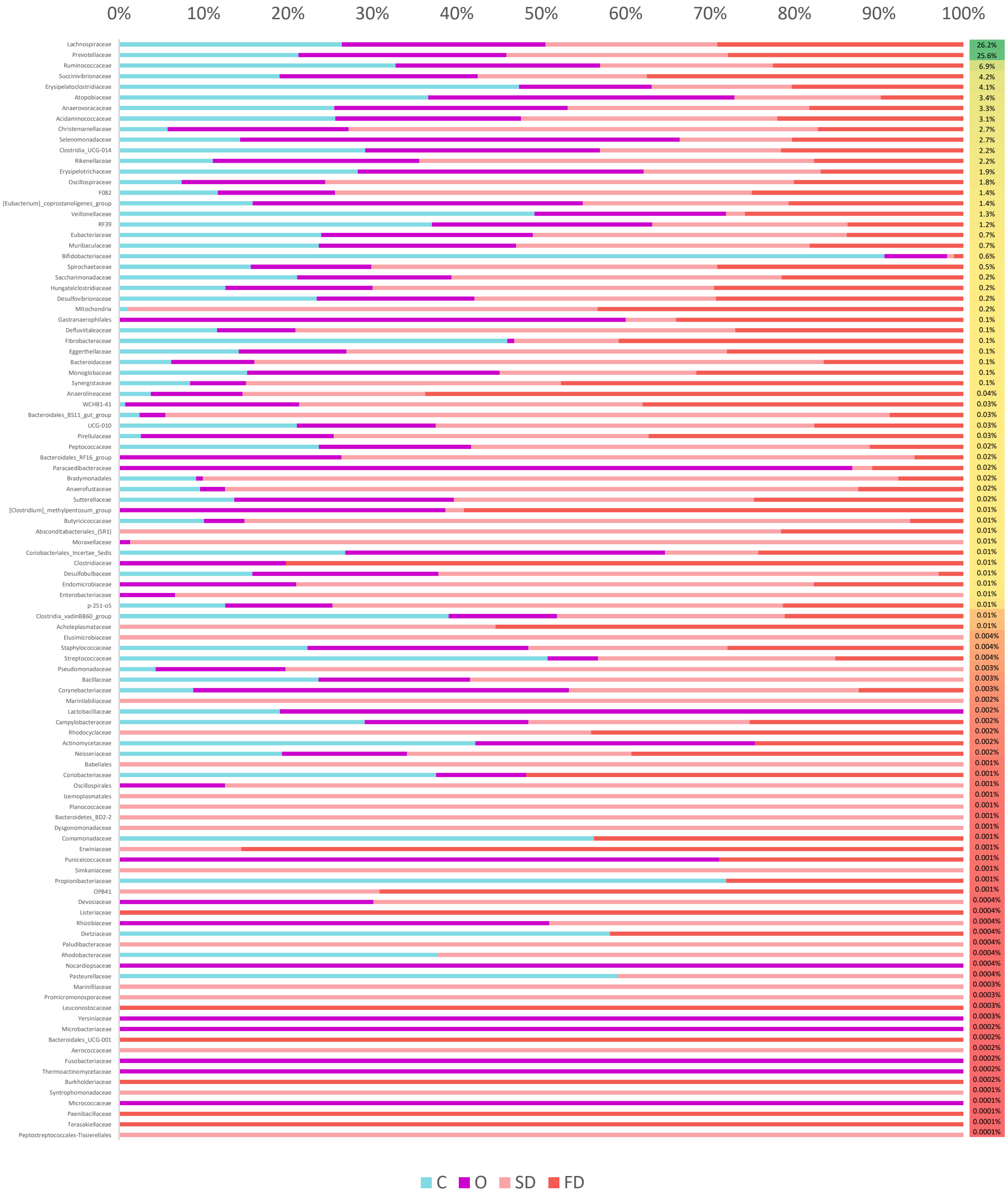

Figure 3.3- Families abundances in the rumen.

The counts were normalized to the minimum. Unknown and uncharacterized families were removed from the data. All 28 animals were included in the data. Diets are the following: C (control) – light blue, O (oil) - purple, SD (spray-dried *Nannochloropsis oceanica*) – light pink, FD (freeze-dried *Nannochloropsis oceanica*) - red. The most abundant family starts from the top of the figure. A colour-scaled bar is displayed on the left side of the graph indicating the relative abundance of each family.

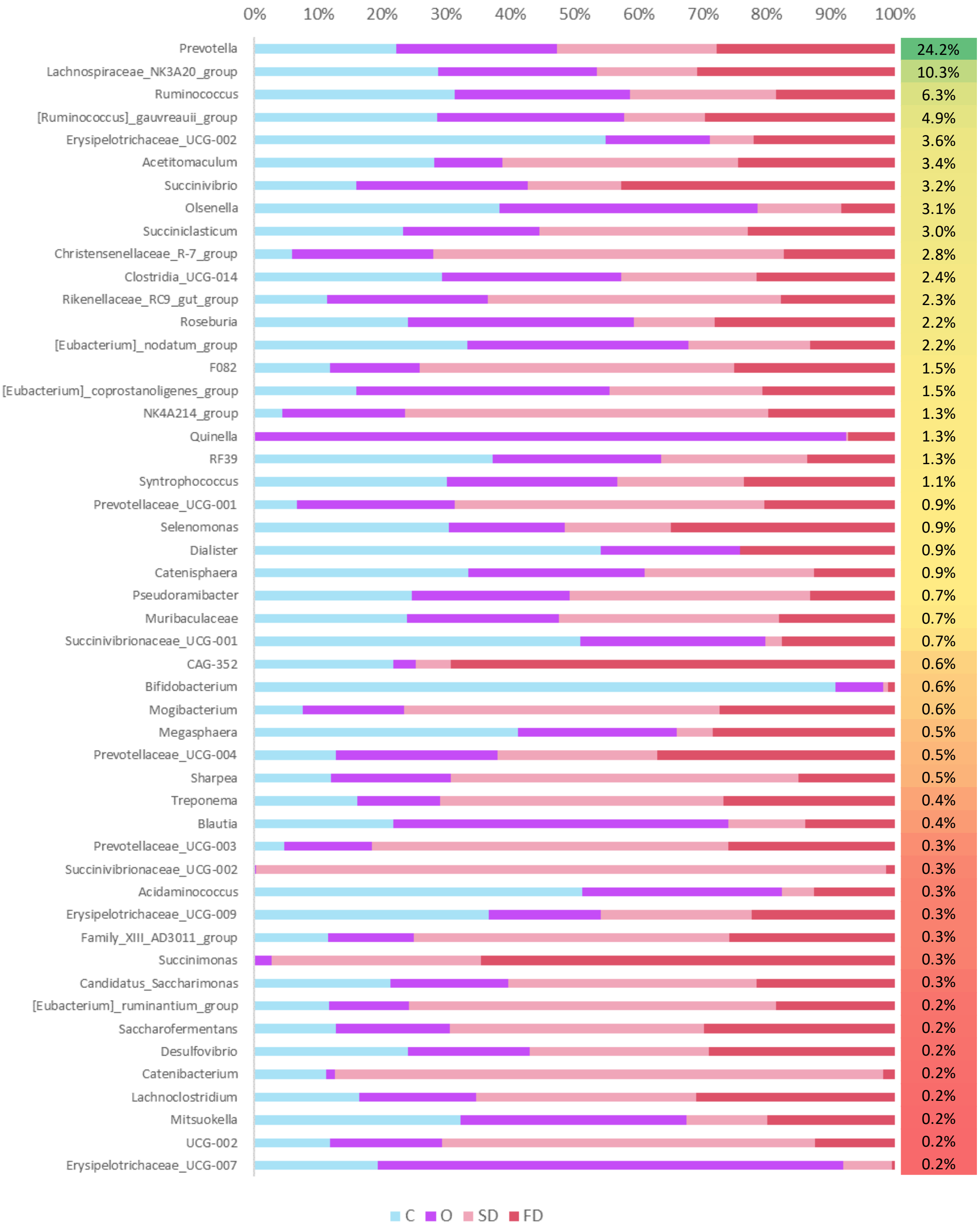

Figure 3.4- 95% Most abundant genera in the rumen.

The read counts were normalized to the minimum. Unknown genera, uncultured genera and misidentified genera were removed from the data. All 28 animals were included in the data. Diets are the following: C (control) – light blue, O (oil) - purple, SD (spray-dried *Nannochloropsis oceanica*) – light pink, FD (freeze-dried *Nannochloropsis oceanica*) - red. The most abundant genera start from the top of the figure. A colour-scaled bar is displayed on the left side of the graph indicating the relative abundance of each genus.

3.3.2. Differences in genera abundances and diversity indices between diets in the rumen.

From 125 selected genera in the rumen, only one significant difference was found between treatments (p = 0.025) being it for *Gastranaerophilales,* that was significantly higher in the O treatment (1.71) when compared to the remaining ones (-1.75) (Table 3.1).

Table 3.1- Differences in genera abundances between diets in the rumen.

Genus	Diets				SEM	*p*-value
	C	O	SD	FD		
[Anaerorhabdus]_furcosa_group	-1.39	-1.66	-0.48	-1.33	0.489	0.626
[Eubacterium]_coprostanoligenes_group	3.39	4.24	3.21	3.24	0.318	0.427
[Eubacterium]_eligens_group	0.38	0.04	-2.29	-1.13	0.956	0.526
[Eubacterium]_hallii_group	-1.39	-0.17	0.91	0.15	0.595	0.402
[Eubacterium]_nodatum_group	4.57	4.22	3.26	3.24	0.356	0.252
[Eubacterium]_ruminantium_group	-0.20	-0.11	1.38	0.45	0.703	0.627
[Eubacterium]_ventriosum_group	-2.06	-0.83	-2.11	-1.23	0.616	0.632
[Eubacterium]_xylanophilum_group	-1.66	-1.40	-1.58	-1.73	0.708	0.997
[Ruminococcus]_gauvreauii_group	4.97	4.80	3.28	4.50	0.504	0.427
[Ruminococcus]_torques_group	-2.00	-2.49	-1.37	-1.70	0.501	0.670
Acetitomaculum	3.7998	3.82	4.17	3.57	0.537	0.934
Acidaminococcus	2.63	0.66	-1.65	-0.60	0.836	0.224
Actinomyces	-2.21	-2.17	-2.94	-2.57	0.258	0.500
Alloprevotella	0.55	0.13	-0.88	0.20	0.625	0.662
Anaerofustis	-2.05	-2.21	-0.99	-1.89	0.429	0.524
Anaeroplasma	-2.69	-2.49	-2.22	-1.91	0.318	0.626
Anaerovibrio	-0.68	0.68	1.59	0.99	0.497	0.252
Anaerovorax	-1.29	-1.84	-0.58	-1.39	0.391	0.500
Atopobium	-0.91	-1.04	0.47	-0.90	0.589	0.552
Bacteroidales_BS11_gut_group	-2.33	-2.11	-0.98	-2.30	0.522	0.552
Bacteroidales_RF16_group	-2.69	-1.44	-1.15	-2.51	0.520	0.460
Bacteroides	-0.92	-0.57	0.91	-0.90	0.415	0.243
Bifidobacterium	0.37	-0.66	-2.30	-1.67	0.910	0.523
Blautia	1.42	1.27	0.78	0.93	0.722	0.945
Bradymonadales	-1.92	-2.39	-0.73	-2.26	0.396	0.252
Butyrivibrio	0.12	0.15	1.26	0.93	0.581	0.662
CAG-352	1.08	-0.22	0.13	0.63	0.918	0.863
Campylobacter	-2.26	-2.29	-2.59	-2.57	0.200	0.714
Candidatus_Saccharimonas	0.95	0.96	1.02	1.09	0.615	0.998
Catenibacterium	-0.07	-1.50	-0.32	-2.06	0.781	0.552
Catenisphaera	3.54	2.32	1.41	1.19	0.776	0.500
Christensenellaceae_R-7_group	2.64	3.26	4.21	3.17	0.477	0.500
Clostridia_UCG-014	4.25	3.98	3.61	3.66	0.405	0.773
Clostridia_vadinBB60_group	-1.52	-2.05	-2.25	-2.20	0.316	0.626

Colidextribacter	-0.77	-1.96	-1.38	-0.99	0.613	0.734
Coprococcus	-0.64	0.09	1.62	0.90	0.628	0.402
Corynebacterium	-2.46	-1.93	-2.58	-2.69	0.347	0.663
Defluviitaleaceae_UCG-011	-1.14	-0.27	0.73	-0.16	0.490	0.402
Desulfobulbus	-1.97	-1.84	-1.80	-2.69	0.382	0.626
Desulfovibrio	1.72	1.43	0.96	1.22	0.504	0.855
Dialister	3.57	1.11	-2.60	1.01	0.906	0.050
Dorea	-0.77	-2.13	-2.94	-2.26	0.490	0.252
Enterorhabdus	-1.49	-1.96	-1.29	-1.17	0.497	0.796
Erysipelotrichaceae_UCG-002	4.34	2.02	0.89	2.53	1.080	0.500
Erysipelotrichaceae_UCG-007	-1.39	-0.55	-1.32	-2.36	0.889	0.734
Erysipelotrichaceae_UCG-009	2.28	1.68	1.11	1.67	0.408	0.568
F082	2.72	2.10	3.89	3.04	0.398	0.252
Faecalibacterium	-1.42	-1.70	-2.94	-1.03	0.617	0.500
Family_XIII_AD3011_group	1.13	1.38	2.26	1.31	0.377	0.500
Family_XIII_UCG-001	-0.69	-0.15	-0.58	-0.97	0.394	0.733
Fibrobacter	-1.22	-2.17	-1.05	-1.25	0.657	0.767
Flexilinea	-2.23	-1.64	-1.35	-1.16	0.605	0.767
Fretibacterium	-2.16	-2.39	-2.56	-2.11	0.492	0.945
Gastranaerophilales	**-2.69^{b}**	**1.71^{a}**	**-1.36^{b}**	**-1.19^{b}**	**0.586**	**0.025**
GCA-900066575	-0.96	-1.47	-1.23	-2.02	0.335	0.500
Howardella	0.58	0.61	-0.52	-0.25	0.350	0.402
Incertae_Sedis	-1.50	-1.71	-2.05	-1.13	0.540	0.795
Lachnoclostridium	0.83	0.85	1.64	1.52	0.458	0.670
Lachnospira	-0.28	-1.44	-1.51	-1.38	0.599	0.662
Lachnospiraceae_FCS020_group	-2.41	-1.72	-2.09	-2.08	0.422	0.828
Lachnospiraceae_FE2018_group	-0.53	-0.23	0.50	0.76	0.575	0.626
Lachnospiraceae_ND3007_group	-1.96	-1.24	-2.54	-2.19	0.700	0.767
Lachnospiraceae_NK3A20_group	5.73	5.76	4.68	5.66	0.275	0.252
Lachnospiraceae_NK4A136_group	0.42	-2.29	-0.94	0.69	0.609	0.224
Lachnospiraceae_UCG-001	0.15	-1.32	-2.01	-0.85	0.685	0.500
Lachnospiraceae_UCG-002	-2.08	-2.17	-0.76	-1.25	0.515	0.500
Lachnospiraceae_UCG-006	-2.23	-1.96	-1.92	-1.83	0.351	0.930
Lachnospiraceae_UCG-008	0.12	-0.65	0.17	0.36	0.509	0.724
Lachnospiraceae_UCG-010	-1.60	-2.26	-2.25	-2.66	0.277	0.402
Libanicoccus	0.01	-0.39	-2.46	-2.16	0.482	0.084
Marvinbryantia	-1.53	-1.47	-1.10	-0.52	0.627	0.773
Megasphaera	2.49	1.00	-1.12	0.61	0.930	0.402
Mitsuokella	0.73	0.68	-1.16	-0.38	0.951	0.666
Mogibacterium	0.27	1.34	2.82	1.80	0.654	0.402
Monoglobus	-0.47	0.33	-0.84	0.29	0.555	0.626
Moryella	-1.63	-0.10	0.92	0.66	0.578	0.252
Muribaculaceae	2.26	2.72	2.26	2.15	0.465	0.913
NK4A214_group	1.60	2.48	3.55	2.79	0.431	0.252

Olsenella	4.63	4.34	3.08	3.16	0.505	0.402
Oribacterium	0.27	-0.14	0.62	0.80	0.320	0.520
p-1088-a5_gut_group	-2.18	-1.44	-1.08	-1.21	0.514	0.664
p-251-o5	-2.12	-2.12	-1.70	-2.04	0.334	0.864
Peptococcus	-1.46	-1.50	-2.01	-2.18	0.430	0.734
Prevotella	6.37	6.56	5.97	6.42	0.239	0.626
Prevotellaceae_Ga6A1_group	-2.54	-1.79	-1.13	-1.69	0.550	0.626
Prevotellaceae_UCG-001	1.22	1.92	2.86	2.55	0.617	0.565
Prevotellaceae_UCG-003	-1.09	0.48	2.01	1.24	0.629	0.243
Prevotellaceae_UCG-004	1.28	1.36	1.96	1.73	0.623	0.927
Prevotellaceae_YAB2003_group	-0.30	0.02	-0.66	0.97	0.634	0.626
Pseudobutyrivibrio	-0.22	-0.46	0.65	0.89	0.552	0.557
Pseudoramibacter	3.15	1.63	1.64	1.78	0.793	0.676
Pyramidobacter	-1.36	-1.30	0.05	-0.84	0.417	0.402
Quinella	-2.69	-0.52	-2.00	-1.20	0.925	0.632
Raoultibacter	-1.82	-1.90	-2.28	-1.96	0.273	0.773
RF39	3.54	3.57	2.63	2.73	0.403	0.534
Rikenellaceae_RC9_gut_group	3.26	3.55	4.07	3.16	0.375	0.626
Roseburia	4.03	3.54	2.04	3.51	0.720	0.557
Ruminococcus	5.09	4.72	4.17	4.07	0.579	0.734
Saccharofermentans	-0.42	0.42	1.59	0.88	0.698	0.552
Schwartzia	-0.33	0.24	-0.12	-0.24	0.551	0.934
Selenomonas	2.36	2.87	2.14	2.22	0.662	0.928
Sharpea	1.03	0.56	0.83	0.29	0.859	0.949
Shuttleworthia	-0.29	0.09	1.08	0.90	0.434	0.442
Slackia	-1.95	-2.23	-2.94	-2.47	0.307	0.500
Solobacterium	-1.86	-0.45	0.86	0.61	0.673	0.258
Sphaerochaeta	-0.44	-0.55	-1.03	-1.89	0.610	0.626
Staphylococcus	-2.14	-2.06	-2.33	-2.32	0.341	0.948
Streptococcus	-1.75	-2.33	-2.35	-2.50	0.367	0.706
Subdoligranulum	-0.57	-0.86	-2.21	-1.46	0.657	0.626
Succiniclasticum	4.48	3.69	3.91	4.07	0.428	0.767
Succinimonas	-2.69	-1.72	-1.25	-1.63	0.806	0.769
Succinivibrio	3.31	2.81	1.33	3.62	0.874	0.568
Succinivibrionaceae_UCG-001	-0.17	-1.35	-1.41	-0.82	1.105	0.925
Succinivibrionaceae_UCG-002	-2.69	-1.91	-1.34	-1.90	0.700	0.767
Sutterella	-1.87	-0.90	-1.29	-1.30	0.401	0.647
Syntrophococcus	3.70	3.42	2.69	2.93	0.336	0.500
Termite_Treponema_cluster	-2.54	-1.86	-2.50	-1.12	0.585	0.591
Treponema	-0.30	0.89	2.53	2.09	0.679	0.252
U29-B03	-1.62	-1.00	0.06	-1.03	0.482	0.461
UCG-002	0.71	0.49	1.75	0.11	0.479	0.460
UCG-004	-1.84	-1.92	-2.94	-1.68	0.418	0.500
UCG-005	0.84	0.12	1.66	0.57	0.402	0.402

UCG-009	-2.04	-2.49	-2.55	-2.37	0.291	0.767
UCG-010	-0.45	-0.76	-0.32	-1.04	0.381	0.734
Veillonellaceae_UCG-001	-2.15	-1.44	-0.23	-1.14	0.642	0.548
WCHB1-41	-2.49	-1.36	-0.43	-0.63	0.523	0.293

The counts were transformed using centred log-ratio in R. Statistical analysis was made using proc mixed by SAS. Data is presented in the format of mean ± standard error of the mean (SEM). Statistically significant results are considered for *p*-value < 0.05. All 28 animals were included in the data. Genus with different letters differ statistically and are highlighted in bold. *p*-value was correct by the BH method in R.

The genera diversity indexes evaluated (Shannon, Simpson, Inverse Simpson, Fisher Alpha, Species Number and Pielou's Eveness) did not differ between diets (Table 3.2). The beta diversity, defined as gamma/alpha -1 was higher in the O diet (0.23) and lower in the C diet (0.12). Both SD (0.18) and FD (0.17) diets presented similar values.

Table 3.2-Rumen genera diversity indexes.

Index	**Diets**				**SEM**	***p*-value**
	C	**O**	**SD**	**FD**		
Shannon	2.57	2.47	2.67	2.52	0.073	0.282
Simpson	0.86	0.85	0.87	0.85	0.014	0.847
Inverse Simpson	7.66	7.56	7.81	7.02	0.775	0.897
Fisher alpha	5.22	5.05	5.34	5.35	0.170	0.588
Species number	41.9	40.71	42.4	42.6	1.22	0.700
Pielou's evenness	0.69	0.67	0.71	0.71	0.018	0.336

All 28 animals were included in the data. The different diets are represented as follow: C (control), O (oil), SD (spray dried *Nannochloropsis oceanica*), FD (freeze-dried *Nannochloropsis oceanica*). Statistical analysis was made using proc mixed by SAS. Data is presented in the format of mean ± standard error of the mean (SEM). Statistically significant results are considered for *p*-value < 0.05.

3.3.3. Correlation between bacterial genera abundances and BH fatty acid indicators.

Spearman correlations were established between EPA and ruminal bacteria, but no significant correlations were found.

Spearman correlations were established between *t*10-18:1 and ruminal bacteria. Results were filtered by a false discovery rate (FDR) value lower than 0.05 and 55 genera were included. After, bacteria were filtered by abundance (calculated as average center log ratio (CLR) transformed value per bacteria) and bacteria with positive CLR abundance values were selected, accounting for 32 bacteria included. After, an index resulting from the multiplication of CLR abundance and Spearman correlation value was calculated and bacteria were selected for an index value greater than 0.5 and lower than -0.5. In the end 15 and 8 bacteria were selected for positive and negative Spearman correlations with *t*10-18:1 (Figure 3.5).

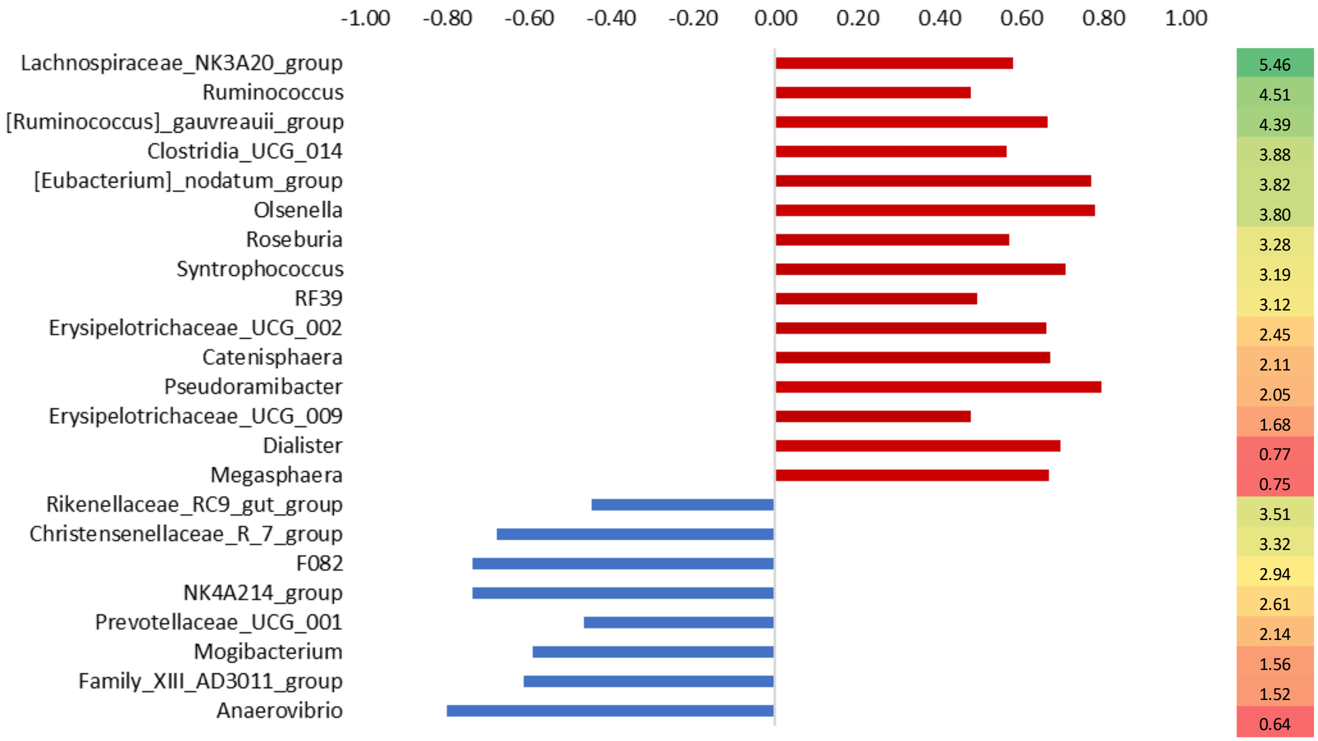

Figure 3.5- Spearman correlations between ruminal bacteria and *t*10-18:1 FA.
Only the selected 15 positive correlations and the 8 negative correlations are represented in the Figure ($p < 0.05$). Positive correlations are represented in red and negative correlations are represented in blue. On the left, CLR abundance scale-colored.

Regarding correlations between *t*11-18:1 and ruminal bacteria, after selecting bacteria with FDR lower than 0.05, no significant FDR values were found for Spearman correlations.

Regarding the *t*10-18:1/ *t*11-18:1 ratio (*t*10-shift), after filtering bacteria with FDR lower than 0.05, 56 bacteria were included and after filtered by abundance, bacteria with positive CLR abundance values were selected and 31 bacteria were included. After selecting for an index value greater than 0.5 and lower than -0.5, 15 and 7 bacteria were selected for positive and negative Spearman correlations. In total, 21 of the total 23 bacteria found were shared between *t*10-18:1 and t10-shift. However, *Desulfovibrio* was exclusively included in the relevant correlations with *t*10-shift and *Anaerovibrio* and *Erysipelotrichaceae_UCG_009* were exclusively included in the relevant correlations with *t*10-18:1.

Regarding the correlations between 18:0 and ruminal bacteria, only one significant FDR value was found, represented by *Lachnospiraceae_UCG_010* (r = 0.61). Although, this bacterium had a CLR abundance of -2.19.

3.3.4. Differences in BCFA and DMA profile between diets in the rumen.

Differences between ruminal BCFA and DMA, between different diets (mg/ g DM), are presented in the Table 3.3. As can be seen, none of the partial sums of both BCFA and DMA significantly differed between treatments. However, individual changes were registered. Regarding BCFA, both *i*-13:0 and *i*-14:0 were higher in the rumen of lambs fed FD diet (*p* = 0.002). Regarding the DMA, DMA 14:0 was higher in the rumen of lambs fed SD diet (*p* = 0.022) and DMA *c*9-18:1 was higher in lambs fed both SD and FD diets.

Table 3.3- Differences between ruminal BCFA and DMA between different diets (mg/g DM).

Lipid specie		Diets				SEM	*p*-value
		C	**O**	**SD**	**FD**		
BCFA	*i*-13:0	0.03^{d}	0.06^{c}	0.08^{b}	0.10^{a}	0.005	0.002
	i-14:0	0.16^{b}	0.17^{b}	0.20^{b}	0.30^{a}	0.020	0.002
	i-15:0	0.15	0.21	0.22	0.15	0.02	0.135
	i-16:0	0.21	0.15	0.11	0.15	0.04	0.291
	i-17:0	0.05	0.08	0.06	0.05	0.02	0.532
	a-15:0	0.74	0.85	0.52	0.55	0.09	0.110
	a-17:0	0.15	0.15	0.07	0.09	0.038	0.243
DMA	DMA *i*-14:0	0.05	0.08	0.12	0.09	0.020	0.243
	DMA 14:0	0.05^{c}	0.10^{ab}	0.17^{a}	0.15^{ab}	0.022	0.014
	DMA *i*-15:0	0.02	0.03	0.04	0.03	0.006	0.291
	DMA *a*-15:0	0.13	0.18	0.21	0.15	0.028	0.243
	DMA 15:0	0.11	0.10	0.07	0.06	0.012	0.126
	DMA *i*-16:0	0.26	0.14	0.14	0.22	0.048	0.291
	DMA 16:0	0.85	0.89	0.53	0.68	0.102	0.126
	DMA 18:0	0.06	0.03	0.03	0.03	0.006	0.051
	DMA *t*-18:1	0.15^{a}	0.10^{b}	0.09^{b}	0.10^{b}	0.015	0.109
	DMA *c9*-18:1	0.03^{b}	0.10^{a}	0.09^{a}	0.10^{a}	0.01	0.002
Partial sums	TDMA	1.71	1.75	1.48	1.61	0.672	0.679
	TBCFA	1.49	1.66	1.26	1.33	0.138	0.215
	a-BCFA	0.89	1.00	0.59	0.57	0.010	0.067
	i-BCFA	0.55	0.59	0.61	0.71	0.171	0.171

Statistical analysis was made using proc mixed by SAS. Data is represented as mean ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$). Statistically significant results are considered for *p*-value < 0.05. The different diets are represented as follow: C (control), O (oil – control plus 1.2% *Nannochloropsis* sp. oil), SD (control plus 12.3% spray-dried *Nannochloropsis oceanica* FD (control plus 9.2% freeze-dried *N. oceanica*). All 28 animals were included. Differences in BCFA (Branched-Chain Fatty Acids) and DMA (Dimethyl Acetals) in the *iso* (i) and *anteiso* (a) are represented.

3.3.5. Correlation between DMA and BCFA, and BH FA indicators.

Spearman correlations were established between EPA and DMA and BCFA. Results were filtered by FDR value lower than 0.05 and 7 significant correlations were found (Figure 3.6-A), being the one with *i*-13:0 the only positive one. Regarding the correlations established with *t*10-18:1, 13 significant correlations were found (Figure 3.6-B), being the highest the negative association between *t*10-18:1 and DMA 14:0. Regarding 18:0, 6 significant correlations were found (Figure 3.6-C), After the positive association with DMA 18:0, the highest association was found between 18:0 and DMA 15:0. Similarly to what was found for the correlations between *t*11-18:1 and ruminal bacteria, there were no significant Spearman correlations between ruminal DMA and BCFA and *t*11-18:1 FA.

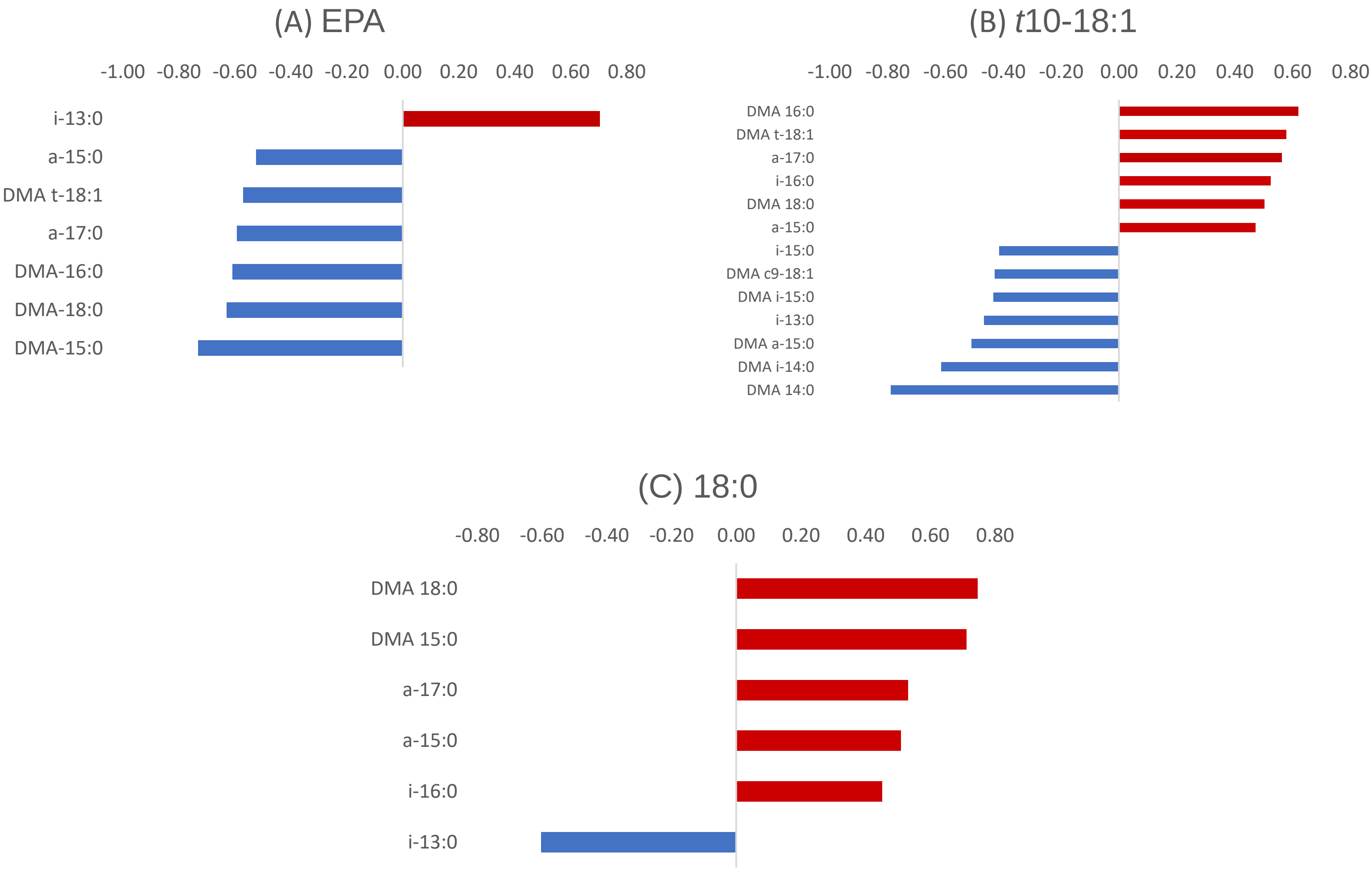

Figure 3.6- Spearman correlations between ruminal DMA and BCFA and EPA (A), *t*10-18:1 FA (B), and 18:0 (C).
Only significant correlations ($p < 0.05$) are represented in the Figure above. Positive correlations are represented in red and negative correlations are represented in blue.

Because *t*10-18:1 presented a higher number of both positive and negative Spearman correlations with both DMA and BCFA, a correlation heatmap was performed (Figure 3.7) with a highlight in Pearson correlation coefficients superior to 0.6. Stronger positive correlations were found, among other, between *Mogibacterium* and *Christensenellaceae_R_7_group*; *Anaerovibrio* and DMA 14:0; *Christensenellaceae_R_7_group* and *Anaerovibrio* and *NK4A214_group*; *F082* and DMA 14:0; *Syntrophococcus* and [*Eubacterium]_nodatum_ group*;

Olsenella and [*Eubacterium]_nodatum_ group* and *Pseudoramibacter*; and *Clostridia_UCG014* and *Catenisphaera*.

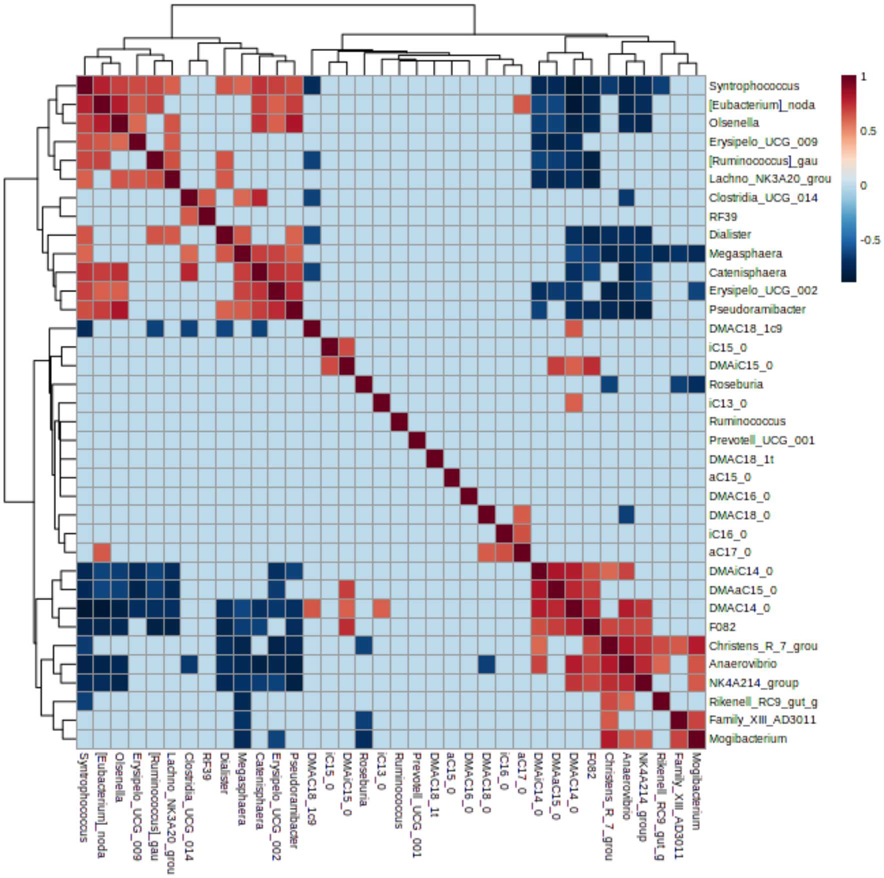

Figure 3.7- Spearman correlation heatmap with DMA, BCFA and bacteria with relevant correlations with *t*10-18:1 with a r = 0.6 cutoff.

A sparse Partial Least Squares Discriminant Analysis (sPLSDA) plot with DMA, BCFA and bacteria with relevant correlations with *t*10-18:1 FA was also performed, and it is represented in the Figure 3.8. The selected variables clearly separated all 4 diets, having SD the narrower distribution and C and O the most overlapping shapes. Component 1 explained around 19% of the variability and Component 2 explained around 29% of the variability.

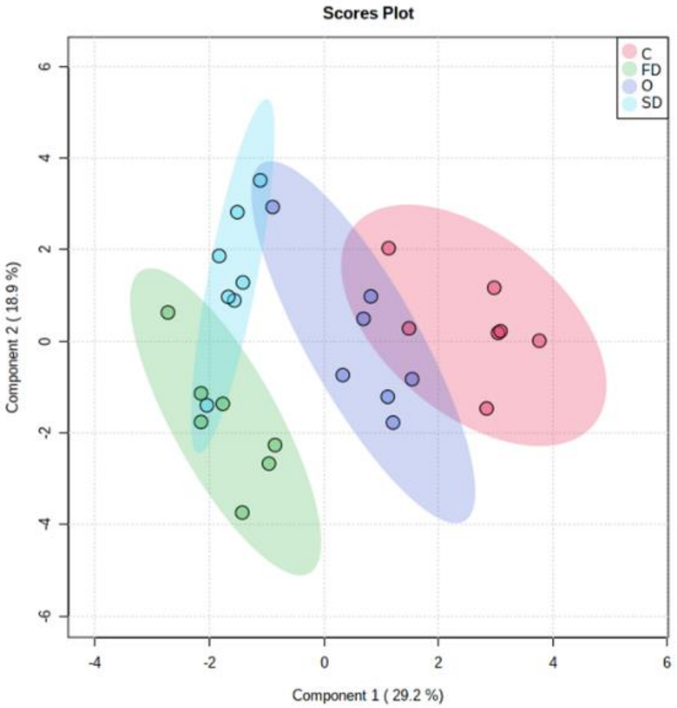

Figure 3.8- sPLSDA scores plot with DMA, BCFA and bacteria with relevant correlations with *t*10-18:1 FA.

A sPLSDA loadings plot with DMA, BCFA and bacteria with relevant correlations with *t*10-18:1 is represented in the Figure 3.9-A to C. Figure 3.9-A represents a more detailed image of the same plot. It is possible to observe a closer relationship between *i*-15:0, DMA *i*-15:0, DMA *a*-15:0 and *F082*; *Dialister*, *Lachnospiraceae_NK3A20_group* and *a*-15:0; and DMA 16:0 and *a*-17:0. In the Figure 3.9-A, there is an interrupted line circle with the most overlapped variables. A more detailed image of those variables is displayed in the Figure 3.9-B. In this last figure, it is possible to observe a closer relationship between *Family_XIII_AD3011_group*, *Rikenellaceae_RC9_gut_group*, DMA *i*-14:0, *Anaerovibrio* and *Syntrophococcus*; and between, *[Ruminococcus]_gauvreauii_group*, *[Eubacterium]_nodatum_group*, *RF39* and *Olsenella*. Figure 3.9-C better illustrates the relationship between *Family_XIII_AD3011_group*, *Rikenellaceae_RC9_gut_group*, DMA *i*-14:0, and include RF39. *Anaerovibrio* and *Syntrophococcus* also group relatively near to the last ones.

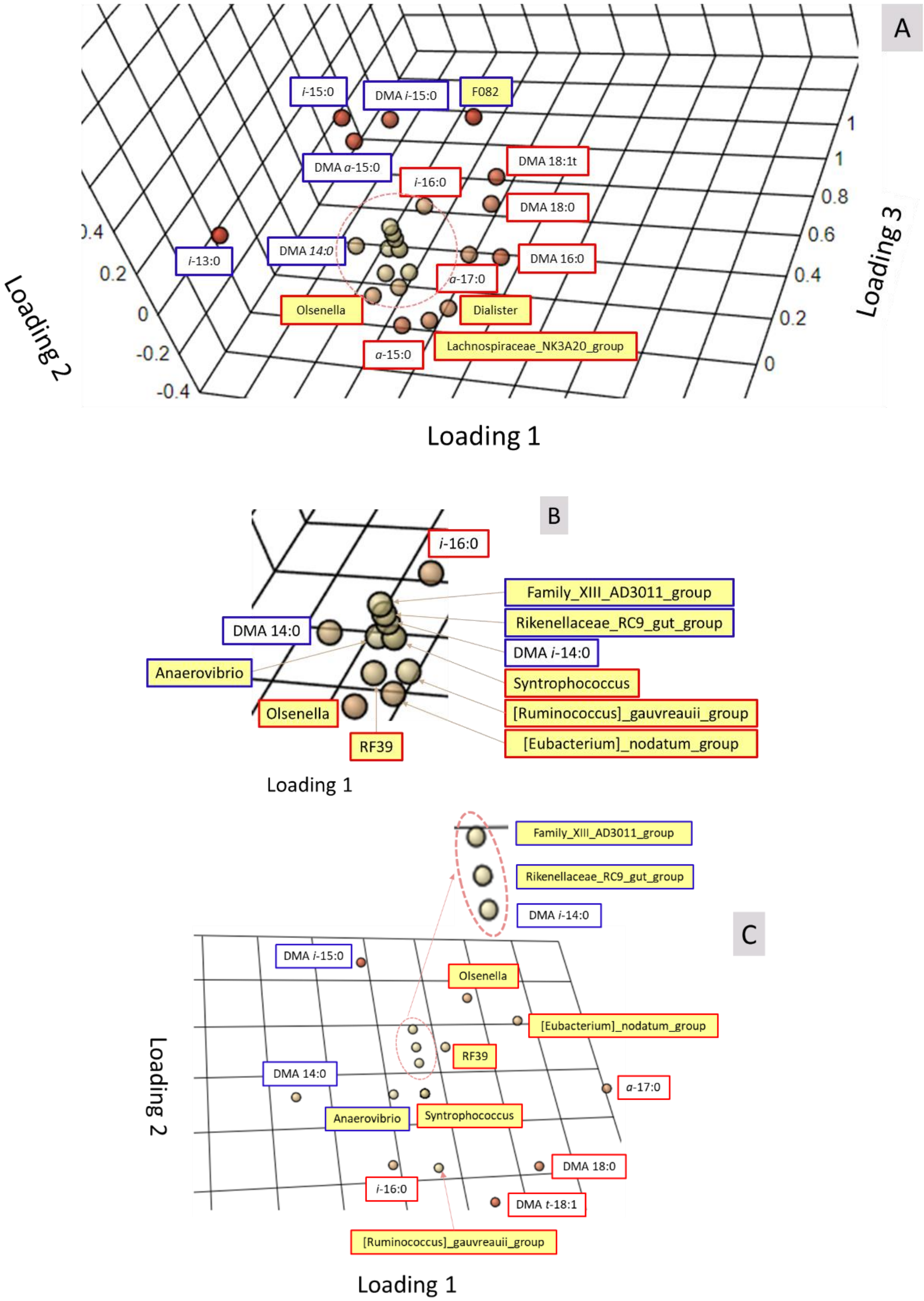

Figure 3.9- sPLSDA loadings plot with DMA, BCFA and bacteria with relevant correlations with *t*10-18:1 FA.

Images A-C represent different levels of zoom: A – 3Dplot with no zoom; B- 3D plot with zoom and tags; C- circled area zoomed from B and tags. Bacterial genus tags are highlighted in yellow. Variables with positive correlations with *t*10-18:1 are outlined in red and variables with negative correlations with *t*10-18:1 are outlined in blue.

3.3.6. Correlations between DMA and BCFA significantly correlated with *t*10-18:1, and bacterial genera.

Spearman correlations were established between ruminal microbiota and DMA and BCFA. Significant FDR values determined 2 significant correlations between *a*-17:0 and [*Eubacterium]_nodatum_group* (r = 0.64) and *Moryella* (r = -0.63). Four significant correlations between *a*-15:0 and *Libanicoccus* (r = 0.72), *Acidaminococcus* (r = 0.57), *F082* (r = -0.57) and *Anaerovibrio* (r = -0.57). Two significant correlations between *i*-15:0 and *U29_B03* (r = 0.63) and *UCG_002* (r = 0.62). A significant correlation between *i*-13:0 and *Libanicoccus* (r = -0.65).

Significant FDR values determined 1 significant correlation between DMA 16:0 and *Howardella* (r = 0.62). Four significant correlations between DMA 18:0 and *Lachnospiraceae_UCG_010* (r = 0.71), *Moryella* (r = -0.65), *Anaerovibrio* (r = -0.63) and *Mogibacterium* (r = -0.58).

Significant FDR values, followed by positive CLR abundance values and an index value greater than 0.5 and lower than -0.5 determined the correlations illustrated from Figure 3.10-A to E for DMA *c*9-18, DMA *a*-15:0, DMA *i*-15:0, DMA *i*-14:0 and DMA 14:0, respectively.

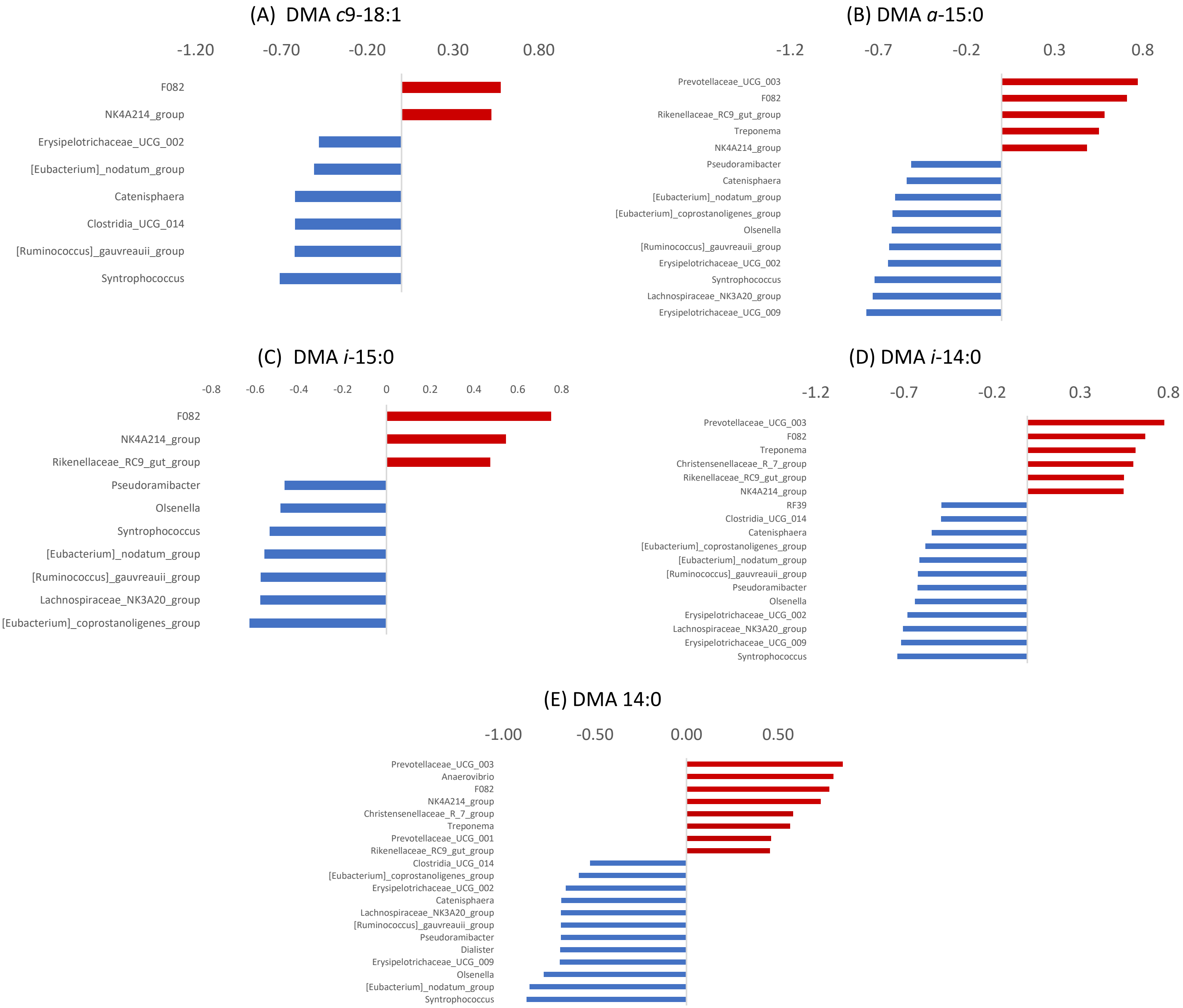

Figure 3.10- Spearman correlations between ruminal DMA significantly correlated with *t*10-18:1: DMA *c*9-18:1 (A), DMA *a*-15:0 (B), DMA *i*-15:0 (C), DMA *i*-14:0 (D), DMA 14:0 (E) and ruminal bacteria.
Positive correlations are represented in red and negative correlations are represented in blue.

3.3.7. Differences in CowPi functional units (FU) abundances between diets in the rumen

The main functions identified (Figure 3.11) with a percentage of occurrence higher than 2% corresponding to 9 different categories were leaded by Transporters (4.4%), followed by Two-component system (3.2%) and DNA repair and recombination proteins (3.2%). The main KEGG (Kyoto Encyclopedia of Genes and Genomes) Classes identified (Figure 3.12) with a percentage of occurrence higher than 4% corresponded to 9 different categories, leaded by Amino Acid Metabolism (10.4%), followed by Carbohydrate Metabolism (10.1%) and Replication and Repair (9.4%).

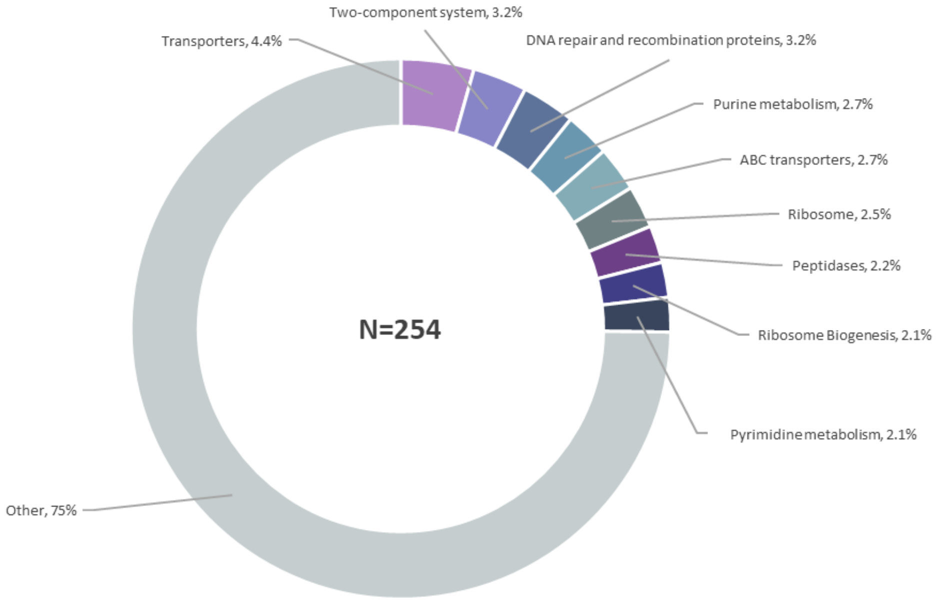

Figure 3.11- Percentage of CowPi main “Function” categories in the rumen.

The N value equal to 254 corresponds to the number of “Function” categories analysed.

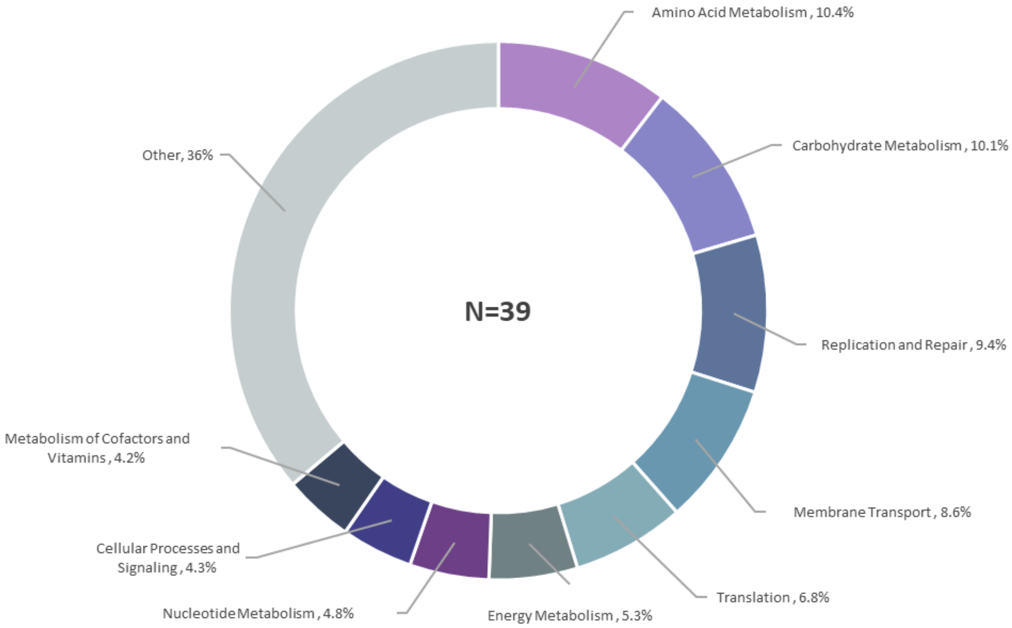

Figure 3.12- Percentage of CowPi main "KEGG Classes" categories in the rumen.
The N value equal to 39 corresponds to the number of "KEGG Classes" categories analysed.

From a total of 254 CowPI identified functional units (FU), 118 differed between diets ($p < 0.05$) (Table A.1. - Annexes), corresponding to 36 different KEGG Classes. However, from those FU, 17 were excluded due to lack of contextual coherence in the results. From those identified FU, seven were enriched in the rumen of lambs fed diets containing *Nannochloropsis* (oil, SD and FD), compiled into five different classes. A total of 70 FU were enriched in the lambs fed C diet, among which were the following pathways: Biosynthesis of unsaturated fatty acids, Fatty acid biosynthesis, Glycerophospholipid metabolism, Lipid biosynthesis proteins and Lipid metabolism. Together, the 70 FU belonged to 26 classes (Figure 3.13). A total of 23 FU were underrepresented in lambs fed the SD diet. On the contrary, 8 FU were found enriched in lambs fed the SD diet, among which was α-linolenic acid metabolism. A total of seven FU were enriched both in C and O fed lambs. Two FU were enriched both in C and FD fed lambs and only one in SD and FD fed lambs.

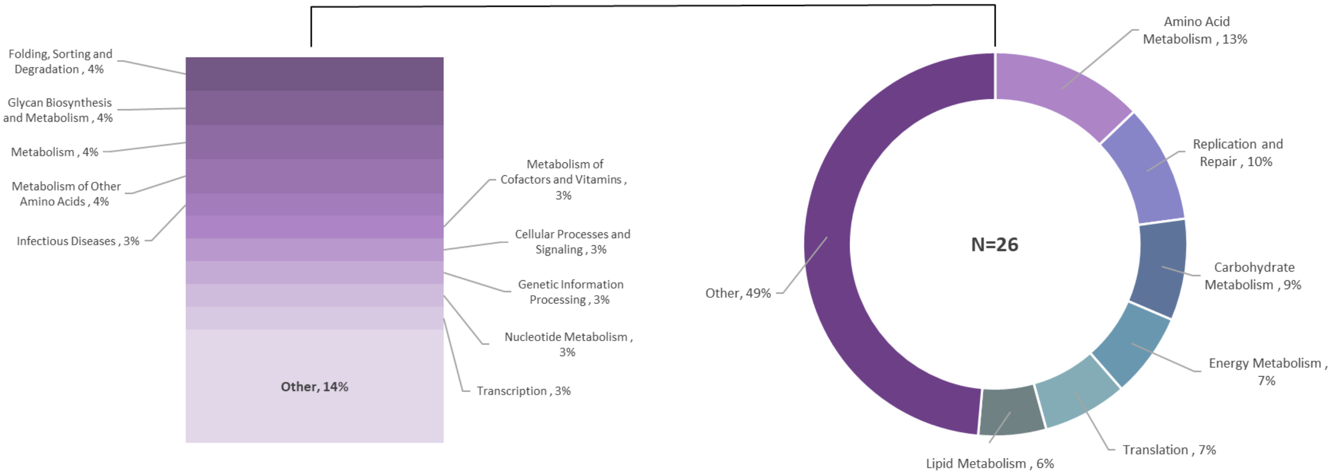

Figure 3.13- KEGG Classes enriched in the C fed lambs.

The N value equal to 26 corresponds to the number of enriched “KEGG Classes” categories analysed in C fed lambs. The dark purple slice correspondent to the class “other”, in the right donut chart, is detailed in the purple colour gradient bar on the left side.

3.3.8. Correlations between CowPi FU and FA indicators.

CowPI transformed dataset was divided into two subsets – one with variables with positive CLR average and the other with variables with negative CLR average. No significant correlations were found between *t*11-18:1, 18:0 and EPA and the variables neither in the positive neither in the negative subset.

Spearman correlations were calculated between the positive subset and *t*10-18:1 FA and 141 significant pathways were found (FDR < 0.05). From those, 115 were selected based on their abundance (CLR abundance > 1). After, 100 were selected based on their r value > 0.5. Finally, 52 were selected based on an abund*corr index > 2. Partial results (only r ≥ 0.77) are illustrated in the Figure 3.14.

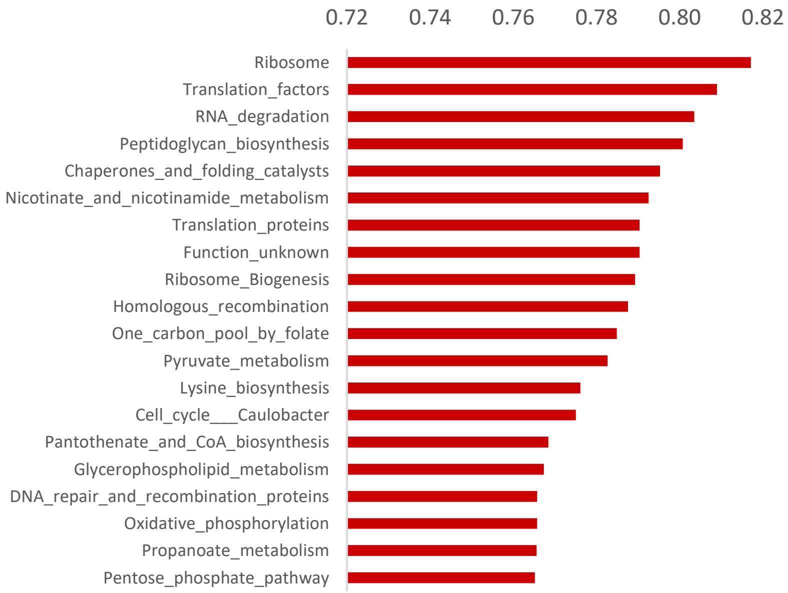

Figure 3.14- Spearman correlations (r ≥ 0.77) between CowPi FU and *t*10-18:1.

3.4. Discussion.

Overall, our results regarding the lack of major ruminal microbial changes among lambs fed different diets is in accordance with the purpose to ensure that feeding *Nannochloropsis* sp. at the present incorporation percentage does not causes any detrimental effects on the lambs' GIT microbiome.

Nevertheless, diet has been found to be the primary factor influencing differences in microbial community compositions, with bacteria being the main differentiation factor, whereas the role of the host was found to be less significant (Henderson et al., 2015). Also, diet seemed to have a greater influence on the rumen microbiome than host species (Henderson et al., 2015), therefore, making comparisons of rumen microbiota across host species possible.

The predominant bacterial phyla found in the rumen of lambs consuming different diets were Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria. In healthy cattle, Firmicutes (up to 81%) have been identified as the predominant phylum, followed by Bacteroidetes (18-26%) (Khalil et al., 2022). The profile of phyla abundances presently found was similar to the one observed in previous works (Mao et al., 2015; Dewanckele et al., 2018; Carreño et al., 2019; Boggio et al., 2021), where the most abundant phyla were Firmicutes, followed by Bacteroidetes and Proteobacteria.

With the exception of *Veillonellaceae*, a family that seems to be proportionally more abundant in sheep, when compared to bovine (Henderson et al., 2015), the most represented bacterial families are compatible to the ones reported in sheep (McLoughlin et al., 2020).

The most abundant genus found were compatible with the most abundant genus found by Wirth et al. (2018), when examining 10 Holstein dairy cow rumen fluid fraction whole metagenome and transcriptome datasets. According to Pereira et al. (2022), the most widely abundant genera in livestock species are *Prevotella*, *Butyrivibrio* and *Ruminococcus*, unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, *Clostridiales* and *Succinivibrio* (Pereira et al., 2022). Also, selecting the most abundant taxa (relative abundance > 2%), Mao et al. (2015), found that predominant genera in cattle gastrointestinal tract also included *Treponema*, *Succiniclasticum*, *Acetitomaculum*, *Mogibacterium* and *Acinetobacter*.

The richness and diversity of the bacterial microbiota in the rumen serve as crucial indicators of the typical biochemical processes occurring in the rumen, with a positive effect of diversity on community robustness (Costa-Roura et al., 2022). Regarding the α-diversity indexes evaluated, there were no significant differences found between animals consuming different diets. Therefore, the putative toxic effects of supplemented n-3 LC-PUFA in the microalgal diets seem not to negatively impact bacterial diversity in the rumen. This might have been due to the used incorporation rate of the microalga biomass (108 g/ kg DM) and oil (12 g/ kg DM) in the base diet, coupled with a relatively short trial period. In studies with DHA Gold (*Schizochytrium spp*. commercial product) supplementation, 8 g/ kg DM did not affect rumen bacterial diversity in dairy sheep (Castro-Carrera et al., 2014), and neither the maternal supplementation of 18.2 g/ kg fresh matter and in early life with 0.28 g/ kg of body weight, affected α-diversity indexes in goats (Dewanckele et al., 2018). No major differences were also found in a study by Mavrommatis et al. (2021) where higher doses composed of 20, 40 and 60 g of *Schizochytrium spp*. were offered daily per animal, with the exception of a lower Shannon index at the species level in the goat's group supplemented with 40 g of *Schizochytrium spp*.

In fact we had previously reported no major differences neither in the profile of volatile fatty acids produced, neither in C18 biohydrogenation between lambs consuming different diets supplemented with *Nannochloropsis* sp*.* (Vítor et al., 2021), therefore we were not expecting major shifts regarding microbiota abundances. Moreover, in sheep, other factors such as, for instance, feed efficiency are likely influenced by compositional changes of the archaeal community, and abundance changes of specific bacteria, rather than major overall shifts within the rumen microbiome may occur (McLoughlin et al., 2020). The archaeal community was not considered in the present study once the sequencing methods were not optimized for the identification of archaea. Therefore, we further focused in finding specific bacterial associations, that might explain the short differences found in the overall ruminal FA profile of lambs in the different groups.

No differences were found in the ruminal bacterial individual abundance between different treatments, except for *Gastranaerophilales* that were significantly higher in the O treatment (1.71) when compared to the remaining ones (-1.75). *Gastranaerophilales* are found in human and other animal guts, but their exact role is unknown. However they are thought to have a beneficial effect for their hosts (Di Rienzi et al., 2013). They belong to class *Melainabacteria*, capable of fermenting a range of sugars (e.g., glucose, starch, and hemicellulose) into butyrate in the gut of herbivores. In lambs, this bacteria has been associated with poor growth performance (Perea et al., 2017). However it has also already been reported as one of the most prevalent bacteria associated with healthy cattle oral cavity (Khalil et al., 2022). In the present study, we could not add meaning to the higher abundance of this genus in the O fed lambs. However, it is very positive that no major changes were seen in the GIT microbiota, as it suggests that *Nannochloropsis* sp. can be used to enhance human health beneficial EPA content in lamb GIT without any negative effects on microbiota.

There are many factors leading to changes in rumen microbial communities, being diet the most obvious factor influencing the rumen microbiome and fermentation (Newbold & Ramos-Morales, 2020). Moreover it is stated that UFA, which are dominant in common fat sources for ruminants, have negative effects on microbial growth, especially in fibrolytic bacteria (Enjalbert et al., 2017), however, changes in rumen microbiota due to an increased concentrate proportion are much higher than effects due to fat addition (Zened et al., 2013a). Also, a decreased abundance *in vivo* of a bacterial genus following a dietary change cannot be unequivocally interpreted as a direct effect of a dietary component, but could also reflect a more global change in nutrient and function partitioning among different rumen microorganisms (Enjalbert et al., 2017). Maybe in our current experiment, neither the inclusion percentage of n-3 LC-PUFA (especially EPA) nor the overall experimental time were enough to produce significant alterations in the overall ruminal microbiome composition. This finding is positive, once EPA was efficiently enhanced both in the rumen (Vítor et al., 2021) and lamb edible tissues (Vítor et al., 2023), without compromising the ruminal bacterial abundances and diversity.

Even though no major differences had been found in the microbial abundances between treatments, we explored existing correlations between bacterial genera and EPA content in the rumen. Previous *in vitro* studies had observed positive and negative responses in terms of bacterial genera abundances when incubated with EPA (Carreño et al., 2019). However ruminal EPA content varied between treatments, no significant correlations were found with specific bacterial genera. Therefore, once we already verified a high animal variability regarding C18 biohydrogenation intermediates in the rumen (Vítor et al., 2021) we decided to also evaluate correlations between bacterial genera and C18 biohydrogenation intermediates (*t*10-18:1 and *t*11-18:1) and 18:0.

The main bacteria group involved in the C18 biohydrogenation are known to be part of the "*Butyrivibrio* group" (Boeckaert et al., 2008b) however, increasing discriminatory capacity for identifying genera positively associated with more complete or more incomplete biohydrogenations is growing due to a more widely use of high throughput sequencing technologies and bioinformatic tools. Therefore, although yet some recent identified genera are not so well defined, every new knowledge contribution will be essential in the future to try to better understand their metabolic roles in the context of different ruminal environments.

Regarding the correlations found between ruminal bacteria and *t*10-18:1 FA in the rumen, the highest positive correlation was found for *Pseudoramibacter*, followed by *Olsenella*, [*Eubacterium]_nodatum_group*, *Syntrophococcus* and *Dialister*. *Pseudoramibacter* had been previously positively correlated to *t*10-18:1 in goats (Dewanckele et al., 2018). This genus is a member of the *Eubacteriaceae* family and it can utilize carbohydrates for energy, while producing fermentation end products butyrate, acetate, formate, and hydrogen (Deusch et al., 2017). A previous study on Holstein steers and goats showed that increased grain feeding significantly elevated the abundance *Pseudoramibacter* in the cecum (Tao et al., 2017) and Enjalbert et al. (2023) found a positive correlation between this genus and *t*10-shift. The association of this genus with carbohydrates fermentation and grain-based diets can constitute a corroborated clue, due to the fact that *t*10-18:1 enrichment is highly associated with these contexts (Dewanckele et al., 2020a). *Olsenella* is a genus composed of bacteria that can ferment carbohydrates to lactic acid (Kraatz et al., 2011). This genus has indeed been previously found enriched in the rumen of animals fed high-grain/ starch-rich diets (Zened et al., 2013a; Kim et al., 2018) and it has already been proposed to be positively related to *t*10-biohydrogenation intermediates (Dewanckele et al., 2020a; Enjalbert et al., 2023) and positively correlated to *t*10-18:1 and negatively with *t*11-18:1 (Daghio et al., 2021). Therefore, our finding corroborates the ones already described. In the study by Dewanckele et al. (2018), *Dialister* was positively correlated with ruminal *t*10-18:1 accumulated or *t*10-shift. Recently, also Enjalbert et al. (2023) positively correlated *Dialister* with *t*10-shift. Correlation analysis based on ruminal bacterial populations and milk (Pitta et al., 2018; Dewanckele et al., 2019), blood (Scholz & Kilian, 2016) or rumen FA profiles (Scholz & Kilian, 2016; Zened et al., 2016) revealed the possible contribution of several bacterial genera in ruminal formation of *trans*-10 intermediates, including *Dialister invisus* E7.25 and *Dialister pneumosintes* Cal 4692-1-74 due to positive correlations between their rumen or buccal abundance and *trans*-10 intermediates in the rumen, milk or blood (Zened et al., 2016; Dewanckele et al., 2018; Dewanckele et al., 2019; Dewanckele et al., 2020a). Therefore, our finding also corroborates the ones already described.

Both in the works of Dewanckele et al. (2018), and in Daghio et al. (2021), *Eubacterium* and [*Eubacterium]_nodatum_group*, were positively correlated with the concentration of *t*10-18:1 and negatively correlated with the concentration of *t*11-18:1. Also, correlation analysis based on ruminal bacterial populations and milk (Pitta et al., 2018; Dewanckele et al., 2019) or rumen FA profiles (Zened et al., 2016; Dewanckele et al., 2018) indicated that also *Syntrophococcus* spp. were positively related to *t*10-biohydrogenation intermediates and also with *t*10-shift (Enjalbert et al., 2023). Therefore, our findings about the positive correlations between *t*10-18:1 and these two genera are also consistent with previous findings.

The highest negative correlations between bacterial genera and *t*10-18:1 were found for *Anaerovibrio*, *NK4A214 group* and *F082*. In the work by Daghio et al. (2021), *Anaerovibrio* and *NK4A214 group* had a positive correlation with the concentration of *t*11-18:1 in the rumen and Gudla et al. (2012), mentioned that DNA abundances for *Anaerovibrio lipolytica* and Butyrivibrio *t*11-18:1-producing bacteria were significantly lower with the low forage diets suggesting that these bacteria are sensitive to low pH conditions. Lower ruminal pH condition are often associated to the occurrence of *t*10-shift, therefore, it is expectable that *t*11-18:1-related bacteria would be decreased (Dewanckele et al., 2020b). Therefore, despite not finding a direct correlation with *t*11-18:1 content in the rumen, a negative correlation with *t*10-18:1 can constitute an indirect clue towards a possible relationship between this genus and the ruminal environment related to *t*11-18:1 production. SILVA identification of *NK4A214 group* stands for *Oscillospiraceae NK4A2014 group*. *Oscillospiraceae NK4A2014* is an uncultured taxon and its role in the rumen is largely unknown (Amat et al., 2021). In the rumen, this genus has been previously positively associated with the presence of *Methanobrevibacter* (Guo et al., 2023*;* Smith et al., 2022). Similarly to what was stated for *Anaerovibrio*, despite we did not find a described negative correlation between this genus and *t*10-18:1, a positive correlation with *t*11-18:1 in the rumen has already been found for *NK4A214 group* (Daghio et al., 2021). In a study by Marie-Etancelin et al. (2021), the genus was found decreased in efficient lambs compared to inefficient lambs, regardless of the diet. The ruminal role of *F082* is still unclear, therefore we could not find previous findings on possible correlations between this genus and RBH indeterminates.

When observing the significant correlations found between ruminal genera and both *t*10-18:1 and the *t*10-shift, it was found that 21 of those correlations were shared between the two FA indicators, being that only 1 was exclusively find for *t*10-shift, corresponding to the positive correlation with *Desulfovibrio*. This positive correlation between *Desulfovibrio* and *trans*-10-

biohydrogenation has been previously reported in correlation analysis based on ruminal bacterial populations and milk (Pitta et al., 2018; Dewanckele et al., 2019) or rumen FA profiles (Zened et al., 2016; Dewanckele et al., 2018).

Dewanckele et al. (2018), found that DHA supplementation of goats increased the ruminal proportions of *t*11-18:1 rather than *t*10-18:1 (Dewanckele et al., 2018), as observed by Vítor et al. (2021), with the supplementation of EPA-supplemented diets in lambs. This can suggest that both goats and lambs are less prone to dietary induced shifts towards the formation of *t*10 intermediates in comparisons with cows (Dewanckele et al., 2018). Because *t*11-18:1 acts as a substrate for the Δ9-desaturase in animal tissues resulting in the formation of the conjugated linoleic acid (CLA) *c*9, *t*11-18:2, its presence is desirable in terms of beneficial health effects for the consumer (Vahmani et al., 2020). Therefore, is would be of major interest to find specific bacteria with positive correlations with the *t*11-18:1 production. According to Huws et al. (2011), bacteria belonging to genera *Anaerovorax*, *Prevotella*, *Lachnospiraceae Incertae Sedis*, *Ruminococcus*, *Butyrivibrio*, and *Pseudobutyrivibrio*, *Tanerella* and unclassified *Bacteroidales*, *Clostridia* and *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae* and *Porphyromonadaceae* might be involved in ruminal *t*11-18:1 formation. However, in the present study, no significant correlations were found between *t*11-18:1 and ruminal bacterial genera. This fact seems to point for the fact that, in our study, *t*10-18:1 seemed to have a more defined associated microbiome, when compared to *t*11-18:1. This can be due to a higher specificity of certain bacteria to produce *t*10-18:1, while a less defined bacteriome is present in the production context of *t*11-18:1. The recent result by Enjalbert et al. (2023) seem to point towards a similar direction.

We also explored significant correlations between 18:0 and ruminal bacteria. Stearic acid is the end product of complete biohydrogenations in the rumen, but when marine oils are fed to ruminants, 18:0 production tends to decrease (Bessa et al., 2015). Therefore, we would expect that bacteria positively correlated with 18:0 would be involved in more complete biohydrogenations and less affected by detrimental effects of oil supplementation in the rumen. Only one positive correlation between *Lachnospiraceae_UCG_010* and 18:0 was found. This genus is a fiber degradation-associated genera (Biddle et al., 2013). Members of *Lachnospiraceae* hydrolyze starch and other sugars to produce butyrate and other short-chain FA and higher abundances are positively correlated with glucose and/or lipid metabolism (Vacca et al., 2020). Little information is known about *Lachnospiraceae_UCG_010* but is has been found enriched in the gut of deer with chronic wasting disease, a neurodegenerative prion disease (Minich et al., 2021). This genus had also been found in the rumen and hindgut of mastitis cows when compared to healthy ones (Zhong et al., 2020). To our knowledge, there is no information on the role of this specific genus in RBH.

Besides gene sequencing, the abundance profile of different lipid species, such as BCFA and DMA can act as a tool to infer differences between ruminal microbiota compositions (Vlaeminck et al., 2005; Alves et al., 2013; Conte et al., 2022). Therefore, we also analysed differences in BCFA and DMA between different treatments and, consistent with the lack of major differences found in the microbiota profile of the different animals using 16S sequencing analysis, no major differences in the abundance of these lipid species occurred in the rumen of lambs fed the different diets.

Although not having found major differences between the lipid species abundance between treatments, we proposed to further explore significant correlations between them and both FA indicators and specific ruminal bacteria. Besides a higher *i*-13:0 verified in the FD fed lambs, a positive correlation between this BCFA and EPA was also found, which could indicate that *i*-13:0 higher abundance in the rumen of FD fed lambs could actually be related to the fact that these animals had the highest EPA content in the rumen of all other treatments (Vítor et al., 2021). Even though no significant correlations were found between EPA and specific bacterial genera, the positive correlation between EPA and *i*-13:0 can be related to a possible association between higher EPA levels and increasing numbers of *i*-13:0-incorporating bacteria. The FA 18:0 was higher in the rumen of C fed lambs, the same animals registering the lowest *i*-13:0 content. Therefore, a negative correlation between 18:0 and *i*-13:0 is consistent in our results. Except for *i*-16:0, all BCFA and DMA positively correlated with 18:0 or with *t*10-18:1, were negatively correlated with the presence of EPA. This probably distinguishes the profile of BCFA and DMA primarily present in more and less complete biohydrogenations, respectively. However, even though a higher presence of BHI usually points towards a lower completeness, in this case, the same BCFA and DMA were positively associated with *t*10-18:1 and 18:0.

When evaluating the correlations between BCFA and DMA and ruminal bacterial, stronger positive correlations were found between *Mogibacterium* and *Christensenellaceae_R_7_group* and we found that these genera were negatively correlated with *t*10-18:1. The same was verified for *Anaerovibrio* and DMA 14:0, for *Christensenellaceae_R_7_group* and *Anaerovibrio* and *NK4A214_group*, and for *F082* and DMA 14:0. Positive correlations were found between *Syntrophococcus* and *[Eubacterium]_nodatum_ group*, between *Clostridia_UCG014* and *Catenisphaera*, and between *Olsenella* and *[Eubacterium]_nodatum_ group* and *Pseudoramibacter*. All these genera were positively correlated with *t*10-18:1. This shows that, bacteria, and DMA and BCFA with individual strong correlations with *t*10-18:1 also show strong correlations between them, both in the case of negative and positive associations.

Also, in the sPLSDA loadings plot with DMA, BCFA and bacteria with relevant correlations with *t*10-18:1 FA, the coherence was overall maintained. All *i*-15:0, DMA *i*-15:0, DMA *a*-15:0 and *F082*, closely associated in the plot, are negatively correlated with *t*10-18:1. All *Dialister*, *Lachnospiraceae_NK3A20_group* and *a*-15:0, and also DMA 16:0 and *a*-17:0, closely associated in the plot, are positively correlated with *t*10-18:1. *[Ruminococcus]_gauvreauii_group*, *[Eubacterium]_nodatum_group*, *RF39* and *Olsenella* are all also positively correlated with *t*10-18:1. However, *Family_XIII_AD3011_group*, *Rikenellaceae_RC9_gut_group*, DMA *i*-14:0, *Anaerovibrio* and *Syntrophococcus* were all grouped in the plot, and whereas the first elements are all negatively correlated with *t*10-18:1, *Syntrophococcus* was positively correlated. However, when observing the correlation heatmap, there was no strong positive correlation between *Synthrophococcus* and these genera.

As mentioned by Vlaeminck et al. (2006), fibrolytic bacteria, are usually enriched in *iso*-FA and starch degrading bacteria, contain relatively greater amount of linear odd-chain FA and a small amount of BCFA. As pointed by Zhang et al. (2020b), ruminal accumulation of OBCFA (15:0, *i*-14:0, *i*-15:0, *a*-15:0 and *a*-17:0) is associated with the abundance of cellulolytic bacteria in lambs. However, regarding the relationship of OBCFA concentrations with bacterial abundance in the rumen, little information is known (Xin et al., 2021). In our study, positive correlations with *iso*-BCFA were established with *U29_B03* (*Rikenellaceae*) and *UCG_002* (*Oscillospiraceae*), both from cellulolytic associated families. On the other side, *Libanicoccus*, exhibited a negative correlation with *i*-13:0. This genus belongs to *Atopobiaceae* family, which encompasses starch-related genera. *U29_B03* seems to play a role in VFA synthesis and energy generation, being it previously found positively associated with increased dietary energy levels in yaks (*Bos grunniens)* (Ahmad et al., 2020). As *t*10-18:1 production is often associated to feed regimens with higher concentrate (Bessa et al., 2015), the positive correlation between *i*-15:0 and *t*10-18:1 and consecutively *U29_B03* is consistent. Also this genus has been previously found positively correlated with *i*-15:0 (Si et al., 2022), corroborating our result. However, in the same study, *U29_B03* was positively correlated with the fat content in dairy cow’s milk (Si et al., 2022). Since *t*10-shift is identified to be related to the occurrence of the milk fat depression syndrome in dairy cows (Bessa et al., 2015), maybe this genus, although with a possible correlation with *t*10-18:1 production, does not contribute to the occurrence of milk fat depression.

The information about the ruminal bacterial composition and lipids associated to their presence is increasingly available, however, metabolic roles associated to the different ruminal bacterial profiles is scarce. In that sense, we also wanted to try to attribute and differentiate metabolic pathways associated to the bacteriome profile found in lambs fed the different diets. For that, we used CowPI tool, a focused version of the PICRUSt provided for the study of the rumen microbiome and the functional profiles predicted are estimated from the metagenomic dataset (Wilkinson et al., 2018). However, studies involving the utilization of CowPI to predict ruminal microorganisms metabolism are almost inexistent (Trabi et al., 2019; Smith et al., 2020). Therefore, there is a need to improve the number of tested datasets in order to better refine and make sense of the results.

Only 7 FU were found enriched in the animals fed *Nannochloropsis* supplemented diets. Moreover, we were unable to attribute meaning to the results obtained, in the context of our work once most of the findings were related to human diseases. Therefore, those relations were not taken into considerations for this discussion. Once again, it is reinforced the lack of associations between EPA ruminal content and specific metabolic pathways enriched in the rumen of lambs supplemented with *Nannochloropsis* sp.

However, when comparing the KEGG Classes enriched in the C fed lambs and in lambs fed diets supplemented with *Nannochloropsis*, it was possible to observe that the enriched pathways point to an overall higher metabolic rate in C lambs, evidenced by the enrichment of pathways related to protein synthesis, nucleic acid replication and energy metabolism. High concentrate diets (50–65% concentrate on a dry matter basis) tend to negatively impact the ruminal microbiota (Zhang et al., 2017a; Hua et al., 2017). In the present study, a proportion of 60:40 was used for concentrate:forage in the basal diet, however, no signs of detrimental dysbiosis were observed and an overall higher microbial metabolic rate was observed in the C fed lambs (lambs fed only the basal diet). There was an enrichment in biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, and lipid biosynthesis proteins also in the lambs fed the C diet. PUFA supplementation was higher in *Nannochloropsis* supplemented diets (9.72 g/ kg DM) when compared to C diet (6.84 g/ kg DM) and it is known that PUFA-supplemented ruminant diets, *de novo* FA synthesis is reduced (Miltko et al., 2019; Urrutia et al., 2020). In this sense, it is consistent that the lipid biosynthesis related pathways were enriched in the lambs fed C and not O, SD and FD diets.

Consistent with the fact that the animals with higher ruminal content of *t*10-18:1 were the ones consuming the C diet, positive strong correlations were found between pathways related to protein synthesis, nucleic acid replication and energy metabolism and *t*10-18:1, once again evidencing a higher metabolic rate. This might be, once again, an indication that indeed that is a strong correlation between concentrate-based diets and the production of *t*10-18:1 in the rumen.

No significant correlations were found between *t*11-18:1 and CowPi FU, which could indicate that *t*10-18:1 rather than *t*11-18:1 could be a more accurately direct indicator of a metabolically different bacteriome in the rumen. This is also corroborated by the fact that no significant correlations were found between specific bacteria, DMA and BCFA, and *t*11-18:1 in the rumen. Also, there were no significant correlations between 18:0 and CowPi FU, which is also consistent with the lack/ almost no correlations found between these FA and rumen bacteria.

Based on the analysis of the data obtained using CowPI, it is clear that there is still a need for further curation in order to fully redirect the results within the context of our study. While the initial findings are promising, namely the ones related to the positive associations found with *t*10-18:1, it is important to note that the presented results are relatively raw, especially those that are included in the table available in the annexes section (Table A.1.). Therefore, it is essential to exercise caution when interpreting these results, as additional refinement and analysis will likely be required in order to draw accurate conclusions.

3.5. Conclusion.

Our results demonstrate that EPA supplementation in lamb's trough the addition of *Nannochloropsis oceanica* to the diets can positively enhance EPA content in the gastrointestinal tract of lambs, without compromising the overall ruminal bacterial abundances and diversity, which is remarkably positive. Also no specific correlations were found between EPA content and specific bacterial genera and metabolic pathways in the rumen of lambs fed *Nannochloropsis* sp. Although we did not find differences in the extension and completeness of the C18 UFA, the lambs had a strong individual variability regarding the type of main intermediate formed (i.e., *t*10 vs. *t*11-18:1) in the rumen. It is not fully known which microbial agents are responsible for this altered BH pattern, therefore, this study providing samples with this individual variability, even within each diet, had the potential to also be a matrix to identify OTU and enriched metabolic pathways associated with *t*10-shift. Our results reinforce previously find associations between bacterial genera and some FA indicators and point for new possible associations that need to be further explored. However, it seems that *t*10-18:1 could be a better target, when compared to *t*11-18:1 and even 18:0, for determining specific bacteriome profiles in the rumen.

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Author contributions

S.P.A., R.J.B.B. and S.H. conceptualization and funding acquisition. A.C.M.V., F.G.S. and S.P.A. conducted laboratory analysis. A.C.M.V., F.G.S., F.R., C.C., R.J.B.B. and S.P.A. performed data analysis. A.C.M.V., R.J.B.B., S.P.A., F.G.S., and S.H. interpretation of results. A.C.M.V. drafted the manuscript. S.P.A., F.G.S., S.H. and R.J.B.B. edited and revised the original draft. All authors revised and approved the final manuscript.

4. CHAPTER 4 | *Nannochloropsis oceanica* microalga feeding increases long chain n-3 polyunsaturated fatty acids in lamb meat.

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Abstract.

To test the hypothesis that lambs fed freeze-dried *Nannochloropsis oceanica* biomass will have a higher deposition of EPA in tissues than those fed other *Nannochloropsis* EPA-sources, we fed 28 lambs with one of four diets: i) C, control, without EPA; ii) O, with 1.2% *Nannochloropsis* oil; iii) SD, with 12.3% spray-dried NO biomass; iv) FD, with 9.2% freeze-dried NO biomass. Dry matter intake, growth, tissues fatty acid composition, oxidative stability and sensory traits of the resultant meat were evaluated. The EPA was highest in tissues of lambs fed SD and FD compared with O but was similar between SD and FD. Total *trans*-18:1 did not differ among treatments, but the *t*10/*t*11–18:1 ratio decreased with all EPA containing diets. EPA diets were also supplemented with Vitamin E preventing the lipid oxidation in EPA-enriched meat and the meat sensory traits were not affected although occasionally some off-flavours were detected in FD meat.

Keywords: fatty acid; hippocampus; prefrontal cortex; dimethyl acetal.

4.1. Introduction.

The n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), namely EPA and docosahexaenoic acid (DHA, 22:6n-3), contribute to preventing and improving chronic human disease outcomes, including metabolic diseases, cardiovascular diseases, neurodegenerative diseases and some types of cancer (Troesch et al., 2020). Although humans can desaturate and elongate α-linolenic acid (ALA, 18:3n-3) into n-3 LC-PUFA, the conversion lacks efficiency, as the conversion of ALA is <5%, justifying the establishment of nutritional requirements of 250 mg per day for EPA plus DHA (FAO, 2010). Consumption of meat enriched with n-3 LC-PUFA can be considered a good alternative to reach n-3 LC-PUFA requirements, particularly in regions where consumption of sea products is low (Givens et al., 2006).

Pasture and forages supply ruminants with ALA, but >90% of the ingested ALA is hydrogenated in the rumen (Glasser et al., 2008). Even if the absorption of ALA increases, the deposition of n-3 LC PUFA in meat is low, ranging from 2 mg/100 g meat up to 40 mg/100 g meat (Bessa et al., 2015). Supplementing ruminant diets directly with n-3 LC-PUFA, by using marine oil sources can improve the efficiency of its deposition in the tissues by bypassing the need for elongation steps after its absorption. However, the majority of the dietary n-3 LC-PUFA will disappear before absorption due to the ruminal biohydrogenation (RBH) (Doreau et al., 2016).

To increase absorption of n-3 LC-PUFA, it will be necessary to ensure these fatty acids (FA) escape RBH. Several techniques have been attempted to protect unsaturated FA against RBH (Gadeyne et al., 2017). The use of some species of microalgae, where n-3 LC-PUFA is naturally protected, is a promising dietary strategy to increase the deposition of these FA in ruminant products (Alves et al., 2018).

Marine microalgae from the *Nannochloropsis* sp. genus show a high capability to store lipids (21–28% dry matter (DM)) mainly in the form of triacylglycerols (TAG), where EPA comprises up to 12% DM and 39% of total FA (Adarme-Vega et al., 2012; Ma et al., 2016). We have previously reported that the EPA present in the *N. oceanica* biomass is partially protected from RBH, when evaluated *in vitro,* and that this protection is more effective if the biomass is freeze-dried rather than spray-dried (Alves et al., 2018). The present experiment was designed to test if the RBH protection described *in vitro,* are replicable *in vivo,* and our results confirm our report in a companion paper (Vítor et al., 2021). Thus, it is expected that the deposition of EPA in meat will increase when animal' diets include freeze-dried *N. oceanica* biomass. Further, the extent to which PUFA are protected against RBH and deposited in meat and subcutaneous fat needs to be examined. Moreover, the impact of *N. oceanica* feeding on meat colour, susceptibility to lipoxidation and the sensory traits, including the presence of off flavours, needs to be evaluated as these are major determinants of meat consumer acceptability. Thus, this study investigated the effect of supplying about 3 g/kg DM of EPA either as *Nannochloropsis* sp. oil, freeze-dried or spray-dried *N. oceanica* biomass in lamb diets on meat quality, including its FA composition. This study provides the first evidence, to our knowledge, of the potential to enhance the human health qualities of lamb meat using microalgae biomass, whilst also examining effects on meat sensory properties.

4.2. Material and methods.

4.2.1. Animals, management, and diets.

Twenty-eight Merino Branco ram lambs of approximately 60 d of age, were transported to INIAV–Santarém, Portugal facilities and assigned to individual pens. The experimental animal procedures were approved by the Ethical and Animal Well-being Commission of the Faculty of Veterinary Medicine, University of Lisbon, Portugal (Protocol FMV/CEBEA 007/2016). Lambs were blocked by initial live weight (LW) into three blocks with an average (± standard deviation) LW of 14.9 ± 1.67, 22.1 ± 1.84 and 26.8 ± 1.73 kg. Treatments consisted of 4 pelleted diets: i) control (C): no added sources of EPA; ii) (O): a mixture of C diet with 12 g/kg of *Nannochloropsis* sp. Oil; iii) (SD): a mixture of C diet with 123 g/kg of spray-dried *N. oceanica* biomass; and iv) (FD): a mixture of C diet with 92 g/kg of freeze-dried *N. oceanica* biomass. All the diets had identical quantities of EPA (≈ 3 g/kg DM). The three experimental diets containing *Nannochloropsis* sp. were supplemented with 1.67 g vitamin E/ kg diet DM.

Fresh frozen *N. oceanica* plates, supplied by Allmicroalgae, Natural products S.A., were freeze-dried in a Scanvac Cool-safe Superior Touch freeze dryer (Scanvac, Denmark) with the following conditions: temperature, −92 °C; initial pressure 0.2 mbar; final pressure 0.07 mbar, at Instituto Superior de Agronomia, University of Lisbon. The spray-dried *N. oceanica* biomass was prepared in an industrial spray-drier at Allmicroalgae facilities. *Nannochloropsis* sp. oil used was obtained from Qualitas Health, USA.

After the first week of adaptation to the facilities and gradual introduction of the diets, lambs were offered the experimental diets twice a day at 0900 and 1800 for 3 weeks. In the first 2 weeks of the experiment, lambs were under restricted feeding at 45 g DM per kg LW. In the last week, animals were fed *ad libitum*.

4.2.2. Slaughter, carcass evaluation and sampling.

After the three-week trial, the animals were slaughtered at the experimental slaughterhouse of INIAV in Santarém, Portugal. After slaughter, the hot carcass weight (HCW) was recorded, and carcasses were stored in a cooling chamber at 10°C for 24 h and the cold carcass weight (CCW) was recorded. The carcasses were then refrigerated at 2°C until 3 days after slaughter. Carcasses were divided in two halves, which were separated in eight joints according to Santos-Silva et al., (2002). In the right side, two 1.5 cm chops were collected, and the *longissimus thoracis* (LT) was used to assess lipid and colour stability during 5 days of storage at 2°C in illuminated cooler.

At day 0 of storage (day 3 *post-mortem*), the colour parameters were determined after 1 h of blooming and then meat samples were vacuum-packed and stored at −80 °C until lipid oxidation analysis. The other chop was on a polystyrene tray, over-wrapped with oxygen permeable polyvinyl chloride film and displayed 5 days (day 8 *post-mortem*). At the end of storage time, the plastic wrap was removed, and the chop was allowed to bloom for 1 h before determining the colour parameters. After that, the samples were vacuum-packed and stored at −80 °C until lipid oxidation and α-tocopherol analysis.

Seventy-two hours after slaughter, the right loins, containing *longissimus lumborum* (LL) were vacuum-packed, stored at 2°C until 10 days *post-mortem* and then frozen at −20 °C, until being used for sensory analysis. At the same time, samples from the left LL muscle and subcutaneous adipose tissue (SAT) from the proximal region of the 12th rib were collected, vacuum-packed and stored at −80 °C for FA analysis.

4.2.3. Analytical determinations.

4.2.3.1. Feed composition.

The chemical composition of the diets was determined as described in detail by Santos-Silva et al. (2016) and is presented in a companion paper (Vítor et al., 2021). Briefly, the basal diet was composed of barley (37%), soybean meal (16%) and dehydrated alfalfa (38%). Diets were isoproteic (19.8%). The ether extract averaged 33.9% in the EPA-sources (ES) diets and 13.3% in the C diet. NDF and ADF averaged 26.4% and 16.6% respectively. The TFA content averaged 13.7 in the C diet and 20.1 g/ kg of diet DM in the C and ES diets (O, SD and FD) respectively.

4.2.3.2. Muscle lipid content and tissues FA analysis.

Lipids from freeze-dried muscle and SAT samples were extracted with dichloromethane and methanol (2:1, v/v) and transesterified into FA methyl esters (FAME) using a combined basic and acid catalysis as described by Alves et al. (2015). The FAME were analysed by gas chromatography with flame ionisation detection (GC-FID) using a Shimadzu GC-2010 Plus (Shimadzu, Kyoto, Japan) equipped with an SP-2560 capillary column (100 m, 0.25 mm i.d., 0.20 μm film thickness: Supelco Inc., Bellefonte, PA, USA). GC conditions were as follows: injector and detector temperatures were maintained at 220 °C and 250 °C respectively; helium was the carrier gas at a constant flow of 1.0 mL/ min; and 1 μL of sample was injected with a split ratio of 50:1. For FA quantification, 1 mL of nonadecanoic acid (19:0) at 1 mg/mL was added as the internal standard. Identification of FAME was achieved by comparing retention times with those of commercial standard mixtures (FAME mix 37 components, Supelco Inc., Bellefonte, PA, USA), and by comparison with published chromatograms (Alves et al., 2013;

Vahmani et al., 2016). Additional identification of the FAME was confirmed by GC coupled to mass spectrometry (MS) using a Shimadzu GC–MS QP2010 Plus (Shimadzu, Kyoto, Japan) equipped with the SP-2560 capillary column. The chromatographic conditions for GC-FID and GC–MS were described in detail by Santos-Silva et al. (2020).

4.2.3.3. Meat α-tocopherol content.

The α-tocopherol content in meat was determined according to a procedure described by Prates et al. (2006), using a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific) as described in detail by Jerónimo et al. (2020).

4.2.3.4. Lipid oxidation analysis.

Evaluation of the meat lipid stability over storage time was performed as detailed in Francisco et al. (2020) following the general procedure proposed by Grau et al. (2000), where meat lipid oxidation was assessed through the quantification of thiobarbituric acid reactive substances (TBARS). Results were expressed as mg of malondialdehyde (MDA)/kg of meat (Grau et al., 2000).

4.2.4. Physical and sensory analysis of meat.

4.2.4.1. Muscle and subcutaneous adipose tissue colour.

Longissimus thoracis muscle and SAT colour were determined with a chromometer (Minolta CR400, Konica Minolta, Portugal) according to CIE L^*, a^*, b^* system, where L^* is lightness, a^* redness and b^* yellowness. Three measurements per sample were recorded. Measurements were made using the C illuminant and 2° standard observers. Hue angle (H*) was calculated as tan-1(b*/a*) x (180/π) and colour saturation (chroma, C*) as $(a^{*2} + b^{*2})^{1/2}$. Meat colour variation during storage time (between days 3- and 8-days *post-mortem*) was calculated as $\Delta E_{(8—3)} = ((\Delta L^*_{8—3})^2 + (\Delta a^*_{8—3})^2 + (\Delta b^*_{8—3})^2)^{1/2}$.

4.2.4.2. Meat cooking loss and sensory evaluation.

Four sessions were carried out to evaluate the sensory quality of the meat. For each one, four LL samples were randomly selected, corresponding to each of the four diets. The loin joints were thawed for 24h in a refrigerator (4°C). The LL muscle was isolated and weighed before being cooked in an electric oven, pre-warmed at 170°C, until samples reach an internal temperature of 72°C, monitored with an internal thermocouple (Thermometer, Omega RDXL4SD, Manchester, USA). After cooking, the samples were weighted and cooking loss was determined by the difference of samples' weight before and after cooking, expressed as a percentage of the initial weight.

The samples were then prepared to be presented to the sensory panel composed of 11 testers from INIAV-Santarém. The LL was trimmed of external connective tissue, cut into 1 × 1 × 1 cm subsamples, and maintained in heated dishes at 40°C, until tasting.

The meat was evaluated based on a numeric scale going from 1 to 8 for odour intensity, juiciness, tenderness, flavour intensity, flavour acceptance and global acceptance, where 8 corresponded to “extremely odorous, juicy, tender, flavoured and pleasant” and 1 corresponded to “extremely inodorous, dry, tough, unflavoured and unpleasant”. For the off flavours, the evaluation was made only on a binary basis where 1 indicated presence and 0 indicated absence.

4.2.5. Statistical analysis.

Feed intake, live weight gain, carcass traits, FA and SAT colour parameters data were analysed using the Proc Mixed procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) as a completely randomised block design, where individual lamb was used as the experimental unit. The model included the treatment and initial LW block as the fixed factors. Orthogonal contrasts were computed for comparing: i) Control diet with the diets containing EPA sources (C vs. ES); ii) O diet with diets containing *N. oceanica* biomass (O vs. BM); and iii) the two diets containing *N. oceanica* biomass (SD vs. FD). Variance heterogeneity between treatments was tested for a level of $p = 0.01$, and when significant, the variance heterogeneity was accommodated in the model. Statistically significant differences were considered when $p < 0.05$.

Data for α-tocopherol, MDA and muscle colour parameters on day 3 and day 8 *post-mortem* were analysed using repeated measurements model. The model considered the effects of treatment, initial LW block, day, and interaction of day with treatment and an unstructured covariance matrix for observations repeated in the same experimental unit. When the treatment × day interaction was not significant only the main effects were presented.

Data from the sensory panel tests were analysed with a mixed model that included treatment, initial LW block as fixed effects and tasting session and panellists as random blocks.

For the presence or absence of off flavours (binary variable), data were analysed with “proc GLIMMIX” using a binary distribution and a Logit transformation as link function and excluding data from C treatment due to zero occurrence.

4.3. Results.

4.3.1. Intake, growth performance and carcass traits.

The experimental diets did not affect the lambs’ LW at 14 d and 21 d (Table 4.1). As expected, the dry matter intake (DMI) remained constant during the restricted feeding period, but even in the *ad libitum* week, there were no differences among treatments in DMI. During the restricted feeding period (day 1 to 14), lambs fed diets with EPA sources (O, SD and FD diets) tended to have lower ($p = 0.089$) average daily gain (ADG) and had a higher ($p = 0.049$) feed conversion ratio (FCR) than those fed Control diet (269 vs. 316 g/d for ADG and 4.95 vs. 3.71 for feed conversion). The O diet also tended ($p = 0.091$) to increase lambs’ FCR compared with SD and FD diets. In the *ad libitum* period, no differences among treatments were detected for ADG or FCR.

Daily intake of crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and FA, across the 3 weeks of the trial are presented in Table 4.2. Control lambs ingested less CP (226 vs. 241 g/d, $p = 0.011$) and tended ($p = 0.095$) to ingest more NDF when contrasted with lambs fed ES diets. The O diet resulted in lower intake of CP (223 vs. 251 g/d, $p < 0.001$) and higher intake of NDF (325 vs. 293 g/d, $p < 0.001$) and ADF (212 vs. 190 g/d, $p < 0.001$) than the diets containing *N. oceanica* biomass. The SD and FD treatments did not differ in CP intake, but NDF and ADF intake tended ($p < 0.10$) to be higher with FD than SD (Table 4.2).

The intake of all FA, except for ALA, was higher ($p < 0.02$) with the ES diets than with the Control diet. Moreover, the Control diet did not supply EPA and 20:4n-6 to lambs. The intake of all FA, except the 16:0, differed ($p < 0.001$) when the O treatment was contrasted with BM treatments, although the intake of all FA except the 18:2n-6 differed ($p < 0.001$) between SD and FD treatments. The FD diet resulted in the lowest EPA intake among the ES diets.

Hot carcass weight (HCW), cold carcass weight (CCW) and chilling losses were not affected by treatments (Table 4.1), but C fed lambs presented a lower ($p = 0.011$) carcass yield than lambs fed ES diets (46.3 vs. 48.2%).

Table 4.1- Productive performance and carcass traits of lambs fed experimental diets.

	Diets[1]				SEM[2]	Contrasts[3]		
Item	**C**	**O**	**SD**	**FD**		**C vs. ES**	**O vs. BM**	**SD vs. FD**
Live weight (kg)								
Day 1 (initial)	21.4	21.7	21.1	21.1	0.778	-	-	-
Day 15	25.8	25.1	24.9	25.1	0.870	0.473	0.885	0.911
Day 22 (final)	28.7	28.6	28.3	28.0	0.749	0.652	0.614	0.717
DM intake (g/d)[4]								
Day 1 to 14	1 022	1 037	1 045	1 037	2.6	0.565	0.890	0.833
Day 15 to 21	1 333	1 381	1 360	1 289	4.8	0.852	0.339	0.292
Average daily gain (g/d)								
Day 1 to 14	316	248	274	284	23.3	0.089	0.288	0.742
Day 15 to 21	371	431	437	424	35.3	0.141	0.994	0.793
Feed Conversion								
Day 1 to 14	3.71	5.70	5.18	3.98	0.522	0.049	0.091	0.110
Day 15 to 21	3.76	3.29	3.22	3.15	0.333	0.142	0.789	0.879
Carcass traits								
Hot carcass weight (kg)	13.3	13.6	13.6	13.5	0.36	0.445	0.998	0.826
Cold carcass weight (kg)	12.7	13.1	13.1	13.1	0.35	0.378	0.909	0.996
Chilling losses (%)	4.1	3.8	4.0	3.8	0.15	0.209	0.669	0.388
Carcass yield (%)	46.3	47.6	48.1	48.8	0.59	0.011	0.258	0.453

[1] – C, control diet with no EPA sources; O, diet with *Nannochloropsis* sp oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass.[2] – Standard error of the mean.[3] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD.[4] – Dry matter intake.

Table 4.2- Average daily nutrient intake (g/d) during the experiment (1 to 21 d).

	Diets[1]				SEM[2]	Contrasts[3]		
Item	**C**	**O**	**SD**	**FD**		**C vs. ES**	**O vs. BM**	**SD vs. FD**
Dry matter	1071	1104	1107	1064	92.1	0.429	0.509	0.183
Crude protein	226	223	253	248	19.9	0.011	<0.001	0.398
NDF	317	325	284	302	23.4	0.095	<0.001	0.050
ADF	205	212	184	196	17.1	0.129	<0.001	0.057
Fatty acids								
14:0	-	0.32	0.59	0.54	0.030	<0.001	<0.001	<0.001
16:0	4.11	5.78	5.71	6.20	0.466	<0.001	0.217	0.005
*c*9-16:1	0.06	1.30	2.49	1.97	0.119	<0.001	<0.001	<0.001
18:0	0.66	0.74	0.60	0.79	0.061	0.015	0.011	<0.001
*c*9-18:1	3.01	3.62	2.80	3.26	0.277	0.014	<0.001	<0.001
*c*11-18:1	0.12	0.23	0.11	0.16	0.014	<0.001	<0.001	<0.001
18:2n-6	6.64	7.90	6.71	6.91	0.607	0.006	<0.001	0.360
18:3n-3	1.44	1.70	1.19	1.53	0.129	0.457	<0.001	<0.001
20:4n-6	-	0.67	0.89	0.75	0.058	-	<0.001	<0.001
20:5n-3	-	2.60	2.71	1.63	0.178	-	<0.001	<0.001
Total	16.1	25.1	24.0	23.7	1.90	<0.001	0.033	0.701

[1] – C, control diet with no EPA sources; O, diet with Nannochloropsis oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass.[2] – Standard error of the mean.[3] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD.
Note: (*cis*).

4.3.2. FA composition of subcutaneous adipose tissue and *longissimus lumborum* (LL) muscle.

4.3.2.1. FA composition of subcutaneous adipose tissue (SAT).

The SAT from animals fed diets containing EPA sources presented higher ($p < 0.05$) proportions of *iso*-15:0, *iso*-17:0, *c*9–16:1, *t*11–18:1, *t*9,*c*12–18:2, *t*11,*c*15−/*t*10,*c*15–18:2, 20:2n-6, 20:3n-6, 20:3n-3, 20:4n-3, EPA, 22:5n-3 (docosapentaenoic acid, DPA), DHA, oxo-18:0, 20:X and lower ($p < 0.05$) proportions of *iso*-16:0, *c*9–17:1, *c*9–18:1 and *c*13–18:1, compared with the SAT from animals fed C diet (Table 4.3). Taking together these changes, the total n-3 PUFA and the total of biohydrogenation intermediates (BHI) 18:2 increased ($p < 0.05$), whereas the *cis*-MUFA and the *t*10/*t*11 ratio decreased in the SAT from animals fed diets containing EPA sources compared to the Control.

Lambs fed diets containing *N.* oceanica biomass (SD and FD) presented higher ($p < 0.05$) SAT proportions of *iso*-15:0, 16:0, *iso*-17:0, *c*7–16:1, *c*9–16:1, 20:4n-6, EPA, DPA, DHA and lower ($p < 0.05$) proportions of *c*15–18:1, and *t*11,*c*15−/*t*10,*c*15–18:2, compared with those fed the diet containing *Nannochloropsis* sp. oil (O diet). Taking together these changes resulted in the increase of linear-SFA and *iso*-BCFA with *N. oceanica* biomass diets compared with O diet.

Lambs fed the FD diet presented higher ($p < 0.05$) SAT proportions of *c*9–16:1, and lower ($p < 0.05$) proportions of *c*12–18:1, *c*13–18:1, *c*9,*t*12–18:2, *c*9,*c*15–18:2, and on the sum of the unidentified 20:1, 20:2, 20:3 and 20:4 FA (20:X) compared to those fed SD diet. These changes did not translate in the differences of the partial FA sums.

Table 4.3- Fatty acid (FA) profile (mg/g total FA) of subcutaneous adipose tissue from lambs fed experimental diets.

FA profile	Diets[1]				SEM[2]	Contrasts[3]		
	C	O	SD	FD		C vs. ES	O vs. BM	SD vs. FD
SFA								
14:0	31.7	39.7	40.5	45.2	3.34	0.081	0.399	0.244
15:0	6.37	6.49	6.22	6.60	0.321	0.884	0.827	0.361
16:0	219	230	247	243	5.6	0.050	0.027	0.499
17:0	21.8	20.7	16.8	18.6	1.38	0.130	0.145	0.236
18:0	151	141	157	152	8.8	0.909	0.168	0.691
20:0	0.97	1.09	1.87	0.89	0.243	0.151	0.334	0.090
22:0	0.08	0.14	0.10	0.09	0.019	0.083	0.105	0.508
BCFA								
iso-15:0	0.84	0.90	1.35	1.17	0.09	0.006	0.010	0.175
iso-16:0	2.24	1.83	2.14	1.82	0.117	0.038	0.258	0.179
iso-17:0	2.94	2.92	4.00	3.57	0.187	0.025	0.002	0.219
iso-18:0	1.98	1.55	1.39	1.30	0.157	0.071	0.200	0.600
anteiso-15:0	2.20	2.04	2.20	2.01	0.13	0.601	0.559	0.317
anteiso-17:0	7.92	6.41	5.88	7.69	1.423	0.444	0.850	0.648

cis-MUFA								
*c*9-14:1	0.94	1.11	1.07	0.98	0.159	0.605	0.607	0.661
*c*7-16:1	4.17	3.92	4.99	4.53	0.269	0.301	0.010	0.370
*c*9-16:1	15.0	18.2	22.8	29.3	1.454	<0.001	<0.001	0.017
*c*9-17:1	9.57	7.84	5.86	6.10	0.698	0.020	0.083	0.741
*c*9-18:1[4]	331	311	290	283	9.0	0.005	0.123	0.454
*c*11-18:1	11.2	9.97	10.3	11.6	0.543	0.366	0.137	0.187
*c*12-18:1	3.43	2.64	4.27	2.42	0.363	0.393	0.190	0.014
*c*13-18:1	1.48	1.01	1.21	0.93	0.081	0.002	0.606	0.007
*c*15-18:1	1.39	1.89	1.45	1.30	0.173	0.508	0.045	0.485
*c*16-18:1	0.71	0.74	1.03	0.75	0.074	0.071	0.157	0.078
*c*9-19:1	1.27	1.19	1.07	1.07	0.053	0.175	0.072	0.911
*c*11-19:1	0.43	0.37	0.33	0.30	0.042	0.080	0.218	0.621
trans-MUFA								
*t*6-/*t*7-/*t*8-18:1	4.40	4.53	4.82	4.75	0.436	0.603	0.657	0.882
*t*9-18:1	10.3	11.4	6.47	6.70	1.460	0.419	0.077	0.802
*t*10-18:1	39.2	31.0	19.0	24.9	3.741	0.060	0.081	0.125
*t*11-18:1	7.60	20.3	27.5	25.4	5.400	0.010	0.615	0.689
*t*12-18:1	1.28	1.77	1.87	1.27	0.307	0.214	0.768	0.075
*t*16-18:1[5]	2.00	2.01	2.76	2.54	0.373	0.334	0.157	0.706
Non-conjugated dienes								
*c*9,*c*15-18:2	0.68	0.64	0.64	0.51	0.048	0.257	0.364	0.023
*c*9,*t*11-/*t*7,*c*9-18:2	7.04	8.60	9.58	9.29	1.175	0.096	0.665	0.835
*c*9,*t*12-18:2	0.58	0.64	0.80	0.61	0.056	0.092	0.414	0.029
*c*9,*t*13-18:2[6]	2.99	2.90	3.47	3.08	0.326	0.691	0.533	0.317
*t*8,*c*13-/*c*9,*t*15-18:2	1.09	1.13	1.24	1.16	0.158	0.675	0.735	0.704
*t*9,*c*12-18:2	0.67	0.92	1.40	1.18	0.170	0.001	0.115	0.534
*t*9,*t*12-18:2	0.32	0.36	0.19	0.20	0.060	0.485	0.125	0.689
*t*11,*t*15-/*t*10,*t*15-18:2	0.22	0.29	0.19	0.21	0.042	0.958	0.152	0.721
*t*11,*c*15-/*t*10,*c*15-18:2	3.38	6.34	3.02	4.31	0.524	0.047	0.010	0.079
Conjugated dienes								
*c*9,*t*11-/*t*7,*c*9-18:2	7.04	8.60	9.58	9.29	1.175	0.096	0.665	0.835
*t*9,*c*11-18:2	0.47	0.34	0.26	0.19	0.055	0.100	0.100	0.089
*t*10,*c*12-18:2	0.28	0.18	0.35	0.16	0.052	0.440	0.246	0.123
n-6 *PUFA*								
18:2n-6	43.6	36.8	35.0	36.3	2.540	0.107	0.704	0.595
20:2n-6	0.30	0.83	0.79	0.79	0.068	<0.001	0.575	0.956
20:3n-6	0.44	1.10	0.97	1.37	0.120	<0.001	0.680	0.058
20:4n-6	2.36	1.64	2.49	3.09	0.261	0.902	<0.001	0.157
22:4n-6	0.43	0.16	0.21	0.17	0.051	0.075	0.331	0.345
n-3 PUFA								
18:2n-3	0.03	0.03	0.04	0.03	0.005	0.086	0.413	0.251
18:3n-3	7.71	7.80	7.18	7.62	0.461	0.782	0.475	0.432
20:3n-3	0.22	2.61	2.02	1.62	0.221	<0.001	0.098	0.272
20:4n-3	0.05	3.40	1.60	2.37	0.331	<0.001	0.086	0.123
20:5n-3	0.36	1.91	3.09	4.16	0.335	<0.001	0.002	0.170
22:5n-3	1.61	2.39	3.42	3.86	0.322	<0.001	0.005	0.447
22:6n-3	0.33	0.38	0.66	0.64	0.096	0.010	0.033	0.898
Other FA								
oxo-18:0	0.40	0.80	0.79	0.82	0.122	0.001	0.964	0.861
Partial sums								
SFA								
L-SFA[7]	436	445	477	474	10.5	0.152	0.034	0.782
iso-BCFA	8.35	7.54	9.53	8.30	0.369	0.787	0.005	0.081
anteiso-BCFA	10.1	8.42	8.14	9.71	1.479	0.440	0.803	0.695
NT-BCFA[8]	18.0	16.6	8.7	8.7	3.61	0.376	0.091	0.996
Total BCFA	35.7	32.2	25.8	26.5	4.50	0.400	0.195	0.859
cis-MUFA	383	360	345	342	9.6	0.013	0.335	0.767
trans-MUFA	67.0	71.6	63.5	65.9	6.74	0.999	0.511	0.748
n-6 PUFA	47.5	40.7	39.5	41.8	2.77	0.182	0.987	0.407
n-3 PUFA	10.5	18.6	18.1	20.3	1.43	<0.001	0.736	0.356

Total PUFA[9]	78.1	87.4	84.3	87.0	3.99	0.089	0.719	0.620
BHI 18:1[10]	73.9	77.8	71.5	71.3	6.63	0.962	0.525	0.980
BHI 18:2[11]	19.0	23.2	22.2	21.7	1.23	0.024	0.387	0.785
20:X[12]	1.63	6.59	7.11	4.90	0.552	<0.001	0.391	0.008
Ratios								
*t*10/*t*11 ratio[13]	3.52	2.40	0.78	1.13	0.603	0.006	0.060	0.679
SCD17[14]	30.3	27.3	25.9	24.4	1.46	0.013	0.254	0.443

[1] – C, control diet with no EPA sources; O, diet with *Nannochloropsis* oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. [2] – Standard error of the mean. [3] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD. [4] – Coelutes with minor quantities of *c*10-18:1, and *t*13-/*t*14-18:1. [5] – Coelutes with minor quantities of *c*14-18:1. [6] – Coeluted peak of *c*9,*t*13-/*c*9,*t*14-/*t*8,*c*12-18:2 and 11-cyclohexyl-11:0. [7] – Linear SFA: Sum of 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 22:0. [8] – Non-terminal BCFA: Sum of 10:0-4Me/10:0-6Me, 12:0-4Me, 12:0-8Me, 14:0-6Me, 14:0-8Me, 14:0-4Me, 14:0-10Me, 14:0-4,8Me/-6,8Me, 16:0-6Me, 16:0-8Me, 16:0-4Me and 16:0-12Me. [9] – Sum of n-3 PUFA, n-6 PUFA, biohydrogenation intermediates (BHI) 18:2, and 20:2, 20:3, 20:4 isomers. [10] – Sum of *t*6/*t*7/*t*8-18:1, *t*9-18:1, *t*10-18:1, *t*11-18:1, *t*12-18:1, *c*12-18:1, *c*13-18:1, *t*16-18:1, *c*15-18:1 and *c*16-18:1. [11] – Sum of *t*11,*t*15-/*t*10,*t*15-18:2, *t*9,*t*12-18:2, *c*9,*t*13-18:2, *t*8,*c*13-/*c*9,*t*15-18:2, *c*9,*t*12-18:2, *t*9,*c*12-18:2, *t*11,*c*15-/*t*10,*c*15-18:2, *c*9,*c*15-18:2 and *c*12,*c*15-18:2. [12] – Sum of 20:1, 20:2, 20:3 and 20:4, excluding n-6 and n-3 C20 PUFA. [13] – Ratio between *t*10-18:1 and *t*11-18:1. [14] – Stearoyl-CoA activity index computed as *c*9-17:1/(*c*9-17:1+17:0) ×100. Note: *t* (*trans*), *c* (*cis*).

4.3.2.2. FA composition of the *longissimus lumborum* (LL) muscle.

Total FA content of muscle did not differ between treatments and averaged 11.1 ± 0.61 g/kg of meat (Table 4.4). The muscle FA profile, expressed as mg/g of total FA, is also shown in Table 4.4. The most abundant FA were *c*9–18:1, 16:0, 18:0 and 18:2n-6, followed by 20:4n-6 and 14:0.

Muscles from animals fed diets containing EPA sources presented higher ($p < 0.05$) proportions of 16:0, *t*9–16:1, *c*9–16:1, *t*9–18:1, *t*11–18:1, *t*15–18:1, *t*9,*c*12–18:2, 20:2n-6, 20:3n-6 and lower ($p < 0.05$) proportions of *anteiso*-17:0, *iso*-18:0, *c*9–17:1, 18:0, *c*9–18:1, *t*10–18:1, *c*13–18:1, 18:3n-6, 20:3n-9 and 22:4n-6, compared with the muscle from animals fed the C diet. The dietary inclusion of EPA sources led to a clear and significant increase of all n-3 PUFA, except for the ALA and DHA. These changes resulted in the increase of the sum of n-3 PUFA (32.7 in C vs 49.5 mg/g FA in ES diets) and a decrease ($p < 0.05$) on the sum of BCFA and *cis*-MUFA.

Lambs fed diets containing *N*. oceanica biomass (SD and FD) presented higher ($p < 0.05$) intramuscular proportions of *iso*-16:0, *c*7–16:1, *c*9–16:1, *iso*-17:0, *t*12–18:1, *t*15–18:1, *c*16–18:1, *t*9,*c*12–18:2, 18:3n-6, 20:0, 20:2n-6, 20:4n-6, 22:4n-6, EPA, DPA and lower ($p < 0.05$) proportions of *c*9–18:1, *t*11,*c*15−/*t*10,*c*15–18:2, 20:3n-3, and 20:4n-3, compared to those fed the diet containing *Nannochloropsis* sp. oil (O diet). Taking together these changes resulted in an increase in the sum of n-3 PUFA (42.9 in O vs 52.8 mg/g FA in BM diets) and total PUFA, and a decrease of *cis*-MUFA and *t*10/*t*11 ratio.

Lambs fed the FD diet presented higher ($p < 0.05$) intramuscular proportions of *c*9–16:1 and *c*12,*c*15–18:2 and lower ($p < 0.05$) proportions of *iso*-16:0, *c*12–18:1, *c*16–18:1, 20:3n-9 and 20:0 compared with those fed SD diet. These changes did not translate in the differences of the partial FA sums.

The most nutritionally relevant FA and FA sums are expressed as mg/100 g muscle and are depicted in Table 4.5. Animals fed diets containing EPA sources presented higher ($p < 0.05$) content of *c*9–16:1, EPA and DPA in muscle compared to those fed the C diet. Compared with C treatment, the EPA and DPA content were 2 and 1.4 times higher in EPA containing treatments. Overall, these changes increased the sum of n-3 PUFA, n-3 LC-PUFA (24.9 in C vs 42.8 mg/100 g FA in ES diets) and EPA + DHA (11.8 in C vs 21.5 mg/100 g FA in ES diets).

Lambs fed diets containing *N. oceanica* biomass (SD and FD) presented higher ($p < 0.05$) intramuscular fat content of 20:4n-6, EPA, DPA and lower ($p < 0.05$) of *c*9–18:1 compared to those fed the diet containing *Nannochloropsis* sp. oil (O) (Table 4.5). These changes increased the sum of n-3 LC-PUFA (38.4 in O vs 45 mg/100 g FA in BM diets), n-6 LC-PUFA (40.1 in O vs 44.8 mg/100 g FA in BM diets), EPA + DHA (18.2 in O vs 23.2 mg/100 g FA in BM diets) and decreased ($p = 0.012$) the sum of *cis*-MUFA (414 in O vs 343 mg/100 g FA in BM diets).

No differences in the content of nutritionally relevant FA or partial FA sums were found between meat from lambs fed SD and FD diets. Nevertheless, the content of EPA tended to be higher ($p = 0.054$) in meat from lambs fed FD when compared to SD.

Table 4.4- Total fatty acid (FA) content (mg/g of meat) and fatty acid profile (mg/g total FA) of *longissimus lumborum* muscle from lambs fed the experimental diets.

Item	**Diets^1**				**SEM^2**	**Contrasts^3**		
	C	**O**	**SD**	**FD**		**C vs. ES**	**O vs. BM**	**SD vs. FD**
Total FA	11.2	11.8	10.2	11.2	0.61	0.913	0.140	0.293
FA profile								
SFA								
12:0	2.43	2.57	2.67	2.48	0.265	0.646	0.969	0.603
14:0	25.5	28.1	28.6	28.2	1.90	0.211	0.905	0.885
15:0	3.40	3.33	3.55	3.71	0.168	0.561	0.159	0.498
16:0	219	231	231	233	4.3	0.017	0.781	0.792
17:0	10.9	10.5	9.4	10.2	0.51	0.162	0.294	0.304
18:0	155	143	144	139	3.3	0.026	0.609	0.299
20:0	1.12	1.01	1.52	1.12	0.104	0.422	0.021	0.010
23:0	1.30	1.23	1.56	1.38	0.211	0.708	0.371	0.546
BCFA								
iso-15:0	0.58	0.49	0.73	0.61	0.071	0.739	0.052	0.237
iso-16:0	1.20	0.90	1.34	1.05	0.089	0.313	0.013	0.027
iso-17:0	2.59	2.48	3.10	2.85	0.182	0.303	0.036	0.324
iso-18:0	1.15	0.90	0.95	0.81	0.058	<0.001	0.789	0.088
anteiso-15:0	1.12	0.96	1.08	0.94	0.089	0.229	0.668	0.275
anteiso-17:0	3.75	2.99	2.98	2.59	0.202	<0.001	0.399	0.173
cis-MUFA								
*c*9-14:1	0.70	0.77	0.80	0.72	0.096	0.588	0.926	0.570
*c*7-16:1	3.07±0.12	2.67±0.08	3.26±0.13	2.95±0.27	-	0.489	0.023	0.317
*c*9-16:1	11.8	13.6	15.8	17.9	0.65	<0.001	<0.001	0.032

*c*9-17:1	5.91	5.10	4.68	4.63	0.293	0.003	0.253	0.897
*c*9-18:1[4]	307	307	269	273	5.7	0.001	<0.001	0.626
*c*11-18:1	16.5	15.8	16.7	18.9	0.87	0.516	0.068	0.088
*c*12-18:1	2.87	2.55	3.71	2.26	0.274	0.920	0.206	<0.001
*c*13-18:1	0.95	0.51	0.56	0.53	0.057	<0.001	0.601	0.763
*c*15-18:1	0.68	0.72	0.70	0.65	0.098	0.932	0.669	0.716
*c*16-18:1	0.71	0.45	1.00	0.69	0.084	0.975	0.001	0.016
19:1	0.61	0.51	0.50	0.48	0.056	0.079	0.746	0.819
trans-MUFA								
*t*9-16:1	0.66±0.15	1.07±0.42	1.46±0.25	1.52±0.24	-	0.006	0.379	0.856
*t*6-/*t*7-/*t*8-18:1	2.16	1.99	2.22	2.04	0.139	0.647	0.427	0.362
*t*9-18:1	2.18	2.52	2.86	2.66	0.155	0.012	0.209	0.393
*t*10-18:1	18.1	14.0	8.4	10.3	2.24	0.011	0.102	0.540
*t*11-18:1	10.0±1.24	15.3±5.48	18.2±2.26	19.0±2.21	-	0.007	0.579	0.811
*t*12-18:1	3.23	3.05	4.21	3.65	0.245	0.172	0.007	0.113
t15-18:1	0.78	0.81	1.41	1.21	0.138	0.032	0.007	0.308
*t*16-18:1[5]	1.19	1.15	1.42	1.34	0.133	0.462	0.172	0.644
Non-conjugated dienes								
*c*9,*c*15-18:2	0.66	0.51	0.56	0.45	0.066	0.061	0.983	0.253
*c*12,*c*15-18:2	0.41	0.65	0.35	0.64	0.094	0.205	0.189	0.038
*c*9,*t*12-18:2	0.40	0.46	0.48	0.48	0.044	0.174	0.711	0.988
*c*9,*t*13-18:2[6]	2.24	2.50	2.56	2.58	0.216	0.231	0.776	0.961
*t*8,*c*13-/*c*9,*t*15-18:2	1.07	1.07	1.19	1.05	0.106	0.817	0.676	0.344
*t*9,*c*12-18:2	0.72	0.76	1.66	1.32	0.219	0.045	0.011	0.271
*t*11,*c*15-/*t*10,*c*15-18:2	2.15	2.98	1.91	2.48	0.276	0.356	0.033	0.159
Conjugated dienes								
*c*9,*t*11-18:2[7]	5.21	6.05	7.07	7.06	0.789	0.108	0.305	0.996
n-6 PUFA								
18:2n-6	90.7	84.4	92.2	90.7	5.07	0.778	0.262	0.837
18:3n-6	1.03	0.67	0.85	0.87	0.053	<0.001	0.007	0.740
20:2n-6	0.93	1.07	1.26	1.28	0.074	0.005	0.046	0.871
20:3n-6	3.53	4.02	4.49	4.87	0.285	0.003	0.077	0.363
20:4n-6	33.0	28.2	36.7	34.9	2.24	0.909	0.011	0.569
22:4n-6	2.36	1.32	1.94	1.59	0.123	<0.001	0.008	0.055
n-3 PUFA								
18:3n-3[8]	9.73	9.99	9.57	9.22	0.445	0.852	0.658	0.588
20:3n-3	0.45	2.11	1.67	1.45	0.155	<0.001	0.007	0.319
20:4n-3	0.56	2.85	1.86	2.20	0.329	<0.001	0.049	0.482
20:5n-3	7.51	11.7	16.7	19.0	1.51	<0.001	0.003	0.277
22:5n-3	11.1	12.4	17.1	17.5	1.15	0.002	0.002	0.791
22:6n-3	3.47	3.84	4.96	4.15	0.517	0.176	0.276	0.283
20:3n-9[9]	4.67	3.38	4.57	3.24	0.367	0.036	0.245	0.016
Partial sums								
L-SFA[10]	418	421	422	419	7.1	0.756	0.990	0.724
BCFA	10.2	8.6	10.2	8.8	0.54	0.043	0.243	0.140
trans-MUFA	39.2	40.2	40.9	42.0	3.65	0.610	0.826	0.828
cis-MUFA	352	350	318	323	5.5	0.013	0.006	0.416
n-6 PUFA	131	120	136	134	7.2	0.905	0.125	0.862
n-3 PUFA	32.7	42.9	51.4	54.2	3.13	<0.001	0.016	0.583
Total PUFA	182	181	210	208	9.2	0.107	0.021	0.887
Ratios								
*t*10/*t*11 ratio[11]	1.76	1.34	0.51	0.63	0.258	0.005	0.021	0.740
SCD17[12]	35.4	32.6	33.3	31.2	1.07	0.023	0.827	0.168

[1] – C, control diet with no EPA sources; O, diet with *Nannochloropsis* oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. [2] – Standard error of the mean. [3] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD. [4] – Coelutes with minor quantities of *c*10-18:1 and *t*13-/*t*14-18:1. [5] – Coelutes with *c*14-18:1 as minor isomers.[6] – Coeluted peak of *c*9,*t*13-/*c*9,*t*14-/*t*8,*c*12-18:2 and 11-cyclohexyl-11:0. [7] – Coeluted peak of *c*9,*t*11-/*t*7,*c*9-/*t*8,*c*10-18:2. [8] – Coelutes with 20:1. [9] – Coelutes with minor quantity of *c*9,*t*11,*c*15-18:3. [10] – Liner SFA: Sum of 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 23:0. [11] – Ratio between *t*10-18:1 and *t*11-18:1. [12] – Stearoyl-CoA activity index computed as c9-17:1/(c9-17:1+17:0)×100. Note: *t* (trans), *c* (cis).

Table 4.5- Intramuscular fat and fatty acid content (mg/ 100 g of meat) from lambs fed experimental diets.

Item	Diets[1]				SEM[2]	Contrasts[3]		
	C	O	SD	FD		C vs. ES	O vs. BM	SD vs. FD
Intramuscular fat	1639	1698	1650	1722	83.7	0.599	0.810	0.542
FA profile								
14:0	28.5	33.9	30.1	32.6	3.64	0.385	0.567	0.627
16:0	244	274	240	261	17.8	0.480	0.285	0.385
18:0	173	170	147	155	9.9	0.188	0.142	0.572
*c*9-16:1	13.1	16.2	16.5	20.0	1.43	0.012	0.248	0.085
*c*9-18:1	344	365	276	308	19.29	0.216	0.005	0.236
18:2n-6	101	98	92	98	4.55	0.361	0.506	0.379
20:4n-6	36.5	32.7	35.7	37.7	1.29	0.463	0.018	0.275
18:3n-3	10.9	12.0	9.8	11.0	0.871	0.945	0.147	0.294
20:5n-3	8.0	13.8	16.6	20.3	1.30	<0.001	0.007	0.054
22:5n-3	12.1	14.5	17.2	18.7	0.96	<0.001	0.007	0.271
22:6n-3	3.84	4.48	4.83	4.54	0.474	0.169	0.727	0.662
Partial sums								
L-SFA[4]	466	500	436	470	32.1	0.950	0.244	0.449
BCFA	11.6	10.3	10.6	10.0	1.17	0.341	0.994	0.734
cis-MUFA	392	414	324	362	22.2	0.324	0.012	0.211
trans-MUFA	43.3	46.5	43.6	46.9	5.54	0.720	0.859	0.680
n-6 PUFA	146	139	136	145	3.59	0.357	0.876	0.264
n-6 LC-PUFA[6]	43.8	40.1	43.4	46.1	1.36	0.688	0.010	0.166
n-3 PUFA	36.2	51.1	52.5	59.4	2.95	<0.001	0.195	0.108
n-3 LC-PUFA[5]	24.9	38.4	42.4	47.6	2.49	<0.001	0.039	0.145
EPA+DHA[7]	11.8	18.2	21.5	24.8	1.63	<0.001	0.021	0.152
PUFA	190	201	198	214	7.52	0.122	0.608	0.122

[1] – C, control diet with no EPA sources; O, diet with Nannochloropsis oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. [2] – Standard error of the mean. [3] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD. [4] – Linear SFA: Sum of 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 23:0. [5] – Long-chain n-3 PUFA: Sum of 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3. [6] – Long-chain n-6 PUFA: Sum of 20:2n-6, 20:3n-6, 20:4n-6 and 22:4n-6. [7] – Sum of 20:5n-3 and 22:6n-3. Note: *t* (*trans*), *c* (*cis*).

4.3.3. Meat colour, α-tocopherol content, and lipid oxidation.

Primary meat colour parameters (CIE, L^*, a^*, b^*) and the chroma parameter were not affected by treatments ($p > 0.05$). However, including EPA sources in the diets of lambs resulted in lower values of Hue angle and ΔE when compared to Control, but no differences between ES treatments were observed. Independently of the diet, L^* and a^* were higher ($p < 0.01$) and b^* and Hue angle were lower ($p < 0.001$) on day 3 compared with day 8 (Table 4.6). No significant interactions between diet and storage time were detected.

The SAT colour parameters did not differ between C and ES diets or between SD and FD diets. However, the values of b^*, chroma, and Hue angle were higher ($p < 0.05$) in *N. oceanica* biomass diets than in the O diet.

Table 4.6- Colour parameters of *longissimus thoracis* muscle and subcutaneous adipose tissue of lambs fed experimental diets.

	Time after slaughter		Diets[1]				Time	Contrasts[2]			T*D
	3 days	8 days	C	O	SD	FD		C vs. ES	O vs. BM	SD vs. FD	
Muscle											
*L**	45.8±0.45	44.8±0.37	45.7±0.77	45.4±0.74	45.3±0.73	45.0±0.72	0.001	0.549	0.800	0.772	0.317
*a**	15.2±0.47	13.9±0.28	13.7±0.62	14.7±0.60	14.9±0.60	14.8±0.59	0.008	0.138	0.852	0.928	0.847
*b**	8.5±0.15	11.6±0.19	10.3±0.28	9.9±0.27	10.0±0.27	10.1±0.26	<0.001	0.342	0.543	0.657	0.447
Chroma	17.5±0.42	18.1±0.32	17.3±0.63	17.8±0.60	18.0±0.59	18.1±0.58	0.129	0.351	0.741	0.946	0.812
Hue angle	29.7±1.03	39.9±0.41	37.4±1.02	33.5±1.00	33.8±1.00	34.4±0.99	<0.001	0.005	0.664	0.686	0.621
∆E	-	-	5.20±0.33	3.96±0.32	4.26±0.32	4.13±0.31	-	0.007	0.549	0.781	-
Subcutaneous adipose tissue											
*L**	-	-	74.1±1.30	73.7±1.25	74.1±1.24	73.7±1.21	-	0.879	0.861	0.810	-
*a**	-	-	5.10±0.79	4.65±0.76	5.26±0.75	3.63±0.74	-	0.506	0.827	0.135	-
*b**	-	-	8.31±0.66	7.53±0.63	10.7±0.63	9.39±0.62	-	0.234	0.004	0.160	-
Chroma	-	-	9.81±0.82	8.89±0.79	12.1±0.79	10.2±0.77	-	0.532	0.029	0.090	-
Hue angle	-	-	58.8±3.21	58.4±3.09	65.8±3.07	68.9±3.00	-	0.133	0.025	0.469	-

[1] – C, control diet with no EPA sources; O, diet with Nannochloropsis oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. [2] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD. Values are means ± standard error of the mean.

An interaction between diets and the number of days *post-mortem* ($p < 0.05$) was observed for both meat α-tocopherol and MDA content (Figure 4.1). The meat α-tocopherol content was lowest at day 3 and day 8 *post-mortem* in C treatment, whereas for EPA sources treatments it was highest at day 3 in O treatment, decreasing at day 8 *post-mortem* in all EPA sources treatments (Figure 4.1-A). The meat MDA content remained low and stable in all treatments at day 3, but at day 8 increased sharply in the C treatment whereas remained low at the ES treatments (Figure 4.1-B).

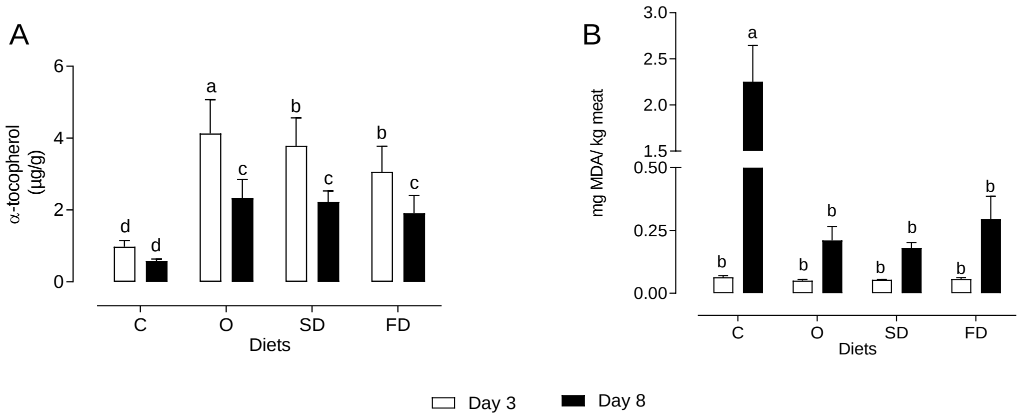

Figure 4.1-Concentration of α-tocopherol (A) and of malondialdehyde (MDA) (B) in meat of lambs fed experimental diets at 3 rd and 8 th day post-mortem (Interaction diet × time, $p < 0.003$).
Experimental diets are represented as follow: C, control diet with no EPA sources; O, diet with *Nannochloropsis* oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. Least square means are presented in each bar with respective standard error of the mean. Different letters (a–d) in each bar indicate significant differences ($P < 0.05$) among means.

4.3.4. Sensory evaluation.

The meat sensory variables presented in Table 4.7 (i.e. odour intensity, succulence, tenderness, flavour intensity, flavour acceptance and global acceptance) were not affected by treatments ($p > 0.05$). Even though detection of off flavours in the meat was very low and null in the C treatment, off flavour detection was significantly higher ($p = 0.022$) for FD treatment than for the O and SD (Figure 4.2).

Table 4.7 - Sensory evaluation of meat from lambs fed experimental diet.

Item	Diets[1]				SEM[2]	Constrasts[3]		
	C	O	SD	FD		C vs. ES	O vs. BM	SD vs. FD
Odour intensity	3.90	3.76	3.97	3.95	0.395	0.969	0.260	0.952
Succulence	5.40	5.66	5.72	5.49	0.334	0.203	0.777	0.311
Tenderness	6.39	6.81	6.45	6.50	0.261	0.255	0.061	0.789
Flavour intensity	4.26	4.03	4.11	4.22	0.401	0.396	0.458	0.612
Flavour acceptance	6.10	6.13	6.01	5.75	0.279	0.365	0.116	0.169
Global acceptance	6.11	6.30	6.17	5.99	0.264	0.751	0.118	0.289

[1] – C, control diet with no EPA sources; O, diet with Nannochloropsis oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. [2] – Standard error of the mean. [3] – C vs. ES, compares C with O, SD and FD together; O vs. BM, compares O with SD and FD together; SD vs. FD, compared SD with FD.

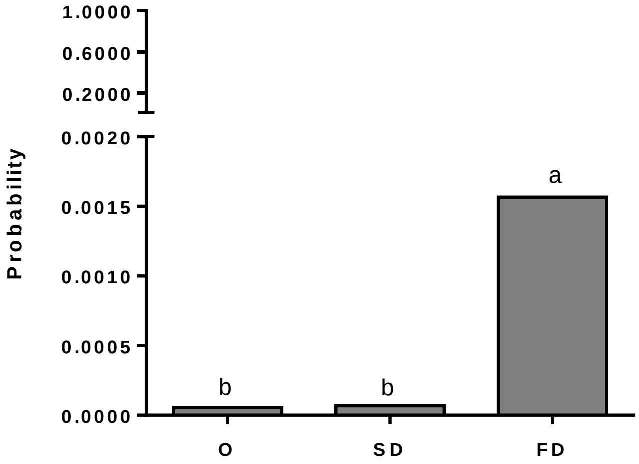

Figure 4.2 -Probability of occurrence of off flavours on sensory evaluation of meat form lambs fed *Nannochloropsis* sources of EPA.

Experimental diets are represented as follow: C, control diet with no EPA sources; O, diet with *Nannochloropsis* oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. Least square means are presented in each bar. Different letters (a–b) in each bar indicate significant differences ($P < 0.05$) among means.

4.4. Discussion.

Previous work from our group (Alves et al., 2018) demonstrated, *in vitro*, that PUFA from FD *N. oceanica* biomass was naturally protected against RBH. The present experiment was designed to confirm if the protective features of FD *N. oceanica* biomass are replicable *in vivo,* and to evaluate to what extent the increased protection against RBH of *N. oceanica* PUFA is translated in the tissues FA profile and other meat quality traits. The results on protection of *N. oceanica* PUFA in the rumen *in vivo* were published in a companion paper (Vítor et al., 2021). The results on intake of diets containing *N. oceanica*, as well as their effects on tissues FA and meat quality traits are presented here.

Feed intake in ruminants is frequently depressed with microalgae supplemented diets, although this effect is highly variable with the microalga used, and as reviewed by Valente et al. (2021), the most frequent explanation for that DMI depression is the low palatability of microalgae, probably due to the occurrence of fish-like odours, their finely powdered physical structure or propensity to lipoxidation.

Low palatability of microalgae has been reported earlier (Papadopoulos et al., 2002; Van Emon et al., 2015; Urrutia et al., 2016; Lamminen et al., 2017) and could explain the adverse effects on intake. Lamminen et al. (2019) reported that *Nannochloropsis gaditana* has low palatability in dairy cattle. As far as we know, this is the first report on the effect of diets containing *N. oceanica* biomass on ruminants' feed intake and our results indicate clearly that diets containing *N. oceanica* biomass were palatable and allowed similar voluntary FI and growth rate to the C and O diets. Thus, probably the palatability of microalgae species might differ widely even for species within the same genus. Alternatively, however, it cannot be overlooked that the palatability issues may have been to some degree avoided due to the pelleting, and addition of vitamin E to diets containing microalgae.

Due to the high cost of spray-dried *N. oceanica* biomass and the difficulty of obtaining enough freeze-dried biomass with lab-scale equipment, we were forced to limit the duration of the experiment to 3 weeks. In the first two weeks, the intake was restricted to about 1 kg DM/day, and the lambs fed the Control diet tended to grow faster than those fed EPA source diets, but this effect disappeared in the third week when the intake was *ad libitum*.

The time on feed was too short to evaluate effects on growth, but, consistent with FI, no noticeable effects were noted, besides an increase in carcass yield in the lambs fed *N. oceanica* biomass diets. The higher sodium content in the *N. oceanica* biomass diets is expected to increase water intake. The increased water intake and the consequent higher digesta flow rate might explain the larger carcass yield observed, as reported by others for lambs fed saline pastures (Friha et al., 2022).

The diets containing EPA sources were designed to supply identical quantities of EPA (≈ 3 g/kg DM), nevertheless the EPA content was lower in the FD diet (1.39 g/kg DM) than in SD (2.20 g/kg DM) or O (2.14 g/kg DM) (Vítor et al., 2021). These differences might be due to losses during handling, feed production, and differences among slurry batches. As FI was similar among treatments, the differences in dietary EPA contents are directly translated in EPA intake, which was about 1 g/day lower with FD than with SD or O treatments. The unprotected

EPA present in O diet was more extensively hydrogenated in the rumen than that present in *N. oceanica* biomass diets (Vítor et al., 2021), explaining why EPA content in tissues was lower in O than in SD and FD treatments. Despite the lower EPA intake in FD lambs, the EPA content in the tissues did not differ or was tendentially higher in the FD than SD treatment, reflecting the higher degree of protection against rumen biohydrogenation of freeze-dried biomass, as the spray-drying method can rupture intact cells due to its high-pressure atomisation process as discussed in Vítor et al. (2021).

The feasibility of using alternative feeds for ruminants depends, among others, on their feeding value, animal production responses and feed costs compared to the conventional feeds (Halmemies-Beauchet-Filleau et al., 2018). For that matter, microalgae cannot yet be regarded as an alternative to crops as a protein source for the feed market due to high production costs mainly associated with technologies needed for cultivation, harvesting, processing/extraction and drying (Saadaoui et al., 2021). However, it is also notable that in only 3 weeks of supplementation, the EPA content of tissues increased so expressively with *N. oceanica* biomass diets. In bovines, the n-3 LC-PUFA are preferentially incorporated in muscle phospholipids instead of neutral lipids of muscle or adipose tissue (Bessa et al., 2015). Lambs also show a similar pattern, although not so strictly, particularly when fed diets supplemented with marine lipids (Cooper et al., 2004; Meale et al., 2014).

The present results confirm that pattern, as despite n-3 LC-PUFA being present in a higher proportion in the lean muscle than in SAT, the response to EPA feeding was larger in SAT than in muscle (i.e., 11.6 times in SAT and 2.5 times in the muscle with FD treatment). The muscle's proportionally smaller n-3 LC-PUFA incorporation is probably explained by the regulatory control mechanism to ensure proper membrane fluidity and functionality (Hagen et al., 2010). In contrast, the proportionally larger deposition in SAT reflects the increased metabolic availability of EPA.

Tissues of lambs fed EPA diets had increased proportions of most C20 FA, including several non-identified isomers (20:X) derived from the RBH of C20 PUFA present in EPA diets. The intermediates formed during EPA biohydrogenation are numerous and not yet fully described (Alves et al., 2018; Toral et al., 2018b). Consistently with the lower RBH of EPA in FD treatment, 20:X (Sum of 20:1, 20:2, 20:3 and 20:4, excluding n-6 and n-3 C20 PUFA) were lower.

Nannochloropsis sp. lipids do not contain detectable amounts of DPA or DHA (Ma et al., 2016; Alves et al., 2018). Thus, the increase of DPA in both tissues, although with lower fold change, indicates the conversion of EPA to DPA (Alvarenga et al., 2015; Drouin et al., 2019).

The concentration of DHA in SAT, although very low, followed the increase of EPA and DPA, which is also indicative of the endogenous n-3 LC-PUFA anabolism. Although being low, DHA enhancement in animal edible tissues is of major importance, due to various health benefits it can have for consumer's health (e.g., brain and visual system) (Calder, 2016; Troesch et al., 2020). Conversely, the concentrations of DHA in the muscle did not follow those of EPA and DPA, suggesting that DHA concentration in muscle membranes remained low due to a predetermined regulation (Calder, 2016). In fact, DHA in muscle is always present in low concentrations, which contrasts with DPA, often the main n-3 LC-PUFA (Curtis & Black, 2013). Similar to EPA and DHA, DPA is also associated with the consumer's health benefits, like heart disease prevention, improvement of lipid metabolism, anti-inflammatory processes, blood metabolites and mental health and cognitive function (Byelashov et al., 2015). DPA can also serve as a reservoir for EPA and DHA conversion (Byelashov et al., 2015; Drouin et al., 2019). Therefore, the inclusion of DPA in n-3 LC-PUFA requirements beyond EPA and DHA is desirable. The minimum recommended dietary allowance for n-3 LC-PUFA is 250 mg/d of EPA + DHA (FAO, 2010), so a 100 g portion of lean loin meat from lambs fed the FD diet provided about 10% of the recommended minimum requirement. If DPA and smaller portions of 20:3n-3 and 20:4n-3 were added to EPA and DHA, 100 g of loin from FD fed lambs would totalize 48 mg of n-3 LC PUFA, which represents circa 20% of the minimum recommended n-3 LC-PUFA daily dose.

According to EU regulations, food can be labelled as a source of omega-3 FA if it contains at least 40 mg of the sum of EPA and DHA per 100 g (EC, 2010). Therefore, meat from FD fed lambs reached 63% of this target. If total n-3 LC-PUFA could be considered, the minimum target of 40 mg n-3 LC-PUFA would have been achieved, and the meat from lambs fed FD diet could be labelled as a source of omega-3 FA. Furthermore, one should consider that lambs were fed the experimental diets for only three weeks, whereas the typical finishing period for light lambs is usually 5 to 8 weeks (Sañudo et al., 1998). Thus it is expectable that longer feeding periods would result in higher n-3 LC-PUFA content in meat (Ponnampalam et al., 2006).

Besides the effects on LC-PUFA, the inclusion of EPA sources in the diets had only a mild effect on FA composition of lamb's muscle and SAT, probably because of the low intake of supplementary FA (i.e. ES diets supplying daily more 8 to 9 g FA than C). One of the most responsive FA was *c*9–16:1, clearly increased in the tissues with EPA-source treatments, certainly because it is a major FA of *Nannochloropsis* lipids (Ma et al., 2016; Alves et al., 2018) being present in a minor amount in the C diet and common feedstuffs. The effects of the *c*9–

16:1 on health and diseases are currently controversial, however, it has been associated with increased insulin sensitivity, decreased hepatic lipid accumulation and expression of proinflammatory markers and adipokines related to the establishment of metabolic abnormalities (Frigolet & Gutiérrez-Aguilar, 2017; Hu et al., 2019).

In general, lipid supplementation of ruminant diets tends to reduce the concentration of 16:0 in tissues as it inhibits the *de novo* FA synthesis in the tissues (Van Leeuwen et al., 2017) and 16:0 is the major output of FA synthase (Drackley, 2000). Nevertheless, our results showed that 16:0 content in the tissues increased with EPA-source diets. This was probably due to the low lipid content of diets and the increased 16:0 intake allowed by EPA-source diets (Vítor et al., 2021), a consequence of the high 16:0 amount in *Nannochloropsis* lipids (Ma et al., 2016; Alves et al., 2018). Increases in 16:0 content in tissues with algal lipids supplementation have been reported (Urrutia et al., 2016; Díaz et al., 2017; Valença et al., 2021) and explained by these lipid sources' richness in 16:0. The enrichment in meat 16:0 is not desirable as it is well established that increased intake of 16:0 is associated with elevated total plasma low-density lipoproteins (LDL) cholesterol and cholesterol concentrations (FAO, 2010).

It is well established that feeding marine oils to ruminants interrupts the complete RBH of C18 unsaturated FA, leading to a strong accumulation of biohydrogenation intermediates (BHI) and a reduced yield of 18:0 (Scollan et al., 2001b; Lee et al., 2005; Boeckaert et al., 2007a; Kim et al., 2008). It is the last step of RBH, that catalyses the hydrogenation of octadecenoates, that is mostly inhibited, being the DHA and the EPA (Abughazaleh & Jenkins, 2004b; Lv et al., 2016; Toral et al., 2017) the active compounds in this process. Thus, it is surprising that in our experiment the inclusion of EPA sources in the diets of lambs did not lead to an increase of *trans*-18:1 isomers in the tissues. Probably, due to the low intake of EPA (i.e. circa 1.6 to 2.7 g/d) and slow release on the *N. oceanica* biomass diets, the EPA concentration in the rumen was not enough to exert the inhibitory effect on RBH. Nevertheless, the absence of *trans*-18:1 isomers accumulation after microalgae lipids supplementation has been reported by others (Urrutia et al., 2016; Rodriguez-Herrera et al., 2017).

Although total *trans*-18:1 in tissues was not affected by the diets, there was an alteration in the *trans*-18:1 isomeric profile, with an increase of *t*11–18:1 and a decrease of *t*10–18:1 when EPA-sources, and particularly with algae biomass, were included in the diets. The consequent decrease on the *t*10−/*t*11–18:1 ratio indicates a reduction of the risk of occurrence of the *t*10-shifted RBH pathways and thus impacting positively the nutritive value of meat as discussed by Alves et al. (2021). The type of *trans*-18:1 isomers produced in the rumen depends greatly on the type of basal diet fed to the ruminant (Bessa et al., 2015) and when high-starch diets are fed, the *t*10–18:1 often becomes the main *trans* monoene isomer as happened in the C treatment. Increasing PUFA intake in those high concentrate diets exacerbates the *t*10-shift (Bessa et al., 2015) and thus it is notable that either *Nannochloropsis* sp. oil or biomass had a protective effect against the occurrence of the *t*10-shift. One plausible explanation can be that these EPA-source diets were also reinforced with vitamin E which has been suggested to inhibit the *t*10-shift (Juárez et al., 2010). However, this putative effect of vitamin E is not well understood, and it is not consistently observed (Alves et al., 2021). Interestingly, *N. oceanica* biomass diets seemed to perform better in decreasing the *t*10-shift than O diet although all three diets were supplemented with the same dose of vitamin E, remaining unclear if there was a direct effect of *N. oceanica* biomass.

Oleic acid (*c*9–18:1) is consistently the major FA of lamb meat, contributing to the present experiment with circa 30% of total FA in tissues, but decreasing with *Nannochloropsis* sp. supplementation. Others also reported a reduction in *c*9–18:1 associated with microalgae lipids feeding (Hopkins et al., 2014; Urrutia et al., 2016). Most of *c*9–18:1 present in the tissues is derived from endogenous Δ-9 desaturation of 18:0 catalysed by the stearoyl-CoA desaturase (SCD) (Bessa et al., 2015). It is well established that PUFA are known as powerful inhibitors of SCD1 gene expression and activity (Mauvoisin & Mounier, 2011), having EPA also an individual effect (Velliquette et al., 2009). Probably, the increased EPA metabolic availability and incorporation in the tissues downregulated the SCD1 expression and hence its activity. The SCD activity is reflected in the relative abundance of their substrates and products, particularly using the pair *c*9–17:1/17:0 as discussed by Bessa et al. (2015). The SCD17 (i.e. SCD activity index computed using the pair *c*9–17:1/17:0) decreased in both muscle and SAT, suggesting that the decrease of *c*9–18:1 with *Nannochloropsis* sp. supplementation can be a reflex of lower SCD activity.

We expected that n-3 LC-PUFA from microalgae would be protected from rumen biohydrogenation and thus extensively incorporated into the tissues. Increased incorporation of PUFA in tissues can increase lipoperoxidation *in vivo* and lead to muscular dystrophies, even if animals are fed with the generally recommended amounts of vitamin E (Radostits et al., 2006). In a previous experiment, we observed weakness and muscle discoloration in lambs fed a DHA rich product (Trevera®) (Soldado et al., 2018; Francisco et al., 2019a). To prevent that, we decided to supply the microalgae diets with a high vitamin E dosage and this fact needs to be considered when comparing the colour and oxidation of the meat of the lambs fed control diet and of those fed EPA-source diets.

The enrichment of the meat in n-3 LC-PUFA observed in the lambs fed EPA-source diets can potentially increase its susceptibility to lipoxidation and alter the colour and sensory traits, nevertheless the vitamin E supplementation was effective in preventing it. The muscle colour from lambs fed diets containing EPA sources was slightly more reddish, as indicated by its lower Hue angle. Meat Hue angle decreases with time *post-mortem*, and changes in meat colour with time are well evaluated through ΔE parameter (AMSA, 2012). The lower Hue angle and ΔE values observed for the meat of lambs fed diets containing EPA sources can be easily explained by the protective effects of vitamin E (Bellés et al., 2019). In fact, O, SD, and FD diets were reinforced with vitamin E and the lambs ingesting those diets showed increased vitamin E content and reduced oxidation. This small but significant change in Hue angle is positive but probably not enough to affect the consumers' preferences (Khliji et al., 2010).

Contrasting with the muscle, the subcutaneous adipose tissue of lambs fed *N*. oceanica biomass diets was more yellowish (i.e. higher *b**, chroma), probably due to the deposition of microalgae-derived pigments. *Nannochloropsis* sp. are yellow-green microalgae that present a high concentration of carotenoid and other pigments (Toyomizu et al., 2001). Others found similar findings in adipose tissue colour of lambs fed with higher microalgae inclusion levels (Phelps et al., 2016).

The sensory evaluation of meat from animals fed marine fat sources is crucial to ensure the absence of off flavours that would prevent meat acceptability by consumers. The presence of "fishy" off flavours in the meat of lambs fed microalgae and fish oil has been reported and correlated with higher levels of n-3 LC-PUFA in the diet (Nute et al., 2007; Phelps et al., 2016; Urrutia et al., 2016). In these conditions, negative flavour/aroma development is due mainly to lipid oxidation (Nute et al., 2007). Thus, the efficacy of vitamin E in preventing the oxidative processes undoubtedly contributed to the maintenance of the sensory traits of meat from lambs fed diets containing EPA. Nevertheless, off flavours were occasionally detected (in <0.2% frequency) in the meat from the 3 EPA source treatments and highest in the FD treatment, where the EPA content tended to be higher. These results suggest that in the absence of supranutritional vitamin E supplementation, the frequency of off flavours detection in meat could be vastly increased and compromise the consumer acceptance.

4.5. Conclusion.

The incorporation of *N. oceanica* into the diets efficiently resulted in a higher content of n-3 LC-PUFA in the meat and SAT of the supplemented lambs, with EPA being the most significant contributor, followed by DPA and DHA, despite the short duration of the experimental trial due to logistic considerations and the fact that the two first weeks had a restricted feed regimen. The meat n-3 enrichment was made without productive performance penalties and with the maintenance of meat quality. EPA content did not differ between lambs fed *N. oceanica* biomass diets despite a lower EPA intake with the FD diet. These findings confirm a better capability of freeze-dried *N. oceanica* to naturally protect EPA from RBH, allowing it to be absorbed and deposited in the tissues. Higher levels of *t*11–18:1 and a tendency for lower levels of *t*10–18:1 were observed in the muscle of lambs fed with diets containing *N. oceanica*, which would be interesting to further explore. The present results are very promising and novel, offering a basis for testing in a large-scale production scenario and potential use on farms. In addition, due to the sizeable reduction in EPA in freeze dried *N. oceanica*, further investigations into why these losses occurred are warranted as this could increase amounts of protected EPA available during feeding.

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Author contributions

R.J.B. Bessa and S.P. Alves conceptualization and funding acquisition. A. Vítor, M. Godinho, A.E. Francisco and J. Santos-Silva conducted the animal experiment. A. Vítor, M. Godinho, L. Fialho, D. Soldado, E. Jeronimo, J. Almeida and S.P. Alves conducted laboratory analysis. A. Vítor, M. Godinho, E. Jeronimo, S.P. Alves and R.J.B. Bessa performed data analysis. A. Vítor, M. Godinho, S.P. Alves, R.J.B. Bessa, N. D. Scollan and S.A. Huws interpretation of results. A. Vítor drafted the manuscript. R.J.B. Bessa and S.P. Alves and edited and revised the original draft. All authors revised and approved the final manuscript

5. CHAPTER 5 | Enrichment of brain n-3 docosapentaenoic acid (DPA) and retinal n-3 eicosapentaenoic acid (EPA) in lambs fed *Nannochloropsis oceanica* microalga.

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Abstract.

N-3 polyunsaturated fatty acids (n-3 PUFA) have special physiological functions in both brain and retinal tissues that are related to the modulation of inflammatory processes and direct effects on neuronal membrane fluidity, impacting mental and visual health. Among them, the long-chain (LC) n-3 PUFA, as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are of special importance. Scarce data are available about the fatty acid (FA) composition of the ruminant brain in response to dietary intervention. However, we decided to examine the brain and retina FA composition of lambs supplemented with an EPA-rich microalga feed for 21 days, as it is known that despite the extensive biohydrogenation of dietary PUFA in the rumen, ruminants can selectively accumulate some n-3 LC-PUFA in their brain and retinal tissues. Twenty-eight male lambs were fed a control diet, or the same diet further supplemented with *Nannochloropsis* sp. microalga. Their brain and retina were collected for FA characterization. Overall, the brain FA profile remained unchanged, with little alteration in n-3 docosapentaenoic acid (DPA) enhancement in both the hippocampus and prefrontal cortex. Retinal tissues were particularly responsive to the dietary intervention, with a 4.5-fold enhancement of EPA in the freeze-dried-fed lambs compared with the control lambs. We conclude that retinal tissues are sensitive to short-term n-3 PUFA supplementation in lambs.

Keywords: fatty acid; hippocampus; prefrontal cortex; dimethyl acetal.

5.1. Introduction.

Fatty acids (FA) are the most abundant organic compounds in the brain. More than 90% of polyunsaturated FA (PUFA) in the mammalian brain are composed of long-chain PUFA (i.e., ≥20 C chain, LC-PUFA) such as arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) (Svennerholm, 1968). Most of these PUFA are esterified in brain membrane phospholipids (PL), and they influence brain functions by altering the biophysical properties of cell membranes (Fenton et al., 2000). N-3 PUFA (n-3 PUFA) have a special importance since they play a role in a variety of physiological functions related to neurogenesis, neurotransmission, and neuroinflammation, contributing to the development, functioning, and ageing of the brain. Furthermore, in humans, n-3 LC-PUFA dietary deficiencies are associated with an increased risk of developing various psychiatric disorders (Lange, 2020) and neurodegenerative diseases (Dyall, 2015). Among these FA, eicosapentaenoic acid (EPA, 20:5n-3) and DHA have been linked to the maintenance of mental health, mediated by the modulation of inflammatory processes and direct effects on neuronal membrane fluidity and receptor function (Lange, 2020). N-3 docosapentaenoic acid (DPA, 22:5n-3) also plays an important role, as it is the second most abundant n-3 LC-PUFA in the brain after DHA. It is suggested to be specifically beneficial for elderly neuroprotection and early-life brain development (Drouin et al., 2019).

In addition to the brain, retinal tissue is also rich in lipids, which comprise approximately 20% of the retina's dry weight. Retinal membrane PL have the highest content of LC-PUFA of any tissue in humans (approximately 33%) (Fliesler & Anderson, 1983; Acar et al., 2012; Albouery et al., 2020). The retina is a tissue with a naturally high content of n-3, particularly DHA, which plays an essential role in optimizing the fluidity of photoreceptor membranes, retinal integrity, and visual function (Querques et al., 2011). DHA also has a protective role in the retina, participating in the anti-inflammatory activity, anti-angiogenesis, anti-apoptosis and providing protection from neurotoxicity (Querques et al., 2011).

The brain lipids of ruminant species, especially cattle, have been well characterized and appear to be very similar to those of the human brain in terms of both content and composition (Christie, 1981). Although they are less well-characterized, the lipids in the brain of sheep also do not seem to differ markedly from the ones found in cattle (Christie, 1981). In ruminants, brain characterization has been traditionally focused on the types of lipids and less on a deeper characterization of FA. Moreover, lipid analyses have been performed primarily in brain homogenates or gross anatomical structures within the brain (Christie, 1981), and not in specific functional regions or tracts of the ruminant brain. Bovine and ovine retinal FA compositions are similar: they are composed of appreciable amounts of palmitic acid (16:0), stearic acid (18:0), DHA and AA (Anderson & Sperling, 1971; Christie, 1981). Thus, despite the extensive biohydrogenation of dietary PUFA in the rumen, ruminants can selectively accumulate n-3 LC-PUFA in their brain and retinal tissues, contrasting with the low deposition of these FA in adipose tissue and muscle (Ponnampalam et al., 2021). Liver stores of n-3 LC-FA were reported to be the primary source of these FA for the brain tissues of rats (Rapoport et al., 2010), even during periods of low dietary intake of these FA (Rapoport et al., 2010; Igarashi et al., 2007b). This allows to maintain adequate levels of DHA and AA in the brains of DHA- and AA-deprived animals (Rapoport et al., 2010; Igarashi et al., 2007b). In a very recent publication, cattle supplemented with calcium salts of fish oil with 11% EPA and 8% DHA had, across most brain regions, greater EPA concentrations when compared to palm-oil-supplemented animals (Rule et al., 2022).

The dietary supplementation of microalgae has been shown to enhance n-3 LC-PUFA along lambs' gastrointestinal tract (Vítor et al., 2021). Moreover, Vítor et al. (2021) showed that the drying method applied to the microalgae strongly influenced the powder architecture and cell wall integrity, consequently affecting the degree of EPA protection against rumen microbes. Therefore, we hypothesise that lambs' brains and/or retinal tissues would be sensitive to the differences in n-3 LC-PUFA absorption due to the changes in rumen biohydrogenation associated with the processing of microalgae biomass. Thus, we collected brain and retina samples from six-month-old lambs used in a previous experiment and conducted a detailed FA composition of those tissues.

5.2. Material and methods.

5.2.1. Animal handling and diets.

The current lamb trial was conducted in compliance with the ARRIVE and international guidelines. The trial was conducted in certified facilities and was approved by an ethical and animal well-being commission, as fully detailed by Vítor et al. (2021). Twenty-eight sixty-day-old Merino Branco ram lambs with an average body weight of 21.8 ± 4.4 kg were housed in INIAV facilities in Santarém, Portugal. The animals were randomly allocated to individual pens (1.52 m^2) with *ad libitum* access to clean water. The lambs were sorted into four experimental groups with seven replicates per group. The experimental diets included a control diet (C diet), consisting of pellets containing dehydrated lucerne, barley and soybean meal and no added sources of EPA, and three diets supplemented with the microalga *Nannochloropsis* sp., designed to provide approximately 3 g of EPA per kg of diet dry matter (DM). The average content of EPA (mg EPA/g product) in each microalgal format was 235 in the *Nannochloropsis* oil, 22.7 in the spray dried *Nannochloropsis oceanica* and 30.8 in the lyophilized *Nannochloropsis oceanica*. *Nannochloropsis* sp. containing diets were composed of the C diet plus 123 g/kg of spray-dried *Nannochloropsis oceanica* biomass (SD diet); 92 g/kg freeze-dried *Nannochloropsis oceanica* biomass (FD diet); and 12 g/kg of *Nannochloropsis* sp. free-oil (O diet) (Table 5.1). The trial had a 3-week duration limitation due to the high cost of the spray-dried *Nannochloropsis oceanica* biomass and the difficulty of obtaining enough freeze-dried biomass with lab-scale equipment.

Table 5.1- Total fatty acid content (g/kg dry matter) and fatty acids (FA) profile (% of total fatty acids) of the experimental diets.

Item	Diets[2]			
	C	O	SD	FD
Total FA[1]	13.7	20.6	19.5	20.2
FA profile				
14:0	n.d.	1.26	2.40	2.28
16:0	25.5	23	23.9	26.2
*c*9-16:1	0.51	5.15	10.2	8.22
17:0	0.66	0.34	n.d.	n.d.
18:0	4.09	2.96	2.51	3.32
*c*9-18:1	18.6	14.5	11.7	13.7
*c*11-18:1	0.73	0.93	0.72	0.69
18:2n-6	41	31.5	28.2	29.1
18:3n-3	8.91	6.80	5.02	6.44
20:4n-6	n.d.	2.58	3.69	3.17
20:5n-3	n.d.	10.40	11.3	6.88
22:0	n.d.	0.58	0.36	n.d.

[1]FA—fatty acids. [2]C—control diet with no EPA sources; O—diet with Nannochloropsis sp. oil; SD—diet with spray dried Nannochloropsis oceanica biomass; FD—diet with freeze-dried Nannochloropsis oceanica biomass; n.d.—not detected. In the FA notation (x:n-), 'x' represents the number of C atoms, ':' the number of double bonds and 'n-' the location, in its carbon chain, of the double bond which is closest to the methyl end of the molecule. *c* stands for cis. Adapted from Vítor et al. (2021).

5.2.2. Slaughter and sample collection.

After the end of the third week, the animals were slaughtered using a captive bolt. This was followed by exsanguination. The brain tissue was removed whole and it retained its shape and landmarks in spite of the captive bolt damage. The brain was cut on a sagittal plane and divided into two hemispheres, which were then frozen at – 80°C. After thawing, the different parts were individualized from the right hemisphere (Figure 5.1), stored in individual bags, frozen at – 80°C and lyophilized. Grey and white matter were collected from two different points in the brain and were considered samples from non-function-specific brain parts, representing only samples from the two histologic and physiological brain areas. The prefrontal cortex (cerebral cortex covering the front part of the frontal lobe), and hippocampus (located in the medial part of the temporal lobe and, on a mid-sagittal section of the brain, posterior to the amygdala extending posteriorly to the splenium of the corpus callosum) were selected as function-specific brain parts.

Immediately after slaughter, the right eyeball of each lamb was removed with a spatula, stored in a bag and frozen at – 80°C. The eyeballs were thawed, and the retina and *tapetum lucidum* (RTL) were individualised (Figure 5.2). The liver was removed from the carcass, and a portion of the left lobe was stored in a bag and frozen at –80 °C. All brain parts, RTL and a portion of the liver were lyophilized prior to fatty acid extraction.

Figure 5.1- Lamb right brain hemisphere.
The four collected parts are highlighted in the figure: prefrontal cortex and hippocampus are filled in blue and yellow, respectively; gray matter is identified with the yellow triangles and white matter with the blue circles.

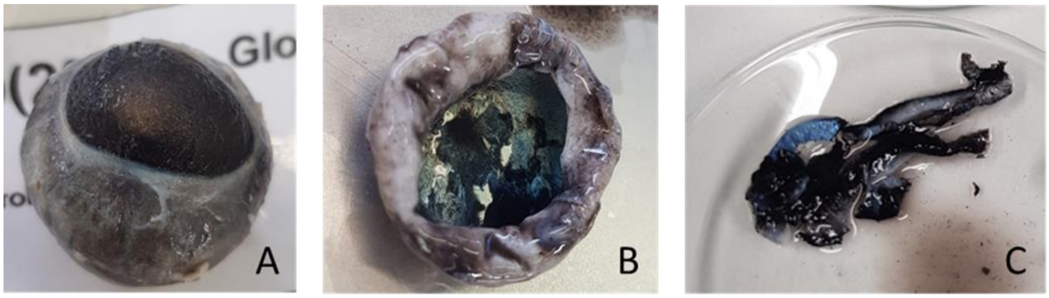

Figure 5.2- Dissection of the right eyeball of one lamb.
From left to right: A - right eyeball; B – removal of eye structures (cornea, iris, and lens) and *tapetum lucidum* evidenced; C – cutting of material used for FA analysis including *tapetum lucidum* and retina tissues.

5.2.3. Fatty acid methyl esters (FAME) and dimethyl acetals (DMA) analysis.

Fatty acid methyl esters (FAME) and dimethyl acetals (DMA) of the brain, RTL tissues and liver samples were prepared by acid-catalysed transesterification in methanol (Christie & Han, 2012). In plasmalogens, the sn-1 position of the glycerol contains a vinyl-ether that releases a DMA in the presence of acid methanol solution. Briefly, approximately 100 mg of lyophilised grinded samples were weighed into reaction tubes. Toluene and 1 mL of internal standard (methyl nonadecanoate - 1 mg/mL) were added, and the samples were placed in an ultrasound bath for ten minutes. A solution of 1.25 M HCl in methanol (3 mL) was added and left to react overnight at 50ºC in a water bath.

FAME and DMA were analysed by gas-chromatography with flame ionization detection (GC-FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with an SP-2560 (100 m × 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA, USA) capillary column. The injector and detector temperatures were maintained at 220ºC and 250ºC, respectively. The carrier gas was helium at a constant flow of 1 mL/min. The GC oven temperature started at 50ºC for 1 min, then increased to 150ºC at 50ºC/min and held for 20 min, increased to 190ºC at 1ºC/min and finally increased to 220ºC at 2ºC/min and held for 40 min. The identification of FAME was achieved by comparison of fatty acid retention times with those of commercial standards (FAME mix, 37 components from Supelco Inc., Bellefont, PA, USA) and with published chromatograms (Alves & Bessa, 2009; Alves et al., 2013). Additional confirmation of FAME and DMA was achieved by electron impact mass spectrometry using a Shimadzu GC–MS QP2010 Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m x 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA, USA) capillary column and similar GC conditions.

5.2.4. Statistical analysis.

FAME and DMA data were analysed as a completely randomised experimental design using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Diet was used as a fixed factor, and the animal was used as the experimental unit. Feed intake data were analysed as a completely randomised design, in which an individual lamb was used as the experimental unit and the model included the treatment as the fixed factor. The least square means and standard error of the mean (SEM) were reported, and the main effects and their interactions were considered significant at $p < 0.05$. The TFA + DMA content is presented in mg/g DM, and the FA individual composition is presented in % of TFA + DMA (g FA/100 g TFA + DMA). The sparse partial least squares discriminant analysis (sPLSDA) was performed using MetaboAnalyst 5.0 software, using the centred log ratio transformed FA data as input.

5.3. Results.

5.3.1. Fatty acid intake.

The feed intake averaged 1.19 ± 0.13 kg (mean ± standard error of the mean) of DM/day during the experiment. It did not differ among treatments ($p > 0.05$) (Vítor et al., 2021). A brief report of individual and total FA intake (mg/day) is presented in Table 5.2.

Table 5.2- Daily fatty acid (FA) intake (mg/day) during the trial.

FA[1]	**Diets**[2]				**SEM**[3]	***p*-value**
	C	**O**	**SD**	**FD**		**C vs. *Nannochloropsis* diets**[4]
14:0	-	320	590	540	0.030	<0.001
16:0	4110	5780	5710	6200	0.466	0.217
*c*9-16:1	60	1300	2490	1970	0.119	<0.001
18:0	660	740	600	790	0.061	0.011
*c*9-18:1	3010	3620	2800	3260	0.277	<0.001
*c*11-18:1	120	230	110	160	0.014	<0.001
18:2n-6	6640	7900	6710	6910	0.607	<0.001
18:3n-3	1440	1700	1190	1530	0.129	<0.001
20:4n-6	-	670	890	750	0.058	<0.001
20:5n-3	-	2600	2710	1630	0.178	<0.001
Total	16,100	25,100	24,00	23,700	1.90	0.033

[1]FA—fatty acid; [2]C— control diet with no EPA sources; O—diet with *Nannochloropsis* sp. oil; SD—diet with spray-dried *Nannochloropsis oceanica* biomass; FD—diet with freeze-dried *Nannochloropsis oceanica* biomass; n.d.—not detected. [3]Standard error of the mean. [4]C vs. *Nannochloropsis* diets, compares C with O, SD, and FD together. In the FA notation (x:n-), 'x' represents the number of C atoms, ':' the number of double bonds and 'n-' the location, in its carbon chain, of the double bond which is closest to the methyl end of the molecule. *c* stands for cis.

Apart from 16:0, the intake of all the FA analysed differed between the control and *Nannochloropsis*-supplemented lambs ($p < 0.05$). The FA 14:0, 20:4n-6 and 20:5n-3 were only present in the *Nannochloropsis*-supplemented diets; therefore, their feed intake was zero in the Control-fed lambs. The total FA intake differed between Control and *Nannochloropsis*-supplemented lambs ($p < 0.05$), being higher in the latter.

5.3.2. Brain fatty acid (FA) and dimethyl acetal (DMA) profile.

The total FA and DMA (TFA + DMA) content (mg/g DM) and composition (g/100 TFA + DMA) of grey and white matter are presented in Table 5.3; those of the hippocampus and prefrontal cortex are presented in Table 5.4. Regarding the grey and white matter, the amount of TFA + DMA did not differ among treatments, averaging 191 and 227 mg/g DM, respectively. Additionally, in both the hippocampus and prefrontal cortex, TFA + DMA content did not differ among treatments, averaging 199 and 208 mg/g DM, respectively.

Table 5.3- Total fatty acid (TFA) and dimethyl acetal (DMA) content (mg/g DM) and composition (% TFA + DMA) of the grey matter and white matter of lambs.

FA and DMA[1]	Grey matter						White matter					
	Diets[2]				SEM[3]	*p*-value	Diets[2]				SEM[3]	*p*-value
	C	O	SD	FD			C	O	SD	FD		
TFA+DMA	189	192	190	193	5.2	0.970	235	227	225	222	4.1	0.163
14:0	0.57	0.55	0.58	0.58	0.025	0.746	0.52	0.52	0.55	0.53	0.018	0.778
15:0	0.11	0.12	0.11	0.13	0.008	0.392	0.09	0.09	0.09	0.11	0.007	0.177
16:0	19	19	19	19	0.4	0.942	13	14	14	15	0.4	0.261
*c*7-16:1	0.42	0.42	0.40	0.42	0.021	0.934	0.35	0.37	0.35	0.36	0.016	0.722
*c*9-16:1	0.42	0.43	0.45	0.45	0.024	0.810	0.26	0.29	0.27	0.29	0.013	0.354
17:0	0.29	0.29	0.27	0.31	0.014	0.445	0.27	0.31	0.28	0.31	0.015	0.181
*c*9-17:1	0.11	0.11	0.10	0.11	0.010	0.940	0.11	0.17	0.14	0.15	0.019	0.223
18:0	21	21	20	21	0.5	0.964	15	16	16	16	0.4	0.176
*c*9-18:1	15	15	15	14	0.5	0.847	22	22	22	21	0.5	0.216
*c*11-18:1	3.4	3.3	3.3	3.3	0.07	0.433	3.0	3.0	2.9	3.0	0.05	0.671
18:2n-6	0.48	0.48	0.50	0.52	0.030	0.673	0.29	0.43	0.39	0.42	0.042	0.111
19:1	0.06	0.07	0.07	0.07	0.006	0.836	0.10	0.10	0.10	0.10	0.008	0.892
20:0	0.32	0.31	0.31	0.32	0.017	0.870	0.69^{a}	0.64^{ab}	0.62^{b}	0.62^{b}	0.019	0.045
18:3n-3	0.04	0.03	0.03	0.04	0.008	0.843	0.13^{a}	0.08^{b}	0.13^{a}	0.09^{b}	0.013	0.026
*c*11-20:1	0.86	0.83	0.88	0.80	0.080	0.902	2.2	2.0	2.1	1.9	0.11	0.372
20:1	0.27	0.27	0.27	0.26	0.021	0.941	0.62	0.62	0.59	0.56	0.035	0.542
21:0	0.06	0.06	0.07	0.07	0.005	0.428	0.09	0.10	0.11	0.10	0.012	0.868
20:2	0.10	0.12	0.11	0.12	0.014	0.761	0.26	0.25	0.24	0.23	0.023	0.803
20:2n-6	0.10	0.11	0.11	0.10	0.019	0.959	0.15	0.14	0.14	0.14	0.022	0.638
20:3n-9	0.54	0.54	0.59	0.49	0.068	0.764	0.69	0.69	0.69	0.65	0.054	0.943
22:0	0.71	0.66	0.64	0.66	0.069	0.915	1.90	1.84	1.68	1.69	0.078	0.140
20:3n-6	0.33	0.39	0.36	0.39	0.020	0.094	0.35	0.42	0.38	0.45	0.030	0.111
22:1	0.33	0.32	0.31	0.30	0.040	0.970	0.80	0.10	0.40	0.37	0.170	0.068
20:3n-3	0.13	0.13	0.12	0.11	0.020	0.941	0.57	0.78	0.62	0.60	0.083	0.334
22:1/ 20:3n-3	0.28	0.20	0.30	0.27	0.064	0.727	0.81	0.88	0.98	0.70	0.067	0.154
20:4n-6	6.3	5.8	5.7	6.2	0.24	0.312	3.8	4.0	3.8	4.2	0.16	0.176
23:0	0.21	0.20	0.21	0.20	0.028	0.992	0.50	0.39	0.39	0.37	0.050	0.262
22:2n-6	0.09	0.09	0.09	0.09	0.018	0.985	0.52	0.48	0.49	0.60	0.119	0.863
20:5n-3	0.26	0.29	0.32	0.32	0.040	0.655	0.71	0.60	0.69	0.69	0.053	0.502
24:0	0.66	0.63	0.61	0.63	0.139	0.997	2.3	2.3	2.2	2.0	0.11	0.115
*c*15-24:1	1.2	1.3	1.4	1.3	0.20	0.951	4.4	3.9	4.2	3.7	0.25	0.251
24:1	0.69	0.20	0.23	0.22	0.200	0.284	0.70	0.65	0.65	0.63	0.038	0.576
22:4n-6	2.5	2.5	2.6	2.9	0.268	0.864	2.8	2.4	2.5	2.7	0.14	0.194

21:5	0.11	0.03	0.03	0.03	0.035	0.334	0.14	0.17	0.12	0.10	0.030	0.448
22:5n-6	0.62	0.41	0.38	0.38	0.066	0.060	0.34	0.25	0.25	0.25	0.037	0.251
26:0	0.15	0.02	0.03	0.04	0.053	0.304	0.08	0.07	0.08	0.08	0.012	0.892
C26:1	0.08	0.10	0.13	0.09	0.049	0.878	0.26	0.34	0.42	0.23	0.036	0.069
22:5n-3	3.0	0.76	1.05	1.21	1.111	0.504	0.51	0.32	0.57	0.65	0.150	0.454
22:6n-3	11	14	14	13	1.07	0.248	4.7	5.2	5.2	5.9	0.40	0.212
DMA												
DMA 16:0	2.2	2.2	2.3	2.2	0.10	0.911	3.7	3.6	3.7	3.5	0.09	0.644
DMA 17:0	0.19	0.16	0.16	0.19	0.013	0.320	0.23	0.24	0.23	0.26	0.012	0.319
DMA 18:0	3.9	4.7	4.7	4.7	0.35	0.275	4.5	4.6	4.6	4.7	0.09	0.543
DMA *c*9-18:1	0.92	1.01	1.11	1.01	0.128	0.784	2.8	2.5	2.3	2.5	0.24	0.458
DMA *c*11-18:1	0.95	0.96	1.00	0.95	0.071	0.941	2.0	1.9	2.0	1.9	0.075	0.645
Partial sums												
C18[4]	39	39	39	39	0.3	0.526	40	41	41	40	0.5	0.207
C18:1[5]	20	0.27	0.27	0.27	9.97	0.418	0.32	0.34	0.33	0.28	0.027	0.355
DMA	15	9.0	9.2	9.1	2.82	0.341	13	13	13	13	0.2	0.596
SFA[6]	38	42	42	42	1.6	0.270	35	36	35	36	0.6	0.207
MUFA[7]	22	22	22	22	0.8	0.901	35	34	34	32	0.8	0.156
cis-MUFA	22	21	21	21	0.9	0.455	32	32	32	30	0.7	0.223
PUFA[8]	24	26	26	26	0.9	0.519	16	16	16	18	0.6	0.159
n-3 PUFA	14	15	15	15	0.5	0.121	6.7	7.0	7.3	8.0	0.41	0.162
n-6 PUFA	12	9.8	9.8	10	0.55	0.101	8.1	8.0	7.8	8.7	0.33	0.320
EPA + DHA	18	14	14	14	2.2	0.414	5.4	5.8	5.9	6.6	0.41	0.232
AA/EPA ratio	21	23	19	21	3.07	0.815	5.7	6.9	5.7	6.2	0.56	0.375

Means within a row with different letters are significantly different ($p < 0.05$). [1]FA and DMA—fatty acids and dimethyl acetals; [2]C—control diet with no EPA sources; O—diet with *Nannochloropsis* sp. oil; SD—diet with spray-dried *Nannochloropsis oceanica* biomass; FD—diet with freeze-dried *Nannochloropsis oceanica* biomass. [3]Standard error of the mean; the value presented corresponds to a pooled sample standard error of the mean. [4]Sum of C18 FA. [5]Sum of C18:1 FA. [6]Sum of saturated FA. [7]Sum of monounsaturated FA. [8]Sum of polyunsaturated FA. In the FA notation (x:n-), 'x' represents the number of C atoms, ':' the number of double bonds and 'n-' the location, in its carbon chain, of the double bond which is closest to the methyl end of the molecule. *c* stands for cis.

Table 5.4- Total fatty acid (TFA) and dimethyl acetal (DMA) (mg/g DM) content and composition (% TFA + DMA) of the hippocampus and prefrontal cortex of lambs.

	Hippocampus						**Prefrontal cortex**					
FA and DMA[1]	**Diets**[2]				**SEM**[3]	***p*-value**	**Diets**[2]				**SEM**[3]	***p*-value**
	C	**O**	**SD**	**FD**			**C**	**O**	**SD**	**FD**		
TFA+DMA	199	196	204	195	4.4	0.569	206	206	211	210	3.0	0.533
14:0	0.52	0.51	0.53	0.48	0.018	0.243	0.59	0.59	0.61	0.63	0.024	0.643
15:0	0.11	0.10	0.09	0.10	0.005	0.185	0.11	0.11	0.10	0.12	0.008	0.493
16:0	17	17	16	16	0.4	0.458	18	17	17	18	0.3	0.923
*c*7-16:1	0.43	0.43	0.40	0.38	0.017	0.101	0.43	0.42	0.40	0.41	0.018	0.649
*c*9-16:1	0.35	0.39	0.38	0.34	0.017	0.174	0.42	0.42	0.41	0.43	0.019	0.863
17:0	0.29	0.28	0.27	0.29	0.009	0.428	0.27	0.29	0.26	0.31	0.019	0.408
*c*9-17:1	0.11	0.12	0.12	0.12	0.009	0.937	0.13	0.13	0.13	0.15	0.010	0.263
18:0	18	19	18	18	0.4	0.642	19	19	19	19	0.4	0.951
*c*9-18:1	17	17	18	17	0.5	0.756	17	17	17	17	0.5	0.905
*c*11-18:1	3.4	3.3	3.2	3.3	0.05	0.325	3.4	3.3	3.2	3.2	0.05	0.107
18:2n-6	0.41	0.45	0.45	0.44	0.027	0.644	0.40	0.42	0.42	0.45	0.035	0.790
19:1	0.08	0.08	0.08	0.09	0.008	0.578	0.08	0.08	0.07	0.08	0.005	0.125
20:0	0.50	0.48	0.49	0.50	0.029	0.968	0.45	0.48	0.44	0.45	0.027	0.705
18:3n-3	0.09	0.08	0.09	0.06	0.013	0.481	0.07	0.08	0.06	0.06	0.008	0.443
*c*11-20:1	1.4	1.3	1.6	1.6	0.14	0.567	1.2	1.2	1.2	1.2	0.07	0.912
20:1	0.38	0.37	0.40	0.39	0.026	0.822	0.38	0.39	0.38	0.37	0.021	0.878
21:0	0.07	0.07	0.08	0.07	0.007	0.904	0.10	0.09	0.08	0.07	0.013	0.660
20:2	0.19	0.17	0.19	0.17	0.012	0.472	0.19	0.20	0.19	0.18	0.018	0.846
20:2n-6	0.12	0.12	0.12	0.15	0.013	0.297	0.08	0.11	0.10	0.11	0.007	0.075
20:3n-9	0.83	0.84	0.77	0.70	0.051	0.221	0.73	0.74	0.69	0.64	0.066	0.673
22:0	1.0	1.1	1.2	1.2	0.12	0.815	1.1	1.2	1.1	1.2	0.07	0.759
20:3n-6	0.37^{b}	0.44^{a}	0.42^{ab}	0.46^{a}	0.023	0.049	0.29^{b}	0.38^{a}	0.35^{a}	0.37^{a}	0.018	0.008
22:1	0.50	0.47	0.58	0.59	0.054	0.348	0.46	0.53	0.48	0.48	0.032	0.536
20:3n-3	0.24	0.21	0.25	0.24	0.022	0.588	0.26	0.29	0.25	0.27	0.022	0.572
22:1/ 20:3n-3	0.59	0.49	0.55	0.57	0.145	0.960	0.43	0.30	0.46	0.46	0.037	0.108
20:4n-6	5.4	5.4	4.9	5.1	0.26	0.476	5.3	5.0	4.9	5.1	0.17	0.346
23:0	0.38	0.33	0.36	0.37	0.030	0.641	0.24	0.25	0.24	0.25	0.022	0.980
22:2n-6	0.18	0.13	0.19	0.19	0.035	0.600	0.18	0.20	0.21	0.20	0.038	0.958
20:5n-3	0.56	0.59	0.63	0.62	0.035	0.580	0.55	0.65	0.63	0.65	0.061	0.596
24:0	1.4	1.4	1.5	1.5	0.14	0.971	1.1	1.3	1.2	1.2	0.10	0.805
*c*15-24:1	2.6	2.1	2.7	2.8	0.28	0.266	2.2	2.3	2.3	2.3	0.17	0.983
24:1	0.39	0.31	0.38	0.40	0.034	0.260	0.43	0.42	0.40	0.44	0.030	0.823
22:4n-6	3.2	2.8	2.8	2.9	0.16	0.235	2.8	2.5	2.6	2.6	0.13	0.329
21:5	0.09	0.08	0.09	0.10	0.014	0.861	0.06	0.07	0.07	0.06	0.008	0.413
22:5n-6	0.50^{a}	0.34^{b}	0.31^{b}	0.31^{b}	0.052	0.047	0.52^{a}	0.38^{ab}	0.34^{b}	0.33^{b}	0.052	0.056
26:0	0.05	0.04	0.05	0.05	0.005	0.286	0.05	0.05	0.05	0.05	0.005	0.675
C26:1	0.26	0.18	0.23	0.25	0.067	0.846	0.52	0.35	0.60	0.57	0.078	0.256
22:5n-3	0.83^{c}	1.14^{b}	1.24^{ab}	1.36^{a}	0.066	<0.001	0.54^{c}	0.82^{b}	0.97^{ab}	1.06^{a}	0.069	<0.001

22:6n-3	9.0	9.6	8.9	9.0	0.38	0.573	10	10	10	10	0.53	0.956
DMA												
DMA 16:0	2.7	2.7	2.8	2.7	0.09	0.717	2.7	2.9	2.8	2.8	0.12	0.910
DMA 17:0	0.20	0.20	0.20	0.22	0.013	0.716	0.19	0.19	0.19	0.21	0.013	0.373
DMA 18:0	4.6	4.7	4.7	4.8	0.08	0.612	4.5	4.7	4.7	4.6	0.09	0.519
DMA *c*9-18:1	1.4	1.5	1.8	1.8	0.17	0.268	1.5	1.6	1.6	1.6	0.11	0.986
DMA *c*11-18:1	1.4	1.4	1.5	1.4	0.05	0.301	1.3	1.4	1.4	1.4	0.08	0.919
Partial sums												
C18[4]	40	40	40	39	0.3	0.303	40	40	40	40	0.3	0.813
C18:1[5]	0.37	0.32	0.33	0.33	0.018	0.265	0.29	0.28	0.26	0.27	0.019	0.807
DMA	10.3	10	11	11	0.3	0.320	10	11	11	11	0.3	0.830
SFA[6]	39	40	39	39	0.6	0.311	40	40	40	40	0.5	0.934
MUFA[7]	27	26	28	27	0.9	0.617	27	27	27	27	0.7	0.979
Cis-MUFA	25	25	26	25	0.8	0.671	25	25	25	25	0.6	0.972
PUFA[8]	22	22	21	22	0.7	0.744	22	22	22	22	0.6	0.952
n-3 PUFA	11	12	11	11	0.3	0.340	12	12	12	12	0.5	0.790
n-6 PUFA	10	9.7	9.2	9.6	0.41	0.362	9.6	9.0	8.9	9.2	0.29	0.309
EPA + DHA	9.6	10	9.5	9.6	0.39	0.600	10	11.0	11	11	0.5	0.960
AA/EPA ratio	9.8	9.2	8.1	8.4	0.72	0.366	10	8.4	8.2	8.3	1.13	0.476

Means within a row with different letters are significantly different ($p < 0.05$). [1]FA and DMA—fatty acids and dimethyl acetals; [2]C—control diet with no EPA sources; O—diet with *Nannochloropsis* sp. oil; SD—diet with spray-dried *Nannochloropsis oceanica* biomass; FD—diet with freeze-dried *Nannochloropsis oceanica* biomass. [3]Standard error of the mean; the value presented corresponds to a pooled sample standard error of the mean. [4]Sum of C18 FA. [5]Sum of C18:1 FA. [6]Sum of saturated FA. [7]Sum of monounsaturated FA. [8]Sum of polyunsaturated FA. In the FA notation (x:n-), 'x' represents the number of C atoms, ':' the number of double bonds and 'n-' the location, in its carbon chain, of the double bond which is closest to the methyl end of the molecule. *c* stands for cis.

No major effects of microalgal supplementation ($p > 0.05$) were observed for any of the FA and DMA in both the grey and white matter. Significant differences in the FA composition were observed in the hippocampus and prefrontal cortex (Table 5.4). In the hippocampus, 20:3n-6 was significantly ($p < 0.05$) lower in the C and SD diets and higher in the O and FD diets. In the prefrontal cortex, this FA was higher in all *Nannochloropsis* supplemented lambs compared to C-fed lambs. Regarding 22:5n-6, it was significantly higher in the hippocampus in the C-fed lambs when compared to the *Nannochloropsis*-supplemented lambs. In the prefrontal cortex, this FA tended ($p = 0.056$) to follow a similar pattern to what was found in the hippocampus. DPA had its lowest values ($p < 0.05$) in the hippocampi and prefrontal cortices of C lambs, and its highest values were found in the tissues of lambs fed with the FD diet. DPA was lower ($p < 0.05$) in these tissues in O-fed lambs, than in SD-fed lambs. *Nannochloropsis* sp. supplemented diets did not affect ($p > 0.05$) the EPA or DHA concentrations in the hippocampus or prefrontal cortex. The sum of EPA + DHA averaged 10% in the hippocampus and 11% in the prefrontal cortex. The total PUFA and n-3 PUFA contents averaged 22% and 12% in both the hippocampus and prefrontal cortex, respectively. The content of TFA + DMA was not affected ($p > 0.05$) by dietary treatment.

5.3.3. Retina and *tapetum lucidum* (RTL).

Similar to what was observed in the brain parts, the TFA + DMA content in the RTL tissues did not differ among treatments, averaging 53 mg/g DM (Table 5.5). However, more treatment effects were observed in RTL tissues than were observed in brain tissues.

In RTL tissues, the *c*16-18:1 content was greater ($p < 0.05$) in SD lambs than in all other treatments. Regarding 20:3n-9, a higher content was found in both the C- and SD-fed lambs when compared to the remaining groups. The content of 20:3n-6 was lower in C-fed lambs when compared to the remaining treatments, and 22:4n-6 was higher in both C- and SD-fed lambs when compared to the remaining treatments. Both EPA and DPA were higher in the microalgae-biomass-fed lambs, although there were no differences between the SD- and FD-fed lambs. When compared to the C treatment, biomass-fed lambs had 4.6 times more EPA and twice more DPA in their RTL tissues.

Table 5.5- Total fatty acid (TFA) and dimethyl acetal (DMA) (mg/g DM) content and composition (%TFA + DMA) of the RTL tissues of lambs.

FA and DMA[1]	Diet[2]				SEM[3]	*p*-value
	C	O	SD	FD		
TFA+DMA	54	61	43	53	8.9	0.591
10:0	0.05	0.06	0.08	0.06	0.014	0.376
12:0	0.26	0.24	0.23	0.25	0.036	0.955
14:0	2.2	1.3	1.6	1.9	0.25	0.069
i-15:0	0.08	0.05	0.05	0.02	0.014	0.063
a-15:0	0.18	0.06	0.07	0.05	0.066	0.433
*c*9-14:1	0.06	0.08	0.09	0.07	0.017	0.518
15:0	0.27	0.27	0.28	0.31	0.026	0.766
i-16:0	0.09	0.07	0.10	0.06	0.018	0.341
16:0	21	21	20	21	0.58	0.608
i-17:0	0.15	0.17	0.17	0.15	0.027	0.881
*c*7-16:1	0.29	0.30	0.25	0.29	0.034	0.717
*c*9-16:1	0.77	0.77	0.66	0.83	0.118	0.752
a-17:0	0.18	0.14	0.09	0.13	0.041	0.488
17:0	0.67	0.70	0.60	0.74	0.038	0.084
*c*9-17:1	0.31	0.32	0.27	0.27	0.033	0.543
18:0	18	18	19	18	0.5	0.591
*t*6/*t*7/*t*8-18:1	0.08	0.11	0.11	0.09	0.019	0.664
*t*9-18:1	0.07	0.12	0.11	0.11	0.018	0.198
*t*10-18:1	0.49	0.53	0.28	0.35	0.096	0.219
*t*11-18:1	0.66	0.87	0.88	0.87	0.114	0.455
*t*12-18:1	0.20	0.22	3.56	0.23	1.784	0.437
*c*9-18:1	29	28	21	27	2.4	0.121
*c*11-18:1	1.8	1.8	2.0	1.9	0.10	0.415
*c*12-18:1	0.09	0.11	0.15	0.10	0.023	0.241
*c*13-18:1	0.06	0.06	0.04	0.05	0.014	0.728
*t*16/*c*14-18:1	0.09	0.13	0.10	0.12	0.025	0.671
*c*15-18:1	0.04	0.04	0.05	0.03	0.014	0.799
tc/*ct*-18:2/ ciclo-17	0.11	0.18	0.16	0.22	0.043	0.370
*c*9,*t*12/ *c*9,*t*15/ *t*8,*c*13-18:2	0.09	0.10	0.07	0.11	0.021	0.626
*c*16-18:1	0.03^{b}	0.03^{b}	0.08^{a}	0.04^{b}	0.012	0.021
*t*9,*c*12-18:2	0.04	0.06	0.07	0.05	0.013	0.385
*t*11,*c*15/ *t*10,*c*15-18:2	0.06	0.16	0.08	0.14	0.033	0.094
18:2n-6	0.31	3.75	4.25	3.98	0.299	0.317
20:0	0.16	0.17	0.20	0.17	0.022	0.675
18:3n-3	0.63	0.67	0.65	0.66	0.038	0.873
*c*9,*t*11-CLA	0.26	0.37	0.30	0.38	0.057	0.339
20:2n-6	0.15	0.16	0.19	0.17	0.020	0.509
20:3n-9	0.29^{a}	0.20^{bc}	0.24^{ab}	0.14^{c}	0.032	0.014
22:0	0.05	0.07	0.07	0.06	0.012	0.414

20:3n-6	0.38^{b}	0.59^{a}	0.70^{ab}	0.58^{a}	0.056	0.004
20:3n-3	0.18	0.22	0.22	0.20	0.029	0.646
20:4n-6	6.4	5.4	6.5	5.6	0.54	0.391
20:5n-3	0.18^{c}	0.59^{b}	0.83^{a}	0.81^{a}	0.057	<0.001
22:4n-6	0.93^{a}	0.62^{b}	0.73^{ab}	0.58^{b}	0.071	0.008
22:5n-3	1.2^{c}	2.0^{b}	2.5^{a}	2.4^{ab}	0.17	<0.001
22:6n-3	5.7	6.8	6.9	6.2	0.90	0.752
DMA						
DMA 16:0	1.2	1.2	1.5	1.2	0.14	0.195
DMA 18:0	1.8	1.9	2.1	1.7	0.18	0.552
DMA 18:1	0.41	0.37	0.43	0.34	0.041	0.360
Partial sums						
C18[4]	37	37	34	36	1.6	0.485
DMA	3.4	3.4	4.0	3.3	0.3	0.351
SFA[5]	42	41	41	42	0.40	0.417
MUFA[6]	34	33	30	32	1.7	0.330
cis-MUFA	32	31	25	30	2.4	0.141
PUFA[7]	20	22	24	22	1.6	0.266
n3-PUFA	7.9	10	11	10	1.0	0.130
n6-PUFA	11	11	12	11	0.9	0.472
EPA + DHA	5.9	7.4	7.7	7.0	0.91	0.491
AA/EPA ratio	37^{a}	9.1^{b}	8.0^{b}	6.9^{b}	2.33	<0.001

Means within a row with different letters are significantly different ($p < 0.05$). [1]FA and DMA—fatty acids and dimethyl acetals; [2]C—control diet with no EPA sources; O—diet with *Nannochloropsis* sp. oil; SD—diet with spray-dried *Nannochloropsis oceanica* biomass; FD—diet with freeze-dried *Nannochloropsis oceanica* biomass. [3]Standard error of the mean; the value presented corresponds to a pooled sample standard error of the mean. [4]Sum of C18 FA. [5]Sum of saturated FA. [6]Sum of monounsaturated FA. [7]Sum of polyunsaturated FA. In the FA notation (x:n-), 'x' represents the number of C atoms, ':' the number of double bonds and 'n-' the location, in its carbon chain, of the double bond which is closest to the methyl end of the molecule. *c* stands for *cis* and *t* stands for *trans*. *i* stands for *iso* and *a* stands for *anteiso*.

The DHA content in RTL tissues did not differ among treatments ($p > 0.05$). In the partial sums evaluated, the AA/EPA ratio differed between treatments, being higher in the C-fed lambs when compared to the remaining treatments ($p < 0.001$). The sum of the EPA + DHA averaged 7% TFA. N-3 PUFA averaged 10 ± 1.0%, corresponding to approximately 45% of the total PUFA. Similar to what was verified in the brain, none of the individual DMA nor the total DMA content differed between treatments in the RTL tissues. Overall, the total DMA content was much lower than that found in the brain, averaging 4%. The fold change in the EPA, DPA and DHA content between FD-fed lambs and C-fed lambs was compared between the brain, RTL and liver (Table 5.6) and previously analysed subcutaneous adipose tissue (SC AT) and *longissimus lumborum* muscle samples (Vítor et al., 2023). The EPA fold change was higher in the SC AT and similar between the liver and RTL.

Table 5.6- Summary table for total fatty acid (TFA) and dimethyl acetal (DMA) (mg/ g DM) content and composition (%TFA+DMA) of the liver tissues of lambs.

FA[1]	**Diets**[2]				**SEM**[3]	***p*-value**
	C	**O**	**SD**	**FD**		
TFA + DMA	80	75	67	71	3.99	0.174
14:0	0.53	0.46	0.52	0.42	0.050	0.312
16:0	15.8	13.8	14.4	14.2	0.561	0.080
*c*9-16:1	1.17	1.07	1.19	1.31	0.151	0.715
17:0	1.56^{b}	1.68^{b}	1.73^{b}	2.32^{a}	0.195	0.039
18:0	24.7	24.8	23.5	24.0	0.946	0.764
*c*9-18:1	18.33^{a}	11.48^{b}	9.95^{bc}	8.11^{c}	0.730	<0.001
*c*11-18:1	2.07	1.95	1.90	2.50	0.298	0.462
18:2n-6	8.06	7.85	7.40	7.21	0.621	0.740
22:0	0.15	0.07	0.08	0.13	0.038	0.318
20:4n-6	7.88^{a}	5.77^{b}	7.01^{a}	7.27^{a}	0.407	0.007
20:5n-3	0.87^{b}	4.25^{a}	4.14^{a}	4.68^{a}	0.387	<0.001
22:5n-3	2.87^{b}	8.18^{a}	8.95^{a}	9.19^{a}	0.478	<0.001
22:6n-3	2.14^{b}	4.19^{a}	4.08^{a}	2.88^{b}	0.318	<0.001
Partial sums						
TC18[4]	59.9^{a}	54^{b}	52.1^{b}	50.9^{b}	1.28	<0.001
SFA[5]	43.2	41.3	40.8	41.7	1.13	0.480
MUFA[6]	28.3^{a}	21.4^{b}	21.4^{b}	20.0^{b}	1.12	<0.001
Cis-MUFA	23.8^{a}	15.7^{b}	14.5^{b}	13.0^{b}	1.06	<0.001
PUFA[7]	26.9^{b}	36^{a}	36^{a}	36.6^{a}	1.83	0.002
n-3 PUFA	6.40^{b}	17.5^{a}	18^{a}	17.7^{a}	0.894	<0.001
n-6 PUFA	18.3	15.8	16.2	16.5	0.880	0.207
EPA + DHA	3.01^{b}	8.45^{a}	8.21^{a}	7.56^{a}	0.573	<0.001

Means within a row with different letters are significantly different (p < 0.05). [1]FA and DMA- fatty acids and dimethyl acetals. [2]C, control diet with no EPA sources; O, diet with *Nannochloropsis* sp. oil; SD, diet with spray-dried *Nannochloropsis oceanica* biomass; FD, diet with freeze-dried *Nannochloropsis oceanica* biomass. [3]Standard error of the mean; the value presented corresponds to a pooled sample standard error of the mean. [4]Sum of C18 FA. [5]Sum of saturated fatty acids. [6]Sum of monounsaturated FA. [7]Sum of polyunsaturated FA. In the FA notation (x:n-), 'x' represents the number of C atoms, ':' the number of double bonds and 'n-' the location, in its carbon chain, of the double bond which is closest to the methyl end of the molecule. *c* stands for *cis*.

5.3.4. sPLSDA analysis.

Figure 5.3 illustrates a total of five sparse partial least squares-discriminant analysis (sPLS-DA) plots, corresponding to four plots that belong to all the brain parts evaluated (Panels A–D) and one plot belonging to RTL tissues (Panel E). It is possible to observe that there is no clear individualization of lambs belonging to the same diet in accordance with their brain FA and DMA compositions (%TFA) in all brain parts. However, in the RTL tissues (Figure 5.3-E), it is possible to clearly separate C-fed lambs from *Nannochloropsis* supplemented lambs based on their retinal FA composition.

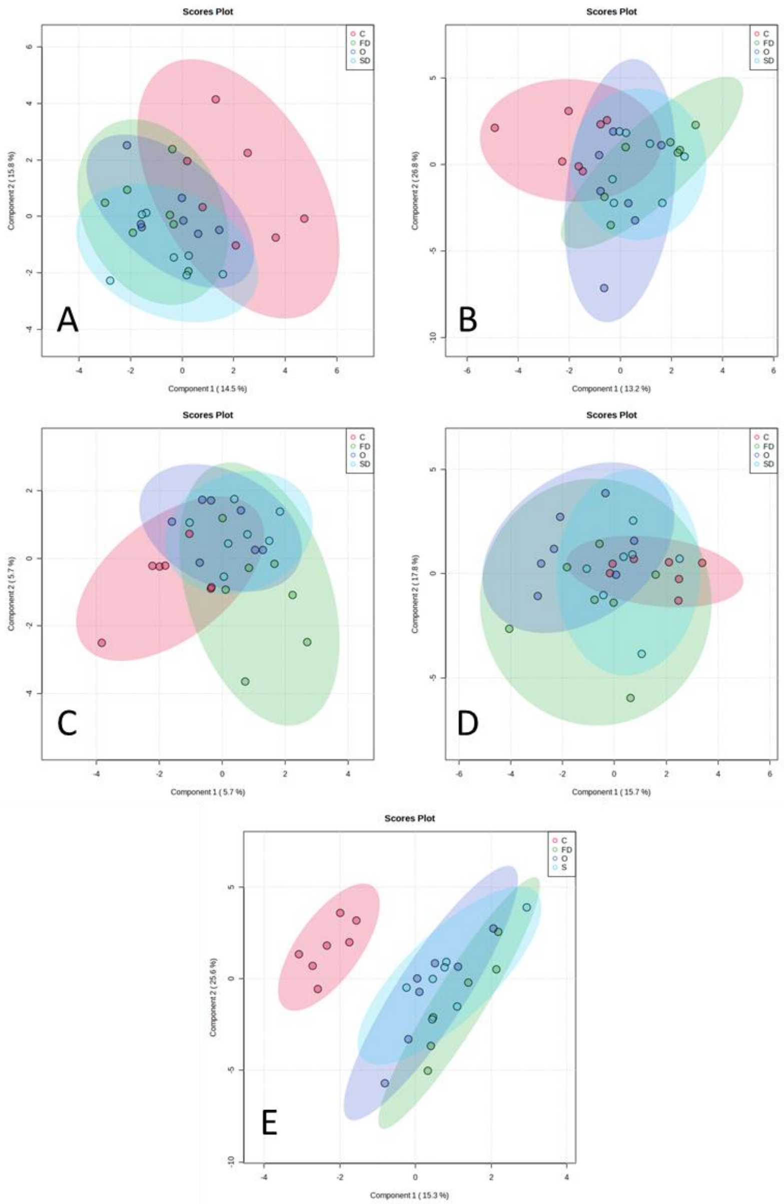

Figure 5.3- sPLSDA analysis including the FA+DMA profile.

The different plots correspond to the different brain parts (A-D) and RTL tissues (E). A: prefrontal cortex, B: hippocampus, C: grey matter, D: white matter; and E: RTL tissues. Diets are represented in different colors: red- C, control diet with no EPA sources; purple - O, diet with *Nannochloropsis* sp. oil; blue - SD, diet with spray-dried *Nannochloropsis oceanica* biomass; and green - FD, diet with freeze-dried *Nannochloropsis oceanica* biomass.

5.4. Discussion.

5.4.1. Brain FA composition.

The classic research on ruminant brain lipids has been based on evaluating the lipid classes and the FA within the lipid classes of whole brain homogenates (Christie, 1981). In the present study, we present the FA and DMA profile (expressed as % of TFA + DMA) of the brain and RTL tissues of lambs fed *Nannochloropsis* sp. lipids.

The most abundant FA found in the ovine brain were 18:0 (≈18%), 16:0 (≈17%), *c*9-18:1 (≈17%), DHA (≈9%) and AA (≈5% of TFA + DMA). A similar pattern was also observed in the bovine brain regions (Rule et al., 2022), with the same five FA also being the most abundant: 16:0 (≈18%), 18:0 (≈20%), *c*9-18:1 (≈24%), AA (≈6%) and DHA (≈9% of total FA). It was shown that linoleic acid (LA), often the major PUFA in muscle, and α-linoleic acid (ALA) were only present at very low levels. In fact, the *longissimus lumborum* muscles of the same animals presented LA and ALA proportions of circa 9 and 1% (Vítor et al., 2023), contrasting with the proportions of ≈0.5% and ≈0.1% observed in the brain, respectively. Despite being the major dietary PUFA, LA can hardly be considered functional in the brain because of its low concentration (<0.5% of TFA). This low concentration is probably due to LA being extensively converted to AA, which plays a role in neurodevelopment (Taha, 2020). Moreover, the majority (≈59%) of LA entering the brain is rapidly β-oxidized (Jr et al., 2006).

We hypothesised that lambs' brains and/or retinal tissues would be sensitive to the differences in n-3 LC-PUFA absorption due to the changes in rumen biohydrogenation associated with processing the microalgae biomass. Namely, FD *Nannochloropsis oceanica* supplemented diets appeared to produce a higher n-3 LC-PUFA enhancement in the lambs' brains when compared to O and SD because freeze-drying better protects the integrity of the microalgal cell wall, reducing the access of ruminal microbiota to the n-3 LC-PUFA inside the cell. In ruminants, almost 90% of dietary lipids reach the duodenum as non-esterified saturated FA (Doreau & Ferlay, 1994), and PUFA are selectively converted into phospholipid forms in the enterocyte (Moore & Christie, 1984). The transport and uptake of EPA and DHA within brain and retina involves their esterification into a lysophospohtidylcholine and a specific transporter (Mfsd2a) (Nguyen et al., 2014; Yalagala et al., 2019; Sugasini et al., 2020). Thus we anticipated that the uptake of EPA into the brain and retina would be efficient and responsive to the intestinal absorption of EPA. However, despite the dietary supplementation of EPA, its proportions in the brain were low (≈0.6%) and did not differ among treatments in any of the brain parts. This contrasts with the response to EPA deposition observed in the *longissimus lumborum* muscle of the same animals, in which EPA rose from 0.8% in the C treatment to 1.7% in the *Nannochloropsis*-supplemented treatments (Vítor et al., 2023). The response in the liver was even more pronounced, with EPA rising from 0.9% up to 4% in the *Nannochloropsis* supplemented treatments (Table 5.6). Thus, in general, the ovine brain was not responsive to dietary EPA supplementation. This contrasts with the results reported by Rule et al. (2022), in which a similar intake of EPA (2.3 g/day) resulted in an EPA enhancement across various brain parts. However, the supplementation period in our study lasted 1/10 of the one in Rule et al. (2022).

As the uptake of EPA and DHA into the brain is similar (Chen & Bazinet, 2015), the lack of EPA enhancement in the lambs' brains in response to the treatments might be explained by the faster β-oxidation of EPA compared to DHA and/or by the extensive elongation to DPA and subsequent desaturation to DHA. Nevertheless, we also did not observe an increase in DHA. The long half-life of DHA in brain tissues can explain the slow turnover of these fatty acids, therefore explaining the lack of their enhancement in the brain (Rapoport, 2001). Moreover, there seems to be evidence that brain DHA and AA levels can be maintained by the liver stores once there is evidence that liver (but not brain) DHA synthesis is upregulated when the dietary content of n-3 PUFA is reduced (Rapoport et al., 2010).

Similar responses to microalgae supplementation in brain FA were observed for the hippocampus and prefrontal cortex, maybe reflecting the extensive hippocampal–prefrontal interactions involved in various cognitive and behavioural functions in animals (Sigurdsson & Duvarci, 2016; Eichenbaum, 2017). Higher amounts of n-3 DPA and dihomo-γ-linolenic acid (DGLA, 20:3n-6) were found in the hippocampi and prefrontal cortices of microalgae-fed lambs. DGLA is an intermediate of the elongation and desaturation of LA, being converted into AA through the activity of the Δ-5 desaturase enzyme (Wang et al., 2012). The increase in DGLA in the brain of lambs supplemented with microalgae was not obvious, as *Nannochloropsis* does not contain relevant amounts of LA and DGLA. DPA, a product of the elongation of EPA, was increased despite the lack of response in EPA and DHA. As it has been proposed that DPA constitutes a storage depot for EPA and DHA (Miller et al., 2013), its enhancement seems desirable. Although DPA was approximately 10 times lower than DHA in the brain, it was more responsive to the dietary supply of n-3 PUFA. The same pattern was also observed in the brain of lambs suckling from ewes fed with linseed (Nudda et al., 2022) and in the hippocampus of bovines fed fish oil (Rule et al., 2022). Most of the beneficial effects of marine oils (mainly fish oils) have been attributed to DHA and EPA (FAO/WHO, 2008; Drouin et al., 2019). However, DPA, which is the intermediate between EPA and DHA in the n-3 LC-PUFA biosynthetic pathway, also presents beneficial biological effects. It reduces platelet aggregation, improves the lipid plasmatic profile, neural health and endothelial cell migration, and assists in the resolution of chronic inflammation (Drouin et al., 2019). Plasmalogens are a subclass of glycerophospholipids that comprise part of biological membranes, including the plasma membrane and the membranes of intracellular organelles, affecting their biophysical properties. They are quantitively important in membranes of neuronal tissues, including the brain and the retina, and are associated with neurological and psychiatric disorders or are involved in the regulation of retinal vascular development, respectively (Saab et al., 2014; Udagawa & Hino, 2022). In the DMA, the backbone at the sn-2 position is mainly bonded to PUFA such as DHA and AA, suggesting its protective role against lipoxidation (Braverman & Moser, 2012; Dorninger et al., 2020). The DMA content of ruminant brains is not often reported (Alfaia et al., 2017). In our study, the high abundance of plasmalogens in the brain can be perceived through the high DMA content (≈10% of TFA + DMA). The average content of DMA was higher in the brain when compared to the retina (≈3.5% of TFA + DMA).

5.4.2. RTL tissue FA composition.

The retina is a thin, highly organised neural tissue lining the posterior aspect of the eye. It is responsible for initiating vision by transducing light into neural signals (Smith et al., 2009). The visual streak area of the retina is a narrow horizontal band. It runs parallel to the ventral edge of the *tapetum* (Lossi, 2022). Therefore, due to anatomical proximity and for practical reasons, we collected both tissues simultaneously. The *tapetum lucidum* is a biologic reflector system that is commonly present in the eyes of vertebrates. It enhances visual sensitivity at low light levels by providing light-sensitive retinal cells with a second opportunity for photon–photoreceptor stimulation. Ovine *tapetum lucidum* belongs to the choroidal fibrous type, and the reflective material is made of collagen (Ollivier et al., 2004) which constitutes 65% of the dry weight of the *tapetum* (Bellairs et al., 1975). When comparing the results with the literature, it is important to consider that the specialized retina lipids in the joint RTL samples will be diluted by the fibrous *tapetum* tissue.

Studies with rodents demonstrated that the FA composition of the retina is influenced by diet (Seddon et al., 2001; Chong et al., 2008; Schnebelen et al., 2009b; Schnebelen et al., 2009a; Tan et al., 2009; Simon et al., 2011; Merle et al., 2011) and, for a given species, the retinal FA composition of each phospholipid class is comparable to that of the brain grey matter

(Bartley et al., 1962; Fliesler & Anderson, 1983). The retina of sheep and cattle have a similar FA composition (Anderson, 1970), and the main FA of bovine retina (% dry weight) are 16:0 (25%), 18:0 (17%), 18:1 (17%) and DHA (23%) (Fliesler & Anderson, 1983). In the present study, 16:0 averaged 20%, 18:0 averaged 18%, *c*9-18:1 averaged 26% and DHA averaged 6.4%. AA averaged 6%, in line with it being the most abundant n-6 PUFA in the retina (Acar et al., 2012; Albouery et al., 2020). Contrary to what was observed in the brain, EPA supplementation increased the EPA content in the RTL tissues. In the C lambs, EPA averaged 0.18% TFA + DMA, which was in line with human EPA retinal content (Bretillon et al., 2008). The EPA content significantly increased in O-fed lambs (0.59% TFA + DMA) and particularly in SD- and FD-fed lambs (0.82% TFA + DMA; + 4.6 times the EPA content in the C-fed lambs). Contrary to the results achieved in the brain, our results showed that the RTL tissues of lambs are very responsive to EPA supplementation. The same magnitude of response was only comparable to what we found in the liver (Figure 5.4).

The 4.6-fold increase was achieved despite the short duration of EPA supplementation. Consistent with a better responsiveness of RTL tissues to the experimental diets, control lambs were clearly separated from the lambs consuming *Nannochloropsis*-supplemented diets in the sPLSDA analysis. This shows, once again, that the RTL tissues seem to have been much more sensitive to dietary intervention. The high responsiveness of the retina is evident in rodent studies, in which EPA contents of 6 to 35 times greater have been reported following EPA supplementation (Shindou et al., 2017; Yalagala et al., 2019). As in the brain, no alterations in DHA content were observed between different treatments. The content of DHA in RTL tissues averaged 6.4% of the total TFA + DMA. This is considerably lower that what was reported in previous studies for ruminants in which the DHA content averaged approximately 20–30% (Anderson, 1970; Fliesler & Anderson, 1983; Lecomte et al., 1996).

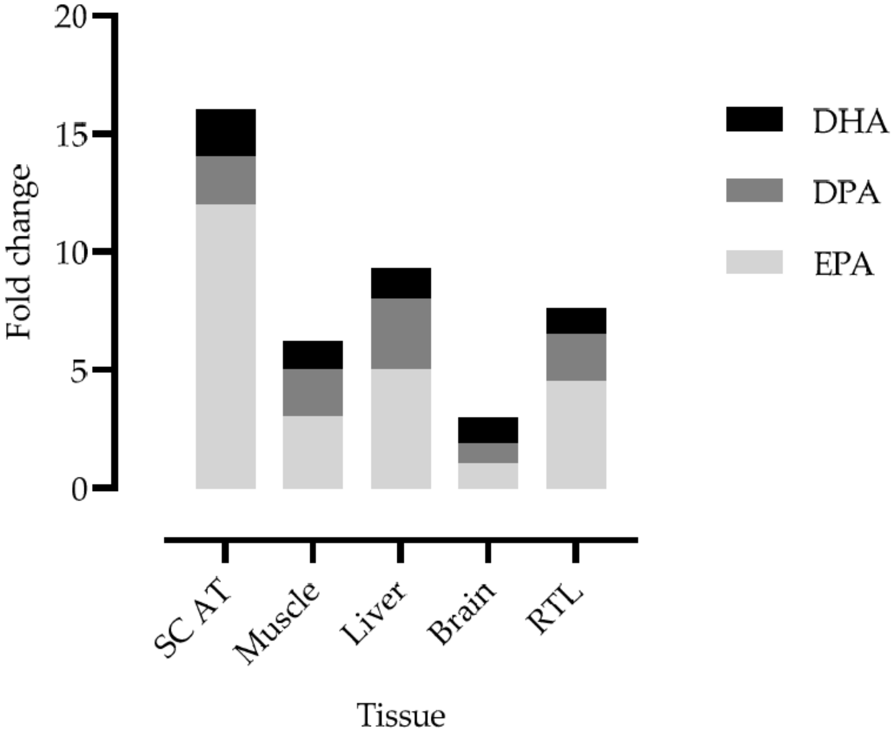

Figure 5.4- EPA, DPA and DHA fold change in lambs' tissues.
The fold change was calculated between the mean value determined in the tissues of freeze-dried *Nannochloropsis oceanica*-fed lambs versus the mean value for control-fed lambs. SC AT: subcutaneous adipose tissue; RTL: retina and *tapetum lucidum*. The reference value for the brain corresponds to the mean of all brain parts.

5.5. Conclusion.

After a short-term EPA supplementation in lambs, achieved through feeding using three different diets containing *Nannochloropsis* sp. microalga, it was possible to conclude that the brain content of EPA was not responsive to dietary supplementation. However, the EPA content in the retina was highly responsive in lambs supplemented with *Nannochloropsis*, especially lambs consuming SD and FD diets. Although we could not confirm an advantage in freeze-drying over spray drying *Nannochloropsis oceanica* with respect to the efficiency of EPA enhancement in the lambs' retinal tissues, we can confirm their advantage over the free oil. Overall, our results suggest that RTL is a good target to evaluate the differences in n-3 LC-PUFA absorption due to the changes in rumen biohydrogenation associated with dietary interventions.

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Author contributions

S.P.A. and R.J.B.B., were responsible for conceptualization and funding acquisition. A.C.M.V., S.P.A. and R.J.B.B. implemented the experiment. A.C.M.V. conducted the animal experiment. A.C.M.V., and S.P.A. conducted the laboratory analysis. J.J.C. monitored and assisted histopathological analysis; A.C.M.V., S.P.A. and R.J.B.B. performed data analysis. A.C.M.V., S.P.A. and R.J.B.B. did the interpretation of results. A.C.M.V. drafted the manuscript. S.P.A., J.J.C., and R.J.B.B. edited and revised the original draft. All authors revised and approved the final manuscript.

6. CHAPTER 6 | *Nannochloropsis oceanica* algaenan: a novel coating material for rumen-protected lipid encapsulation.

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Abstract.

The ruminal biohydrogenation (RBH) of polyunsaturated fatty acids (PUFA) results in the production of various RBH intermediates and saturated FA (SFA). This process and its extension restrict the amount of PUFA that can be accumulated in the tissues of ruminants. Due to the protection conferred by the cell wall of some microalgae species, microalgal intracellular lipids are proposed to be naturally protected against RBH. However, the key-components of the cell wall, responsible for such degree of protection, are still uncertain. Thus, the aim of this project was to develop a method to protect PUFA from the microbial BH using an effective non-toxic, naturally protected technology, based on *Nannochloropsis oceanica* cell wall constituents. For that, we developed an adapted method to extract the algaenan from the *Nannochloropsis* cell wall and used it for the development of biopolymer-based capsules. Different algaenan extraction procedures were tested and optimized, and their impact on the structure of the algaenan was evaluated by structural analysis. Several biopolymer encapsulation techniques were tested using the algaenan as coating biopolymer and n-3 PUFA as the encapsulated substrate. The most suitable techniques were chosen based on the practicability of the method and physical-chemical properties of the microcapsules. The most promising products were tested for its PUFA-protection efficacy against ruminal biohydrogenation *in vitro*. The developed microcapsules were incubated with rumen inoculum in an *in vitro* batch system and dry matter disappearance and the FA profile of each capsule was evaluated until 24h post incubation. Linoleic (LA) and α-linolenic (ALA) acids followed an expected metabolization profile, which did not occur for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The results regarding EPA and DHA metabolization after 24h of incubation were inconclusive and, therefore, experimental procedures need to be repeated.

Keywords: capsule; microalga; cell wall; polyunsaturated fatty acids.

6.1. Introduction.

Some algal groups, such as chlorophytes, eustigmatophytes and dinoflagellates, contain highly aliphatic polyester heteropolymers called algaenans in their cell wall structures (Zhang & Volkman, 2017). Due to a steric protection effect of the molecular structure, algaenans are highly acid and base-resistant (Largeau & Leeuw, 1995). Their high resistance to microbial and chemical degradation allows them to be selectively preserved over time, contributing to the formation of some kerogen fractions of source rock deposits (Allard et al., 2002). Because they can be preserved with little structural change, they are essentially important for geochemical studies (De Leeuw & Largeau, 1993).

Pioneer works of Atkinson et al. (1972) have characterized the algaenans' non-hydrolysable and highly insoluble nature. They were first isolated from the cell walls of *Botryococcus braunii* (Berkaloff et al., 1983), a green freshwater microalga and they were first studied by both pyrolytic and spectroscopic methods (De Leeuw & Largeau, 1993). In those characterizations, it was possible to observe that the macromolecule was based on a three-dimensional network composed of ether-linked, long $(CH2)_n$ polymethylenic chains (n up to 30) with few hydroxyl and ester functions present, producing a steric network, that was indeed a protection from chemical reagents (De Leeuw & Largeau, 1993).

Algaenans differ from other resistant biomacromolecules found in spores and higher plants, as sporopollenin, cutin and cutan (De Leeuw et al., 2006). In some green algae, algaenans are located in trilaminar outer walls, that produce a two electron-dense layer sandwiching and electron-lucent one structure (Brunner & Honegger, 1985) often designated as "trilaminar sheats" (TLS), with 10-20 nm thick. Even though the presence of algaenans is associated to TLS occurrence, there is no dependent relationship (De Leeuw & Largeau, 1993).
Whereas in *B. braunii* algaenans are major constituents of the total biomass (10-33%) (Berkaloff et al., 1983; Derenne et al., 1989) they can represent as low as 0.6-0.3% of the total biomass in *Prototheca wickerhamii* and *Chlorella fusca*, respectively (Atkinson et al., 1972; Puel et al., 1987).

Nannochloropsis is a genus characterized for having a very resistant cell wall, containing algaenans (Scholz et al., 2014). In *Nannochloropsis*, algaenans form an outer cell wall layer protecting an inner cellulose layer (Scholz et al., 2014). In a work by Zhang & Volkman (2017), after stepwise pyrolysis of *Nannochloropsis oculata* cell wall, constituents of different pyrolyzates revealed that the algaenan structure was composed of repeating, interlinked C32 n-alkan-1,17-diols, whereas the free plus bound lipids were dominated by the C32 n-alkan-1,15-diol (Zhang & Volkman, 2017) (Figure 6.1).

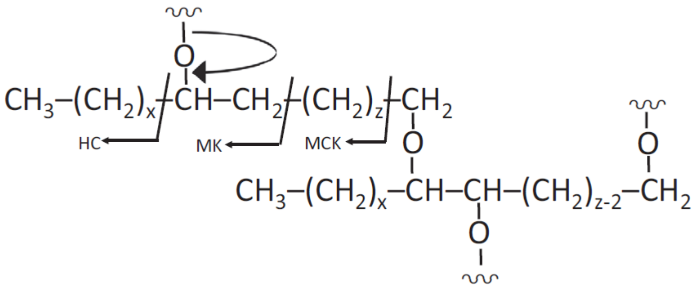

Figure 6.1- Structural units of algaenan proposed by Gelin et al. (1997a) in *Nannochloropsis*.

Proposed origins of the major products (n-alkenes/n-alkanes, n-alkan-2-ones and mid-chain ketones) from pyrolysis of the structural units of algaenan proposed by Gelin et al. (1997a) in *Nannochloropsis*. X and Z are used to designate variable numbers of CH_2 units. Retrieved from Zhang & Volkman (2017).

Due to the recalcitrancy of *Nannochloropsis* cell wall, this microalga was recently proposed as a natural protected source of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) in the rumen (Alves et al., 2018; Vítor et al., 2021). Previously developed encapsulation methods reduced the amount of biohydrogenated n-3 PUFA in the rumen (Gadeyne et al., 2017), but in general they still lack efficiency or possess some degree of toxicity. Among the encapsulation methods, the use of alginate and chitosan polymers has been showing promising results in ruminal protection of biomolecules in *in vitro* incubations (Olloqui et al., 2018; Gawad & Fellner, 2019; Besharati et al., 2022). However, there is still space for improvements. Although the already documented high resistance of algaenan to microbial degradation (Derenne et al., 1992; Largeau & Leeuw, 1995), there are no records of algaenan applications in rumen encapsulation methods. Therefore we proposed to develop an encapsulation method to protect an n-3 LC-PUFA-rich fish oil in ruminal *in vitro* incubations using *N. oceanica* algaenans. Also, due to the fact that microalgae are still an expensive resource in animal feed, the success of a biotechnological product based on algaenans can potentially add value to microalgal utilization in ruminant production.

6.2. Materials and methods.

6.2.1. *Nannochloropsis oceanica* algaenan isolation.

Freeze-dried *N. oceanica* was used as raw material for algaenan isolation procedures since the group had already confirmed that freeze-drying is the most effective method to dry *N. oceanica* biomass in terms of cell wall integrity maintenance (Vítor et al., 2021). The algaenan isolation protocol involved chemical and mechanical treatment steps to successively remove labile components present in the microalgae cells, such as proteins, carbohydrates, and lipids (Zelibor et al., 1988; Blokker et al., 1998; Allard et al., 1998) (Figure 6.2). The algaenan constituted the remaining residue at the end of the procedure, which was freeze-dried and stored for characterization.

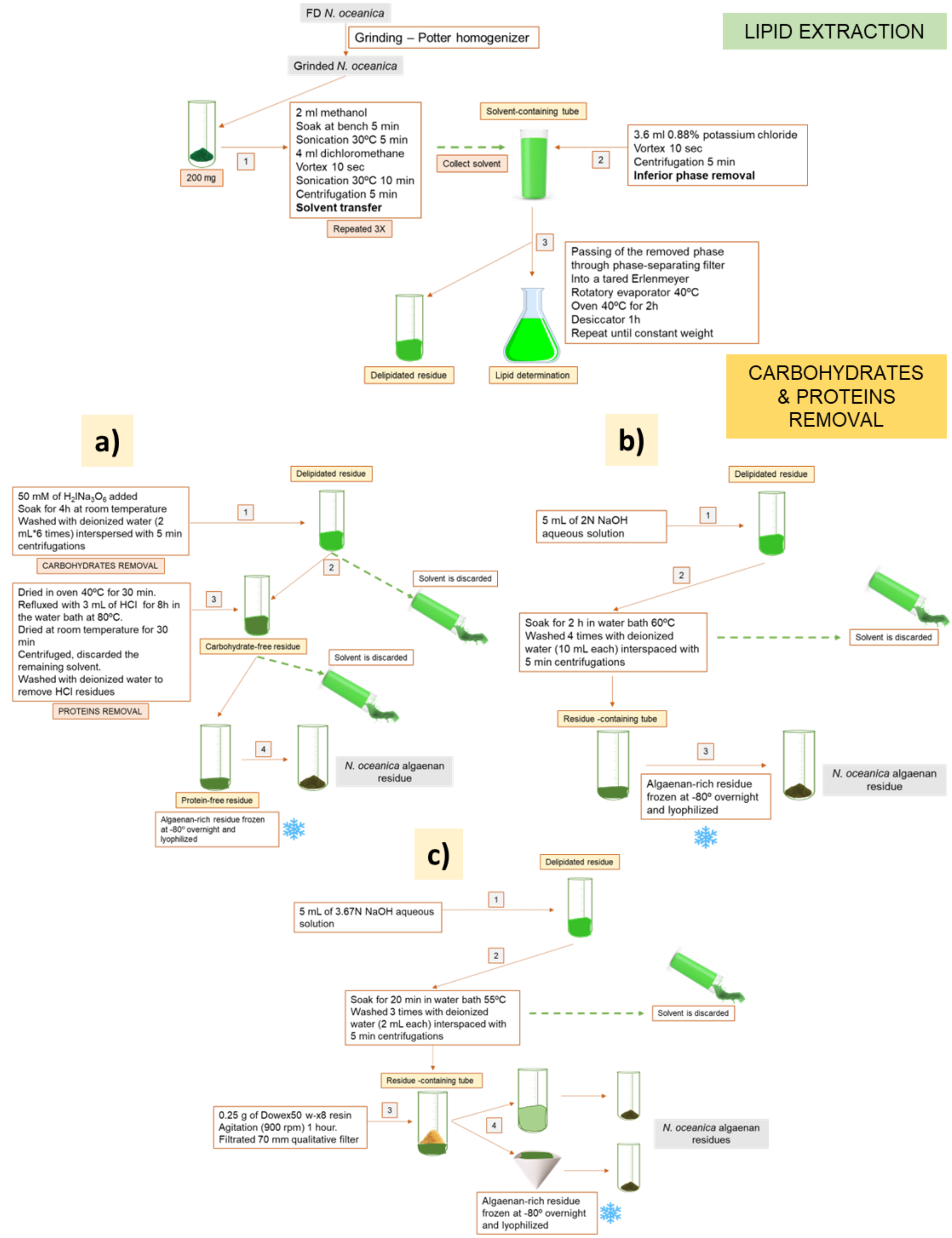

Figure 6.2- Fractionation scheme followed for obtaining *N. oceanica* algaenan.
The Figure schematizes the lipid extraction procedure and the three tested procedures for carbohydrate and protein removal: a) sodium paraperiodate method; b) NaOH long method and c) NaOH short method. Numbers indicated in the grey squares represent the order of the consecutive steps in the flow.

6.2.1.1. Lipid extraction.

Two hundred milligrams of lyophilized *N. oceanica* were weighed and grinded using a 10 mL Potter homogenizer. Three repeated lipid extractions were performed using Folch et al. (1957) adapted method. Two hundred milligrams of the macerated biomass were placed in 16 mL glass tubes and 2 mL of methanol were added. Samples were left to soak for 5 minutes in the bench, after which they were placed in a sonicator water bath (Elmasonic S 60H, Singen, Germany) at 30ºC for 5 minutes. After, 4 mL of dichloromethane were added, and samples were vortexed for 10 seconds. They were after replaced in the sonicator water bath for 10 minutes at the same temperature. Samples were centrifuged (Astor 8 New, Poncarale, Italy) for 5 minutes and the maximum solvent was transferred into another 16 mL tube. Following, 6 mL of dichloromethane:methanol (2:1) (with the addition of 25 mg/L of BHT) were added to the tube containing the solid phase and it was vortexed for 10 seconds. The same tube was again placed in the sonicator water bath for 10 minutes and centrifuged for 5 minutes, after which more solvent was collected into the solvent containing tube. This last step was repeated once again except that the sonicating time was reduced to 5 minutes. This totalled 3 periods of vortexing – sonicating – centrifuging – solvent collection. After these periods, 3.6 mL of a 0.88% potassium chloride solution were added into the solvent containing tubes. These tubes were vortexed for 10 seconds and centrifuged for 5 minutes. The water-rich top phase was used for the next steps (carbohydrates and proteins removal) and the bottom phase (organic phase containing the lipid extract) was collected and passed through a phase-separating filter to a previously tared 50 mL Erlenmeyer, that was placed in a rotary evaporator (Heidolph Hei-Vap, Heidolph Instruments, Schwabach, Germany) at 40 ºC. Erlenmeyers were then placed in an oven at 40 ºC for 2 hours and 1 hour in the desiccator. This last procedure was repeated until constant weight.

6.2.1.2. Algaenan extraction methods by carbohydrates and proteins removal.

Carbohydrates and proteins needed to be removed from the delipidated microalgae residue to isolate algaenans. For that, three methods: Sodium paraperiodate method (a), NaOH short method (b) and NaOH long method (c), were tested. Replicas containing lipid-free residue were subjected to one of the methods, originating different final algaenan-rich residues (Figure 6.3). All the tubes subjected to carbohydrates and proteins removal steps were protected from the direct light, being wrapped with aluminium foil.

(a) Sodium paraperiodate ($H_2INa_3O_6$) method.

Sodium paraperiodate ($H_2INa_3O_6$) is an oxidizing agent that selectively oxidizes specific carbohydrates and aminoacids (Halsall et al., 1947). The method using sodium paraperiodate was adapted from Yuan et al. (2018). Briefly, 50 mM of sodium paraperiodate solution (adjusted to pH 4) were added to the residue and left to soak for 4h at room temperature. After that, consecutive washes, to remove sodium paraperiodate residues, were made with deionized water (2 mL* 6 times) interspersed with 5 min centrifugations.

After the carbohydrate-removing step, proteinaceous material was removed with HCl, using an adaptation of Zelibor et al. (1988) method. The residue free of carbohydrates was extracted with 5 mL of dichloromethane:methanol (1:1) for 1 hour, centrifuged for 5 min and the solvent was discarded. The tube was placed in the stove at 40ºC for approximately 3 hours until the residue was dry. After, it was washed with deionized water (5 mL), centrifuged, and discarded to remove any residues of the solvent. The tube was again placed to dry in an oven at 40ºC for 30 min. After, the residue was refluxed with 3 mL of HCl for 8h in the water bath at 80ºC. Finally, the residue was left drying at room temperature for 30 min, after which it was centrifuged, and the remaining solvent was discarded. The final residue was washed with deionized water to remove HCl residues. In the last step, the residue was once again extracted with 5 mL of dichloromethane: methanol (1:1) for 1 hour and the solvent was discarded. The remaining was frozen at -80º overnight and lyophilized.

(b) NaOH long method.

This method resulted from an adaptation of Obeid et al. (2015) method, developed to isolate *Scenedesmus* algae algaenan. To each test rube containing the lipid-free residue, 5 mL of 2N NaOH aqueous solution was added. Tubes were left for 2h in the water bath at 60ºC and then washed 4 times with deionized water (10 mL each) interspaced with 5 min centrifugations. All the solvent was discarded, and the remaining was frozen at -80º overnight and lyophilized.

(c) NaOH short method.

This method resulted from an adaptation of García-Martínez et al. (2016) method applied to remove carbohydrates and proteins from *Chlorella vulgaris*. To each test tube containing the lipid-free residue, 5 mL of 3.67N NaOH aqueous solution was added. The test tube was placed in a water bath for 20 min at 55ºC and the residue was washed 3 times with deionized water (2 mL) interspaced with 5 min centrifugations. After, 0.25 g of Dowex50 w-x8 ion exchange resin (Supelco®) was added to exchange residual sodium (Obeid et al., 2015), and the tube was left for agitation (900 rpm) for 1 hour. The content was filtrated through a 70 mm qualitative filter. Both the filtrate and the filter content (the filter content was placed in an oven at 40ºC overnight to dehydrate) were stored, frozen at -80ºC overnight and lyophilized and both residues were further used for algaenan characterization.

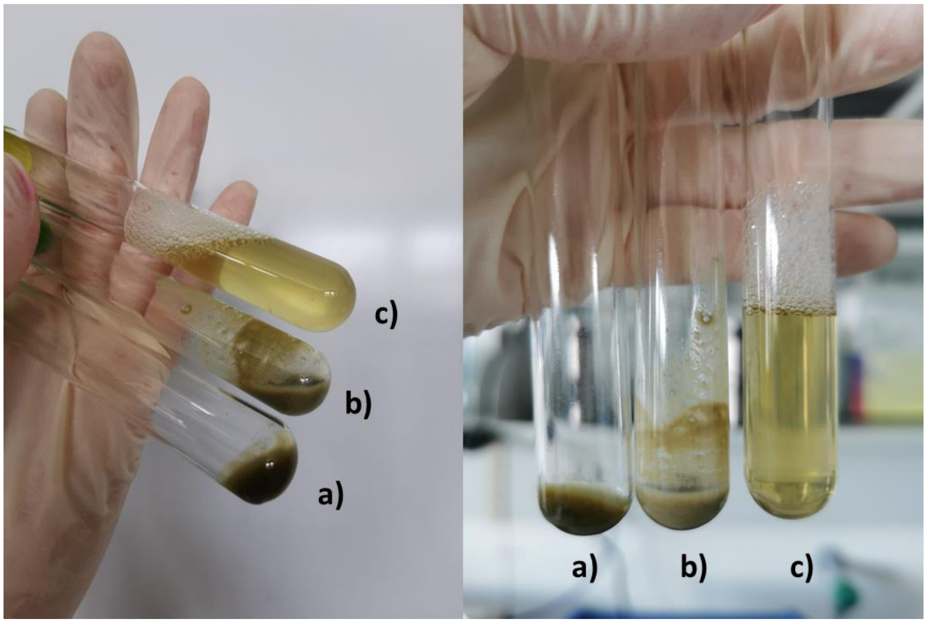

Figure 6.3- Carbohydrates and protein removal methods

The two images illustrate the various aspects of the residual resulting from the different methods used. In both images: a- proteins removal using sodium paraperiodate adapted method from Yuan et al. (2018); b- carbohydrates and proteins simultaneous removal using NaOH long adapted method from Obeid et al. (2015); and c- carbohydrates and proteins simultaneous removal using NaOH short adapted method from García-Martínez et al. (2016).

6.2.2. Algaenan-rich residue characterization using instrumental methods.

The different algaenan-rich residues derived from different carbohydrates and proteins extraction methods were characterized using instrumental methods of analysis at CICECO – Aveiro Institute of Materials, using Fourier transform infrared spectrometry (FTIR), elemental analysis (EA) and thermogravimetric analysis (TGA).

6.2.2.1. Fourier transform infrared spectrometry (FTIR).

Fourier Transform Infrared (FTIR) spectra of samples were acquired using a Golden Gate single reflection diamond attenuated total reflectance system in a Bruker IFS-55 spectrometer. Spectra were recorded at the absorbance mode from 4000 cm^{-1} to 400 cm^{-1} wavenumber (mid-infrared region) with a resolution of 4 cm^{-1}. Five replicates (64 co-added scans) were collected for each sample.

6.2.2.2. Elemental analysis.

Elemental analyses of samples were performed with a TruSpec 630-200-200 CHNS Analyzer. The temperature of combustion furnace and afterburner was 1075°C and 850°C, respectively. Detection method applied: carbon – infrared absorption, hydrogen – infrared absorption, nitrogen – thermal conductivity.

6.2.2.3. Thermogravimetric analysis (TGA).

Thermogravimetric analysis (TGA) was conducted on a TGA-50 Shimadzu equipment under air flow from room temperature to 700°C and with a heating rate of 10°C/min.

6.2.3. Development of an encapsulation method using *N. oceanica* algaenan.

6.2.3.1. Algaenan extraction using NaOH long method.

To develop the encapsulation method, higher algaenan quantities were extracted using the NaOH long method (Figure 6.4), which was selected after algaenan characterization and also because it delivered a higher algaenan yield and, in terms of practicality, it was less time consuming. Thus, several batches were produced with an average yield of the algaenan residue of about 12%.

Figure 6.4- Freeze-dried algaenan residue.

6.2.3.2. Preliminary encapsulation formulas (Capsules EXP 1).

Different capsules using alginate, and alginate + chitosan as polymer base, with or without algaenan and fish oil were produced (Figure 6.5).

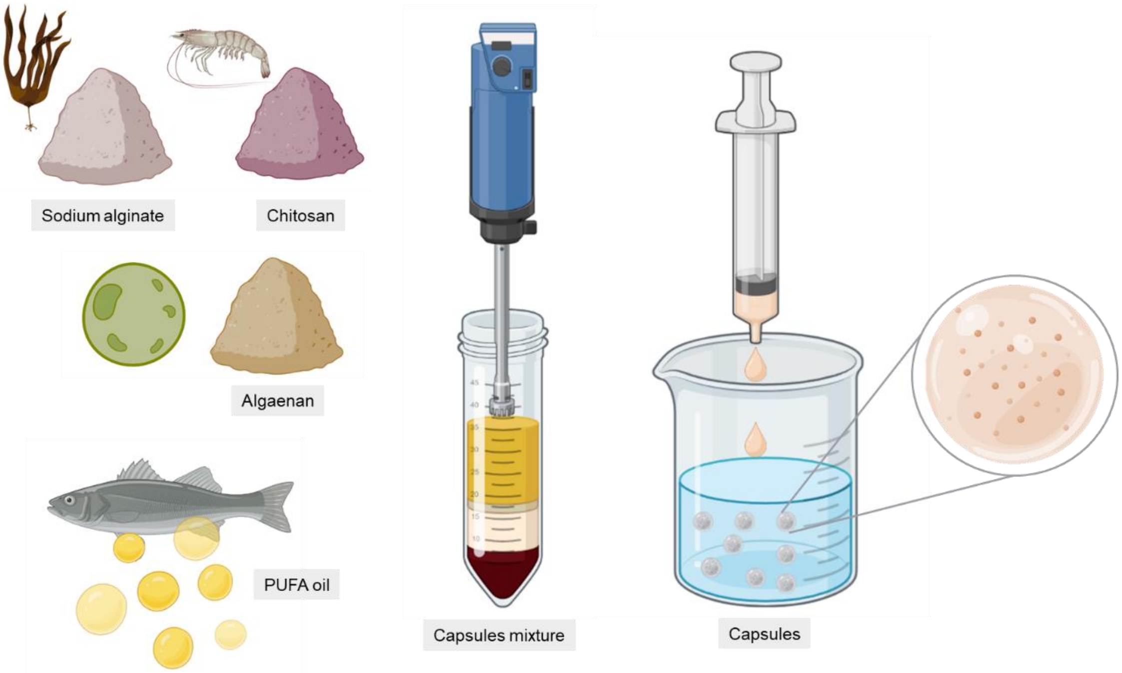

Figure 6.5- Capsules EXP 1 scheme.

Sodium alginate, extracted from brown algae cell walls, and chitosan, found in the exoskeleton of crustaceans such as shrimp were used as the base soluble polymers. Algaenan, extracted from the cell wall of *N. oceanica* was include in the capsules to increase the degree of polyunsaturated fatty acids (PUFA) protection. An eicosapentaenoic acid-rich fish oil was used as the encapsulated agent. The ingredients were mixed in order to create the emulsion for the capsule fabrication. Capsules were obtained by dripping the emulsion in a solution of $CaCl_2$. Created with BioRender.com.

Sodium alginate (Sigma-Aldrich) and chitosan (Sigma-Aldrich) of high molecular weight and > 75 % deacetylation were used. The protocol used to formulate alginate capsules was adapted from Olloqui et al. (2018), which performed an encapsulation of fish oil into alginate beads to be tested in a ruminal-intestinal *in vitro* digestion model. The protocol used to formulate alginate + chitosan capsules was adapted from Gawad & Fellner (2019), who evaluated glycerol encapsulated with alginate-chitosan polymers in a gut-simulated environment. The different capsules and experiment were designed as follows (Table 6.1):

Table 6.1- Capsules first experiment design (Capsules EXP 1).

Capsules	**Sodium alginate**	**Chitosan (0.4%)**	**Glucose syrup (20%)**	**PUFA (Fish oil, 15%)**	**Algaenan (0.3%)**
AP	X (2.5%)	-	X	X	-
APa	X (2.5%)	-	X	X	X
CP	X (0.8%)	X	-	X	-
CPa	X (0.8%)	X	-	X	X
Free PUFA	-	-	-	X	-

The designation of each capsule is as follows: AP (capsule containing alginate and PUFA oil); APa (capsule containing alginate, PUFA oil and algaenan); CP (capsule containing alginate + chitosan and PUFA oil); CPa (capsule containing alginate + chitosan, PUFA oil and algaenan). The percentages of inclusion of each ingredient are presented as % (w/v) of the emulsion and ingredient inclusion is marked with an “X”. Glucose syrup was added as a stabilizer. Free PUFA corresponds to the use of the PUFA-rich oil in the *in vitro* planning, in order to constitute a positive control for FA metabolization.

Alginate capsules mixture was prepared in 10 mL of distilled water and homogenized with an Ultra-Turrax T25 basic (IKA-WERKE) at 15.000 rpm at room temperature for 10 minutes. The mixture was transferred to a 100 mL glass syringe (FORTUNA OPTIMA) and passed through a 22 G needle. The emulsion was dripped in a 500 mL glass Becker containing a 0.5 mol/L solution of $CaCl_2$. The capsules were left in agitation (700 rpm) for about 20 min to harden. Capsules were strained and washed with distilled water. Finally, capsules were dried in an oven at 40ºC for 4 hours. After, they were refrigerated in a sealed container.

Alginate and chitosan capsules mixture was prepared in successive steps. Sodium alginate and chitosan pose difficulties in mixing because they have opposite charges. When combined, they can form a gel-like substance due to a process called ionotropic gelation (Szliszka et al., 2009), and inconsistent mixing may cause clumps or inconsistent gel formation. To enhance mixing, chitosan was dissolved in an acidic solution to reduce its positive charge before mixing with sodium alginate. Therefore, chitosan was added to 10 mL of 1% acetic acid solution and was left agitating at 700-900 rpm overnight. Alginate was gradually added to the chitosan solution with constant stirring, being left agitating at 700-900 rpm for 48 hours. PUFA oil and algaenan were added and left agitating at 1000 rpm for 1 hour. After, the mixture was homogenized with an Ultra-Turrax T25 basic (IKA-WERKE) at 15.000 rpm at room temperature for 5 minutes. The emulsion was transferred to a 100 mL glass syringe (FORTUNA OPTIMA) and passed through a 22G needle. The emulsion was dripped in a 500 mL glass Becker containing a 3% solution of $CaCl_2$. Capsules were strained and washed with distilled water. The capsules were left in agitation (700 rpm) for about 1 hour to harden. Finally, capsules were left to dry at room temperature for 24h and dried in an oven at 40ºC for 3 hours. After, they were refrigerated in a sealed container. These capsules were used in a preliminary *in vitro* study with strained rumen fluid as described in section 6.2.6.

6.2.3.3. Encapsulation formulas with target concentration of EPA (Capsules EXP 2).

In order to obtain a 0.05 mg EPA/ mL of incubation medium as described in a work by Alves et al. (2018) or 0.3 mg of EPA per tube (total 6 mL of medium per tube), the EPA content in each type of capsule was taken into consideration. Therefore, the previous developed capsules were re-done and a final *in vitro* was planned (Table 6.2).

Table 6.2- Capsules of the second experiment (EXP 2).

Capsules	Sodium alginate	Chitosan (0.42%)	Glucose syrup (20%)	PUFA (Fish oil, 15%)	Algaenan (0.3%)
AP	X (2%)	-	X	X	-
APa	X (2%)	-	X	X	X
A	X (2%)	-	X	-	-
CP	X (0.83%)	X	-	X	-
CPa	X (0.83%)	X	-	X	X
C	X (0.83%)	X	-	-	-
FO	-	-	-	X	-

The designation of each capsule is as follows: AP (capsule containing alginate and PUFA oil); APa (capsule containing alginate, PUFA oil and algaenan); A (capsule containing alginate); CP (capsule containing alginate + chitosan and PUFA oil); CPa (capsule containing alginate + chitosan, PUFA oil and algaenan); C (capsule containing alginate + chitosan). The percentages of inclusion of each ingredient are presented as % (w/v) of the emulsion and ingredient inclusion is marked with an "X". Glucose syrup was added as a stabilizer. Free PUFA (FO) corresponds to the use of the PUFA-rich oil in the *in vitro* planning, in order to constitute a positive control for FA metabolization.

The reformulation of alginate and chitosan capsules was done in order to improve the capsule-making technique and the number of capsules per tube were adjusted to the same amount of EPA per tube (0.3 mg).

Alginate-based capsules containing algaenan contained 2% (w/v) alginate (320 mg), 0.3% algaenan (48 mg), 20% glucose syrup (3.2 g) and 15% PUFA oil (2.4 g) dissolved in 10 mL of distilled water. Three different formulas were made using alginate as a polymer base: a) alginate + PUFA oil (AP); b) alginate + PUFA oil + algaenan (APa); and c) alginate only (A). Alginate + chitosan-based capsules containing algaenan contained 0.83% (w/v) alginate (99 mg), 0.42% chitosan (50 mg), 0.3% algaenan (36 mg) and 15% PUFA oil (1.8 g) dissolved in 10 mL of a 1% solution of acetic acid. Three different formulas were made using alginate + chitosan as a polymer base: a) chitosan and alginate + PUFA oil (CP); b) chitosan and alginate + PUFA oil + algaenan (CPa); and c) chitosan and alginate only (C). Mixing was done using an Ultra-Turrax T25 basic (IKA-WERKE) at speed 3 (13.500 rpm min) for 5 minutes. The mixture of each formulation was poured into a 100 mL glass syringe (FORTUNA OPTIMA) and passed through a 22 G needle. The alginate-based emulsion was dripped into a 0.5 mol/L $CaCl_2$ solution, and the alginate + chitosan-based emulsion was dripped into a 3% $CaCl_2$ solution. The capsules were left in agitation (700 rpm) for about 20 min to harden. After, they were washed with distilled water and frozen at -80ºC. After, they were lyophilized in a Scanvac Cool-safe Superior Touch freeze dryer (Scanvac, Denmark) with the following conditions: temperature, − 92°C; initial pressure 0.2 mbar; final pressure 0.07 mbar, for 4 days. Figure 6.6 illustrates the aspect of the different experimental capsules after lyophilization.

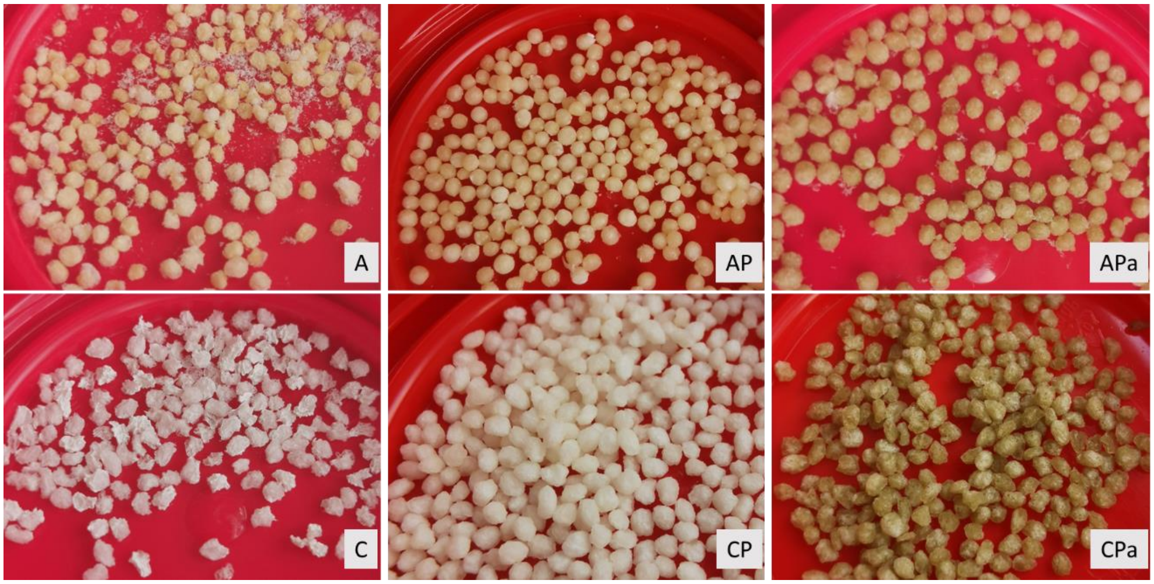

Figure 6.6 - Experimental capsules (EXP 2).
The capsules in the upper row correspond to the ones incorporating sodium alginate as the base polymer: A – sodium alginate; AP – sodium alginate and PUFA; APa – sodium alginate, PUFA and algaenan. The capsules in the lower row corresponded to the ones incorporating a mixture of sodium alginate and chitosan as the base polymers: C – chitosan and sodium alginate; CP – chitosan, sodium alginate and PUFA; and CPa – chitosan, sodium alginate, PUFA and algaenan.

6.2.4. Characterization of the capsules EXP 2 using experimental methods.

Samples from each capsule type were characterized by Fourier transform infrared spectroscopy (FTIR), carried out in a Bruker Vertex 70 model equipment preparing KBr pellets at 1% (w/w). FTIR spectra were obtained with a resolution of 4 cm−1, with 120 scans between 4000 and 400 cm−1. For characterization by X-ray diffraction (XRD), a Shimadzu diffractometer (Labx-XDR 6000 model) was used operating at 40 kV and 30 mA, with Cu Kα radiation (λ = 1.5406) and range from 3 to 75° 2θ with a sweep speed of 2°/min-1.

6.2.5. Scanning electron microscopy (SEM) characterization of the capsules EXP 2.

In order to observe further details into the capsules surface, scanning electron microscopy (SEM) images were taken of both alginate and alginate + chitosan complete capsules (biopolymer + PUFA-rich oil + *N. oceanica* algaenan). Images were obtained using a HITACHI TM3030 Plus Tabletop Microscope. Wall details photographs were taken with 180x magnification and the photographs showing the overall capsule aspect were taken with 60x magnification, using an accelerating voltage of 5 keV.

6.2.6. *In vitro* batch incubations.

About 500 mL of ruminal fluid was collected from a sheep immediately after its sacrifice and immediately transferred to the laboratory in a sealed thermostatic box at 39°C. The pH of the ruminal fluid was measured immediately (Digital pH meter; JP Selecta S.A, Barcelona, Spain) and ranged from 6.19 to 7.06 throughout the study. Rumen fluid was directly strained through four layers of cheesecloth and diluted (1:4, v/v) in the medium of Goering and Van Soest (Goering & Soest, 1970) under CO_2 flux. The ruminal buffered solution (6 mL) was distributed into Hungate tubes containing about 60 mg of a commercial total mixed ration (TMR) composed of 60:40 concentrate:forage ratio - Control group, or the TMR supplemented with capsules - EXP 2: (1) alginate capsules (A); (2) alginate + commercial n-3 PUFA oil capsules (AP); (3) alginate + commercial n-3 PUFA oil + *N. oceanica* algaenan capsules (APa); (4) alginate + chitosan capsules; (5) alginate + chitosan + commercial n-3 PUFA oil capsules (CP); (6) alginate + chitosan + commercial n-3 PUFA oil + *N. oceanica* algaenan capsules (CPa); and (7) commercial n-3 PUFA free oil (FO). Free oil (TG1000 Fish Oil with 35% EPA and 25% DHA from Biofil® without being encapsulated in one capsule) was taken into consideration to constitute a positive control, in terms of RBH, for the *in vitro* incubations.

The quantity of the substrate used for the *in vitro* incubations was adjusted to have a concentration of 0.05 mg/mL EPA per tube, as in a previous work by Alves et al. (2018). For batch incubations, Hungate tubes were filled with CO_2 and closed with a butyl rubber stopper and screw cap, then tubes were incubated in a water bath (Unitronic, J.P. Selecta, Barcelona, Spain) for 0, 2, 4, 10, and 24 hours at 39°C with gentle agitation (40 rpm). After incubation, tubes were directly frozen and stored at −20 °C. The allocation of tubes to the treatments, incubation time, order of filling with buffered ruminal fluid, and position in the water-bath were randomized. The incubation procedure was replicated over 3 consecutive weeks with one tube per treatment and time. Samples were freeze-dried (ScanVac CoolSafe, LaboGene ApS, Lynge, Denmark), and stored at −20 °C until analysis.

6.2.7. FA transesterification.

Fatty acid methyl esters (FAME) and dimethyl acetals (DMA) from each incubation tube were prepared by direct transesterification with 0.5M sodium methoxide in methanol as alkaline catalyst followed by the addition of 1.25M hydrogen chloride in methanol as acid catalyst (Alves et al., 2013). Methyl nonadecanoate (1 mg/mL) was used as internal standard and added in the beginning of the transesterification. Both FAME and DMA were analyzed by gas chromatography with flame ionization detection (GC-FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m × 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA, USA) capillary column. The chromatographic conditions were as follow: injector and detector temperatures were set at 250°C and 280°C, respectively; helium was used as the carrier gas at 1 mL/min constant flow; the initial oven temperature of 50 °C was held for 1 min, increased at 50 °C/min to 150 °C and held for 20 min, increased at 1 °C/min to 190 °C and then increased at 2 °C/min to 220 °C and held for 40 min.

6.2.8. Statistical analysis.

EPA disappearance (Equation 6.1) at each incubation time was analyzed using the MIXED procedure of SAS and considering the *in vitro* experiment runs as random block and the EPA-source as the single fixed effect. When needed, the group option of the repeated statement was included in the model to accommodate the variance heterogeneity. Least square means and standard error of the mean are reported, and treatment or contrast effects were considered significant at $p < 0.05$. Taking into account the three *in vitro* replicas, EPA disappearance was calculated over the different incubation time points (Equation 6.1), as in the work by Alves et al. (2018).

$$\text{EPA Disappearance (\%)} = [(\text{EPA0}-\text{EPAt})/\text{EPA0}] \times 100 \qquad \textbf{(6.1)}$$

Equation 6.1 - EPA Disappearance (%)

In the Equation 6.1, EPA is expressed in %TFA at time 0 (EPA0) and time t (EPAt, t = 2, 4, 10, 24 hours).

6.3. Results

6.3.1. Characterization of the algaenan extracted residue.

The FTIR spectra of the first algaenan samples, produced using different protein and carbohydrates removal extraction methods are presented in Figure 6.7.

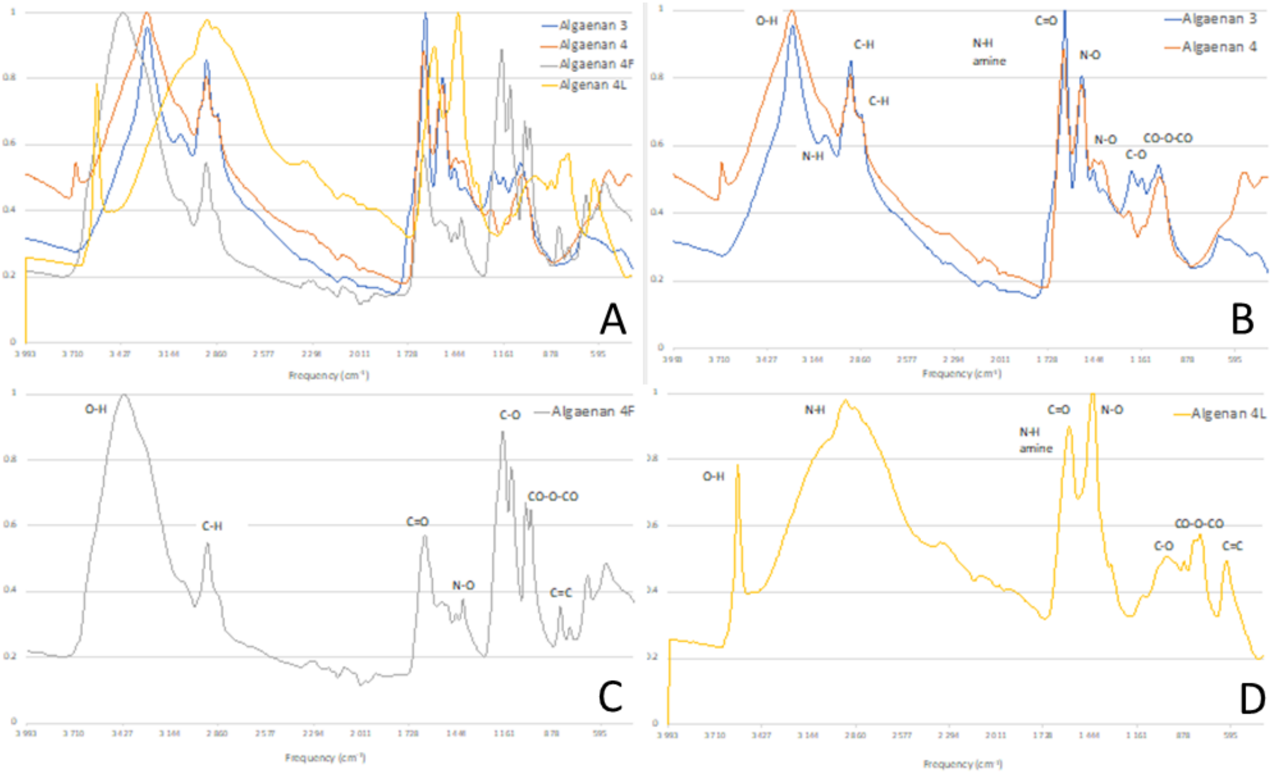

Figure 6.7- FTIR spectra of the algaenan extracted residue.
Algaenan 3 (paraperiodate treatment), Algaenan 4 (NaOH long treatment), Algaenan 4F (NaOH short treatment – Filter content), Algaenan 4L (NaOH short treatment – Liquid filtrate content). Panel A: overlap of the four algaenan residues spectra; Panel B: overlap of algaenan 3 and 4; Panel C: Algaenan 4F; Panel D: Algaenan 4L.

The samples corresponding to Algaenan 3 (obtained from NaOH short method) and 4 (NaOH long method) show similar spectra (Figure 6.7-B), revealing a similar structure. It was possible to observe the presence of a broad band in the region of O–H and N–H stretching vibrations (≈3600–3000 cm$^{–1}$), as well as the C–H stretching vibrations related to the occurrence of aliphatic structures (2900 and 2850 cm^{-1}), which are characteristic of the algaenan structure. The presence of two peaks at approximately 1620 and 1530 cm$^{–1}$ can correspond to C=O and N-O stretching vibrations, respectively, which is usually indicative of the presence of proteins. In addition, the peaks between 1200-900 cm$^{–1}$ are attributed to C-O and CO-O-CO stretching vibrations.

The spectrum of the algaenan 4F (the filter-retained content of NaOH short method after Dowex50 w-x8 addition) revealed the presence of the characteristic bands of O–H (≈3600–3000 cm–1), only one band characteristic of C–H stretching (2900 cm$^{–1}$), and the bands attributed to the C=O, C-O, and CO-O-CO stretching vibrations, which seems to indicate a decrease on protein content and a concomitant increase in the characteristic bands of the algaenan structure (Figure 6.7-C).

Algaenan 4L (Figure 6.7-D, obtained from the filtrated content resulting from the NaOH short method after Dowex50 w-x8 addition) revealed a completely different spectrum comparing with the other samples analysed with main bands/peaks at 3500, 2900, 1560, and 1420, which are attributed to O-H, N-H, C=O, and N-O stretching vibrations.

The presence of carboxyl and carbonyl groups characterised by the absorption band at 1710 cm$^{–1}$ and 1735 cm$^{–1}$, respectively, were not observed in the samples, which is characteristic from algaenan samples (Allard & Templier, 2000).

Elemental analysis was also done in algaenan samples, with exception of algaenan 3. The algaenan 4 was composed of 35% of C, 5.8% of H, and 7.5 of N (Table 6.3). The high content of N could be due to the presence of protein, as observed by the FTIR analysis. Otherwise, sample 4F revealed high content of C (39%) but low content of N (0.3%), indicating the lower content of protein. The sample 4L had lower content of organic material with only 8% of C and 1.5% of N. Algaenan 3 was not analysed in terms of elemental analysis due to insufficient sample quantity.

Table 6.3- Elemental composition of algaenan samples.

Sample	%C	%H	%N
Algaenan 4	35.3 ± 0.08	5.8 ± 0.03	7.5 ± 0.03
Algaenan 4F	38.8 ± 0.10	4.5 ± 0.03	0.3 ± 0.03
Algaenan 4L	8.0 ± 0.13	6.0 ± 0.16	1.5 ± 0.04

Algaenan 4 (NaOH long method); Algaenan 4F (the filter-retained content of NaOH short method after Dowex50 w-x8 addition); Algaenan 4L (obtained from the filtrated content resulting from the NaOH short method after Dowex50 w-x8 addition). %C – percentage of carbon atoms; %H – percentage of hydrogen atoms; %N – percentage of nitrogen atoms.

The thermal stability of the different algaenan samples was studied by thermogravimetry (TGA) from 20°C to 700°C under air atmosphere. TGA and derivate thermogravimetry (DTG) curves were obtained for each sample Figure 6.8.

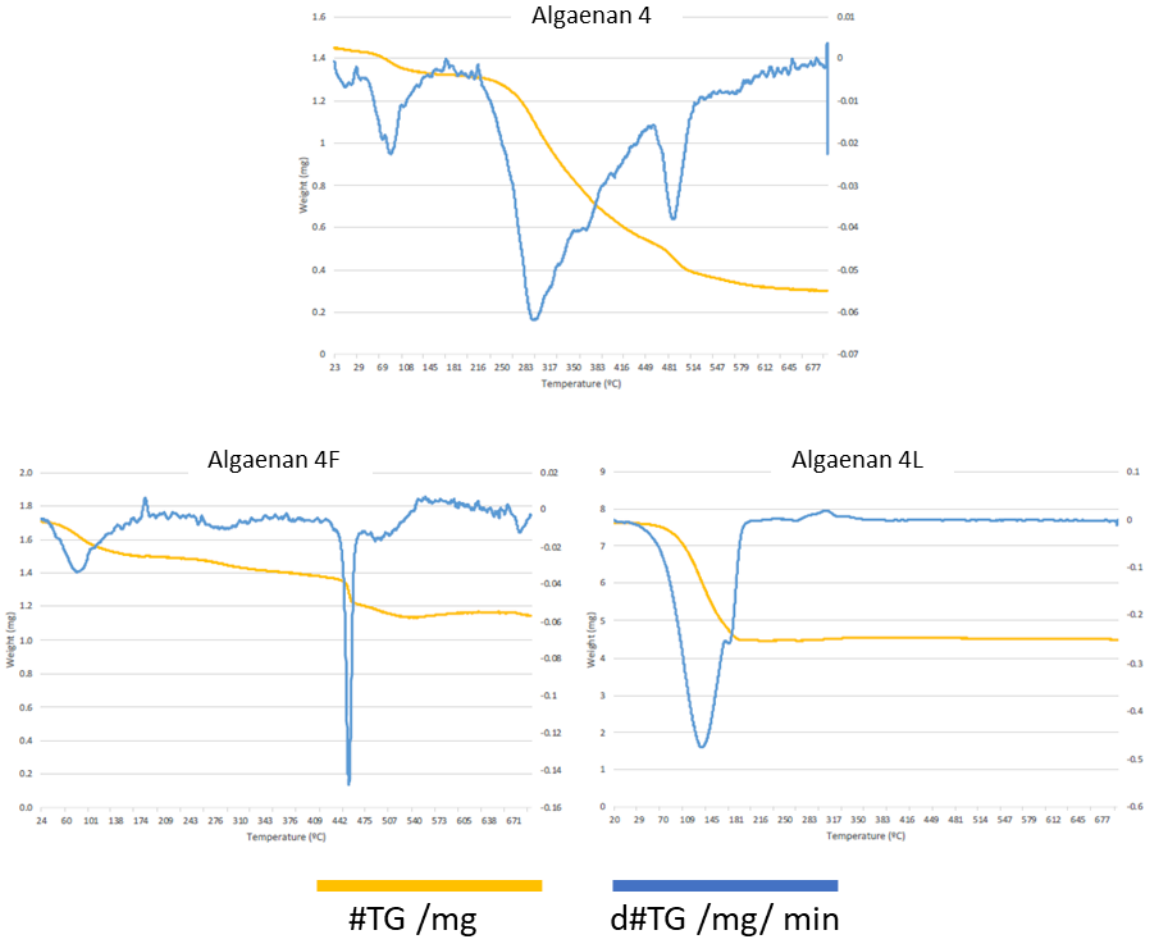

Figure 6.8-TGA and DTG curves of algaenan samples obtained under air atmosphere.

The algaenan 4 lost weight in three major steps. The first step happened below 120°C and that is attributed to the evaporation of water molecules entrapped in the structure. The second phase occured approximately between 250 – 400°C, corresponding to the elimination of oxygen functionalities due to the dehydration, depolymerization, and pyrolytic decomposition with vaporization and elimination of volatile products. The last weight loss step started approximately at 450 °C and refers to the degradation of carbon structure, which have a highly stable structure and resistance to temperature. Around 22% of the sample weight remained after the 700°C combustion. Algaenan 4F revealed two main steps of weight loss, at around 100°C and 450°C, attributed to the evaporation of water molecules and the degradation highly stable structure of carbon, respectively. This could indicate a higher content of algaenan in the sample. However, this sample revealed a high content of inorganic material, since 70% of the weight remained after 700°C. This has probably to do with a present contamination with the cationic-change resin used. Algaenan 4L revealed a completely different profile with only one weight loss step at around 130 °C, remaining 60 % of the weight sample at 800 °C. This means that the sample is composed by a high percentage of inorganic material, which is in accordance with the elemental analysis. Once again, Algaenan 3 was not taken into consideration for TGA analysis due to insufficient sample quantity.

The two additional batches of algaenan samples extracted using NaOH long method, and used for the encapsulations, were also submitted to FTIR analysis. Their spectrum is presented in Figure 6.9. It was again observed the presence of a broad band in the region of O–H and N–H stretching vibrations (≈3600–3000 cm^{-1}), as well as the C–H stretching vibrations related to aliphatic structures (2900 and 2850 cm^{-1}), which are characteristic of the algaenan structure. Although, once again, it was detected the presence of two bands at approximately 1620 and 1530 cm^{-1} that can correspond to C=O and N-O stretching vibrations, respectively, which are usually indicative of the presence of proteins. In addition, the bands between 1200-900 cm^{-1} are attributed to C-O and CO-O-CO stretching vibrations, also present in algaenan structure described in literature (Allard & Templier, 2000; Simpson et al., 2003; Zhang & Volkman, 2017). Therefore, this sample seemed to be identical to the samples named algaenan 3 and 4, from the previously analysed batches, but with a lower protein content.

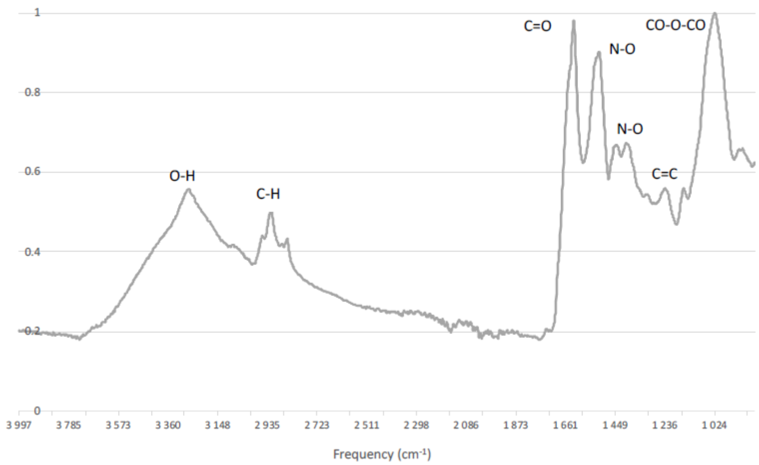

Figure 6.9- FTIR spectra of the Algaenan 4 samples (NaOH long treatment) used for the encapsulation experiments.

6.3.2. Characterization of the EXP 2 capsules.

Figure 6.10 shows the FTIR results obtained for the algaenan powder, and the capsules based on alginate and alginate plus chitosan with PUFA oil and algaenan incorporation.

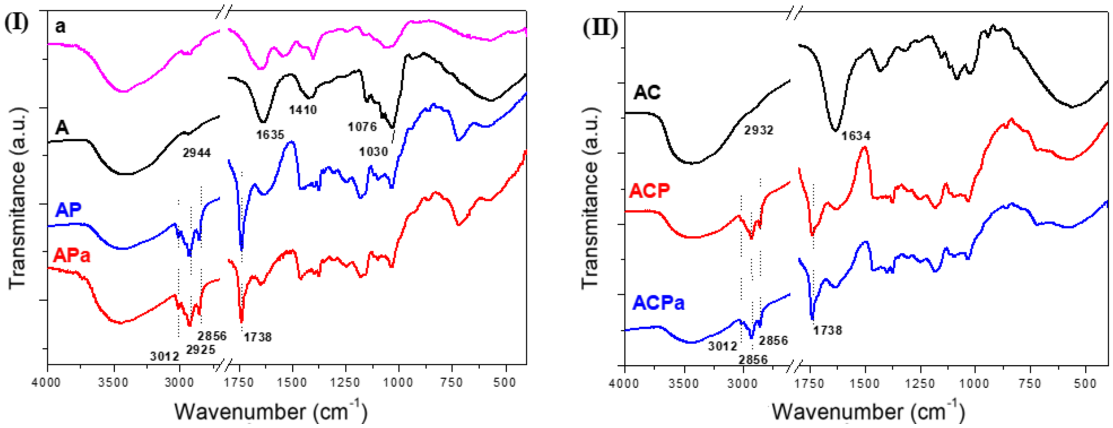

Figure 6.10- FTIR spectra of the EXP 2 capsules.

FTIR spectrum for (I) the isolated algaenan (a); the capsules containing Alginate (A); capsules containing Alginate and PUFA Oil (AP); the capsules containing Alginate, PUFA Oil and algaenan (APa). For (II) the capsules containing alginate and chitosan (AC); capsules containing alginate, chitosan and PUFA oil (ACP); the capsules containing alginate, chitosan, PUFA oil and algaenan (ACPa).

The spectrum of the capsules containing only alginate (A in Figure 6.10-I) shows a broad band between 3700-3000 cm^{-1} related to OH stretching with strong intra and/or intermolecular hydrogen bonding. The overlapping of the symmetric and asymmetric stretching vibrations of C-H of the alginate is responsible for the band at 2944 cm^{-1}. At 1635 and 1410 cm^{-1}, the asymmetric and symmetric vibration modes of the carboxylate ions (COO^-) are observed, respectively. The molecular vibration at 1076 cm^{-1} can be attributed to C–O stretching of the pyranoside ring.

The capsule containing alginate and chitosan (AC in Figure 6.10-II) showed minor changes compared to the spectrum of the capsule containing only alginate. Likewise, when compared with sample A, an enlargement of the bands that appear in the region between 3700-2600 cm^{-1} can be noticed, overlapping the bands attributed to OH and CH groups, probably due to the intermolecular interactions formed between the biopolymers. Additionally, the intermolecular interactions with the C=O double bond of the acetylated units ($–CONH_2$ groups) cause an increase in intensity and widening of the band in the region of 1634 cm^{-1}.

The FTIR spectra referring to the capsules containing PUFA oil (AP and APa in Figure 6.10- I; ACP and ACPa in Figure 6.10-II) showed new bands characteristic of lipids, such as those that appear in the range of 3050–2700 cm^{-1}, attributed to the C–H stretching modes of the methyl and methylene groups, with the multiple double bonds in PUFA evidenced by the band observed at 3012 cm^{-1}, due to the stretching of the cis =C–H bond (Vasile et al., 2016). Another characteristic PUFA band present in the capsules appears at 1738 cm^{-1}, which can be attributed to the C=O stretching vibration of the triglyceride ester functional groups (Hosseini et al., 2021; Lucarini et al., 2018).

Figure 6.11 shows the X-ray diffractograms obtained for the isolated algaenan and the capsules obtained.

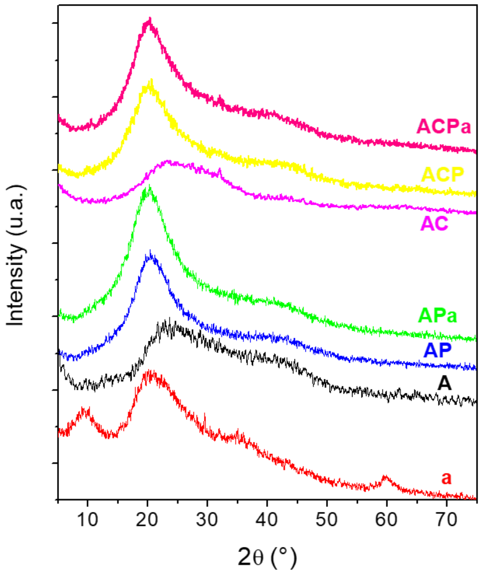

Figure 6.11- X-ray diffraction of the EXP 2 capsules.
Results obtained by X-ray diffraction of powdered algaenan (a); alginate capsules (A); alginate capsules and PUFA oil (AP); alginate capsules, PUFA oil and algaenan (APa); alginate and chitosan capsules (AC); alginate, chitosan and PUFA oil (ACP) capsules; alginate, chitosan, PUFA oil and algaenan (ACPa) capsules.

The diffractogram for the isolated algaenan powder sample (Figure 6.11-a) shows a pattern with a partially crystalline characteristic due to its relatively regular chain due to the existing inter and intramolecular interactions, showing three reflections with broad peaks at 9.3°; 20.9° and 59.6° 2θ. The x-ray diffraction pattern for the alginate-based capsule (Figure 6.11-A) shows a broad signal in the region between 15-50° 2θ, which confirms an amorphous character for the formed capsules. Similar amorphous behaviour is observed in the capsule containing alginate and chitosan (Figure 6.11-AC), in which a decrease in the intensity of the amorphous region between 35-50° 2θ is observed when compared to the diffractogram of sample A. This can be explained by the intermolecular interactions formed between the biopolymers in the composition of the AC capsule.

The introduction of PUFA oil in the composition of the capsules generates a new x-ray diffraction pattern with semicrystalline characteristics, with a peak at 20.1° 2θ and an amorphous region between 30-50° 2θ.

6.3.3. Scanning electron microscopy (SEM) of the capsules EXP 2.

The SEM images of both alginate and alginate + chitosan reformulated capsules containing algaenan revealed differences in the overall aspect of the capsule’s surface (Figure 6.12). Alginate capsules revealed a better maintenance of the spherical shape and an overall good surface integrity maintenance. However, a few pore-like structures (Figure 6.12-b) could be observed in the surface of the capsules. The alginate + chitosan capsules revealed a more irregular pyramidal-like shape and when observing the details of the surface of these capsules (Figure 6.12-d) it was possible to observe an apparent higher capsule integrity maintenance, due to the absence of surface continuity solutions. Also, the surface of these capsules revealed a more shining “glass-like” appearance.

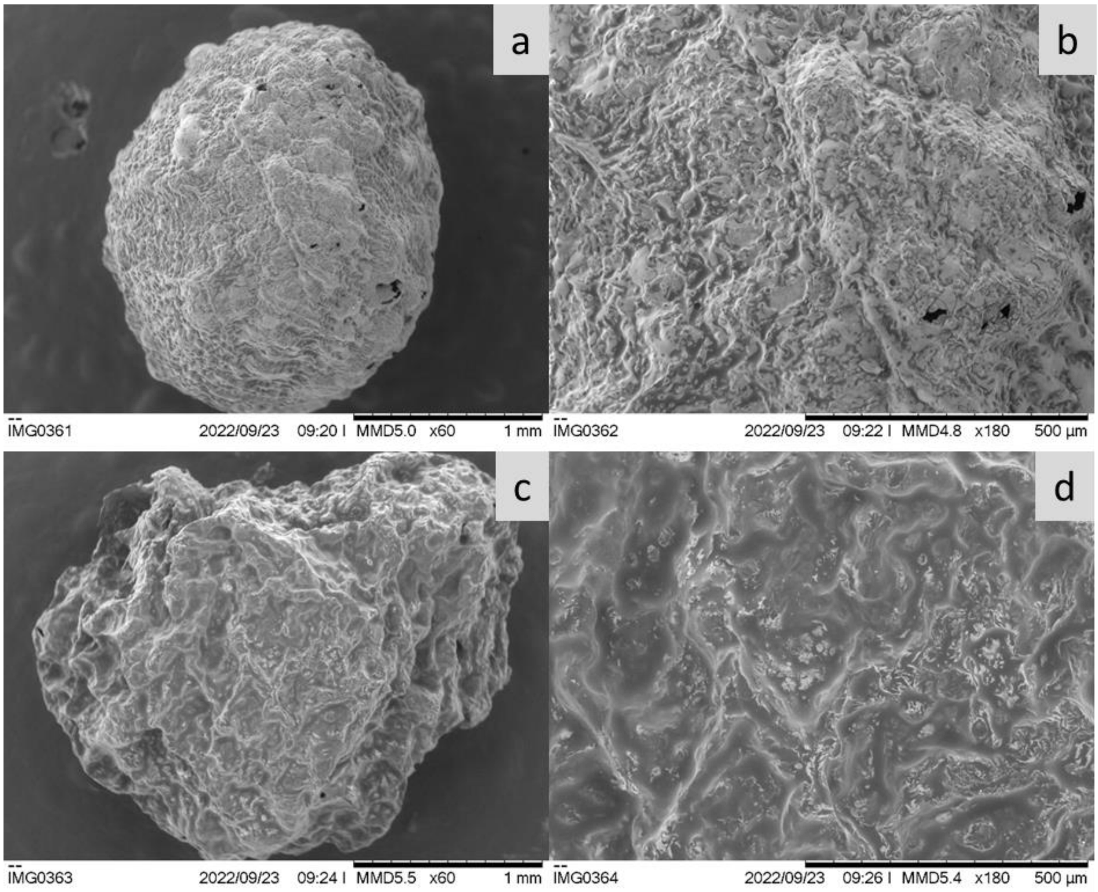

Figure 6.12- SEM of alginate (APa) and alginate + chitosan (CPa) capsules containing PUFA-rich oil and *Nannochloropsis oceanica* algaenan.
The top row of the image illustrates an alginate-based capsule aspect (APa), whereas the bottom row illustrates an alginate + chitosan capsule aspect (CPa). In the image, a - alginate capsule (APa); b- alginate capsule (APa) – surface details; a - alginate + chitosan capsule (CP); and d - alginate + chitosan capsule (CPa) – surface details.

6.3.4. N-3 LC-PUFA of the experimental capsules

The concentration of EPA and DHA as proportion of total FA (%TFA) of the capsules produced during the preliminary encapsulation experiment with different polymers or with algaenan in their composition are presented in Figure 6.13.

Relatively to the FA composition of the incubated capsules, it was possible to observe that, overall, EPA content was equally higher in both alginate and alginate + chitosan capsules (Figure 6.13-A). The same was verified for DHA. Relatively to the algaenan incorporation in the capsules, it was possible to observe that there were no differences between the EPA and DHA content (%TFA) between capsules containing or not the algaenan in its composition (Figure 6.13-B).

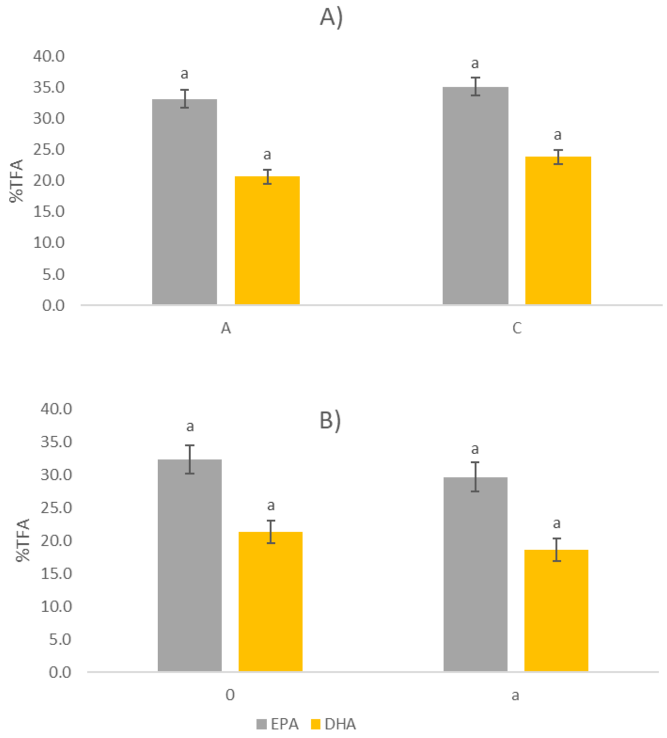

Figure 6.13- EPA and DHA content (%TFA) in the capsules EXP 1. A) Effect of polymer composition; and B) Effect of algaenan inclusion.
The capsules are the following: alginate (A) and alginate + chitosan (C). '0' stands for the capsules not incorporating algaenan and "a" stands for the capsules containing algaenan in the formulation. Least square means are presented in each bar with respective standard error of the mean. Same letters (a) in each bar indicate lack of significant differences ($p > 0.05$) among means.

When evaluating all the formulations used to develop capsules EXP 1, it was possible to observe that both EPA and DHA contents did not differ between capsules (Table 6.4).

Table 6.4- EPA and DHA content (%TFA) of the capsules EXP 1.

FA	AP	APa	CP	CPa	SEM	*p*-value
EPA	33.4	32.9	36.4	33.8	2.19	0.692
DHA	21.1	20.3	25.9	21.8	1.32	0.188

Least square means are presented in each bar with respective standard error of the mean (SEM). The capsules are the following: alginate with PUFA and no algaenan (AP); alginate with PUFA and algaenan (APa); chitosan + algaenan with PUFA and no algaenan (CP); chitosan + algaenan with PUFA and algaenan (CPa).

The EPA and DHA contents of the capsules EXP 2, using the different polymers were determined before conducting the *in vitro* experiments and are presented in Table 6.5.

Table 6.5- EPA, DHA and total PUFA content (mg/g DM) of the capsules EXP 2.

Item (mg/g DM)	**AP**	**APa**	**CP**	**CPa**	**SEM**	***p*-value**
EPA	73.8^b	71.3^b	202.1^a	188.7^a	12.0	0.003
DHA	46.6^b	43.3^b	127.3^a	121.4^a	7.91	0.003
Total PUFA	143.7^b	139.5^b	395.1^a	374.8^a	23.03	0.002

The designation of each capsule is as follows: AP (capsule containing alginate and PUFA oil); APa (capsule containing alginate, PUFA oil and algaenan); CP (capsule containing alginate + chitosan and PUFA oil); CPa (capsule containing alginate + chitosan, PUFA oil and algaenan). Free oil theoretical contents are the following: EPA- 350 mg/g DM, DHA – 250 mg/g DM, and Total PUFA – 700 mg/g DM. (corresponds to the use of the PUFA-rich oil in the *in vitro* planning, in order to constitute a positive control for FA metabolization.

As can be observed in the Table 6.5, even though having been formulated with the same amount of EPA-rich oil, all EPA, DHA and Total PUFA contents varied between different capsule formulations ($p < 0.05$). In general, alginate-based capsules (either containing or not the algaenans) registered lower EPA, DHA and Total PUFA content, when compared to the alginate + chitosan-based capsules.

Because there was an estimated target concentration of 0.05 mg EPA/ mL of incubation medium for the experimental *in vitro*, the number of capsules of each polymer was adjusted per tube.

6.3.5. *In vitro* batch incubations

6.3.5.1. Preliminary *in vitro* with capsules EXP 1.

The results of the dry matter (DM) disappearance of the *in vitro* batch incubations with ruminal fluid during 24h with each capsule produced in the preliminary experiment did not differ ($p > 0.05$) from control tubes and averaged 10.4% (Figure 6.14).

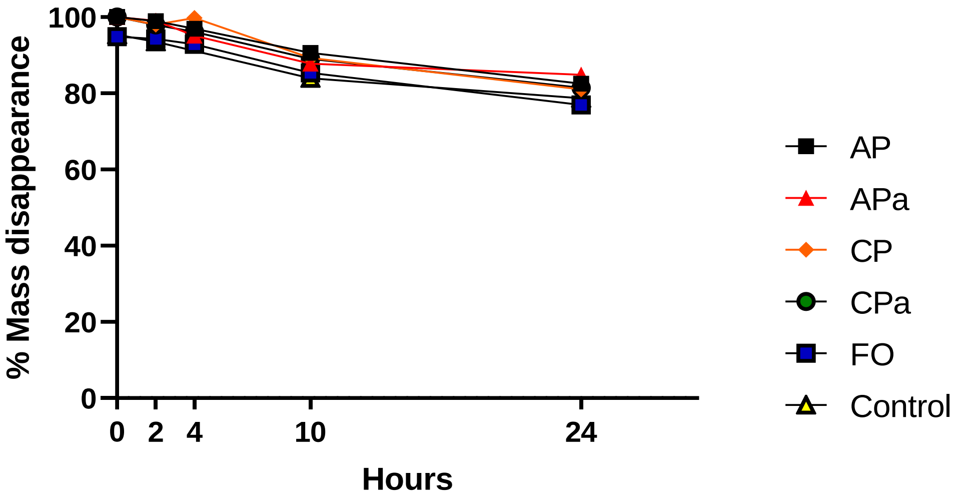

Figure 6.14- Dry matter (DM) disappearance (%).
The capsules are the following: alginate with PUFA and no algaenan (AP); alginate with PUFA and algaenan (APa); chitosan + algaenan with PUFA and no algaenan (CP); chitosan + algaenan with PUFA and algaenan (CPa).

Regarding the *in vitro* results, it was possible to observe that there was not a clear EPA and DHA metabolization in the formulated capsules composed of alginate and chitosan + alginate as base polymers (Figure 6.15). Although LA and 18:0 followed a normal RBH pattern, especially evident in the FO, EPA and DHA curves seemed to maintain a tendency for a flat line across the several incubation timepoints. Also, due to the FO behaviour through the incubation period, it could not be validated as a positive control for the RBH of the encapsulated oil.

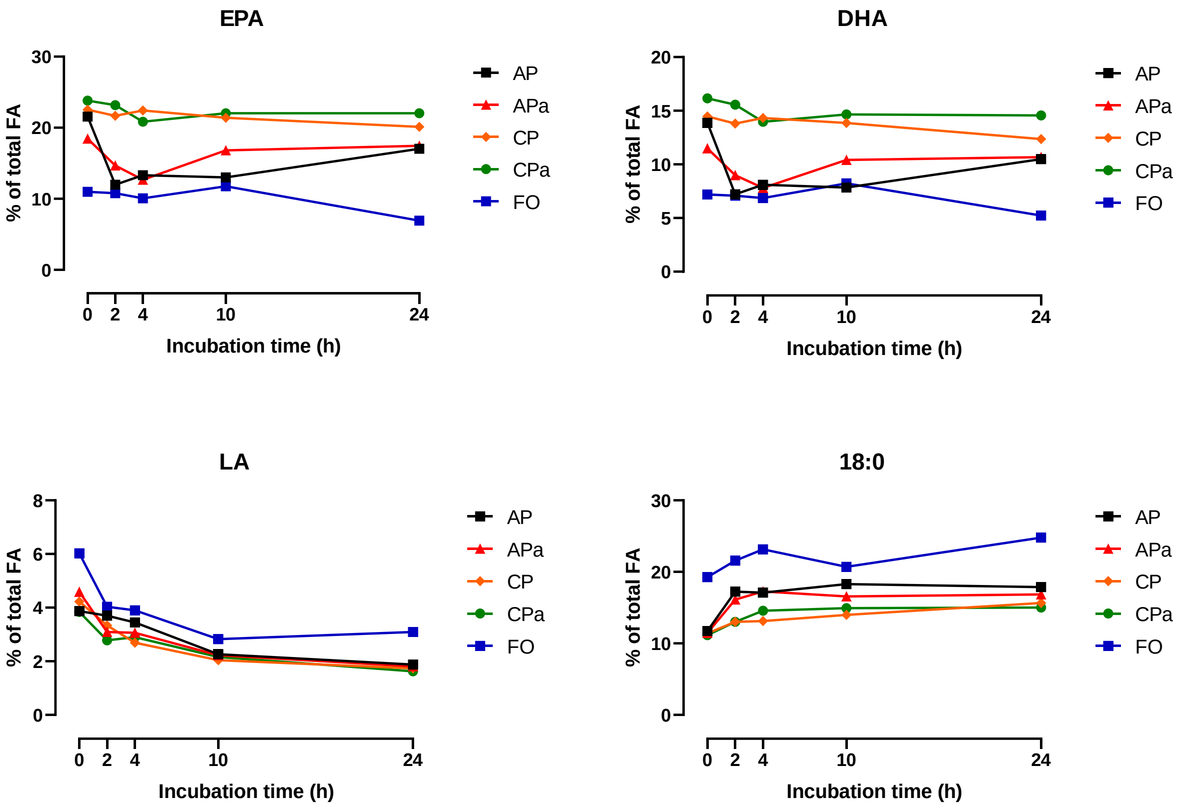

Figure 6.15- EPA, DHA, LA and 18:0 %TFA preliminary *in vitro* results.
The capsules are the following: alginate with PUFA and no algaenan (AP); alginate with PUFA and algaenan (APa); chitosan + algaenan with PUFA and no algaenan (CP); chitosan + algaenan with PUFA and algaenan (CPa). FO stands for free oil.

6.3.5.2. Second *in vitro* with capsules EXP 2.

After the preliminary *in vitro* experiment, another *in vitro* was planned to evaluate the efficacy of the second capsules produced with a target EPA concentration. For that, a series of 3 *in vitro* batch incubation with ruminal inoculum were done in similar conditions. During the incubation period, the dry matter (DM) disappearance in tubes containing EPA sources did not differ ($p > 0.05$) from control tubes, and it averaged 8%, 10% and 7% in the first, second and third *in vitro* replicas respectively (Figure 6.16).

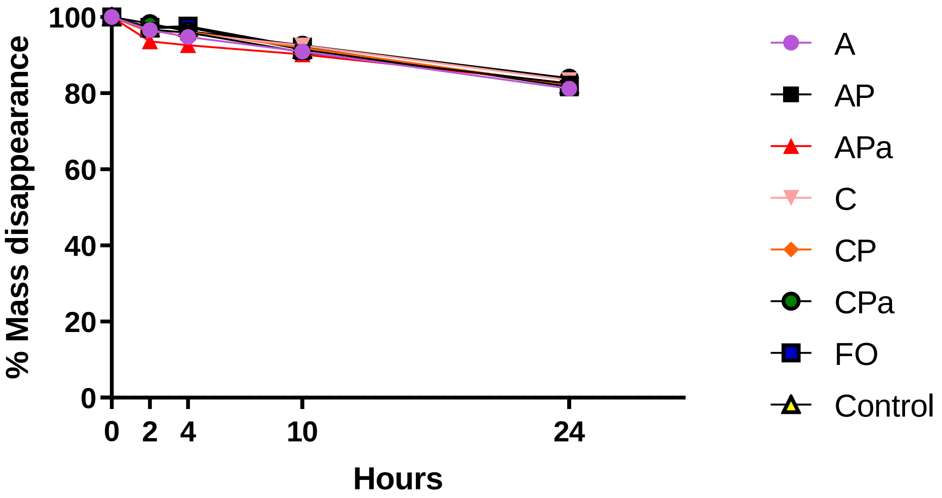

Figure 6.16- Dry matter (DM) disappearance (%) of the 3 *in vitro* replicas.
The three *in vitro* replicas (IV1, IV2 and IV3) are represented in the Figure. The capsules are the following: alginate and no PUFA (A); alginate with PUFA and no algaenan (AP); alginate with PUFA and algaenan (APa); chitosan + algaenan and no PUFA (C); chitosan + algaenan with PUFA and no algaenan (CP); chitosan + algaenan with PUFA and algaenan (CPa). FO stands for free oil (positive control – free PUFA) and control (negative control – no PUFA).

When including all tested capsules (AP, APa, CP, CPa) and the free oil (FO), EPA disappearance (average ± SEM %TFA) significantly varied with the substrate (p = 0.020). The disappearance was higher in the CP capsules (42.9 ±16.81), medium in all AP, APa and FO, and lower in CPa (-20.1 ± 16.81) capsules. Also, when evaluating capsules with and without algaenan, capsules containing algaenan registered a lower EPA disappearance (-14.9 ± 14.88, p = 0.031) when compared to the ones not containing the algaenan (19.8 ± 14.88). However, when evaluating only the type of polymer in each capsule (alginate or chitosan + alginate), there were no differences (p =0.236).

If removing the chitosan + alginate capsules (Figure 6.17-b), EPA disappearance differed with the substrate (p <0.001) being it higher in the FO (23.7 ± 6.92) when compared to AP and APa (-6.5 ± 6.92). There was also a significant difference regarding different timepoints (p = 0.039), being EPA disappearance higher at 24 h (11.5 ± 7.28) and lower at 4h post incubation (-7.6 ± 7.49). Also, the presence of algaenan significantly decreased EPA disappearance (p = 0.041) being it lower in the capsules containing algaenan (-9.7 ± 7.49) and higher in the capsules not containing algaenan (-3.3 ± 7.49). Regarding this, also timepoints differed in these capsules (p = 0.015), being the EPA disappearance higher at 24h (-0.19 ± 7.76) and lower at 4h (-15.2 ± 7.76).

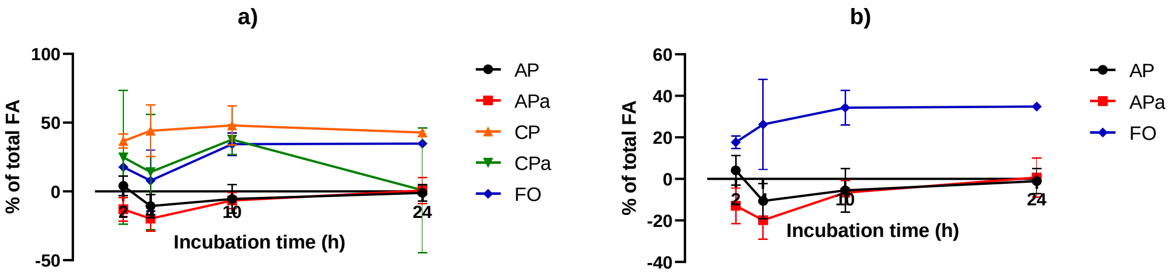

Figure 6.17- EPA disappearance (%TFA).

The a) image illustrates EPA disappearance in all capsules and in free oil (FO), and image b) illustrates EPA disappearance only in alginate capsules and FO. The capsules are the following: alginate with PUFA and no algaenan (AP); alginate with PUFA and algaenan (APa); chitosan + algaenan with PUFA and no algaenan (CP); chitosan + algaenan with PUFA and algaenan (CPa). FO stands for free oil (positive control – free PUFA).

When evaluating all capsules at specific timepoints, it was possible to conclude that EPA disappearance did not differ between treatments at 2h and 4h post-incubation times. However, at 10h post-incubation, capsules that had alginate as a base (p = 0.009) registered a lower EPA disappearance (6.0 ± 10.35) when compared to the remaining substrates.

When evaluating only the capsules with alginate as a base polymer and FO at specific timepoints, it was possible to better observe differences between the encapsulated EPA and the free EPA in the FO. At 2h post-incubation, the capsules containing algaenan registered a lower (p = 0.047) EPA disappearance (-12.9 ± 6.648) when compared to the ones without algaenan (4.1 ± 6.648). However, the posterior timepoints did not register this tendency. At 10h *post* incubation, the EPA disappearance was higher (p = 0.0235) in the FO (34.3 ± 8.03) when compare with the capsules (-6.0 ± 6.69). At 24h *post* incubation, the same pattern was maintained, and the FO registered the highest EPA disappearance (34.6 ± 6.32) when compared to the capsules (-0.19 ± 5.52).

Contrary to what was verified for EPA and DHA, LA and ALA behavior at various timepoints post incubation was the expected. Both LA and ALA decreased over time during the incubation time (Figure 6.18).

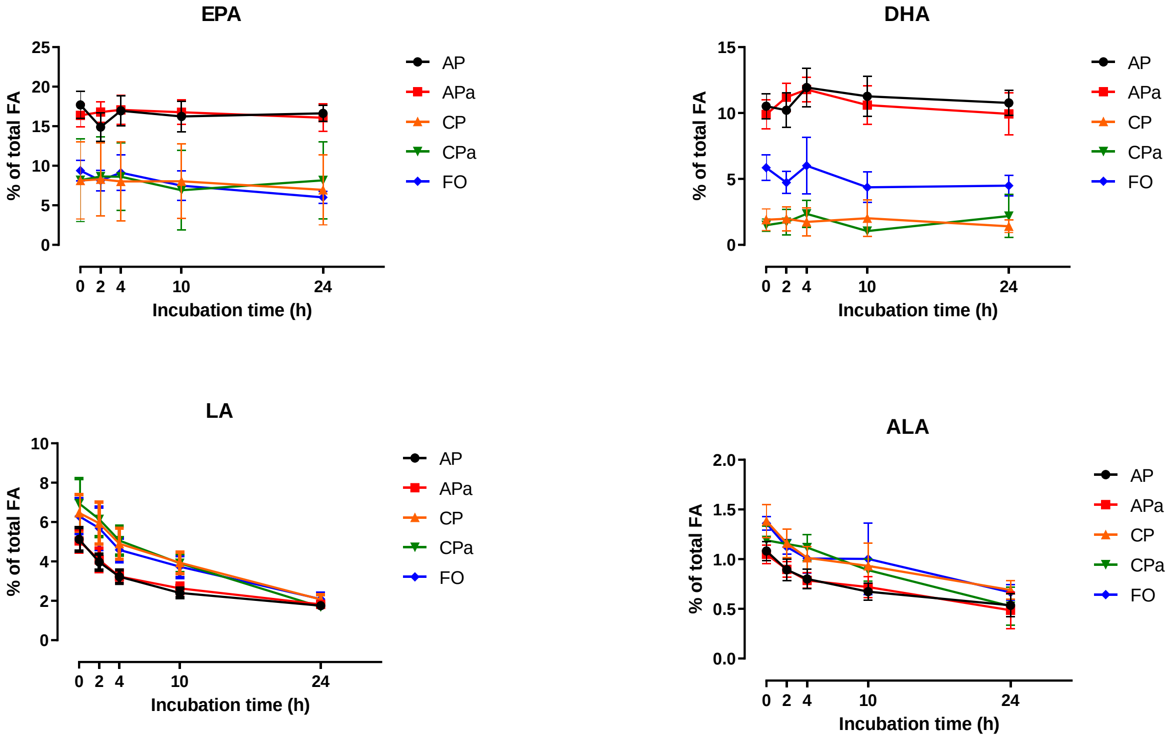

Figure 6.18- EPA, DHA, LA and ALA (%TFA).

The capsules are the following: alginate with PUFA and no algaenan (AP); alginate with PUFA and algaenan (APa); chitosan + algaenan with PUFA and no algaenan (CP); chitosan + algaenan with PUFA and algaenan (CPa). FO stands for free oil (positive control – free PUFA).

6.4. Discussion.

We proposed to develop an encapsulation method to protect an n-3 LC-PUFA-rich fish oil, in ruminal *in vitro* incubations, using *N. oceanica* algaenans. Firstly, we successfully isolated the algaenan from *N. oceanica*. The success of the isolation was confirmed through algaenan characterization using instrumental methods of analysis. Indeed, due to the insolubility of algaenan in various solvents, methods for its identification have been established by integrating multiple pieces of information obtained from invasive and non-invasive methods, which provide bulk chemical information. Thus, Fourier transform infrared spectrometry (FTIR) (Allard et al., 1998; Blokker et al., 1998), elemental analysis (EA), 1D and 2D 13C nuclear magnetic resonance spectrometry (NMR) (Zelibor et al., 1988; Blokker et al., 1998; Obeid et al., 2014; Biller et al., 2015), pyrolysis gas chromatography–mass spectrometry (py–GC–MS) (Zelibor et al., 1988; Blokker et al., 1998; Obeid et al., 2014; Biller et al., 2015; Obeid et al., 2015; Zhang &

Volkman, 2017) and thermogravimetry (TGA) (Valdés et al., 2013; Zhao et al., 2022) are amongst the most used.

In this work, the TGA results regarding the algaenan 4 seem to be similar to those found by Valdés et al. (2013) where weight loss was actually a superposition of at least three processes, namely dehydration between 25 and 180°C, devolatilization at 180–500°C and solid residue decomposition between 500 and 900°C. In a study by Zhao et al. (2022), the thermogravimetric curve indicated that *Nannochloropsis* biomass loss increased with temperature, in three stages. In the first, up to 110°C, the main cause of biomass loss was water volatilization. In the second, from 110°C to 410°C, most of the biomass loss occurred between around 290°C and 380°C and in the third (>410°C), the rate of loss decreased until the biomass remained constant. Zhao et al. (2022) identified a band, that also occurred in our algaenan 4 sample, between 400 and 500ºC that may correspond to lipids (Zhao et al., 2022), given that their pyrolysis occurs at higher temperatures than carbohydrates and proteins (Bach & Chen, 2017). Also in the study by Zhao et al. (2022) is possible to observe a similar FTIR spectrum of the one obtained by us in the present study. In that same study, the absorption peak at 1000–1200 cm^{-1} resulted from C–O single-bond vibration (Zhao et al., 2022), being pointed to possibly reflect the structural and characteristic peak of cellulose and hemicellulose, the glycosidic bond, or the ether bond between lignin and hemicellulose (Binod et al., 2012). In our samples, the peaks between 1200-900 cm^{-1}, attributed to C-O and CO-O-CO stretching vibrations, are also present in algaenan structures described in literature (Allard & Templier, 2000; Simpson et al., 2003; Zhang & Volkman, 2017). The absorption at 1650 cm^{-1} may reflect C=C double bond extension. The O–H stretching near 3300 cm^{-1} and the CH_2 stretching near 2930 cm^{-1} are characteristic cellulose absorption peaks (Binod et al., 2012). The O–H absorption peak at 3410 cm^{-1} probably reflects the cellulose hydrogen bond (Nasirpour et al., 2014). Additionally, we found N–H stretching vibrations (≈3600–3000 cm–1), as well as the C–H stretching vibrations related to the occurrence of aliphatic structures (2900 and 2850 cm-1), also characteristic of the algaenan structure. Both in the study by Zhao et al (2022) and in our samples, there was a strong band near 1740 cm^{-1}, caused by (C=O) ester group stretching which may be derived from lipids and algaenan. Elemental analysis results of algaenan 4 were also similar to those found by others (Pan et al., 2010; Valdez et al., 2011; Neves, 2014) in *Nannochloropsis* residues after lipids extraction.

When characterizing the capsules, the stretching vibration (C-O-C) observed in sodium alginate capsules FTIR was observed at 1030 cm^{-1} and is related to its polysaccharide structure (Gawad & Fellner, 2019). The capsules containing alginate and chitosan showed minor changes compared to the spectrum of the capsule containing only alginate. Previously published works on chitosan report that absorption bands characteristic of this isolated biopolymer appear in regions similar to the bands attributed to capsules containing alginate, which makes it difficult to observe such bands due to overlaps (Branca et al., 2016; Braz et al., 2018; Gierszewska et al., 2019). In addition, the small amount of chitosan incorporated in the formulation in relation to the other constituents may explain the difficulty in observing changes in the spectra.

The x-ray diffraction pattern for the alginate-based capsule confirmed an amorphous character for the formed capsules, as observed in other works that previously reposted the obtainment of alginate beads (Shahid et al., 2021; Wu et al., 2022; Lotfy et al., 2023). The introduction of PUFA oil in the composition of the capsules generated a new x-ray diffraction pattern with semicrystalline characteristics, which can be explained by the intermolecular interactions formed between the PUFA oil and the respective biopolymers that make up the capsules. The lack of significant changes in the diffraction pattern of the samples containing algaenan can be explained by the relatively low amount of this constituent in relation to the others. Therefore, we successfully confirmed algaenan isolation and the incorporation of the algaenan in the different polymer-based capsules did not produce significant structural changes in the formulations.

So far, algaenans have never been used in the development of rumen protection technologies and we presently successfully incorporated the algaenan in an emulsion to produce alginate, and alginate and chitosan capsules. The alginate and chitosan were selected as being non-toxic, biocompatible, and cheap biopolymers (Nedovic et al., 2011) with potential to be used to develop a novel rumen-protection technology. These polymers were used as polymer-based matrix, once both had already been used in rumen protection studies (Olloqui et al., 2018; Gawad & Fellner, 2019; Besharati et al., 2022). The capsule-making protocols were optimized, and capsules were developed to be tested in an *in vitro* batch system with ruminal fluid.

Regarding the *in vitro* planning, in order to obtain a 0.05 mg EPA/ mL of incubation medium as used by Alves et al. (2018), or 0.3 mg of EPA per tube (total 6 mL of medium per tube), the EPA content in each type of capsule was taken into consideration and the quantity of capsules in each tube was adjusted to totalize the same amount of EPA. In the first performed *in vitro* (preliminary *in vitro*), the average EPA+DHA content per tube was 1685 µg in the alginate capsules, 2860 µg in the chitosan + alginate capsules and 660 µg in the free oil. The n-3 LC-PUFA content in the capsules far surpassed the ones pointed by Aldai et al. (2012) for optimal DHA content per tube in *in vitro* incubations with mixed ruminal bacteria. In the study by

Aldai et al. (2012) it is stated that lower levels of DHA addition (≤ 300 µg per tube or 50 µg/mL) extensive metabolization can occur. In the same study, DHA depletion was strongly decreased when the DHA content per tube passed from 100/300 µg per tube to 800 µg per tube and in incubations with 1500 µg of DHA per tube, very little metabolism of DHA was evident after 6h. It is known that high concentrations of free FA are toxic for the rumen microbes and Maia et al. (2007) pointed that at 50 µg/mL of PUFA an inhibition of the growth of several ruminal bacteria can occur. In the preliminary *in vitro*, total PUFA content in the fermenters averaged 365 µg/mL in the alginate capsules, 593 µg/mL in the chitosan + alginate capsules and 158 µg/mL in the free oil. Therefore, we hypothesized that the high n-3 LC-PUFA and total PUFA content in each tube might have been deleterious for the bacteria, inhibiting the metabolization of both EPA and DHA, illustrated in our results by the flat lines of these two FA across the incubation period for both capsules composed of alginate and chitosan + alginate (Figure 6.15).

In the second *in vitro*, EPA+DHA content averaged 960 µg per tube (160 µg/ mL) in the alginate capsules, 120 µg per tube (20 µg/ mL) in the chitosan + alginate capsules and 310 µg per tube (52 µg/ mL) in the free oil. Regarding Total PUFA, alginate capsules averaged 1055 µg per tube (176 µg/ mL), chitosan + alginate capsules averaged 245 µg per tube (41 µg/ mL) and free oil tubes averaged 470 µg per tube (78 µg/ mL). Apparently, the amount of n-3 LC-PUFA in the alginate + chitosan capsules would have been adequate do produce a response in terms of EPA and DHA metabolization. Therefore it is unknown for us the reason why EPA and DHA did not show metabolization in the alginate + chitosan capsules, once LA an ALA registered an expected metabolization pattern (Figure 6.18).

The overall results obtained were not conclusive when considering all the capsules included. We considered that several factors could have negatively impacted the success of the experiment:

a) Our *in vitro* system allowed us to use between 1-2 capsules per tube, introducing a large variability in terms of encapsulated EPA proportion per tube. This can be seen by the different EPA content at the starting of the *in vitro* (Figure 6.18). The hand manufacture process of making the capsules by a dripping manual method can introduce an error that corresponds to differences in EPA content per capsule.

b) The overall freeze-drying period of the capsules might have been insufficient to efficiently dry the capsules, especially the alginate + chitosan-based ones. The remaining humidity might have contributed to accelerate the oxidation of those capsules and negatively impact the reproducibility of our results between *in vitro* replicas. Contrary to what was observed for the alginate capsules, alginate + chitosan capsules appeared to have oxidized after 2-3 months of use, supported by the fact that there was a marked change of colour in those capsules (Figure 6.19). Regarding chitosan oxidation, the destruction of its structure can mainly contribute to the increase in it solubility. Also, negative charge increase in chitosan structure above a certain level can lead to adverse effect on the binding capacity (Yoo et al., 2005). Therefore, oxidized chitosan capsules might have lost integrity and the ability to protect the encapsulated oil in the *in vitro* incubations, also interfering with the correct interpretation of EPA disappearance.

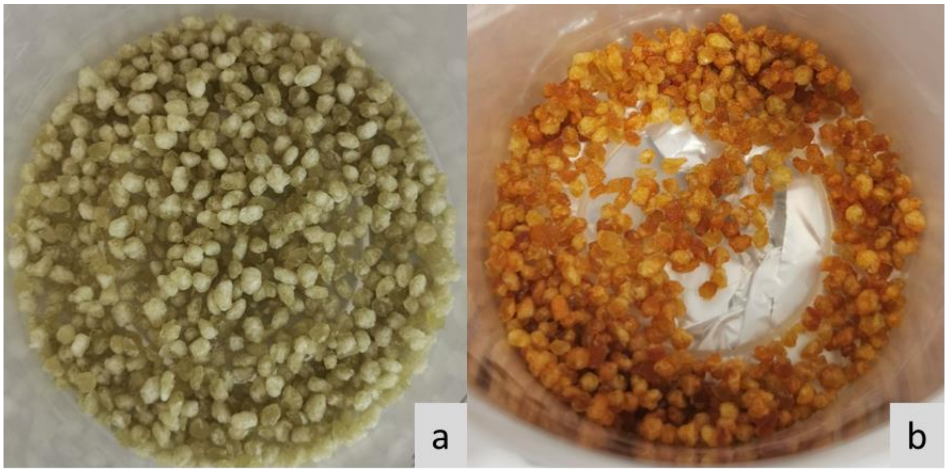

Figure 6.19- Aspect of alginate + chitosan capsules (CPa) immediately after being FD (a) and after 2-3 months of storage (b).

c) Chitosan can react with FA and, over time. Maybe the transesterification method used (reaction with sodium methoxide (0.5 M) in methanol at 50°C for 15 min followed by addition of hydrogen chloride (1.25 M) in methanol at 80°C for 20 min) was insufficient to efficiently extract the FA, and namely EPA, from the capsules. Chitosan is an amine-rich linear polysaccharide formed from the partial deacetylation of alkylated chitin. It is composed of duplicate units of D-glucosamine and *N*-acetyl-D-glucosamine linked by glycoside bonds β (1–4) (Mwangi et al., 2016). Between various methods to produce chitosan-based particles to stabilize emulsions, in the hydrophobic deformation method, chitosan amine groups are bonded with carboxyl groups of compounds containing alkyl groups, and it seems that emulsifying properties of chitosan could be improved by increasing the number of fatty acid carbons bonded to chitosan (Tabatabaei et al., 2022). The modification of the hydrophilic chitosan chains with hydrophobic compounds (such as carboxylic acids, including FA) can result in products with an amphiphilic behavior - scheme of preparation of chitosan-based amphiphilic materials is presented in

Figure 6.20. (El Fray et al., 2012). The amphiphilic chitosan derivatives are able to self-assemble and form nanoparticles (micelles) under appropriate conditions (Muzzarelli & Muzzarelli, 2005).

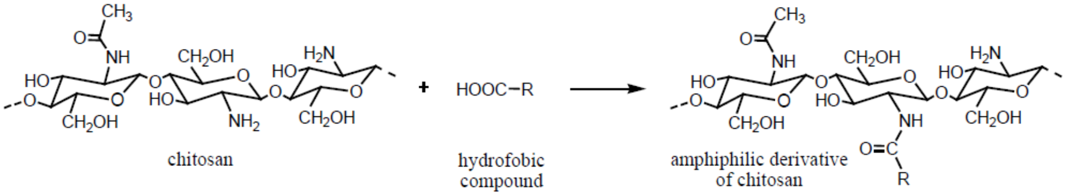

Figure 6.20- Chemical modification of chitosan at primary amino group to obtain amphiphilic derivative.

From El Fray et al. (2012).

In a micelle, the chitosan derivative hydrophobic groups are directed towards the interior of the cluster and the polar head group is directed towards the solvent (Holmberg et al., 2002). In the study by El Fray et al. (2012) the reaction of chitosan with FA, an active ester intermediate was formed in the presence of a catalyst which reacts with carboxyl groups of fatty acids (Figure 6.21-A). The intermediates can react with primary amine groups of chitosan to form an amide bond (Lee et al., 1998)(Figure 6.21-B). The amide groups became stronger for modification products and there was a diminished concentration of amino groups of chitosan after reaction with acids (El Fray et al., 2012).

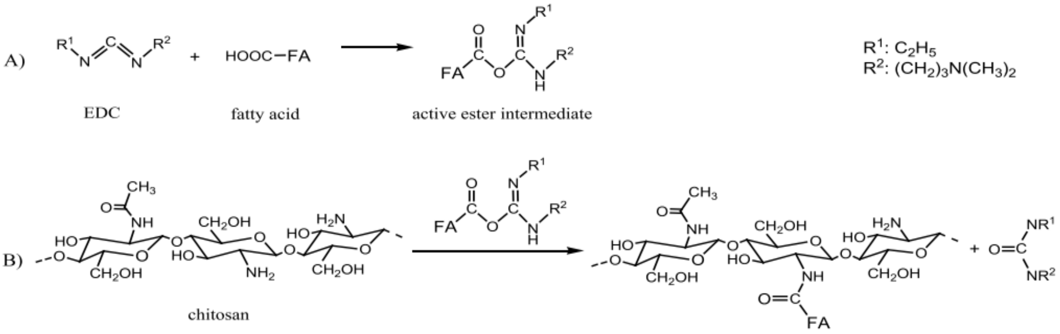

Figure 6.21- Schematic representation of fatty acid activation (A), and its reaction with chitosan (B).

EDC stands for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, a water-soluble carbodiimide coupling reagent that can be used in aqueous phase to facility the activation of a carboxyl group. From El Fray et al. (2012).

It is unknown to us if the eventual amide bonds formed between chitosan and the FA from the encapsulated PUFA oil, during storage period, could have markedly influenced the transesterification of FA from those capsules and, consequently, EPA quantification.

d) A commercial oil was used as n-3 PUFA rich-source to encapsulate (TG1000 Fish Oil with 35% EPA and 25% DHA from Biofil®). The oil contained 0.5% of vitamin E, so maybe the presence of vitamin E helped to stabilize the oil in the *in vitro* incubations, retarding its metabolization and making it difficult to use it as a positive control.

e) Regarding the alginate-based capsules, an intense isomerization of both EPA and DHA, when compared to the alginate + chitosan capsules (Figure 6.22) was observed. No isomerization was observed in the free oil samples. It is unknown to us if the strong isomerization that has occurred could have influenced the overall EPA accurate quantification in those capsules. Both photoisomerization or iodine isomerization (Liu et al., 2019) could have occurred. Besides the protection of the tubes with the aluminum foil against direct sunlight, it is unknown if small light exposure periods occurring during the *in vitro* preparations (namely weighting of the materials) could have impacted EPA and DHA isomerization.

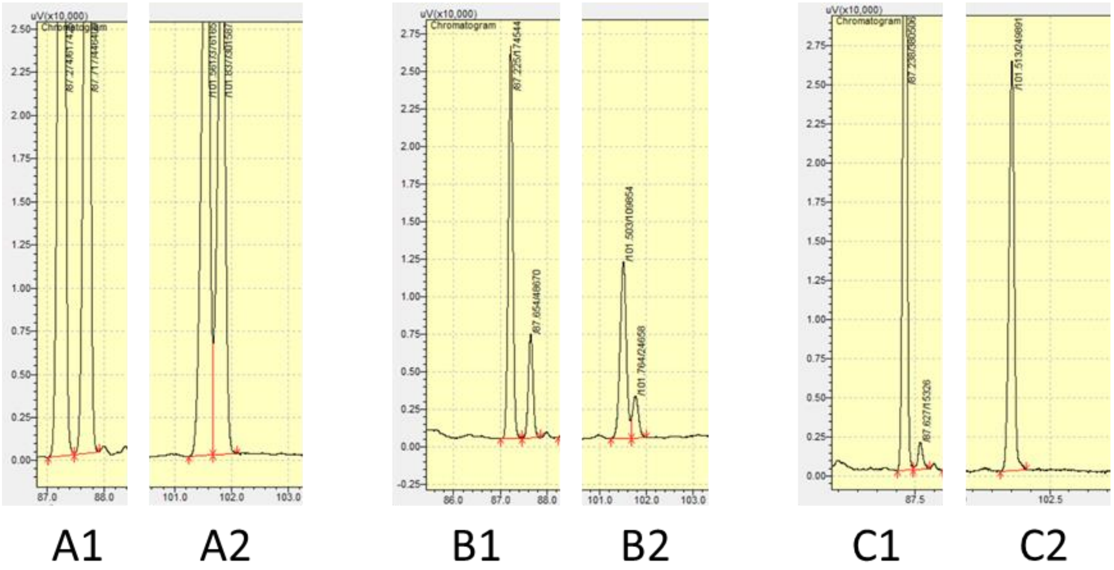

Figure 6.22- Alginate and alginate + chitosan capsules, and free oil EPA and DHA peaks. The chromatograms present correspond to 3 different samples from the IV 1 but represent the same treatment samples across the 3 *in vitro* replicas. In the left (A) chromatograms correspond to EPA (A1) and DHA (A2) peaks present in the alginate + PUFA capsules; the chromatograms in the middle (B) correspond to EPA (B1) and DHA (B2) peaks present in the alginate + chitosan + PUFA capsules; and the chromatograms in the right (C) correspond to EPA (C1) and DHA (C2) peaks present in the free oil.

Sodium alginate is extracted from the cell walls of brown algae, which include the strongest accumulators of iodine known among living systems, ranging from 0.05–5% dry weight (Küpper & Carrano, 2019). Sodium alginate has the ability to adduct with iodine (Takahashi, 1984), however, according to the product description corresponding to the one purchased by Sigma-Aldrich and used in our study, iodine content is not mentioned. If present, iodine could have promoted an EPA and DHA isomerization, more markedly in the alginate-based capsules when compared to the alginate + chitosan ones, due to a higher sodium alginate incorporation percentage in the first ones. Nevertheless, because we did not measure the iodine content in our samples, we could not conclude about its presence.

6.5. Conclusion.

The development of novel rumen-protection technologies is of major interest, once the already available ones present several constrains to their efficiency. *N. oceanica* algaenan was successfully extracted from *N. oceanica* freeze-dried biomass using three different methods. Chemical characterization pointed towards the already documented descriptions for typical algaenan structures, therefore we could confirm its isolation. Alginate, and chitosan were used as matrixes for the development of two types of rumen-protected capsules, encapsulating an EPA-rich oil. Structural analysis of the capsules revealed that incorporating algaenans did not cause major alterations. We tested for the first time a rumen-resistant encapsulation method using microalgal algaenans, however, the results regarding the *in vitro* protection efficiency were inconclusive. It is unknown for the authors the reasons why EPA and DHA *in vitro* metabolization did not occur, therefore there is a need to adapt and repeat experimental procedures.

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Author contributions

A.C.M.V, S.P.A. and R.J.B.B., were responsible for conceptualization and funding acquisition. A.C.M.V. implemented the experiment. A.C.M.V, C.N., LC.B.L., L.R.B. and E.C.F. conducted the laboratory analysis. A.C.M.V. performed data analysis. A.C.M.V. and S.P.A. did the interpretation of results. A.C.M.V. drafted the manuscript. All authors revised and approved the final manuscript.

7. CHAPTER 7 | General discussion, future perspectives, and conclusion.

The scientific research associated to novel feed ingredients, namely microalgae, for ruminant nutrition is increasing with the concurrent aims of improving the nutritional profile of ruminant meat and to ameliorate the production system sustainability.

In a previous preliminary *in vitro* work from our group (Alves et al., 2018) it was possible to conclude that *Nannochloropsis oceanica* showed a strong potential to be used as a natural dietary source of eicosapentaenoic acid to ruminants and there was an intention to further test eicosapentaenoic acid protection degree *in vivo*. The studies presented on this thesis began with the aim to test the eicosapentaenoic acid (EPA) protection degree *in vivo*, coupling it with the study of the posterior transfer efficiency to the edible tissues of the animals (meat and edible fats) and the impact on the ruminal microbiome.

Regarding the improvement of the meat and edible fats nutritional profile, the main goal to be achieved was to contribute to the improvement of the consumer health by enhancing recognized health-beneficial n-3 fatty acids, such as EPA, docosapentaenoic (DPA) and docosahexaenoic acid (DHA), without compromising the animal productivity and health. In our studies, the dietary intervention using *N. oceanica* in lambs' diets demonstrated to efficiently enhance the n-3 fatty acids in the gastrointestinal tract, and consecutively in meat and edible fats, without compromising the animal productive indicators. In this thesis, the success of *N. oceanica* in efficiently enhancing n-3 fatty acids in lamb tissues is reported to be associated to the degree of maintenance of its cell wall structure.

EPA concentration in the rumen of lambs fed diets supplemented with freeze-dried *N. oceanica* was about 50% higher than lambs fed the spray-dried or free oil supplemented diets. Nevertheless, the high levels of EPA in cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine. Therefore, the microalgae wall might have conferred a certain degree of protection, that remained functional until the end of the gastrointestinal tract. The supplementation with *Nannochloropsis*-containing diets also affected the ruminal biohydrogenation of C18 fatty acids, mitigating the shift from the *trans*-10 biohydrogenation pathways to the *trans*-11 pathways compared to the control diet. Overall, our results demonstrate that freeze-dried *N. oceanica* biomass is a natural rumen-protected source of EPA to ruminants.

After the confirmed success of freeze-dried *N. oceanica* in enhancing EPA in the gastrointestinal tract of lambs, we evaluated the efficiency of EPA deposition in the animal tissues. The EPA content was highest in the muscle and subcutaneous fat of lambs fed spray-dried and freeze-dried *Nannochloropsis* diets compared to the ones fed the diet incorporating the free oil. FD fed lambs registered 20 mg of EPA per 100 g of muscle, approximately 2.5 times the content registered in the C fed lambs. With regards to the EPA+DHA daily recommendations for humans, the meat of lambs fed the FD diet attained 25 mg of EPA+DHA per 100 g of muscle, approximately double the amount registered in the C fed lambs. Consequently, 100 g of FD fed lambs meat achieved approximately 10% of the minimum recommended daily dose of EPA+DHA for humans (250 mg).

It is important to take into consideration that when cooking the meat, the cooking method and degree of doneness are important factors to take into consideration, but in general, when trimmed meat is cooked, it leads to higher losses of the water-soluble phospholipids that are present in the membranes, reducing the content of n-3 LC-PUFA (Knight et al., 2004). However, due to cultural preferences, lamb is traditionally cooked in whole pieces and when it is prepared with the bone and fat still present, heating enables the infiltration of FA from the adjacent intermuscular and subcutaneous fat and the introduction of lipids from the surrounding adipose tissues (Knight et al., 2004). This reinforces the importance of enhancing n-3 LC-PUFA in the subcutaneous adipose tissues of lamb, which we have succeed with the introduction of *N. oceanica* biomass in the diets. As a result of these cultural cooking preferences, the amount of FA present in cooked lamb is often higher than what is indicated in raw lamb. In roasted lamb, assuming an approximate increase of 20% in n-3 LC-PUFA (West & Chrystall, 1989), considering the same portion of 100 g, FD fed lambs would provide a roasted piece of meat with an average content of 30 mg of EPA + DHA (12% of the minimum daily dose of 250 mg).

Furthermore, there is promising potential to further increase the n-3 LC-PUFA content in cooked lamb products in the future, particularly in the context of the increasing interest in transformed meat products like sausages or hamburgers. Considering the fast-paced lifestyle that many people lead today, these products offer practicality and convenience in cooking and consuming meat. Moreover, transformed meat products have the potential to include other trimmed fats from the carcass and even internal organs like lamb liver, which can substantially enhance the EPA+DHA content that can be consumed. With ongoing advancements in technology and production methods, it is likely that the potential for increased n-3 LC PUFA content in cooked lamb products will only continue to grow in the future with more convenient alternatives for the consumer.

A similar tendency to what was verified in the gastrointestinal tract was, therefore, maintained in the muscle and fat. However we could not confirm a statistically significant advantage of the freeze-dried *Nannochloropsis* diet over the one containing spray-dried *N. oceanica,* in terms of EPA deposition, it is important to consider that the FD diet contained less EPA/ kg diet DM. Yet, numerically, both EPA content in muscle and fat were higher in FD fed lambs. This finding can be regarded as a possible reinforcement of the higher efficiency of EPA protection in FD *N. oceanica*, supporting our initial hypothesis. Total *trans*-18:1 did not differ among treatments, but the *t*10/*t*11–18:1 ratio (*t*10-shift) decreased with all *Nannochloropsis* containing diets. *Nannochloropsis* diets were also supplemented with Vitamin E preventing the lipid oxidation in EPA-enriched meat and the overall meat sensory traits were not or significantly affected.

After the dietary intervention, the ruminal microbiome composition of the lambs was evaluated. Overall minor changes were detected in the *Nannochloropsis*-fed lambs when compared to the control ones, as important differences in terms of phyla, family and genera were not detected among treatments. However, we had previously found that despite not having found differences in the extension and completeness of the C18 UFA in the rumen, the lambs had a strong individual variability regarding the type of main intermediate formed (i.e., *t*10 vs. *t*11-18:1). Therefore we also evaluated potential associations between specific ruminal bacterial genera and the tendency for a higher/ lower t10-shift. The microbial agents responsible for the altered biohydrogenation pattern are not fully known. Our study in Chapter 3 offers valuable samples with individual variability regarding the predominant biohydrogenation pattern, even within each diet. Therefore, these samples have the potential to serve as a matrix for identifying the OTU’s and enriched metabolic pathways associated with *t*10-shift. We found that *t*10-18:1 may be a more suitable target, when compared to *t*11-18:1 and even 18:0, for determining specific bacteriome profiles in the rumen.

We also aimed to evaluate the degree of response of EPA deposition in both brain and retina of lambs, once these are the tissues with highest n-3 LC-PUFA content and concluded that the dietary intervention could efficiently enhance beneficial n-3 fatty acids in the animal brain and retina. Overall, the brain FA profile remained unchanged with minimal enhancement of n-3 DPA in both hippocampus and prefrontal cortex of lambs. However, retinal tissues were particularly responsive to the dietary intervention, with a relevant EPA enhancement, particularly in the lambs fed the spray-dried and freeze-dried microalga. We could conclude that retinal tissues seem to be sensible to short-period n-3 PUFA supplementations in lambs and they reinforce the results obtained for the gastrointestinal tract, muscle, and fat.

As evidenced in the various chapters of this thesis, the success of freeze-dried *N. oceanica* in protecting and, therefore, enhancing EPA and its elongation product, DPA, in the animals' tissues was attributed to its unique cell wall structure. Algaenans were proposed to play an important role in n-3 fatty acids protection against ruminal biohydrogenation *in vivo*. The high production cost of microalgae remains a significant challenge to their use in commercial feed production, primarily stemming from the costly technologies involved in cultivation, harvesting, processing/extraction, and drying. Therefore, exploring and implementing alternative products or methods to improve the utilization of microalgae in ruminant production could enhance the value of microalgae utilization in the system, providing potential benefits for both the feed industry and animal production. Therefore, we undertook the challenge of isolating and applying *N. oceanica* algaenans to a newly developed rumen encapsulation method for delivering n-3 fatty acids to ruminants. At the present stage, as investigated in this present thesis in the Chapter 6, we could only test the capsules in an *in vitro* preliminary study with, yet inconclusive results regarding n-3 PUFA protection degree and metabolization after 24 hours of incubation. It remains unknown to us if the high variability in the *in vitro* system, the insufficient duration of the freeze-drying of the capsules, transesterification efficiency issues, or the potential role of vitamin E could have played a detrimental cumulative effect in our results. Nevertheless, to the best of our knowledge, this study represents the first attempt to include algaenans in an encapsulation method aimed at protecting EPA in the rumen. Therefore, we believe that these results provide a valuable indication of the potential protective effect of algaenans on EPA ruminal metabolization.

Overall we efficiently demonstrated *in vivo* that *N. oceanica* can be considered a new natural strategy for the protection of a source of polyunsaturated fatty acids from the rumen microbiome, especially when freeze-dried. Furthermore, we tested for the first time the potential of integrating a novel protection method in the future, using only a part of its structure. We believe that these innovative methods can add value to the utilization of microalgae in ruminant production in a long-term.

Looking into the future, there are topics that need to be further explored and clarified. One such topic is the role of vitamin E in mitigating the occurrence of *trans*-10 shift, once in the present study, *trans*-10 shift mitigation effect could not be unequivocally attributed to either *N. oceanica* biomass or vitamin E. An ongoing CIISA innovation project aims to clarify the roles of *N. oceanica* biomass and vitamin E, either individually or in combination, in preventing the occurrence of the *trans*-10 shift in lambs fed a concentrate-based diet. Additionally, there is an aim to further investigate encapsulations incorporating algaenan, adjusting the formulations to be tested in future *in vitro* experiments, including the use of pure EPA as an encapsulating agent instead of a commercial fish oil. Furthermore, there is interest in investigating the effect of vitamin E on the reduction of n-3 LC-PUFA biohydrogenation *in vitro*. The spectrum of encapsulation applications in the production and nutrition of ruminants will be expanded by attempting the encapsulation of bioactive substances-enriched oils to reduce methane emissions, thus exploring additional potential benefits. For that, two different models will be tested: encapsulations designed as slow-release formulations and encapsulations designed as ruminal bypass formulations. Both models will be tested in a recently installed Gas Endeavour® system – a system developed for online measurements of low gas flows from any gas-displacing process at laboratory scale. While the proven success in using alginate and chitosan for encapsulation was verified, there is a plan to further test natural waxes as another potential encapsulating agent. These future studies have the potential to improve animal health and productivity while reducing the environmental impact of livestock production.

In conclusion, the scientific research on the use of novel feed ingredients, particularly microalgae, for ruminant nutrition has shown significant promise in improving the nutritional profile of ruminant meat with a promising possibility of also contributing to the sustainability of the production system. The results of this thesis provide valuable insights into the potential benefits of incorporating *Nannochloropsis oceanica* into ruminant diets to increase the content of beneficial n-3 fatty acids, such as EPA, DPA, and DHA, without compromising animal health and productivity. Feeding lambs with *N. oceanica* has the potential to improve consumer health by offering a natural source of these essential fatty acids in meat products. It is important to note that while increasing the consumption of ruminant meat as a means to increase n-3 intake offers several advantages, it must be done with a balanced approach. Alongside the benefits of n-3 fatty acids, there is the need to consider the additional intake of saturated and *trans*-fatty acids, which can have adverse effects on health. Therefore, as we explore innovative ways to enhance the nutritional profile of ruminant meat, it is equally vital to maintain a holistic perspective on dietary choices and their potential impacts on overall health. Furthermore, the investigation into the impact on the ruminal microbiome and the potential associations between specific bacterial genera and ruminal biohydrogenation patterns contributes to our understanding of the complex interactions within the ruminant digestive system. The research also delves into the possibility of encapsulating algaenans from *N. oceanica* to protect EPA in the rumen, offering a novel method for enhancing the utilization of microalgae in ruminant production. While the results from *in vitro* studies are inconclusive at this stage, they provide a promising foundation for future research in this area. Looking ahead, there are still several questions to be addressed, including the role of vitamin E in mitigating the *trans*-10 shift and further exploration of encapsulation methods using algaenans. In summary, the findings presented in this thesis open new avenues for improving ruminant nutrition, consumer health, and the sustainability of the livestock industry. The potential benefits of incorporating microalgae, particularly *N. oceanica*, are substantial, and ongoing research will continue to uncover innovative methods to maximize these advantages.

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Annexes

Table A.1. Differences in CowPi FU abundances between diets in the rumen.

FU	Diets				SEM	*p*-value
	C	O	SD	FD		
ABC transporters	**4.36**^{a}	**4.23**^{a}	**4.05**^{b}	**4.20**^{ab}	**0.055**	**0.021**
Adipocytokine signaling pathway	1.37	1.34	1.27	1.31	0.054	0.716
Alanine, aspartate and glutamate metabolism	**3.58**^{a}	**3.46**^{ab}	**3.32**^{c}	**3.44**^{bc}	**0.043**	**0.012**
Amino acid metabolism	1.00	1.20	1.10	1.13	0.077	0.465
Amino acid related enzymes	**3.99**^{a}	**3.89**^{ab}	**3.73**^{c}	**3.86**^{b}	**0.043**	**0.011**
Amino sugar and nucleotide sugar metabolism	**3.79**^{a}	**3.68**^{ab}	**3.50**^{c}	**3.60**^{bc}	**0.060**	**0.034**
Aminoacyl-tRNA biosynthesis	**3.71**^{a}	**3.59**^{ab}	**3.43**^{c}	**3.57**^{b}	**0.046**	**0.011**
Aminobenzoate degradation	0.26	0.17	0.24	0.09	0.100	0.712
Antigen processing and presentation	**0.53**^{a}	**0.44**^{a}	**0.27**^{b}	**0.40**^{a}	**0.044**	**0.012**
Apoptosis	-0.36	-0.38	-0.34	-0.22	0.250	0.985
Arachidonic acid metabolism	-0.40	-0.38	-0.24	-0.28	0.100	0.669
Arginine and proline metabolism	**3.49**^{a}	**3.43**^{a}	**3.27b**	**3.39**^{a}	**0.038**	**0.012**
Ascorbate and aldarate metabolism	**1.47**^{a}	**1.39**^{ab}	**1.16**^{c}	**1.28**^{bc}	**0.062**	**0.029**
Bacterial chemotaxis	2.52	2.60	2.53	2.77	0.105	0.440
Bacterial invasion of epithelial cells	-0.49	-0.67	-0.61	-0.41	0.141	0.669
Bacterial motility proteins	3.04	3.10	2.94	3.25	0.107	0.357
Bacterial secretion system	2.42	2.38	2.24	2.34	0.047	0.124
Bacterial toxins	0.81	0.76	0.50	0.68	0.093	0.227
Basal transcription factors	-9.56	-10.31	-8.67	-10.33	0.645	0.348
Base excision repair	**2.39**^{a}	**2.24**^{b}	**2.03**^{c}	**2.21**^{b}	**0.048**	**0.008**
Benzoate degradation	1.87	1.75	1.62	1.65	0.091	0.344
Biosynthesis and biodegradation of secondary metabolites	0.74	0.76	0.66	0.75	0.056	0.650
Biosynthesis of 12-, 14- and 16-membered macrolides	-3.56	-4.16	-3.71	-3.47	0.360	0.650
Biosynthesis of ansamycins	**0.32**^{a}	**0.22**^{a}	**-0.05**^{b}	**0.25**^{a}	**0.075**	**0.026**
Biosynthesis of siderophore group nonribosomal peptides	-0.23	-0.23	-0.39	-0.28	0.093	0.650
Biosynthesis of type II polyketide backbone	-1.39	-2.28	-2.59	-2.22	0.308	0.124
Biosynthesis of unsaturated fatty acids	**1.63**^{a}	**1.53**^{ab}	**1.3**^{c}	**1.43**^{bc}	**0.051**	**0.008**
Biosynthesis of vancomycin group antibiotics	1.20	1.14	1.02	1.13	0.061	0.348
Biotin metabolism	1.12	1.05	0.97	1.15	0.050	0.132
Bisphenol degradation	0.30	0.39	0.19	0.39	0.063	0.206
Butanoate metabolism	2.96	2.91	2.77	2.88	0.044	0.082
Butirosin and neomycin biosynthesis	0.31	0.35	0.27	0.29	0.086	0.980
C5-Branched dibasic acid metabolism	**1.79**^{a}	**1.72**^{a}	**1.52**^{b}	**1.68**^{a}	**0.050**	**0.021**
Caprolactam degradation	**-2.48**^{b}	**-2.26**^{b}	**-1.49**^{a}	**-2.11**^{b}	**0.190**	**0.022**
Carbohydrate digestion and absorption	-0.22	-0.55	-1.18	-0.66	0.282	0.237
Carbohydrate metabolism	2.07	2.08	2.03	2.05	0.064	0.985

Carbon fixation in photosynthetic organisms	**2.73^{a}**	**2.64^{a}**	**2.45^{b}**	**2.61^{a}**	**0.042**	**0.008**
Carbon fixation pathways in prokaryotes	**3.30^{a}**	**3.21^{a}**	**3.09^{b}**	**3.21^{a}**	**0.036**	**0.012**
Carotenoid biosynthesis	-1.81	-1.79	-2.12	-1.54	0.263	0.609
Cell cycle	-9.72	-10.31	-8.98	-10.33	0.523	0.348
Cell cycle - Caulobacter	**3.07^{a}**	**2.99^{ab}**	**2.81^{c}**	**2.94^{b}**	**0.041**	**0.008**
Cell division	**1.20^{a}**	**1.14^{a}**	**0.94^{b}**	**1.11^{a}**	**0.045**	**0.011**
Cell motility and secretion	**1.09^{a}**	**0.94^{b}**	**0.79^{c}**	**0.96^{ab}**	**0.047**	**0.011**
Cellular antigens	-2.22	-2.52	-2.27	-2.34	0.232	0.856
Chaperones and folding catalysts	**3.54^{a}**	**3.43^{a}**	**3.27^{b}**	**3.43^{a}**	**0.046**	**0.012**
Chloroalkane and chloroalkene degradation	1.23	1.16	0.92	1.06	0.079	0.126
Chlorocyclohexane and chlorobenzene degradation	-0.82	-0.93	-0.83	-0.57	0.150	0.507
Chromosome	**3.89^{a}**	**3.77^{b}**	**3.63^{c}**	**3.77^{b}**	**0.040**	**0.008**
Citrate cycle (TCA cycle)	2.58	2.54	2.43	2.54	0.038	0.086
Cyanoamino acid metabolism	2.36	2.33	2.19	2.30	0.063	0.352
Cysteine and methionine metabolism	**3.39^{a}**	**3.26^{b}**	**3.09^{c}**	**3.21^{bc}**	**0.045**	**0.008**
Cytoskeleton proteins	**2.43^{a}**	**2.28^{ab}**	**2.08^{c}**	**2.24^{b}**	**0.054**	**0.008**
D-Alanine metabolism	**1.37^{a}**	**1.30^{ab}**	**1.07^{c}**	**1.21^{b}**	**0.045**	**0.008**
D-Arginine and D-ornithine metabolism	-1.40	-1.54	-2.17	-1.87	0.204	0.115
D-Glutamine and D-glutamate metabolism	**1.62^{a}**	**1.49^{ab}**	**1.38^{b}**	**1.49^{b}**	**0.043**	**0.021**
DNA repair and recombination proteins	**4.54^{a}**	**4.41^{ab}**	**4.25^{c}**	**4.39^{b}**	**0.044**	**0.008**
DNA replication	**2.99^{a}**	**2.87^{b}**	**2.71^{c}**	**2.87^{b}**	**0.041**	**0.008**
DNA replication proteins	**3.56^{a}**	**3.44^{a}**	**3.29^{b}**	**3.45^{a}**	**0.041**	**0.009**
Dioxin degradation	0.65	0.36	0.13	0.10	0.177	0.228
Drug metabolism - cytochrome P450	-6.93	-6.34	-5.01	-5.55	0.618	0.255
Drug metabolism - other enzymes	**2.39^{a}**	**2.32^{a}**	**2.19^{b}**	**2.30^{ab}**	**0.043**	**0.042**
Electron transfer carriers	-2.45	-2.85	-2.73	-2.64	0.201	0.650
Energy metabolism	**3.06^{a}**	**2.99^{ab}**	**2.84^{c}**	**2.95^{b}**	**0.035**	**0.008**
Ether lipid metabolism	-10.04	-8.15	-9.14	-8.63	0.667	0.348
Ethylbenzene degradation	-2.07	-1.82	-1.88	-1.87	0.204	0.878
Fatty acid biosynthesis	**2.80^{a}**	**2.66^{ab}**	**2.49^{c}**	**2.58^{bc}**	**0.048**	**0.008**
Fatty acid metabolism	2.03	1.91	1.78	1.82	0.061	0.086
Flagellar assembly	1.95	2.06	1.92	2.31	0.146	0.357
Flavone and flavonol biosynthesis	-0.09	0.04	-0.22	-0.18	0.117	0.539
Fluorobenzoate degradation	-10.12	-8.79	-9.40	-10.33	0.496	0.237
Folate biosynthesis	**2.08^{a}**	**1.94^{b}**	**1.73^{c}**	**1.96^{ab}**	**0.048**	**0.008**
Fructose and mannose metabolism	**2.97^{a}**	**2.91^{a}**	**2.72^{b}**	**2.83^{ab}**	**0.055**	**0.041**
Function unknown	**2.75^{a}**	**2.64^{ab}**	**2.42^{c}**	**2.61^{b}**	**0.043**	**0.008**
Galactose metabolism	2.77	2.72	2.60	2.67	0.056	0.340
General function prediction only	**3.90^{a}**	**3.79^{a}**	**3.64^{b}**	**3.79^{a}**	**0.039**	**0.008**
Geraniol degradation	**-2.78^{c}**	**-2.27^{bc}**	**-1.46^{a}**	**-2.12^{b}**	**0.203**	**0.008**
Germination	-0.68	-0.45	0.01	-0.40	0.203	0.237
Glutamatergic synapse	**0.86^{a}**	**0.70^{ab}**	**0.53^{b}**	**0.74^{a}**	**0.068**	**0.042**
Glutathione metabolism	**1.68^{a}**	**1.55^{b}**	**1.42^{c}**	**1.55^{b}**	**0.037**	**0.008**
Glycan biosynthesis and metabolism	0.23	0.30	0.11	0.21	0.102	0.703

Glycerolipid metabolism	2.14	2.03	1.86	2.00	0.074	0.163
Glycerophospholipid metabolism	2.84^{a}	2.64^{b}	2.43^{c}	2.65^{b}	0.054	0.008
Glycine, serine and threonine metabolism	**3.25^{a}**	**3.15^{ab}**	**3.03^{b}**	**3.12^{b}**	**0.042**	**0.029**
Glycolysis / Gluconeogenesis	**3.20^{a}**	**3.07^{ab}**	**2.89^{b}**	**3.00^{b}**	**0.063**	**0.035**
Glycosaminoglycan biosynthesis - chondroitin sulfate	**0.44^{a}**	**0.06^{b}**	**-0.23^{b}**	**0.08^{b}**	**0.109**	**0.011**
Glycosaminoglycan degradation	0.04	0.21	-0.10	0.02	0.100	0.315
Glycosphingolipid biosynthesis - ganglio series	-3.50	-3.35	-3.82	-3.37	0.324	0.784
Glycosphingolipid biosynthesis - globo series	-0.62	-0.62	-0.58	-0.64	0.115	0.989
Glycosyltransferases	**2.75^{a}**	**2.62^{a}**	**2.39^{b}**	**2.58^{a}**	**0.059**	**0.011**
Glyoxylate and dicarboxylate metabolism	**2.78^{a}**	**2.65^{b}**	**2.48^{c}**	**2.64^{b}**	**0.043**	**0.008**
Hepatitis C	-9.72	-10.31	-8.98	-10.33	0.523	0.348
Histidine metabolism	**2.93^{a}**	**2.83^{ab}**	**2.68^{c}**	**2.79^{bc}**	**0.048**	**0.029**
Homologous recombination	**3.35^{a}**	**3.24^{ab}**	**3.07^{c}**	**3.22^{b}**	**0.041**	**0.008**
Inorganic ion transport and metabolism	0.83	0.67	0.45	0.55	0.121	0.273
Inositol phosphate metabolism	1.00	0.95	0.99	1.07	0.062	0.650
Ion channels	0.15	0.00	-0.10	0.00	0.074	0.237
Isoquinoline alkaloid biosynthesis	0.47	0.46	0.33	0.40	0.060	0.428
Limonene and pinene degradation	-2.07	-1.84	-1.58	-2.09	0.154	0.173
Linoleic acid metabolism	0.24	0.35	0.15	0.35	0.069	0.273
Lipid biosynthesis proteins	**3.19^{a}**	**3.07^{ab}**	**2.90^{c}**	**3.01^{bc}**	**0.045**	**0.008**
Lipid metabolism	**1.60^{a}**	**1.41^{b}**	**1.25^{b}**	**1.41^{b}**	**0.062**	**0.018**
Lipoic acid metabolism	-0.50	-0.34	-0.30	-0.27	0.106	0.563
Lipopolysaccharide biosynthesis	2.20	2.23	1.91	2.26	0.165	0.539
Lipopolysaccharide biosynthesis proteins	2.75	2.70	2.44	2.69	0.117	0.320
Lysine biosynthesis	**3.36^{a}**	**3.24^{ab}**	**3.08^{c}**	**3.19^{bc}**	**0.047**	**0.010**
Lysine degradation	0.60	0.51	0.24	0.28	0.114	0.157
Lysosome	-0.01	0.10	-0.18	-0.12	0.115	0.396
Membrane and intracellular structural molecules	**2.74^{a}**	**2.67^{ab}**	**2.58^{b}**	**2.71^{a}**	**0.034**	**0.038**
Metabolism of cofactors and vitamins	**0.76^{a}**	**0.71^{a}**	**0.60^{b}**	**0.75^{a}**	**0.034**	**0.024**
Metabolism of xenobiotics by cytochrome P450	-7.03	-6.34	-5.01	-5.55	0.625	0.203
Methane metabolism	3.43	3.33	3.25	3.32	0.047	0.113
Mineral absorption	**-0.69^{a}**	**-1.13^{a}**	**-1.75^{b}**	**-1.15^{a}**	**0.162**	**0.008**
Mismatch repair	**3.19^{a}**	**3.06^{b}**	**2.90^{c}**	**3.05^{b}**	**0.042**	**0.008**
N-Glycan biosynthesis	**0.77^{a}**	**0.47^{b}**	**0.20^{b}**	**0.43^{b}**	**0.098**	**0.015**
NOD-like receptor signaling pathway	**0.55^{a}**	**0.51^{a}**	**0.33^{b}**	**0.47^{a}**	**0.043**	**0.025**
Naphthalene degradation	0.39	0.10	-0.20	-0.22	0.184	0.140
Nicotinate and nicotinamide metabolism	**2.89^{a}**	**2.77^{a}**	**2.63^{b}**	**2.76^{ab}**	**0.046**	**0.018**
Nitrogen metabolism	**3.05^{a}**	**2.91^{b}**	**2.77^{c}**	**2.90^{bc}**	**0.047**	**0.014**
Nitrotoluene degradation	0.72	0.89	0.80	0.94	0.065	0.175
Non-homologous end-joining	-4.18	-4.40	-3.26	-3.73	0.286	0.085
Novobiocin biosynthesis	**1.67^{a}**	**1.58^{ab}**	**1.40^{c}**	**1.49^{bc}**	**0.044**	**0.008**
Nucleotide excision repair	**2.42^{a}**	**2.26^{b}**	**2.08^{c}**	**2.23^{b}**	**0.044**	**0.005**
Nucleotide metabolism	**2.22^{a}**	**2.06^{ab}**	**1.94^{b}**	**2.05^{b}**	**0.056**	**0.033**

One carbon pool by folate	**3.04^{a}**	**2.93^{a}**	**2.78^{b}**	**2.90^{ab}**	**0.048**	**0.026**
Other glycan degradation	-1.08	-0.92	-0.65	-0.81	0.171	0.389
Other ion-coupled transporters	**3.63^{a}**	**3.51^{b}**	**3.37^{c}**	**3.51^{b}**	**0.038**	**0.008**
Other transporters	2.07	2.03	1.91	2.01	0.055	0.275
Others	3.02	2.92	2.83	2.92	0.052	0.166
Oxidative phosphorylation	**3.20^{a}**	**3.08^{ab}**	**2.90^{c}**	**3.05^{b}**	**0.048**	**0.010**
PPAR signaling pathway	1.66	1.61	1.49	1.56	0.048	0.175
Pantothenate and CoA biosynthesis	**3.04^{a}**	**2.95^{ab}**	**2.76^{c}**	**2.90^{b}**	**0.040**	**0.007**
Pathogenic Escherichia coli infection	-10.32	-10.31	-10.26	-10.08	0.184	0.805
Penicillin and cephalosporin biosynthesis	-1.78	-1.82	-1.67	-2.44	0.251	0.222
Pentose and glucuronate interconversions	2.90	2.84	2.69	2.85	0.067	0.238
Pentose phosphate pathway	**3.11^{a}**	**2.96^{b}**	**2.80^{c}**	**2.96^{b}**	**0.043**	**0.006**
Peptidases	**4.14^{a}**	**4.05^{a}**	**3.90^{b}**	**4.04^{a}**	**0.044**	**0.023**
Peptidoglycan biosynthesis	**3.35^{a}**	**3.20^{b}**	**3.02^{c}**	**3.16^{bc}**	**0.049**	**0.008**
Peroxisome	**2.07^{a}**	**1.96^{ab}**	**1.85^{b}**	**1.93^{b}**	**0.041**	**0.023**
Phenylalanine metabolism	1.73	1.77	1.63	1.67	0.046	0.233
Phenylalanine, tyrosine and tryptophan biosynthesis	**3.42^{a}**	**3.30^{ab}**	**3.12^{c}**	**3.24^{bc}**	**0.046**	**0.008**
Phenylpropanoid biosynthesis	1.69	1.72	1.62	1.68	0.090	0.904
Phosphatidylinositol signaling system	0.79	0.78	0.70	0.79	0.036	0.318
Phosphonate and phosphinate metabolism	-0.31	-0.30	-0.73	-0.53	0.120	0.102
Phosphotransferase system (PTS)	**2.40**	**2.22**	**1.81**	**1.94**	**0.147**	**0.072**
Photosynthesis	**2.48^{a}**	**2.27^{b}**	**1.98^{c}**	**2.23^{b}**	**0.064**	**0.005**
Photosynthesis - antenna proteins	-4.83	-4.88	-4.70	-4.84	0.225	0.951
Photosynthesis proteins	**2.48^{a}**	**2.27^{b}**	**1.99^{c}**	**2.23^{b}**	**0.064**	**0.005**
Plant-pathogen interaction	**1.75^{a}**	**1.68^{a}**	**1.46^{b}**	**1.70^{a}**	**0.045**	**0.008**
Polycyclic aromatic hydrocarbon degradation	**-8.93^{b}**	**-6.57^{a}**	**-5.52^{a}**	**-6.70^{a}**	**0.418**	**0.005**
Polyketide sugar unit biosynthesis	2.19	2.11	1.94	2.05	0.061	0.103
Pores ion channels	3.04	3.10	3.00	3.12	0.122	0.910
Porphyrin and chlorophyll metabolism	2.66	2.74	2.66	2.73	0.062	0.738
Prenyltransferases	1.83	1.76	1.60	1.77	0.066	0.167
Primary bile acid biosynthesis	-0.88	-1.29	-1.36	-1.35	0.189	0.314
Propanoate metabolism	2.75	2.65	2.53	2.64	0.051	0.074
Proteasome	0.62	0.62	0.55	0.62	0.053	0.741
Protein export	**2.53^{a}**	**2.42^{ab}**	**2.27^{c}**	**2.39^{bc}**	**0.045**	**0.015**
Protein folding and associated processing	**2.87^{a}**	**2.77^{a}**	**2.56^{b}**	**2.72^{a}**	**0.053**	**0.015**
Protein kinases	3.30	3.35	3.35	3.38	0.066	0.899
Protein processing in endoplasmic reticulum	0.82^{a}	0.59^{b}	0.36^{c}	0.60^{b}	0.072	0.008
Proximal tubule bicarbonate reclamation	**-2.02^{b}**	**-1.95^{b}**	**-1.42^{a}**	**-1.44^{a}**	**0.135**	**0.015**
Purine metabolism	**4.36^{a}**	**4.24^{ab}**	**4.07^{c}**	**4.21^{b}**	**0.046**	**0.008**
Pyrimidine metabolism	**4.07^{a}**	**3.96^{ab}**	**3.83^{c}**	**3.95^{bc}**	**0.042**	**0.015**
Pyruvate metabolism	**3.24^{a}**	**3.11^{ab}**	**2.97^{b}**	**3.05^{b}**	**0.058**	**0.047**
RNA degradation	**2.84^{a}**	**2.68^{b}**	**2.49^{a}**	**2.65^{b}**	**0.047**	**0.006**
RNA polymerase	**1.77^{a}**	**1.65^{ab}**	**1.47^{c}**	**1.61^{b}**	**0.041**	**0.006**
RNA transport	0.64	0.61	0.51	0.66	0.058	0.319

Replication, recombination and repair proteins	**2.40^{a}**	**2.23^{b}**	**2.05^{c}**	**2.21^{b}**	**0.054**	**0.008**
Restriction enzyme	**1.63^{a}**	**1.47^{a}**	**1.25^{b}**	**1.49^{a}**	**0.059**	**0.008**
Retinol metabolism	-7.37	-6.49	-5.25	-5.76	0.570	0.126
Riboflavin metabolism	1.71	1.71	1.55	1.72	0.051	0.131
Ribosome	**4.31^{a}**	**4.17^{b}**	**4.01^{c}**	**4.15^{b}**	**0.043**	**0.007**
Ribosome Biogenesis	**4.11^{a}**	**3.97^{b}**	**3.81^{c}**	**3.96^{b}**	**0.046**	**0.008**
Ribosome biogenesis in eukaryotes	**0.60^{a}**	**0.44^{ab}**	**0.26^{c}**	**0.43^{b}**	**0.052**	**0.008**
Secondary bile acid biosynthesis	-0.88	-1.29	-1.36	-1.35	0.189	0.314
Secretion system	**2.99^{a}**	**2.94^{a}**	**2.76^{b}**	**2.93^{a}**	**0.046**	**0.025**
Selenocompound metabolism	**2.62^{a}**	**2.51^{a}**	**2.36^{b}**	**2.49^{ab}**	**0.050**	**0.027**
Signal transduction mechanisms	2.57	2.60	2.52	2.58	0.043	0.605
Small cell lung cancer	**-9.03^{b}**	**-7.68^{a}**	**-6.77^{a}**	**-7.69^{a}**	**0.436**	**0.029**
Sphingolipid metabolism	-0.49	-0.41	-0.16	-0.29	0.120	0.314
Sporulation	2.13	2.20	2.21	2.20	0.083	0.904
Staphylococcus aureus infection	0.65	0.59	0.38	0.50	0.100	0.333
Starch and sucrose metabolism	**3.62^{a}**	**3.54^{a}**	**3.37^{b}**	**3.48^{ab}**	**0.051**	**0.033**
Steroid biosynthesis	-9.74	-8.92	-8.61	-8.92	0.605	0.649
Steroid hormone biosynthesis	-3.33	-2.97	-2.46	-2.29	0.388	0.312
Streptomycin biosynthesis	2.59	2.52	2.40	2.49	0.052	0.155
Styrene degradation	-2.11	-2.23	-2.00	-2.55	0.161	0.168
Sulfur metabolism	**2.33^{a}**	**2.21^{ab}**	**2.05^{c}**	**2.19^{b}**	**0.043**	**0.008**
Sulfur relay system	1.71	1.57	1.39	1.56	0.068	0.061
Synthesis and degradation of ketone bodies	-0.01	-0.24	-0.35	-0.21	0.120	0.318
Taurine and hypotaurine metabolism	1.47	1.37	1.29	1.41	0.062	0.318
Terpenoid backbone biosynthesis	**2.92^{a}**	**2.80^{ab}**	**2.62^{c}**	**2.78^{b}**	**0.045**	**0.008**
Tetracycline biosynthesis	1.14	0.90	0.77	0.87	0.104	0.157
Thiamine metabolism	**2.54^{a}**	**2.45^{a}**	**2.27^{b}**	**2.47^{a}**	**0.051**	**0.022**
Toluene degradation	0.59	0.62	0.54	0.72	0.097	0.664
Transcription factors	**4.01^{a}**	**3.88^{ab}**	**3.69^{b}**	**3.82^{b}**	**0.066**	**0.043**
Transcription machinery	3.39	3.38	3.29	3.39	0.038	0.300
Transcription related proteins	-3.86	-4.20	-4.11	-3.27	0.528	0.649
Translation factors	**2.83^{a}**	**2.71^{ab}**	**2.54^{c}**	**2.69^{b}**	**0.044**	**0.008**
Translation proteins	**3.34^{a}**	**3.21^{b}**	**3.01^{c}**	**3.16^{b}**	**0.044**	**0.005**
Transporters	**4.85^{a}**	**4.72^{ab}**	**4.54^{c}**	**4.67^{bc}**	**0.061**	**0.033**
Tropane, piperidine and pyridine alkaloid biosynthesis	1.32	1.31	1.17	1.24	0.041	0.103
Tryptophan metabolism	1.12	0.97	0.86	0.94	0.060	0.074
Two-component system	4.42	4.41	4.34	4.44	0.046	0.530
Tyrosine metabolism	**1.66^{a}**	**1.58^{ab}**	**1.37^{c}**	**1.44^{bc}**	**0.065**	**0.038**
Ubiquinone and other terpenoid-quinone biosynthesis	1.55	1.48	1.28	1.53	0.112	0.389
Ubiquitin system	-6.69	-6.27	-8.19	-7.33	0.784	0.396
Valine, leucine and isoleucine biosynthesis	**2.98^{a}**	**2.88^{ab}**	**2.71^{c}**	**2.82^{b}**	**0.038**	**0.006**
Valine, leucine and isoleucine degradation	1.73	1.68	1.58	1.62	0.053	0.314
Various types of N-glycan biosynthesis	-10.32	-10.31	-10.26	-10.33	0.130	0.982

Vitamin B6 metabolism	1.89	1.88	1.71	1.79	0.048	0.103
Xylene degradation	0.56	0.25	0.00	-0.02	0.196	0.220
Zeatin biosynthesis	0.82	0.71	0.59	0.71	0.053	0.074
alpha-Linolenic acid metabolism	**-4.87^{b}**	**-4.36^{ab}**	**-3.69^{a}**	**-4.19^{ab}**	**0.249**	**0.049**
beta-Alanine metabolism	1.52	1.55	1.41	1.54	0.038	0.095
beta-Lactam resistance	-1.12	-0.85	-0.40	-0.52	0.175	0.064
mRNA surveillance pathway	-9.62	-10.31	-8.79	-10.33	0.600	0.314
mTOR signaling pathway	-9.72	-10.31	-8.98	-10.33	0.523	0.314
p53 signaling pathway	**-9.03^{b}**	**-7.68^{a}**	**-6.77^{a}**	**-7.69^{a}**	**0.436**	**0.029**

The counts were transformed using centred log-ratio in R. Statistical analysis was made using proc mixed by SAS. Data is presented in the format of mean ± standard error of the mean (SEM). Statistically significant results are considered for *p*-value < 0.05. All 28 animals were included in the data. FU without meaning in the experimental context were excluded. FU with different letters differ statistically and are highlighted in bold. *p*-value was correct by the BH method in R.