UNIVERSIDADE DE LISBOA

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SPIRULINA AND *CHLORELLA VULGARIS* AS INGREDIENTS IN THE DIET OF WEANED PIGLETS: A VALORISATION APPROACH USING FEED ENZYMES

CÁTIA FALCÃO MARTINS

Orientador: Professor Doutor José António Mestre Prates

Coorientadores: Professor Doutor João Pedro Bengala Freire

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

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Faculdade de Medicina Veterinária da Universidade de Lisboa, 28 de Setembro de 2023

Assinatura: Cátia Falcão Martins

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RESUMO

Espirulina e *Chlorella vulgaris* como ingredientes na dieta de leitões na fase de pós-desmame: uma abordagem de valorização utilizando enzimas alimentares

A produção mundial de suínos enfrentará nos próximos anos desafios associados à alimentação, sobretudo devido ao elevado custo e impacto ambiental da importação de matérias-primas convencionais. Torna-se por isso evidente a necessidade de matérias-primas mais sustentáveis. As microalgas, pelas suas características de produção podem ser encaradas como uma alternativa sustentável. As suas paredes celulares recalcitrantes diminuem a biodisponibilidade dos seus nutrientes para os suínos, tendo sido associadas a enzimas que consigam degradá-las, nomeadamente as "Carbohydrates-Active Enzymes" (CAZymes). A *Arthrospira platensis* (Espirulina) e a *Chlorella vulgaris* são as duas microalgas mais estudadas, como suplemento, na alimentação de suínos. Desta forma, numa abordagem inicial, o objetivo deste estudo foi avaliar a incorporação de 10% de Espirulina em dietas de leitões na fase de pós-desmame, suplementadas com duas CAZymes (Rovabio® Excel AP ou lisozima), sobre as performances produtivas, digestibilidade dos nutrientes das dietas, estado de saúde, metabolismo hepático e qualidade da carne. As performances zootécnicas foram afetadas negativamente por esta incorporação, verificando-se um aumento significativo da viscosidade da digesta e uma diminuição da digestibilidade da fração proteica. A utilização de lisozima, em contraste com a da Rovabio® Excel AP, mostrou-se mais eficiente na degradação das paredes celulares, no entanto, as proteínas libertadas não foram absorvidas, uma vez que o crescimento destes animais foi igualmente prejudicado. A qualidade da carne não foi afetada pela incorporação de Espirulina, individualmente ou combinada com enzimas alimentares, assim como não se verificaram alterações no metabolismo hepático apesar da melhoria no potencial antioxidante hepático, sobretudo ao nível dos carotenoides totais. Numa segunda abordagem, foi realizado um ensaio com leitões na fase de pós-desmame para apurar o valor nutritivo de dietas com 5% de *Chlorella vulgaris*, suplementadas com duas CAZymes (Rovabio® Excel AP ou mistura de quatro CAZymes recombinantes testadas *in vitro* em estudo prévio). A inclusão desta microalga afetou negativamente a digestibilidade dos nutrientes, mas sem efeito direto no crescimento dos animais. A qualidade da carne não foi afetada por esta inclusão. O potencial antioxidante sistémico, a resposta imunitária e o metabolismo lipídico hepático dos leitões foram melhorados nos grupos alimentados com *Chlorella vulgaris*. A valorização nutritiva da carne e a não afetação do estado de saúde dos animais pela inclusão destas microalgas nas suas dietas são as evidências benéficas para as continuar a considerar como ingrediente nas dietas de leitões. Adicionalmente, será fulcral a redução do seu custo de produção, assim como o estudo do seu valor nutritivo.

Palavras–chave: leitões, Espirulina, *Chlorella vulgaris,* digestibilidade, enzimas

ABSTRACT

Spirulina and *Chlorella vulgaris* as ingredients in diets of weaned piglets: a valorization approach using feed enzymes

Worldwide swine production will face challenges in the coming years associated with feeding, mainly due to the high cost and environmental impact of importation of conventional feedstuffs. Therefore, the need for sustainable alternative feedstuffs is clear. Microalgae, due to their production systems, may be a sustainable alternative. However, their recalcitrant cell walls decrease nutrient bioavailability for pigs. To solve the association with feed enzymes, namely Carbohydrates-Active enZymes (CAZymes), has been equated. *Arthrospira platensis* (Spirulina) and *Chlorella vulgaris* are the two most studied microalgae, as a supplement, in swine feeding. Thus, in an initial approach, the aim of this Thesis was to evaluate the inclusion of 10% Spirulina in diets of *post*-weaned piglets, supplemented with two CAZymes (Rovabio® Excel AP or lysozyme) on growth performance, nutrient digestibility, health status, liver metabolism and meat quality traits. Growth performance was negatively affected by this dietary inclusion, with a significant increase in digesta viscosity and a lower protein digestibility. The use of lysozyme, in contrast to Rovabio® Excel AP, proved to be more efficient in the degradation of cell walls; however, the released proteins were not absorbed, as animal growth was impaired. Meat quality traits were not affected by the inclusion of Spirulina, individually or supplemented with feed enzymes, as well as no changes were observed on hepatic metabolism despite the improvement in hepatic antioxidant potential, especially in terms of total carotenoids. On a second approach, a *post*-weaning piglet trial was performed to determine the nutritional value of diets with 5% *Chlorella vulgaris*, supplemented with two CAZymes (Rovabio® Excel AP or a mixture of 4-CAZymes previously tested *in vitro*). *Chlorella vulgaris* inclusion negatively affected the digestibility of nutrients but without direct effects on growth performance, which can be explained by the compensatory mechanisms observed (greater development of the intestinal mucosa and predominance of beneficial bacteria on intestinal microflora). Meat quality traits were not affected by such dietary inclusion. Systematic antioxidant potential, immune response and hepatic lipid metabolism were improved in the groups fed with *Chlorella vulgaris*. In conclusion, the results indicate that microalgae have the potential to be exploited as ingredients in swine diets, providing meat nutritional enhancement and improving the health status of the animals. However, reducing the production cost and further knowledge of their nutritional value is necessary.

Keywords: piglets, Spirulina, *Chlorella vulgaris*, digestibility, enzymes

RESUMO ALARGADO

Espirulina e *Chlorella vulgaris* como ingredientes na dieta de leitões na fase de pós-desmame: uma abordagem de valorização utilizando enzimas alimentares

As projeções atuais indicam que a população mundial está em crescimento e essa tendência deverá persistir ao longo do século. Além do aumento da população mundial, existirá também um crescimento do poder de compra *per capita*, o que inevitavelmente aumentará a procura por produtos de origem animal. A carne de porco é uma das mais consumidas em todo o mundo, o que trará desafios para a produção mundial de suínos. Um dos custos mais elevados desta produção está associado à alimentação dos animais, que depende principalmente da utilização de bagaço de soja e milho, como principais fontes de proteína e energia, respetivamente. A importação destes ingredientes tem um custo económico e ecológico bastante elevado. Torna-se, pois, premente a procura por matérias-primas alternativas mais sustentáveis. As microalgas podem ser vistas como uma alternativa promissora para a alimentação de suínos devido à sua interessante composição nutricional e às suas características de produção, com baixo impacto ambiental, incluindo a vantagem de utilizar terras sem aproveitamento agrícola. No entanto, elas também apresentam desafios, especialmente relacionados com a complexidade da composição das suas paredes celulares, o que limita a disponibilidade de nutrientes para os suínos. Para superar essa limitação, os estudos realizados têm explorado o uso de enzimas alimentares, especificamente as Carbohydrate-Active enZymes (CAZymes). A *Arthrospira platensis* (comumente conhecida como Espirulina) e a *Chlorella vulgaris* têm sido as microalgas mais estudadas como suplemento em dietas de suínos (incorporação nas dietas inferior a 5%).

Nesta perspetiva, numa abordagem inicial, o objetivo desta Tese consistiu na avaliação dos efeitos resultantes da incorporação de 10% de Espirulina na dieta de leitões na fase de pós-desmame, juntamente com a suplementação com duas CAZymes (Rovabio® Excel AP e lisozima), sobre as performances produtivas, a digestibilidade dos nutrientes das dietas, o estado de saúde dos animais e a qualidade da carne produzida. Foram utilizados 40 leitões, provenientes de cruzamentos entre marrãs Large White x Landrace com varrascos Pietrain, com um peso inicial de 12,0 ± 0,89 kg (média ± desvio padrão). Os animais foram alocados a um dos quatro tratamentos experimentais (n =10): dieta controlo à base de cereais e bagaço de soja (controlo), dieta com 10% de Espirulina (SP), dieta com 10% de Espirulina suplementada com 0,005% de Rovabio® Excel AP (SP+R) e dieta com 10% de Espirulina suplementada com 0,01% de lisozima (SP+L). Nos parques de crescimento, onde os animais foram alojados 2 a 2, o acesso à alimentação foi *ad libitum*, com registo das quantidades fornecidas. Os animais foram pesados semanalmente, permitindo o cálculo posterior de parâmetros zootécnicos. Nas duas últimas semanas do ensaio, foi adicionado óxido de crómio às dietas, e na última semana, foram recolhidas fezes para determinar a digestibilidade total

aparente. Após quatro semanas de período experimental, os animais foram abatidos, tendo sido recolhidas amostras de sangue para análise dos principais metabolitos sanguíneos, amostras de fígado para análise da composição química hepática e a expressão dos genes hepáticos, conteúdo intestinal para avaliar a viscosidade e amostras de músculo para determinar a qualidade da carne e a sua composição química. Os resultados deste estudo revelaram que as performances de crescimento foram negativamente afetadas pela incorporação da microalga em estudo, com uma redução média de 9,1% no peso final, em comparação com os animais do grupo controlo. O coeficiente de utilização digestiva da matéria seca, matéria orgânica, energia digestível e proteína bruta foi diminuído com a incorporação de 10% de Espirulina. No entanto, a lisozima mostrou melhorar a utilização digestiva da matéria seca, matéria orgânica e energia digestível, com valores de digestibilidades no grupo SP+L semelhantes aos do grupo controlo. Assim, a lisozima, em contraste com a utilização de Rovabio® Excel AP, mostrou ser mais eficiente na degradação da parede celular da Espirulina. No entanto, as proteínas libertadas não foram eficientemente absorvidas pelos animais, com o grupo SP+L a apresentar uma utilização digestiva da proteína e um crescimento prejudicados em comparação com o grupo controlo. Da mesma forma, verificou-se um aumento da viscosidade da digesta ao nível do duodeno, jejuno e íleo em todos os grupos com incorporação de Espirulina, em comparação com o grupo controlo. Além disso, nestes grupos, foi observado um aumento do comprimento relativo do intestino delgado, um mecanismo fisiológico desenvolvido para aumentar a área de absorção de nutrientes. A incorporação desta microalga não afetou as características de qualidade da carne. Além disso, não foram identificadas alterações consideráveis no metabolismo hepático, embora tenha sido verificada uma melhoria no potencial antioxidante sanguíneo, possivelmente relacionada com o aumento significativo dos níveis de carotenoides totais no fígado.

Num segundo ensaio experimental, procurou-se avaliar o efeito da incorporação de 5% de *Chlorella vulgaris* na dieta de leitões na fase de pós-desmame, juntamente com a suplementação com duas CAZymes (Rovabio® Excel AP e uma mistura de 4 CAZymes previamente testada *in vitro* como eficaz na degradação da parede celular desta microalga), sobre a digestibilidade dos nutrientes das dietas, a morfologia e microbiota intestinal, a saúde dos animais e a qualidade da sua carne. O ensaio de digestibilidade realizado envolveu a utilização de 44 leitões, descendentes de marrãs Large White x Landrace cruzadas com varrascos Pietrain, com um peso inicial de 11,2 ± 0,46 kg. Esses animais foram alojados em gaiolas metabólicas, permitindo fazer, individualmente, a alimentação equitativa entre grupos e a recolha da excreta total. Com base nos dados recolhidos, foi possível calcular os principais índices zootécnicos e a digestibilidade total aparente. Os leitões foram alimentados com uma das quatro dietas experimentais: dieta base com cereais e bagaço de soja (controlo; n = 11),

dieta com 5% de *C. vulgaris* (CH; *n* = 10), dieta com 5% de *C. vulgaris* suplementada com 0,005% de Rovabio® Excel AP (CH+R; *n* = 10) e dieta com 5% de *C. vulgaris* suplementada com 0,01% da mistura de 4 CAZymes (CH+M; *n* = 11). Após 21 dias de período experimental, os animais foram abatidos, tendo sido recolhidas amostras de sangue para análise dos principais metabolitos sanguíneos, amostras de fígado para determinar a composição química hepática e os principais metabólitos hepáticos, conteúdo intestinal para avaliar a viscosidade e microbiota, tecido intestinal para determinar a sua morfologia e amostras de músculo para avaliar a qualidade da carne e a sua composição química. Os resultados indicaram que a incorporação de 5% de *C. vulgaris* teve um impacto negativo na digestibilidade dos nutrientes, embora não tenha afetado diretamente o crescimento dos animais. Essa aparente contradição nos resultados pode ser explicada pelos mecanismos compensatórios observados nos leitões, tais como o desenvolvimento da mucosa intestinal e a predominância de bactérias benéficas na microbiota intestinal. É importante ressalvar que as características da carne analisadas não foram afetadas pela inclusão de *C. vulgaris* na dieta, tendo existido ainda um aumento do teor de ácidos gordos polinsaturados ómega 3 e do de carotenoides totais na carne. Além disso, os leitões alimentados com *C. vulgaris* apresentaram melhorias no potencial antioxidante sistémico, na resposta imunitária e no metabolismo lipídico hepático. Os resultados sugeriram que as enzimas alimentares utilizadas não tiveram um papel relevante com este nível de incorporação de *C. vulgaris*, já que não tiveram impacto na maioria dos parâmetros analisados.

No futuro, seria interessante estudar o valor nutritivo destas microalgas e testar misturas enzimáticas eficientes, equacionado a sua associação com peptidases. Os resultados observados, como a melhoria nutricional da carne e a inexistência de efeitos adverso na saúde dos animais, são promissores e podem promover a continuidade da exploração das microalgas como ingredientes na dieta de leitões. Contudo, é fundamental aguardar a redução do custo de produção destas microalgas para continuarmos a considerá-las como substitutos viáveis das matérias-primas convencionais, como o milho e a soja, na dieta de suínos.

LIST OF PUBLICATIONS

This PhD Thesis was based on the following publications:

1. **Martins, C. F.**, Ribeiro, D. M., Costa, M., Coelho, D., Alfaia, C. M., Lordelo, M., Almeida, A. M., Freire, J., Prates, J. (2021). Using microalgae as a sustainable feed resource to enhance quality and nutritional value of pork and poultry meat. *Foods (Basel, Switzerland)*, *10*(12), 2933.

2. **Martins, C. F.**, Assunção, J. P., Santos, D. M. R., Madeira, M. S., Alfaia, C. M., Lopes, P. A., Coelho, D., Lemos, J. P., Almeida, A. M., Prates, J.A.M., Freire, J. P. B. (2021). Effect of dietary inclusion of Spirulina on production performance, nutrient digestibility and meat quality traits in *post*-weaning piglets. *Journal of Animal Physiology and Animal Nutrition*, 105, 247–259.

3. Madeira, M. S. M., Lopes, P. A. A. B., **Martins, C. F.**, Assunção, J. M. P., Alfaia, C. M. R. P. M., Pinto, R. M. A., Prates, J. A. M. (2021). Dietary *Arthrospira platensis* improves systemic antioxidant potential and changes plasma lipids without affecting related hepatic metabolic pathways in *post*-weaned piglets. BMC Veterinary Research, 17, 158.

4. **Martins, C. F.**, Trevisi, P., Coelho, D. F., Correa, F., Ribeiro, D. M., Alfaia, C. M., Pinho, M., Pestana, J. M., Mourato, M. P., Almeida, A. M., Fontes, C. M. G. A., Freire, J. P. B., Prates, J. A. M. (2022). Influence of *Chlorella vulgaris* on growth, digestibility and gut morphology and microbiota of weaned piglet. *Scientific Reports,* 12, 6012.

5. **Martins, C. F.**, Pestana, J. M., Alfaia, C. M., Costa, M., Ribeiro, D. M., Coelho, D., Lopes, P. A., Almeida, A. M., Freire, J. P. B., Prates, J. A. M. (2021). Effects of *Chlorella vulgaris* as a feed ingredient on the quality and nutritional value of weaned piglets' meat. Foods, 10, 1155.

6. **Martins, C. F.,** Lopes, P. A., Palma, M., Pinto, R. M. A., Costa, M., Alfaia, C. M., Pestana, J. M., Coelho, D., Ribeiro, D. M., Viegas, I., Almeida, A. M., Freire, J. P. B., Prates, J. A. M. (2022). Impact of dietary *Chlorella vulgaris* and feed enzymes on health status, immune response and liver metabolome in weaned piglets. Scientific Reports, 12, 16816.

The author also published the following articles related to this project:

1. **Martins, C. F.**, Ribeiro, D. M., Matzapetakis, M., Pinho, M. A., Kuleš, J., Horvatić, A., Guillemin, N., Eckersall, P. D., Freire, J. P. B., Almeida, A. M., Prates, J. A. M. (2022). Effect of dietary Spirulina (*Arthrospira platensis*) on the intestinal function of *post*-weaned piglet: An approach combining proteomics, metabolomics and histological studies. Journal of Proteomics, 269, 104726.

2. Costa, M., Madeira, M., Coelho, D., **Martins, C. F.**, Mourato, M., Prates, J. A. M. (2022). Dietary *Chlorella vulgaris* with a specific enzyme mixture enriches pork in potassium and improves its sodium to potassium ratio. British Food Journal, 124(12), 4644-4652.

3. Ribeiro, D. M., Coelho, D., Osório, H., **Martins, C.**, Bengala, J. P. F., Almeida, J., Moreira, O., Almeida, A. M., Prates, J. A. M. (2022). Effect of dietary incorporation of *Chlorella vulgaris* and CAZyme supplementation on the hepatic proteome of finishing pigs. Journal of Proteomics, 256, 104504.

4. Ribeiro, D. M., **Martins, C. F.**, Kuleš, J., Horvatić, A., Guillemin, N., Freire, J. P. B., Eckersall, P. D., Almeida, A. M., Prates, J. A. M. (2021). Influence of dietary Spirulina inclusion and lysozyme supplementation on the *longissimus lumborum* muscle proteome of newly weaned piglets. Journal of Proteomics, 244, 104274.

5. Alfaia, C. M., Pestana, J. M., Rodrigues, M., Coelho, D., Aires, M. J, Ribeiro, D. M, Major, V. T., **Martins, C. F.**, Santos, H., Lopes, P. A., Lemos, J. P. C., Fontes, C. M. G. A., Lordelo, M. M, Prates, J. A. M. (2020). Influence of dietary *Chlorella vulgaris* and carbohydrate-active enzymes on growth performance, meat quality and lipid composition of broiler chickens. Poultry Science, 100 (2), 926-937.

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LIST OF ABBREVIATIONS AND SYMBOLS

®	Registered trademark
µg	Microgram
µL	Microliter
a*	Redness (colour dimension)
ADF	Acid Detergent Fibre
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
ADL	Acid Detergent Lignin
ANOVA	Analysis of Variance
ALA	Alpha-linolenic (18:3*n*-3)
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
b*	Yellowness (colour dimension)
bp	Base pairs (in nucleic acids)
BW	Body Weight
C2	Acetic acid
C3	Propionic acid
C4	Butyric acid
C5	Valeric acid
CAZymes	Carbohydrate-Active Enzymes
CF	Crude Fat
CIE	Commission Internationale de l'Éclairage
CIISA	Centro de Investigação Interdisciplinar em Sanidade Animal
CP	Crude Protein
C. vulgaris	*Chlorella vulgaris*
cm	Centimetre
d	Day
DHA	Docosahexaenoic acid (22:6*n*-3)
DM	Dry Matter

DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid (22:5*n*-3)
E.C.	Enzyme Commission (of IUBMB)
EPA	Eicosapentaenoic acid (20:5*n*-3)
EU	European Union
FA	Fatty acids
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FID	Flame Ionization Detector
g	G force (acceleration)
g	Gram
GC	Gas Chromatography
GGT	Gamma-glutamyltransferase
GLA	γ-linolenic acid
GLM	Generalized Linear Mixed
GPX	Glutathione Peroxidase
HCl	Hydrochloric Acid
HDL	High Density Lipoproteins
His	Histidine
HOMA-IR	Homeostasis Model Assessment using the Insulin Resistance Index
hPa	HectoPascal
HPLC	High Performance Liquid Chromatography
iC5	Isovaleric acid
i.d.	Inner diameter
ISA	Instituto Superior de Agronomia
kcal	Kilocalorie
kg	Kilogram
L*	Lightness (colour dimension)
LA	Linoleic acid (18:2*n*-6)
LC-PUFA	Long-Chain Polyunsaturated Fatty Acids
LDA	Linear discriminant analysis

LDL	Low Density Lipoproteins
LEfSe	Linear discriminant analysis effect size
LL	*Longissimus lumborum*
LW	Live weight
m	Metre
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
Mm	Milimolar
mm	Millimetre
MUFA	Monounsaturated Fatty Acids
n-6:*n*-3	Total *n*-3 fatty acids: total *n*-6 fatty acids ratio
NDF	Neutral Detergent Fibre
nM	Nanomolar
nm	Nanometre
NMR	Nuclear Magnetic Resonance
ºC	Degree Celsius
OECD	Organisation for Economic Cooperation and Development
OM	Organic Matter
p	Probability
PCA	Principal Component Analysis
pH	Negative decimal logarithm of the hydrogen ion activity in a solution
PLS	Partial Least Squares analysis
PUFA	Polyunsaturated Fatty Acids
PUFA:SFA	Polyunsaturated fatty acids: saturated fatty acids ratio
rpm	Rotation per minute
s	Second
SAS	Statistical Analysis System
SEM	Standard Error of the Mean
SFA	Saturated Fatty Acids
TAC	Total Antioxidant Capacity

TAG	Triacylglycerols
TBARS	Thiobarbituric Acid Reactive Substances
TSP	5.0 M 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt
TTAD	Total Tract Apparent Digestibility
UV-Vis	Ultraviolet-visible
vs.	*Versus*
v/v	Volume *per* volume
VFA	Volatile Fatty Acids
VLDL	Very Low-Density Lipoproteins
WBSF	Warner-Bratzler Shear Force
WHO	World Health Organization
λ	Wavelength

Chapter 1 – INTRODUCTION

Projections indicate that global population will double by 2050, especially urban population, with a growing income. Consequently, cereal and meat production need to increase to meet a foreseeable increased demand for foodstuffs. Corn and soybean meal are the basis of feeds for monogastric animals, which are in high demand by the consumer (FAO 2017). The lack of sustainability of such crops due to droughts, climate change and competition with human nutrition are the main reasons for the need to find alternatives. Ideally, novel feed resources should have high nutritional value, be able to optimize the use of land and water and assure animal product quality in a sustainable system (Poppi and McLennan 2010). Regarding pig production, although there are market oscillations across the years, the future tendency is that there will be an increase in world production (Eurostat 2020). In modern swine production, farmers must bet in the sustainability of their farms, investing in technology and, subsequently, optimizing daily monitoring of the whole process: gestation, farrowing, nursery and growing/finishing. During the weaning phase, piglets are particularly susceptible to digestive and respiratory pathologies resulting from the imbalance between the animals' immunity and environmental infection (Pluske et al. 1997; Weary et al. 2008). The use of antibiotics for preventive or therapeutic purposes of such pathologies is not presently well accepted. It is thus, crucial to use different strategies to reduce or prevent their use.

Microalgae are a promising source of protein and other nutrients as well as bioactive compounds for both food and feed. They do not require arable land and are produced in photobioreactors or raceway ponds using, for instance, saltwater or wastewater. Additionally, they can be used as bio-sequesters of carbon dioxide, with promising scenario for greenhouse gas emissions reduction. This can be done for instance by integrating microalgal production into biorefineries (Chaudhary et al. 2018). The nutritional profile of microalgae, amongst other factors, vary considerably according to species and numerous other factors. In general, microalgae are characterized by protein, carbohydrate and lipid contents that are, at least, comparable to those of conventional feedstuffs. In this context, the use of microalgae in animal feed has been an object of study as nutrient source but also as a source of bioactive compounds that can improve animal immune response, disease resistance, antiviral and antibacterial action and gut function and stimulate probiotic colonization (Madeira et al. 2017). Microalgae are furthermore a relevant source of pigments, vitamins, minerals and fatty acids (FA), particularly eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which are known to improve meat quality of pigs and broilers (Madeira et al. 2017). Presently, the production of microalgae is, in general, very expensive, but these costs will likely be reduced in the coming years due to simplification of technology production and increasing microalgae

productivity (Acién et al. 2012). Nevertheless, microalgae have recalcitrant cell walls that render them largely indigestible for monogastric animals, it is thus necessary to develop novel technologies to improve microalgal nutrient utilization (Austic et al. 2013; Lum et al. 2013).

The nutritional value of lipids from pork is low due to the low levels of beneficial omega-3 (*n*-3) long-chain polyunsaturated fatty acids (LC-PUFA), EPA and DHA (Madeira, Pires et al. 2013). Therefore, during the last decades, the scientific trend has been to improve the sensory quality and nutritional value of meat by controlling its fatty acid profile (Hocquette et al. 2010). The enriched concentrations of *n*-3 LC-PUFA by microalgae represent a largely untapped natural resource with well-known beneficial health implications for both humans and animals (Calder 2012). The use of microalgae in swine and poultry feeding has been shown to improve meat quality. Lower levels of microalgae supplementation seem to have some advantages in meat quality without negatively impacting animal growth. However, there is an inconsistent relationship between higher levels of supplementation and meat quality traits (Martins, Ribeiro et al. 2021).

In line with the above-described context, our work explores the combination of high dietary incorporation of microalgae with exogenous enzymes in weaned piglets as a novel alternative to conventional feedstuff. We aimed to cover their nutritional needs, to reinforce immunity and to reduce the use of antibiotics in pig rearing, thus contributing to the development of new, more sustainable and highly health products.

This PhD Thesis is divided into nine chapters, as herein explained. Chapter 1 is the general introduction. Chapter 2 revises the scientific literature on *post*-weaning in pork industry, the feedstuffs used at this critical stage, the microalgae as a novel alternative, specifically the two species under study, *Arthrospira platensis* and *Chlorella vulgaris*, as well as the biggest disadvantage of their use and the need to develop new technologies to improve microalgal nutrient utilization. At the end of this chapter, the objectives of the work are described. Chapters 3, 4, 5, 6 and 7 are adapted from peer-reviewed scientific manuscripts published in international peer-reviewed journals in the framework of this PhD project. Specifically, chapters 3 and 4 address the effect of dietary inclusion of *Arthrospira platensis* and the supplementation with the exogenous Carbohydrate-Active enZymes (CAZymes), respectively, on growth performance, nutrient digestibility and meat quality traits of weaned piglets and on blood health markers, immune function, oxidative status and hepatic lipids of weaned piglets. The effect of dietary inclusion of *Chlorella vulgaris* and the supplementation with the exogenous CAZymes are described in chapters 5, 6 and 7. Particularly, the effect on performance, nutrient digestibility and gut morphology, fermentation products and microbiota profile (chapter 7), meat quality traits (chapter 6), blood health markers, immune function, oxidative status and hepatic lipids of weaned piglets (chapter 7). Finally, chapter 8 consists on a general discussion of the results obtained in each of the five previous chapters. It comprises

an integrated discussion of the results. Finally, chapter 9 presents the conclusions of our work and addresses its future perspectives.

Chapter 2 – SCIENTIFIC BACKGROUND AND OBJECTIVES

2.1. *Post*-weaning in pork industry

The *post*-weaning period in piglets comprises from the moment of the actual weaning until the beginning of the growing-finishing phase. The most used *post*-weaning period starts with 28 days old piglets (moment of actual weaning) until 60 days of age. Weaning is considered one of the most challenging periods in swine production, with significant impacts on pig welfare and growth performance (Xiong et al. 2019). There are multiples stress factors associated with the weaning stage, such as complex social changes (separation from the sow and litter), abrupt changes in diets and in the environment. All these stressors occur at a time when the animals still have immature immune and digestive systems, low thermoregulation, predisposing them to opportunistic pathogens. Therefore, it usually causes low feed intake, poor weight gain, occurrence of diarrhoea and/or increased morbidity and mortality (Pluske et al. 1997; Weary et al. 2008).

The nutrition of young animals is a huge component of successful farming, being one of the most important areas that must be worked to achieve optimal animal performance. The young animal with the right nutrition will have better performance at later stages. The industry has increasingly come to recognize the importance of investing in nutrition for young animals. Highlighting feed as the most expensive component of the pig industry (typically around 60-70% of the production costs). Collins et al. (2017) examined the production performance and financial outcomes associated with diet complexity at weaning for piglets of different weaning weigh classes. Authors confirmed the impact of weaning weight on lifetime growth, suggesting that a high complexity/cost feeding program should focus on weaning weight less than 6.5 kg at 27 days of age to maximize financial returns.

Novel feed formulation programs allow a formulation based on nutrients and not in ingredients, which adds suppleness to diet formulation, maintaining accurate nutrition and low production costs as feedstuff costs fluctuate. Improved digestion and absorption of nutrients, rapid development of healthy microbiota in the gut, immune modulation to enhance disease resistance are all important factors to guarantee the success in the piglet's phase (Xiong et al. 2019). Thus, obtaining an optimal diet for weaning piglets implies the balance between pig performance and feed costs, something that changes almost on a weekly basis.

Piglets have nutritional requirements supported by diets with high nutrient density, palatability and digestibility. In general, a balanced diet targets the requirements of piglets, focusing on the balance between amino acids and energy, calcium and phosphorus, as well as electrolyte balance (Wu et al. 2020). During the transition from sow milk to solid feed, the

primary source of digestible energy changes from fat to carbohydrates, and the digestible protein changes from highly digestible milk protein to less digestible plant protein (Xiong et al. 2019). The correct amount of protein is thus critical in *post*-weaned piglet diets. Too much protein can lead to increased growth of unwanted bacteria when nondigested protein reaches the large intestine leading to *post*-weaning diarrhoea. It is important to consider the digestion rates and the increasing of feed transit from stress and gut health disorders to support the complete protein digestion (Xiong et al. 2019). The main amino acid that limits the performance of piglets is lysine that is required for tissue growth and for feed protein utilization efficiency. Glutamine has been proposed as a conditionally essential amino acid for weaned piglets because the main source of this amino acid is the sow milk. This amino acid is essential for maintaining the integrity of the enterocytes and their decline will be associated with gut atrophy (Thacker 1999). The reduction in absorptive capacity due to a reduction in the surface area of intestinal villi, require the provision of diets containing highly digestible and not antigenic components to the intestinal lining of the gut.

Only feedstuffs with known high digestibility should be used in diets fed to recently weaned pigs. Feedstuffs with anti-nutritional factors (such as non-starch polysaccharides, aromatic amino acids or oxidized lipids) should be avoided. The gut microbiome in young animals is strongly influenced by feed composition. The presence of anti-nutritional factors shifts the gut microbiome from symbiotic to pathogenic bacteria, promoting inflammation and bacterial fermentation, which can increase the risk of diarrhoea and nutrition-related diseases (Pluske et al. 1997). Soybean is a common feedstuff in pig diets, but the level of anti-nutritional factors in soybeans can limit their application (He et al. 2015). Antigenic proteins in soybeans lead to specific antigen–antibody response and delayed hypersensitivity reaction that stimulate the immune system. Xiong et al. (2019) demonstrated that vitamin C and lipoic acid might attenuate the induced hypersensitivity of the soybean protein globulin. In addition, some feed industries use an enzymatic treatment to produce soy-based feed ingredients for young animals with significantly lower anti-nutritional effects when compared to conventional soybean meal. This type of ingredients, often termed “clean”, promote the development of a healthy gut, increasing nutrient absorption, body weight gain, decreasing feed conversion ratio and the need for antibiotics, consequently increasing the return on investment (He et al. 2015).

After enzymatic digestion in the small intestine, the energy from starch present in cereal grains is release and absorbed in the form of glucose (Noblet et al. 1989). Thus, the primary energy source is starch. However, piglets use complex carbohydrates less efficiently; some raw starches are incompletely digested in the small intestine and pass to the large intestine, where they are fermented by bacteria, and causing diarrhoea. Dried skim milk is an important source of lactose in piglets’ diets to solve this problem (Thacker 1999).

There is a misuse of fat by weaned piglets, associated to the reduction of pancreatic lipase activity at weaning. Diets with high inclusion of milk-based ingredients are difficult to pellet and the inclusion of fat is often used to facilitate the pelleting process. Therefore, the added lipids must necessarily be compatible with the piglets´ ability to digest lipids (Thacker 1999).

Another drawback of the weaning phase is the inability of piglets to secrete gastric acid, which causes a high gastric pH that reduces the efficiency of protein digestion. Furthermore, it can lead to an increase in digestive disorders as gastric pH plays a role in preventing the movement of viable bacteria from the environment into the upper small intestine (Thacker 1999).

Several feed additives have been used to restore gut microbial balance associated with weaning transition in piglets. Zinc oxide at pharmacological doses has been until recently, widely used in the swine industry as an effective tool to manage *post*-weaning diarrhoea, due to the positive effects along the entire gastrointestinal tract. However, it has a negative environmental impact and can negatively affect pig health by the accumulation of zinc in the liver, pancreas and kidney (Thacker 1999). Hence, since July 2022, EU legislation limited the use of zinc oxide in animal production (Bonetti et al. 2021). Due to the problems inherent to the use of zinc oxide, there is an emerging need for alternatives. Such need is also associated with the global overuse of antibiotics in this *post*-weaning phase of piglets, which increased the risk of bacterial developing resistance to commonly used antibiotics (Wang et al. 2015). Plant extracts with essential oils have antimicrobial, antioxidant or antiviral properties that has been associated with an increase in the *Lactobacillus* bacterial group and a decrease in *Escherichia coli* and total coliforms, and their use seems to improve growth performance in weaned piglets (Marcin et al. 2006; Neil et al. 2006). Proanthocyanidins have been suggested as an effective antibiotic alternative, so Han et al. (2016) tested them in weaned piglets using grape seed proanthocyanidins as opposed to colistin. These authors found that proanthocyanidins with half-dose colistin was equivalent to the integral dose of antibiotic, supporting a reduction in intestinal oxidative stress by increasing diversity and improving the intestinal microbial in weaned piglets. Organic acids such as citric, propionic, lactic or fumaric acids, have bacteriostatic and bacterial effects. Several authors described their effects on the microbiota from the stomach to the colon in a broad manner, reporting an increase in *Lactobacillus* in weaned piglets (Ma et al. 2021; Xiang et al. 2021). Huang et al. (2015) proving that dietary sodium butyrate improved the performance of weaned piglets and reduced the incidence of diarrhoea by modulating intestinal permeability and bacterial communities in the ileum and colon. Fermentable carbohydrates, such as beet pulp, wheat starch, lactulose or inulin, which stimulate microbial fermentation, have been tested and demonstrated to promote a stable and healthy gut microbiota ecosystem, reducing the severity of pathogenic infection-

associated symptoms in weaned piglets (Correa-Matos et al. 2013). Probiotics are microorganisms that have been studied as an alternative to the use of antibiotics due to their ability to inhibit or kill pathogens in the gastrointestinal tract and improve the microbial balance in the intestine and regulate intestinal mucosa immunity (Yang et al. 2015). For example, several authors highlight *Lactobacillus reuteri* for its beneficial effects on expression of tight junction proteins and the changing the pH to the better environmental for favourable bacterial species (Hou et al. 2015; Yang et al. 2015). Chen et al. (2009) suggested antimicrobial peptides cecropin AD to mediate immune status improving pig performance and reduce diarrhoea incidence. Reducing the level of crude protein in diets to limit the frequency and severity of digestive problems in piglets has been an adopted strategy but with the associated problem that there may be an inadequate supply of amino acids to support tissue protein synthesis. Nevertheless, Yin et al. (2010) tested the inclusion of L-leucine in low-protein diets of weaned piglets and found increased protein synthesis in intestine, kidney and pancreas. Arginine is a non-essential amino acid in swine feeding, although has attracted interest as it plays an important role in many physiological and biological processes particularly in the gastrointestinal tract. In weaned piglets, dietary arginine supplementation reduced *Escherichia coli* lipopolysaccharide-induced intestinal mucosal damage, which may be related to decreased expression of intestinal inflammation (Liu et al. 2008). Sulfur amino acids, such as methionine and cysteine, play and important role in intestinal digestion, absorption and metabolism. In accordance, the small intestine of methionine-supplemented piglets has been shown to have improved mucosa integrity (Chen et al. 2014). Finally, the use of feed flavours supplement is often employed to facilitate the transition and increase feed intake of piglets (Thacker 1999).

2.2. Microalgae: definition and properties

The broad spectrum of microalgae includes more than 100,000 species divided into four distinct groups: eukaryotic diatoms (*Bacillariophyceae*), green algae (Chlorophyceae), golden algae (*Chrysophyceae)*, and blue-green algae (*Cyanophyceae*) (Madeira et al. 2017). They are mostly autotrophic, since carbon dioxide is the carbon source and sunlight the energy source, but there are also heterotrophic microalgae that use organic carbon instead of sunlight as energy source. The latter are easily cultivated in bioreactors and easily used for biomass production. Microalgae can grow in non-arable lands such as coastal lands, desert or semiarid areas. Their cultivation requires freshwater, saltwater or wastewater from agricultural, domestic, or industrial origins (Chaudhary et al. 2018).

Microalgae have an interesting composition of proteins, carbohydrates, lipids, vitamins, minerals and bioactive compounds, particularly carotenoids. This composition on macro-

and micronutrients depends on several factors, like species, strain, growth conditions and biomass status (whole or defatted algae meal) (Valente et al. 2021). Spirulina (*Arthrospira* sp.), *Chlorella* sp. and *Schizochytrium* sp. have been the most widely used microalgae in animal production. Spirulina is well known as a source of protein, which varies between 60 and 70% of dry weight. It is also a rich source of antioxidants, such as β-carotene and vitamin E, and has a high content of FA, mainly γ-linolenic acid (GLA) (Holman and Malau-Aduli, 2013). The latter can lead to an increase in the level of this FA in pork (Altmann et al. 2019). The range of variation for carbohydrates, crude fat and ash contents are on a dry weight basis: 17.8–22.6%, 1.8–7.3% and 6.5–9.5%, respectively (Madeira et al. 2017).

Chlorella sp. has a protein concentration in the range of 50–60% of dry weight and is considered an important source of cobalamin (vitamin B12) (Gutiérrez-Salmeán et al. 2015; Madeira et al. 2017). Comparatively to Spirulina, it has similar contents of carbohydrates and ash and a higher level of crude fat (12.6 points more, on average) and of *n*-3 PUFA (9 points more, on average) (Madeira et al. 2017).

Schizochytrium sp. is of particular interest for its oils, which are particularly rich in DHA. Several studies used it as a feed supplement under the form of DHA-Gold extract (Valente et al. 2021). With a lower protein content than the other microalgae (about 12.1% of dry weight), it is known by its higher crude fat content (38.0 to 71.1% of dry weight).

The nutritive value of microalgae for livestock production depends on algal species and proximate composition, as well as the adaptation of animals to such feedstuff (Madeira et al. 2017). Microalgae have been successfully used in feeding trials in ruminants, rabbits, broilers and pigs (Peiretti and Meineri, 2011; Kulpys et al. 2009; Kang et al. 2013; Simkus et al. 2013). However, and as microalgae have recalcitrant cell walls that grant resistance to predation and desiccation, several authors have reported issues that limit their use. Indeed, such recalcitrance is the result of the presence of a diverse and complex matrix of cross-linked insoluble carbohydrates (Gerken et al. 2013). Thus, the microalgal cell wall can be largely indigestible by monogastric animals and, so, it is important to develop novel technologies to improve microalgal nutrient utilization and ease the cost-effective use of microalgae for the animal feed industry (Austic et al. 2013; Lum et al. 2013). CAZymes have been studied to enhance the digestion of dietary complex carbohydrates from microalgae (Coelho et al. 2019; Coelho, Lopes et al. 2020) and applied them in pig and poultry feeding trials to prove their effectiveness (Coelho, Pestana et al. 2020; Pestana et al. 2020; Alfaia et al. 2021).

In parallel, there is the issue of the inefficient available technology for microalgae production that strongly limits their use. Indeed, the production of microalgae is, in general, very expensive. However, such costs will likely be reduced in the coming years due to optimization of production technology and increasing microalgae productivity (Acién et al. 2012). These authors referred, for instance, the differentiation of final price of biomass related

to cultivation strategies and water supply. For example, small-scale for specialized applications has a higher price (more than 4€ per kg of microalgae) than wastewater cultivation combined with carbon dioxide capture from industrial flow gases (Acién et al. 2012).

After production, microalgae have to be dried and ground to a powder so they can be incorporated into animal diets. This procedure extends the shelf life of the product and facilitates transport and storage of microalgae products, as well as the formulation itself. Drying processes (freeze drying, spray drying, etc.) are, however, extremely costly and raise sustainability issues due to the use of energy, obtained mostly from fossil fuels.

2.3. Microalgae in pig nutrition

Cereal grains and soybean meal are the main feedstuffs used in swine and poultry feeding, the two most consumed meats and of key relevance to food security worldwide. Such crops, particularly soybean meal, are grown mostly in North and South America and transported over large distances creating sustainability concerns and, furthermore, are in direct competition with human nutrition. Alternatives to these ingredients are, thus, a pressing need to ensure the sustainability of swine and poultry production. Microalgae seem to be a viable alternative due to their interesting nutritional composition. Different researchers have addressed the use of microalgae in monogastric feeding over the last decade, particularly their use as a supplement, whilst their use as a feed ingredient has been comparatively less studied. Most of these studies demonstrated the ability of microalgae to promote benefits to animal growth, improvements in the immune system and meat quality (Madeira et al. 2017).

2.3.1. Growth performance of pigs fed with microalgae

The studies present in the literature are about growth performance of pigs fed with mostly use microalgae as a feed supplement. Their use as a feed ingredient is still very limited nonetheless, has grown in recent years.

Simkus et al. (2013) studied 0.2% of *Arthrospira platensis* in 85-day-old crossbreds of Landrace and Yorkshire pigs until 95 kg and concluded that the average daily gain (ADG) was 9.26% higher than in the control group. Grinstead et al. (2000) found inconsistent results for zootechnical parameters when weaned piglets fed 0.2%, 0.5% and 2% of Spirulina for 28 days, with minimal improvement on growth performance. In an identical line of research, Nedeva et al. (2014) tested the inclusion of 0.15% and 0.2% of the same microalga to piglet diets (from 12.2–12.5 to 30.9–33.9 kg live weight (LW)) and found a significant increase on growth, while feed conversion ratio (FCR) decreased. Contrarily and with a higher level of dietary inclusion, Furbeyre et al. (2017) demonstrated that 1% of Spirulina in weaned piglets with 9.1 kg for 14

days did not affect their growth performance. Neumann et al. (2018) who studied the complete replacement of soybean meal by a microalga meal from *Arthrospira platensis*, using in the final diets 21 and 13% for piglets and growing pigs, respectively, discovered a depressed dietary protein quality when amino acid supplementation was incomplete, without affecting the body weight of animals.

Testing another microalga as an ingredient, Coelho, Pestana et al. (2020) found that dietary incorporation of 5% *Chlorella vulgaris* in diets of finishing pigs (from 59.1 to 100 kg LW) did not affect their growth performance. Earlier, in a first approach with sewage-grown microalgae, Hintz and Heitman (1967) stated that there was no influence on growth performance by the addition of 10% of *Chlorella* and *Scendescmus* to replace fish meal and with added B-vitamin in diets of growing pigs (27.2 to 63.5 kg LW). They found similar results when used 5% of this microalgae mixture in finishing pigs (63.5 to 90.7 kg LW). Furbeyre et al. (2017) tested 1% of *C. vulgaris* in weaned piglets with 9.1 kg for 14 days and found no effect on growth performance of animals. The same authors found similar results in another study using oral supplementation with *C. vulgaris* (385 mg/kg LW) in piglets of 14 days of age (4.9 kg LW) until 14 days after weaning (Furbeyre et al. 2018). As a feed supplement, Baňoch et al. (2012) examined 0.0002% of *C. vulgaris* in diets of finishing pigs (from 30 to 115 kg LW) and no significant differences were observed between the experimental groups. In the same year, Yan et al. (2012) conducted a trial with pigs from 26.6 to 53.0 kg LW with 0.1 and 0.2% dietary incorporation of fermented *C. vulgaris* and found an increase in the ADG of 3.4% in average to the control diet, without no differences on live weight, average daily feed intake (ADFI) and FCR.

Schizochytrium sp. is a microalga that has been studied as a source of DHA mainly in pork quality traits, but all authors evaluated pig growth as the initial basis of their studies. To assess the safety/toxicity of this microalga for pigs, Abril et al. (2003) used 1.10 and 5.51% inclusion levels in weaned castrated male pigs with 9.07 kg for 27 days and 0.39 and 1.94% during 13 days and concluded that there was no influence on the growth performance of these animals. Sardi et al. (2006) used 0.25% of *Schizochytrium* over 4 or 8 weeks and 5% over the 4 weeks prior to slaughtering barrows of 118-160 kg and detected no effect of this dietary inclusion on growth performance. Meadus et al. (2011) studied the inclusion of 0.06, 0.6, and 1.6% of this microalga biomass in diets of finishing pigs (from 80 to 160 kg LW) and found a positive correlation between the increase in DHA of diets and the ADG. De Tonnac et al. (2016, 2018) studied 0.9, 1.9 and 3.7% of *Schizochytrium* sp. in diets of pigs from 64.6 and 50.7 kg to 115 kg LW and found no significant effects on pig performances (LW, ADFI and performance ratios). With higher dietary level of *Schizochytrium* sp., 7% (piglet diet) and 5% (fattening diet), the results indicated that LW and ADG were not affected by microalga supplementation (Kalbe et al. 2019). Moran et al. (2017) started with a lower dietary level, 0.25 and 0.50% of

Aurantiochytrium limacinum, in diets of pigs from 27.9 kg LW until 112 days of age and found no impact on productive parameters, with no change in ADG, ADFI and FCR. Later, these same authors used a higher level (1%) and found similar results (Moran, Morlacchini et al. 2018).

2.3.2. Metabolism of pigs fed with microalgae

There are few studies focusing on this topic, but it is important and further research is needed to understand how the dietary inclusion of microalgae influences animal metabolism. Most studies highlight the use of microalgae as a source of *n*-3 PUFA to take advantage of the immunomodulatory/prebiotic properties to deal with *post*-weaning stress (Valente et al. 2021). Using Spirulina and *Chlorella vulgaris* (both at a 1% of dietary inclusion), Furbeyre et al. (2017) found that piglets (from 9.1 to 20.1 kg LW) had higher villus heights in the jejunum compared with control group. This increase reflected an enhancement in absorptive function, suggesting a positive effect of these two microalgae supplementation on mucosa restoration or development after weaning (Furbeyre et al. 2017). The increase in total tract digestibility for energy, dry matter (DM), organic matter (OM) and neutral detergent fibre (NDF) was denoted for piglets receiving diets with microalga and was associated with an earlier or greater restoration of intestinal structure and function. Additionally, Spirulina supplementation showed no effect on diarrhoea Incidence, while a significance decrease in its incidence was recorded in piglets supplemented with *Chlorella*. Afterwards, the authors studied the supplementation via drinking water and found that the mucosal architecture of the jejunum was unaffected by Spirulina or *Chlorella* administration, although Spirulina reduced the occurrence of diarrhoea in piglets during the first two weeks *post*-weaning (Furbeyre et al. 2018). Yan et al. (2012) studied a lower dietary inclusion of *Chlorella* (0.1 and 0.2%) and described that fermented *Chlorella* at the level of 0.1% could improve the nutrient digestibility and faecal microbial shedding (lower *Escherichia coli* and higher Lactobacillus) in growing pigs. The authors described the improvement in immune status as a possible explanation to the beneficial effect observed on the nutrient digestibility.

The implication of 5% *C. vulgaris*, individually and supplemented with two carbohydrase mixture on health and liver metabolism of pigs was assessed by Coelho et al. (2022). They used entire male pigs from 59.1 to 101 kg LW and found a strong immunosuppressive effect promoted by the microalga, which increases pig´s susceptibility to infections. These results are contradictory to those found so far, where *Chlorella* has been described as an immunoglobulin stimulant by producing B cells in the gut-associated lymphoid tissue and increasing IgA, IgG and IgM concentrations in the plasma of animals. In studies with broilers and *Chlorella*, increased plasma levels of IgA and IgG (Kang et al. 2013) and IgG and IgM (An et al. 2016)

were reported. Nevertheless, Coelho et al. (2022) also showed in their work, regarding hepatic lipids that pigs fed *C. vulgaris* diets had an increase in hepatic *n*-3 PUFA content. Previouly, Nedeva et al. (2014) described Spirulina at 0.15 and 0.2% inclusion in the diet of piglets (from 12.2–12.5 to 30.9–33.9 kg LW) as having no effect on liver function, without any significant differences in the liver enzymes levels. These authors also referred that piglets fed with 0.2% of Spirulina had 15 and 13% higher numbers of red blood cells and hemoglobin content, respectively, compared to the control group. De Tonnac et al. (2016) studied the dietary inclusion of 0.94, 1.85, 2.74 and 3.61% of *Schizochytrium* sp. and showed a linear decrease of activities and gene expression of malic enzyme and fatty acid synthase in the liver with dietary DHA. Thus, the authors suggested that the effects of dietary DHA on lipogenesis were moderated, with the liver being the tissue in which the lipogenic activity and gene expressions were most influenced by the amounts of *n*-3 PUFA or DHA in the diets. In contrast, they found the subcutaneous adipose tissue to be less influenced by diet composition, although other authors have previously referred the tissue as the main site of *de novo* lipogenesis after weaning.

2.4. Meat quality and nutritional value

2.4.1. General overview

The global consumption of meat is projected to increase by 14% in 2030, mainly poultry meat and pork (OECD/FAO 2021). Pork is one of the most commonly consumed meats worldwide, and the most commonly consumed in Europe. Thus, it is an important source of protein and fat for human diet. However, the lipid nutritional value of this meat is poor due to the low levels of the beneficial *n*-3 LC-PUFA, EPA and DHA (Madeira, Pires et al. 2013). Therefore, during recent years, the scientific community has been trying to improve the sensory qualities and nutritional value of pork by controlling its FA profile (Hocquette et al. 2010).

The health benefits of increasing intake of *n*-3 FA are mainly associated with a reduced risk of cardiovascular disease and an improvement in cognitive functions in childhood and older age. To meet the recommended daily requirements of EPA and DHA in humans, it is necessary to consume products rich in these beneficial FAs (Ruxton et al. 2007). Algae have been used in animal feed to increase the *n*-3 FA content of animal products (Moran, Morlacchini et al. 2018). Tissues from monogastric animals, such as pigs, are susceptible to FA changes through dietary modification and this is a viable strategy to increase *n*-3 FA in their products. However, the addition of *n*-3 LC-PUFA can lead to adverse effects, particularly by increasing the lipid peroxidation of meat products. Lipid peroxidation decreases the nutritive

value of meat and generates oxidation products (malondialdehyde and volatile compounds) causing off-flavour, off-tasting and colour changes (Morrissey et al. 1998). To control these problems, some authors advocate limiting polyunsaturated fatty acids (PUFA) content in swine diets and/or associating it with antioxidants, such as vitamin E and selenium (Wood et al. 2004).

2.4.2. Production of pork with dietary microalgae

As mentioned, microalgae contain high amounts of *n*-3 LC-PUFA and, thus, represent an unexploited natural resource with well-known beneficial health effects for both humans and animals (Calder 2012). In particular, the increase of *n*-3 PUFA content in meat and meat products is the most referenced parameter in several studies on the subject (Mooney et al. 1998; Sardi et al. 2006; Meadus et al. 2011; Ribeiro, Lordelo et al. 2013; Ribeiro et al. 2014; Yan and Kim 2013; Baeza et al. 2015; Bonos et al. 2016; De Tonnac et al. 2016, 2017, 2018; Moran et al. 2017; Vossen et al. 2017; Moran, Currie et al. 2018; Moran, Morlacchini et al. 2018; Altmann et al. 2019; Pestana et al. 2020; Coelho, Pestana et al. 2020) Furthermore, due to the inefficiency of lipid extraction process during biofuel production, the residual fibre obtained as a by-product has a high content of *n*-3 LC-PUFA and, thus, could be a valuable sustainable feed source (Pôjo et al. 2021).

The literature related to the effects of different microalgae on quality traits and nutritional value of pork is summarized in Table 2.1.

The inclusion of Spirulina, as a supplement, was studied by Simkus et al. (2013), who used daily feeding of 2 g of fresh microalga biomass plus forage for each pig weighing from 30.6 to 96.4 kg. The ADG was 9.26% higher in microalgae-fed pigs than controls with no effect on backfat thickness, but the amount of intramuscular fat in meat decreased by 0.33% (Simkus et al. 2013). The authors did not examine the FA composition and sensory properties, which, afterwards, were evaluated by Altmann et al. (2019). According to these authors, the dietary inclusion of the same microalga at high levels (6.60–12.5%) leads to stronger overall odours and a more astringent aftertaste in meat compared to the control group. This could be attributed to a different FA composition with higher PUFA contents, mainly 18:3*n*-3 and 18:3*n*-6 in the subcutaneous fat, with no effect on lipid peroxidation.

The same research group, through report by Coelho, Pestana et al. (2020), studied the effects of 5% of *C. vulgaris* in the diets of growing pigs and demonstrated an improvement in the nutritional value of pork, through an increase in total carotenoids and *n*-3 PUFA content. Furthermore, they simultaneously demonstrated that the use of carbohydrases had a minor impact on meat quality, with no influence on growth performance. Seemingly, at this level of incorporation, there is no need for the use of enzymes to improve pig's digestive function. With

the same microalga, but with a lower incorporation level (0.0002%), Baňoch and colleagues in 2012, using female pigs with an initial weight of 30 kg LW for 3 months, found no significant effect on colour, pH, cooking loss, drip loss and lipid oxidation of pork.

Sardi et al. (2006) reported that moderate levels of *Schizochytrium* sp. (0.50%) in barrows' diets increased DHA content of loin and backfat. Although the supplementation with 0.25% for 4 or 8 weeks caused similar levels of DHA enrichment (50 and 40 mg DHA/100 g of *longissimus lumborum* (LL), respectively) compared to the control group, 0.5% of microalga for 8 weeks led to higher content of DHA (70 mg/100 g LL). The treatments did not affect animal performance, meat pH values, meat colour or iodine amount in subcutaneous fat and EPA content in LL and backfat. In parallel, Vossen et al. (2017) investigated the effects of dietary supplementation with 0.3, 0.6 and 1.2% of *Schizochytrium* sp. for 45 days in finishing pigs from 75 to 110 kg and observed an increase of DHA content in pork products (loin and dry cured ham). Nonetheless, lipid peroxidation increased in processed products as a consequence of DHA enrichment, which was not found in fresh meat. Previously, Meadus et al. (2011) using the same microalga and similar incorporation levels (0.06, 0.6 and 1.6%) in pigs reported a linear increase of DHA content in bacon. In this study, off-odour and off-flavours were detected by a trained sensory panel in bacon from pigs fed the highest inclusion level, which were associated with higher lipid peroxidation. The same authors performed a study with the direct injection of DHA into pork loins and established an increase of 146 mg DHA/100 g serving meat without any undesirable taste being detected by the trained sensory panel (Meadus et al. 2013).

Moran et al. (2017) and Moran, Morlacchini et al. (2018) using *Schizochytrium* sp. (*Aurantiochytrium limacinum*) also performed two different studies. Firstly, they used lower levels of dietary incorporation (0.25 and 0.50%) for pigs with an initial weight of 27.9 kg for 114 days and found an enrichment of DHA in LL and backfat. Subsequently, the authors studied either the effect of reducing the feeding period (last month before slaughter) or a higher inclusion level (1%) of microalga. Significant changes in FA profiles of pork LL and backfat were observed, pointing out the increase of DHA content in the microalgae-fed group. Thus, they found similar increases in DHA content in LL and backfat over long and short feeding periods and these findings demonstrated that this microalga effectively increased the *n*-3 PUFA content of pork.

De Tonnac et al. (2018), studied the FA composition of different tissues (*longissimus thoracis*, LL, *semimembranosus* and diaphragm) enriched with dietary *n*-3 PUFA from *Schizochytrium* sp. (0.9, 1.9 and 3.7%) in finisher pigs (from 50.7 to 115 kg). They found that DHA deposition depends on tissue location, where adipose tissues located in extremities revealed higher *n*-3 and *n*-6 PUFA than tissues in the middle of the carcass. Specifically, the percentages of 20:4*n*-6, 20:5*n*-3 and 22:6*n*-3 increased in tissues from pigs fed with microalga.

However, this high PUFA content in microalgae-fed pigs increased pork's lipid peroxidation and fish odour, discernible by a trained sensory panel (De Tonnac et al. 2017). The authors concluded that a limit below 1.5% of microalga incorporation should be used in swine feed to avoid negative effects on the oxidation susceptibility and sensory parameters of pork. In a previous study by the same authors, using finisher pigs (from 64.6 to 115 kg) and 0.94, 1.85, 2.74 and 3.61% of microalga demonstrated that increasing DHA intake down-regulates the activities and gene expressions of key lipogenic enzymes involved in FA metabolism, mainly in the liver (De Tonnac et al. 2016). Kalbe et al. (2019) tested a higher inclusion level (5%) of *Schizochytrium* sp. in diets of finisher pigs and highlighted the accumulation of DHA and EPA in *longissimus thoracis* and *semitendinosus* of microalgae-fed pigs without significant effects on meat quality traits. However, protein content increased in the *longissimus thoracis* muscle due to DHA-rich microalga supplementation, which can induce muscle protein synthesis (Wei et al. 2013).

Importantly, several authors have indicated that long-term algae supplementation with lower concentrations had similar effects on enrichment levels than short-term supplementation with higher concentrations. Concerning the pork´s fatty acid composition, there were no differences between the lowest level of supplementation over a longer period and the highest level of supplementation over a shorter period (Sardi et al. 2006; Vossen et al. 2017; Moran et al. 2017; Moran, Morlacchini et al. 2018). The most common studies in this area have focused on supplementation with microalga *Schizochythrum* sp., which has shown increasing levels of EPA and DHA in pork without negatively impacting swine productivity (Sardi et al. 2006; Meadus et al. 2011; Vossen et al. 2017; De Tonnac et al. 2018, 2017, 2016; Kalbe et al. 2019). Figure 2.1 summarizes the main effects of dietary microalgae on pork quality traits and nutritional value.

Table 2.1. Summary of main effects of dietary microalgae on pork quality traits.

Microalga	Inclusion level	Animal - Initial weight LW - Final weight LW or Trial duration	Main findings	References
Arthrospira platensis	0.200%	Grower pigs - 30.6 kg - 96.4 kg	Microalga had no effect on protein, color, pH, cooking loss, tenderness and backfat thickness; Decreased the amount of intramuscular fat in meat	Simkus et al. 2013
Arthrospira platensis	8.3 and 12.5% (1st period - 25-50 kg LW), 6.6 and 9.9% (2nd period - 51-75 kg LW) and 9.5 % (3rd period - more than 75 kg LW)	Barrows - 25 kg - 110 or 122 kg	Microalga influenced the FA composition of backfat with increased PUFA levels. Meat quality not compromised	Altmann et al. 2019
Chlorella vulgaris	5,00%	Grower pigs - 59.1 kg - 101 kg	Microalga improved total carotenoids and *n*-3 PUFA content in meat	Coelho, Pestana et al. 2020
Schizochytrium sp.	0.250% (for 4 or 8 weeks) and 0.500% (for 4 weeks)	Barrows - 118 kg - 160 kg	Microalga had no effect on backfat thickness; Feeding 0.50% microalga over 4 weeks prior to slaughter increased the DHA content and decreased of *n*-6:*n*-3 ratio	Sardi et al. 2006
Schizochytrium sp.	0.300, 0.600 and 1.20%	Finisher pigs - 75 kg - 110 kg	Microalga increased lipid oxidation and EPA and DHA contents in dry-cured hams; No effect on proximate composition, color, pH and TBARS values of loins	Vossen et al. 2017
Schizochytrium sp.	0.06, 0.60 and 1.6%	Finisher pigs - 80 kg - 110 kg	Microalga increased DHA content and lipid oxidation of bacon. Over 0.6% inclusion, consumer acceptability was reduced due to the development of off-flavors during and after cooking bacon	Meadus et al. 2011
Schizochytrium sp.	0.250 and 0.500%	Grower pigs - 27.9 kg – 17 weeks	Microalga increased the DHA content of loin and backfat	Moran et al. 2017
Schizochytrium sp.	1.00%	Finisher pigs - 117 kg -140 kg	Microalga increased the EPA, DHA and *n*-3:*n*-6 ratio in *Longissimus lumborum*	Moran, Morlacchini et al. 2018
Schizochytrium sp.	0.900, 1.90 and 3.70%	Finisher pigs - 50.7 kg - 115 kg	Microalga increased 20:4, 20:5 and 22:6 *n*-3 contents in tissues studied; DHA deposition depends on tissue location	De Tonnac et al. 2018, 2017
Schizochytrium sp.	0.940, 1.85, 2.74 and 3.61%	Finisher pigs - 64.6 kg - 115 kg	Increase dietary DHA reduced the activity of lipogenic enzymes in the liver and inhibited the expressions of genes involved in FA metabolism	De Tonnac et al. 2016
Schizochytrium sp.	7.00% (piglet diet)/ 5.00% (grower pig diet)	Grower pigs - 9.46 kg - 104 kg	Microalga increased DHA in *Longissimus thoracis* and *semitendinosus* muscle	Kalbe et al. 2019

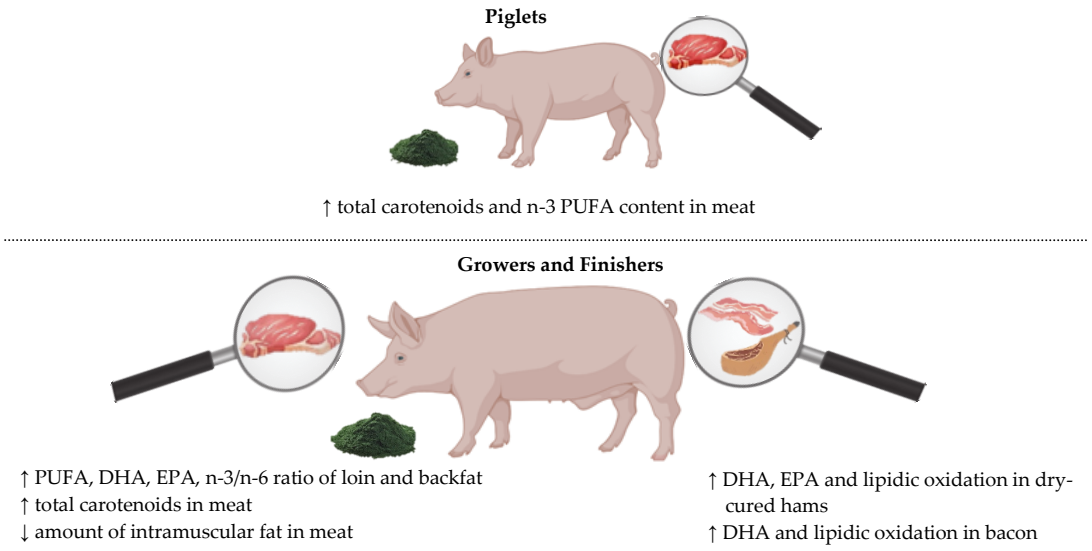

Figure 2.1. Main effects of dietary microalgae on pork.

2.5. Recalcitrant cell walls of microalgae and exogenous CAZymes

The biggest drawback to the use of microalgae in monogastric nutrition is the presence of a recalcitrant cell wall, preventing their full nutritional utilization. This feature is particularly relevant if microalgae are included as an ingredient with a high percentage, and not as a feed supplement (Madeira et al. 2017).

The recalcitrant cell walls confer resistance against predators and difficult environmental conditions such as desiccation during growth, and are refractory to breakage and drying and, consequently, hamper the extraction processes of valuable compounds (Acton 2013). They are rich in polysaccharides and the chemical structure (the degree of sulphation, linkages, type and number of chains) is influenced by factors related to the biology of the algae, such as species, strain, cultivation conditions and processing strategies used (Valente et al 2021). Technological applications in microalgae biomass depend on the composition and cell wall structure, which in turn influences the efficacy of cell-disruption methods to recover high-value intra-cellular compounds, such as lipids and carotenoids (Lee et al. 2017). The low amounts of hemicelluloses and lignin in microalgal cells make them a suitable source of carbohydrates to produce bioethanol as an alternative to crops such as corn or soybean meal (Chaudhary et al. 2018). Recently, polysaccharides and oligosaccharides extracted from microalgae have gained interest due to their biological activities and potential to be used as functional foods (Valente et al. 2021). Antiviral, antibacterial, antioxidant, anti-inflammatory, immunomodulatory, antitumor, anticoagulant, antithrombotic are some of the health benefits reported from microalgal carbohydrates (De Jesus Raposo et al. 2016). Their prebiotic effects

have also been reported, as these compounds are nondigestible in the gastrointestinal tract, being able to influence the growth and/or activity if the bacterial population in the colon (Caporgno and Mathys 2018).

The techniques for microalgae cell disruption can be grouped into four categories: mechanical (bead beating, milling, ultrasonication, high-pressure homogenization and spray-drying), thermal (microwave, autoclaving and freezing), chemical (organic solvent, osmotic) shocks and acid-alkali reactions) and biological processes (microbial degradation and enzymatic reactions) (Günerken et al. 2015). A pre-treatment method of the microalgae cell wall is always necessary to ensure the high yield of extraction processes, reducing the solvent used and energy consumption (Valente et al. 2021). Demuez et al. (2015) reviewed the enzymatic cell disruption treatments employed in biorefinery processes from microalgae, and suggested a new alternative, namely microalgae autolysis. The same authors referred the mild reaction conditions (mild temperatures and pH conditions), the absence of inhibiting by-products, the high selectivity of the reactions (can degrade a specific chemical linkage), as advantages of enzymatic methods for microalgae cell disruption. Consequently, it is less energy consuming and more environmentally sustainable compared to conventional mechanical or chemical methods.

Enzymes (singly or in mixture of two or more) have been applied and demonstrated the feasibility to disrupt of microalgae cell walls (Valente et al. 2021). For example, Wu et al. (2017) used a mixture of lysozyme, cellulase, peptidase and pectinase and obtained a lipid extraction with a 73.1% yield. The specificity of the selected enzymes plays an important role in the efficiency of microbial cell degradation, leading to the frequent application of enzymes mixtures (Demuez et al. 2015). To compose this mixture, commercial enzymes are commonly used, due to their high concentration and in-depth characterization, making the enzymatic cocktails very expensive. Córdova et al. (2019) studied a three commercial enzyme mixture to disrupt the *Chlorella sorokiniana* cell wall and proved its effectiveness for the disruption by the releasing soluble organic compounds. Just for the reason of reducing the cost of these cocktails, several authors have referred alternatives such as the production of these enzymes by another microorganisms (on-site production), and the expression of these enzymes directly by the microalgae to degrade (Demuez et al. 2015).

Exogenous CAZymes, or carbohydrases, mainly xylanases and beta-glucanases, are a class of feed additives for swine and poultry that have been used to improve nutrient utilization. These enzymes are produced by microorganisms and are organized in a CAZy database into families according to amino acid sequences and similar structures, associated with catalytic or functional areas (www.cazy.org). Therefore, they are organized in the following classes: glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases, carbohydrate binding modules and auxiliary activities. Their utilization is widely

accepted to supplement cereal-based diets, being a cost-effective strategy to improve feed nutritive value. However, in terms of their effectiveness to disruption the cell walls of microalga it remains in the field of research with the emergence first of *in vitro* assays (Coelho et al. 2019; Coelho, Lopes et al. 2020) and then the practical application with nutritional trials (Coelho, Pestana et al. 2020; Pestana et al. 2020). One of the reasons for the scarcity of information is that most animal trials consider the dietary inclusion of microalgae as a supplement, where the dietary supplementation with exogenous carbohydrases does not seem to be necessary.

Spirulina (*Arthrospira platensis*) has a fragile cell wall, composed by a simple packet of several layers, mostly of peptidoglycan and lipopolysaccharide nature, without cellulose (Sotiroudis and Sotiroudis 2013). It has been reported that lysozyme is an antimicrobial enzyme capable of hydrolysing the peptidoglycan of bacterial cell walls (Cowieson and Kluenter 2019). Coelho et al. (2019) demonstrated that lysozyme in combination with α-amylase can partially degrade Spirulina cell wall *in vitro*, allowing the release of bioactive compounds with important nutritional value. Knowing the α-amylase is produced endogenously by monogastrics, these authors performed *in vivo* trials in poultry with Spirulina and lysozyme to test their practical applications. They concluded that the incorporation of 15% Spirulina in combination with lysozyme decreased the growth performance of birds, with the consequence gelation of the microalga indigestible proteins. Thus, they proved that the cell wall of Spirulina was successfully broken by the use of lysozyme and suggested a combination of this enzyme with a specific peptidase, probably from a marine organism, to improve the digestibility of the released proteins and to avoid their gelation (Pestana et al. 2020).

Chlorella has a more rigid cell wall and its composition and structure, not yet fully elucidated, depict drastic variations in a single strain under different growth conditions. Some species have only a single microfibrillar layer; others have two layers with the microfibrillar layer proximal to the cytoplasmatic membrane and a mono or trilaminar outer layer (Yamada and Sakaguchi 1982). Coelho, Lopes et al. (2020) investigated in an *in vitro* trial the efficacy of a four-enzyme mixture, composed by an exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme, to degrade *C. vulgaris* cell wall and proved disruption of a significant extent, with the release of valuable nutrients. The same authors tested the dietary incorporation of 5% *C. vulgaris* supplemented with this mixture of carbohydrases in diets of finishing pigs and found that at this level of microalga incorporation the use of exogenous enzymes did not improve its digestive utilization. Also, in broilers diets with 10% *C. vulgaris* this mixture of CAZymes were studied by Alfaia et al. (2021), who confirmed that it caused negligible degradation of cell walls without an improvement of nutrients released.

2.6. Objectives

The main aim of the present study was to develop an approach, based on the combination of microalgae and exogenous enzymes, to make the dietary inclusion of the two most relevant microalgae, *Arthrospira platensis* and *Chlorella vulgaris*, viable as novel feedstuffs in weaned piglet diets. In fact, these microalgae have an interesting nutritional value, but also bioactive compounds that can improve the animal immune response, disease resistance, antiviral and antibacterial action and gut function and stimulate probiotic colonization. Moreover, its richness in pigments, vitamins, minerals and fatty acids may improve meat quality. However, the recalcitrant cell wall of microalgae is a well-known disadvantage, especially for swine, leading us to the use of CAZymes able to disrupt them and release their nutrients improving the nutritive utilization by animals. Thus, our hypothesis was that the incorporation of microalgae combined with specific exogenous enzyme mixes in piglet diets is an effective alternative to cover their nutritional needs and helps the recovery of animals from the critical weaning phase, without compromising productive performance and improving meat quality. The effects and underlying mechanisms were asssessed through the determination of growth performance, nutrient digestibility, health status, liver metabolism, and meat quality traits.

Therefore, with this work we specifically aimed:

1. To assess the effects of incorporating 10% Spirulina in piglet diets and its supplementation with two exogenous CAZymes (Rovabio® Excel AP or lysozyme), on piglets´ growth performance, nutrient digestibility and meat quality traits (Chapter 3).
2. To study the influence of 10% Spirulina feed inclusion in piglet diets and its supplementation with two exogenous CAZymes (Rovabio® Excel AP or lysozyme) on piglets´ blood health markers, immune function, oxidative status and hepatic lipids (Chapter 4).
3. To evaluate the dietary impact of 5% *Chlorella vulgaris* inclusion and the combination with two exogenous CAZymes (Rovabio® Excel AP or the 4-carbohydrase mixture tested *in vitro* by our team), on piglets' performance, nutrient digestibility and gut morphology, fermentation products and microbiota profile (Chapter 5).
4. To study the dietary incorporation of 5% *Chlorella vulgaris* and the combination with two exogenous CAZymes (Rovabio® Excel AP or the 4-carbohydrase mixture tested previously *in vitro*), on meat quality characteristics of piglets (Chapter 6).
5. To assess the dietary impact of 5% of *Chlorella vulgaris* and the combination with two exogenous CAZymes (Rovabio® Excel AP or the 4-carbohydrase mixture above mentioned), on blood health markers, immune function, oxidative status and hepatic lipids and metabolites in weaned piglets (Chapter 7).

Chapter 3 – EFFECT OF DIETARY INCLUSION OF SPIRULINA ON PRODUCTION PERFORMANCE, NUTRIENT DIGESTIBILITY AND MEAT QUALITY TRAITS IN *POST*-WEANING PIGLETS

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Contribution of Cátia Martins to this paper:
Cátia Martins performed the animal trial and sampling. Additionally, conducted laboratory analysis - digestibility study with proximal analysis for diets and faeces and meat quality parameters measurements (namely, determination of fatty acid composition and meat lipid oxidation). Finally, Cátia Martins collaborated in the interpretation of results and wrote the draft manuscript.

Abstract

The effect of Spirulina (*Arthrospira platensis*), individually or in combination with two commercial carbohydrases, in piglet diets was assessed on growth performance, nutrient digestibility and meat quality traits. Forty *post*-weaned male piglets from Large White × Landrace sows crossed with Pietrain boars with an initial live weight of 12.0 ± 0.89 kg were used. Piglets were assigned to one of four dietary treatments (n = 10): cereal and soya bean meal base diet (control), base diet with 10% Spirulina (SP), SP diet supplemented with 0.005% Rovabio® Excel AP (SP+R) and SP diet supplemented with 0.01% lysozyme (SP+L). Animals were slaughtered after a 4-week experimental period. Growth performance was negatively affected by the incorporation of Spirulina in the diets, with an average decrease of 9.1% on final weight, in comparison with control animals. Total tract apparent digestibility (TTAD) of crude protein was higher ($p < 0.05$) in the control group than in other groups. In addition, lysozyme increased TTAD of crude fat and acid detergent fibre, relative to the SP and control groups, respectively. In addition, the incorporation of Spirulina, individually and supplemented with enzymes, did not impair meat quality traits. Surprisingly, no protective effect against lipid oxidation was observed with the inclusion of Spirulina in pork after 7 days of storage. This study indicates that growth performance of *post*-weaning piglets was impaired by the incorporation of 10% Spirulina in the diets, which is mediated by an increase in digesta viscosity and a lower protein digestibility, as a consequence of the resistance of microalga proteins to the action of endogenous peptidases. In addition, it also indicates that lysozyme, in contrast to Rovabio® Excel AP, is efficient in the degradation of Spirulina cell wall in piglet's intestine. However, the digestion of proteins liberated by Spirulina cell wall disruption is still a challenge.

Keywords: digestibility, lysozyme, meat, piglets, Spirulina

3.1. Introduction

Cereal grains and soya bean meal are, respectively, the main energy and protein sources used in pig feeding (FAO 2004). Such ingredients are primarily produced in North and South America and used throughout the world in animal production. The high economic and environmental costs associated with the production and transport of such feedstuffs over large distances and their direct competition with human consumption have important implications for the sustainability of feed and animal production (Manceron et al. 2014). Therefore, alternative sources of protein with balanced amino acid compositions are urgently needed, as are sources of *n*-3 LC-PUFA, vitamins, minerals, carotenoids and bioactive compounds of interest in animal feeding (Florou-Paneri et al. 2014).

Microalgae have been characterized by having high protein, carbohydrate and fat contents, in many cases comparable or even higher than conventional feedstuffs, such as soya bean meal (Lum et al. 2013). One of the most used microalgae in both food and feed production is Spirulina (*Arthrospira platensis*). Spirulina is a cyanobacterium, also known as a blue-green microalga, and therefore an autotrophic prokaryote. It has relatively low cost and a high nutritional value, in particular high protein (50% to 70% of dry weight) and interesting lipid (5% to 14% of dry weight) contents (Hoseini et al. 2013; Gutiérrez-Salmeán et al. 2015; Madeira et al. 2017). However, microalgae in general have recalcitrant cell wall carbohydrates, with very difficult digestion and used by monogastrics, such as pigs (Tibbetts, 2018). Although the microalgae cell wall structure and composition are complexes and poorly studied, it is known that they have rigid components embedded within a polymeric matrix, containing cellulose and an additional tri-laminar sheath containing algaenan, a compound that confers resistance to enzymatic degradation (Popper and Tuohy 2010; Gerken et al. 2013). To circumvent this problem, CAZymes that lyse cell wall complex polysaccharides may be advantageous to improve nutrient utilization of microalgae (Sander and Murthy 2009). Lysozyme is a CAZyme that cleaves the peptidoglycan of prokaryote cell walls, thus leading to a better exposure of proteins and pigments to the endogenous repertoire of digestive enzymes (Oliver and Wells 2015; Al-Zuhair et al. 2016).

Piglet feeding, from weaning to two months of age, is one of the most challenging aspects in swine industry. In fact, it is well known that an adequate feeding of piglets is mandatory to overcome weaning stress and to efficiently reach slaughter weight. Furthermore, it is known that body composition at the end of *post*-weaning period influences growth performance during the growing–finishing phase, as well as body composition when pigs reach 100 kg live weight (Fix et al. 2010; Collins et al. 2017). Interestingly, in Portugal and Spain, there is a tradition of consumption of spit-roasted piglet, a delicacy product particularly valued by consumers (Correia et al. 2017). Examples of such gourmet products include *Leitão de*

Negrais and *Leitão da Bairrada* in Portugal, and *Cochinillo Asado* and *Cochinillo Segoviano* in Spain. Spit-roasted piglet production has become a booming industry in the recent years, due to the product added value and its high demand, constituting an alternative to standard pork production that uses carcasses of animals typically slaughtered at 100 kg live weight. Interestingly, production and meat quality traits of such production systems have still to be characterized.

In line with the above-mentioned, the aim of our study was to assess the effect of incorporating 10% Spirulina in piglet diets, supplemented or not with two exogenous CAZymes (a commercial mixture of carbohydrate-degrading enzymes and lysozyme), on growth performance, nutrient digestibility and meat quality traits in piglets. It was hypothesized that Spirulina, supplemented or not with exogenous enzymes (Rovabio® Excel AP or lysozyme), can be a viable source of nutrients, in particular of proteins, for feeding *post*-weaning piglets, with the main focus on their growth performance, digestibility and meat quality. Such information is of value to the scientific community and to the piglet and pig production industries.

3.2. Material and Methods

3.2.1. Experimental design: animal and diets

Forty male *post*-weaned piglets, sons of Large White × Landrace sows crossed with Pietrain boars, weaned at 28 days of age and with an initial live weight of 12.0 ± 0.89 kg (mean ± *SD*), were obtained from a commercial farm in Central Portugal. After an adaptation period of 2 days, piglets were evenly distributed into four homogeneous groups of 10 piglets, as previously described (Correia et al. 2017). Briefly, they were randomly allocated into pens (1.9 × 1.1 m) equipped with one stainless steel nipple and one creep feeder. The floor was made of plastic slats. All groups had a similar average weight. Piglets had *ad libitum* access to feed and water. Each group received one of the four experimental diets: cereal and soya bean meal-based diet (control), control diet with 10% of Spirulina (SP), control diet with 10% of Spirulina supplemented with 0.005% of Rovabio® Excel AP (Adisseo, Antony, France; SP+R) and control diet with 10% Spirulina supplemented with 0.01% of lysozyme (Sigma 62971; Sigma-Aldrich Ltd.; SP+L).

Diets were balanced for crude protein and essential amino acids. Freeze-dried Spirulina (*A. platensis*) powder provided by Sopropeche (Wimille, France) was used. The CAZymes used were chosen according to the study of Coelho, Lopes et al. (2020), and the incorporation level followed producer recommendations. The ingredient composition of the

diets is described in detail in Table 3.1, and their chemical composition is fully presented in Table 3.2. For further information on the analysis conducted on feeds, see details below.

Table 3.1. Ingredients and feed additives of the experimental diets (g/kg, as fed basis).

	Diets			
	Control	**SP**	**SP+R**	**SP+L**
Wheat	439	460	460	460
Corn	150	170	170	170
Soya bean meal 48	250	110	110	110
Whey powder	100	100	100	100
Sunflower oil	30	30	30	30
Spirulina	0	100	100	100
Rovabio® Excel AP	—	—	0.050	—
Lysozyme	—	—	—	0.100
L-Lysine	5	6	6	6
DL-Methionine	1	1	1	1
L-Threonine	1	—	—	—
Calcium carbonate	5	6	6	6
Dicalcium phosphate	13	12	12	12
Sodium chloride	3	2	2	2
Vitamin-mineral complex[1]	3	3	3	3

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L - Spirulina diet supplemented with lysozyme.

[1]Premix provided per kg of complete diet: vitamin A, 6,500 UI; vitamin D3, 1,500 UI; vitamin E, 15 mg; vitamin K3, 1 mg; vitamin B1, 1 mg; vitamin B2, 3 mg; vitamin B6, 2 mg; vitamin B12, 0.02 mg; pantothenic acid, 10 mg; nicotinic acid, 15 mg; folic acid, 0.5 mg, biotin, 0.03 mg; betaine, 115 mg; vitamin C, 20 mg; Copper, 100 mg; iron, 100 mg; iodine, 0.5 mg; manganese 50 mg; selenium, 0.15 mg; zinc, 100 mg; butylated hydroxytoluene, 3 mg.

Table 3.2. Chemical composition of Spirulina and experimental diets.

		Diets			
	SP powder	**Control**	**SP**	**SP+R**	**SP+L**
Metabolizable energy (kcal/kg DM)[1]	—	3,738	3,809	3,798	3,818
Proximate composition (g/100 g, as fed basis)					
DM	93.8	89.8	90.0	90.0	90.0
CP	60.1	17.9	18.1	17.9	17.8
NDF	—	14.6	11.9	11.9	11.8
ADF	—	4.21	3.97	4.06	3.94
Ash	6.70	5.11	4.76	4.89	4.75
Crude fat	6.84	5.28	5.62	5.87	5.80
Amino Acid composition (g/100 g, as fed basis)					
Alanine	5.21	0.957	1.14	1.14	1.13
Arginine	4.67	1.38	1.26	1.26	1.22
Aspartate	6.50	2.13	1.88	1.86	1.81
Cysteine	0.577	0.374	0.327	0.324	0.326
Glutamate	9.39	4.41	4.02	4.01	3.89
Glycine	3.38	0.909	0.910	0.907	0.899
Histidine	1.03	0.548	0.458	0.456	0.439
Isoleucine	4.15	1.03	1.03	1.03	1.02
Leucine	6.00	1.70	1.69	1.69	1.66
Lysine	3.19	1.48	1.45	1.50	1.53
Methionine	1.59	0.410	0.484	0.457	0.447
Phenylalanine	2.95	1.06	0.963	0.956	0.930
Proline	2.43	1.41	1.29	1.30	1.26
Serine	3.46	1.14	1.06	1.06	1.03
Threonine	3.32	0.913	0.854	0.850	0.846
Tryptophan	1.13	0.295	0.293	0.290	0.294
Tyrosine	3.00	0.736	0.735	0.730	0.710
Valine	4.66	1.14	1.17	1.16	1.16
Fatty acid composition (% total FA)					
12:0	0.000	0.123	0.122	0.146	0.140
14:0	1.01	0.396	0.479	0.520	0.531
16:0	37.6	13.5	17.8	18.3	19.3

16:1*c*9	13.5	0.140	0.817	0.875	0.836
18:0	1.00	3.17	3.19	3.32	3.62
18:1*c*9	1.47	24.1	21.1	20.5	20.1
18:1*c*11	0.21	1.30	1.23	1.28	1.27
18:2*n*-6	17.0	48.6	43.1	42.0	39.1
18:3*n*-3	0.000	4.55	4.17	4.31	4.22
20:0	0.160	0.364	0.325	0.324	0.323
20:1*c*11	0.000	0.298	0.572	0.559	0.820
22:0	0.000	0.365	0.365	0.365	0.421
Pigments (µg/g)					
β-Carotene	233	0.160	3.57	3.14	2.15
Chlorophyll *a*[2]	1,197	2.70	108	112	132
Chlorophyll *b*[3]	45.0	4.97	14.6	13.0	17.5
Total chlorophylls[4]	1,242	7.67	122	125	149
Total carotenoids[5]	697	2.41	11.6	12.6	13.0
Total chlorophylls and total carotenoids[6]	1,939	10.1	134	138	162
Diterpene profile (µg/g)					
α-Tocopherol	24.6	7.41	12.0	12.3	12.9
β-Tocopherol	0.907	0.676	0.254	0.215	0.213
γ-Tocopherol	0.932	1.05	0.997	1.05	0.925
α-Tocotrienol	n.d.	1.09	0.504	0.923	0.994
Estimation of the mineral composition (%)					
Ca	0.120	0.727	0.704	0.704	0.704
P	1.30	0.624	0.662	0.662	0.662
Na	0.450	0.187	0.192	0.192	0.192

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L - Spirulina diet supplemented with lysozyme.
—Difficulties in application of the standard procedure.
n.d.—not detected.
[1]Metabolizable energy (kcal/kg DM) = 4412–11,06 × Ash (g/kg DM) + 3,37 × Crude Fat (g/kg DM) − 5,18 × ADF (g/kg DM) (Noblet et al. 1989).
[2]Chlorophyll *a* = 11.24 × A662 nm − 2.04 × A645 nm.
[3]Chlorophyll *b* = 20.13 × A645 nm − 4.19 × A662 nm.
[4]Total chlorophylls (Ca + b) = 7.05 × A662 nm + 18.09 × A645 nm.
[5]Total carotenoids (Cx + c) = (1,000 × A470 nm − 1.90 × Ca − 63.14 × Cb)/214.
[6]Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

3.2.2. Animal performance and sampling

Throughout the experiment, supplied feed was recorded daily, whereas refusals and piglets were weighed weekly, just before feeding, in order to calculate ADFI, ADG and FCR. All animals were slaughtered, after an experimental period of 28 days, using electrical stunning followed by exsanguination, according to standard procedures used in commercial abattoirs. Gastrointestinal tract was removed, and the length of the small and large intestines was recorded. Samples of *longissimus lumborum* muscle were collected from the right side of the carcass, vacuum packed and stored at −20°C until further analysis to assess the meat quality.

To measure the viscosity of small intestine contents, samples were collected from the duodenum plus jejunum and from the ileum, and centrifuged for 10 min at 18,144 g, and the viscosity of sample's supernatant was measured, in duplicate, using a viscometer adjusted to 6 rpm and 23ºC (Model LVDVCP-II; Brookfield Engineering Laboratories).

3.2.3. Faecal scores and total tract apparent digestibility

The faeces were observed daily, on each pen, to evaluate consistency, according to the following scale: 0 (normal), 1 (soft faeces) or 2 (diarrhoea).

In order to evaluate the digestibility of the four diets, an external marker (chromium oxide) was used, as described by Clawson et al. (1955). During the last two weeks of the experiment, the marker was added to the diets in a proportion of 0.5%. After one week of adaptation, the faeces of each pen were collected twice a day for five consecutive days. Faeces were stored at −20ºC until further analysis. Chromium oxide in the diets and faeces was evaluated according to Bolin et al. (1952), for the estimation of total tract apparent digestibility (TTAD) of DM, OM, crude protein (CP), crude fat (CF), NDF, acid detergent fibre (ADF) and energy.

3.2.4. Diets and faeces analysis

The faeces were dried at 60°C for 72 hr. Diets and dried faecal samples were ground in 1-mm-diameter mesh mill and analysed, in duplicate, for DM, ash, CP (automated Kjeldahl method) and CF contents, following the methods described by AOAC (2000). NDF and ADF were performed sequentially using crucibles system by Van Soest et al. (1991). Energy was calculated by complete combustion of diets and faeces in an adiabatic calorimeter (Parr 1261; Parr Instrument Company).

The determination of amino acids in diets and Spirulina were performed following the pre-approved protocol by the European Commission (2009), and the results are shown in

Table 3.2. For cysteine and methionine, an oxidation with performic acid was performed before hydrolysis. The hydrolysis of the samples consisted of an attack with hydrochloric acid at 110ºC for 23 h. The hydrolysed amino acids were separated using ion-exchange chromatography and determined by photometric detection after reaction with ninhydrin. Tryptophan was hydrolysed using an alkaline solution of barium hydroxide at 110°C for 20 h and measured by High performance liquid chromatography (HPLC) with fluorescent detection.

Fatty acid methyl esters (FAME) of the experimental diets were analysed by one-step extraction and transesterification, using heneicosaenoic acid (21:0) methyl ester as the internal standard (Sukhija and Palmquist 1988).

The pigments of diets were measured according to Teimouri et al. (2013), with slight modifications. Briefly, the samples were extracted with acetone and stored under agitation during overnight. The solutions were centrifuged at 3,345 g during 5 min, and then, absorptions were measured by UV-Vis spectrophotometry (Ultrospec 3100 pro; Amersham BioSciences). The pigment content was calculated using the equations previously described by Hynstova et al. (2018).

The quantification of tocopherols and tocotrienols in the diets involved a direct saponification, a single n-hexane extraction and analysis of the extracted compounds by normal-phase HPLC using fluorescence detection, as described in our previous publication (Prates et al. 2006).

3.2.5. Meat colour and pH measurements

Meat colour was measured on the cut surface of *longissimus lumborum* section, 24 h *post-mortem*, using a colorimeter (Minolta CR-300; Konica Minolta). Two measurements per sample were recorded according to the CIE lightness (L*), redness (a*) and yellowness (b*) system, after 1 h of air exposure and as previously described by Madeira, Costa et al. (2013). The pH of *longissimus lumborum* muscle samples at 24 h *post-mortem* was measured using a pH

meter equipped with a penetrating electrode (HI8424; Hanna Instruments).

3.2.6. Meat lipid oxidation

The extent of meat lipid oxidation was evaluated at days 0, 3 and 7 *post-mortem* (storage at 4°C), by measuring TBARS, following the spectrophotometric method described by Grau et al. (2000). TBARS values were calculated, in duplicate, from a standard curve constructed with 1,1,3,3-tetraethoxypropane, as a precursor of malonaldehyde, and the results were expressed as mg of malonaldehyde per kg of meat (Madeira et al. 2014).

3.2.7. Cooking loss and shear force determinations

Frozen meat samples were thawed at 4°C overnight, weighed and cooked in a water bath at 80°C until reaching an internal temperature of 78°C, using a thermocouple (Lufft C120; Lufft) adjusted with a blank test. After two hours of cooling at room temperature, samples were weighed and longitudinally cut in the fibre axis into 8–10 cores, with a 1-cm2 cross-sectional area for cooking loss and shear force determinations respectively. The cooking loss, expressed in percentage, was calculated as the difference in the weights before and after cooking divided by the initial weight of the sample, which corresponds to the water loss in the thermal process. The Warner-Bratzler shear force (WBSF) was measured using the conditions described by Madeira et al. (2013).

3.2.8. Sensory analysis

Meat sensory characteristics were evaluated by a trained sensory panel in four sessions. The twelve panellists were selected and trained according to Cross et al. (1979). For each session, meat samples were thawed, cooked and prepared according to the study mentioned above. The attributes evaluated were tenderness, juiciness, flavour, off-flavour and overall acceptability, using an eight point-scale, as previously described by Madeira, Costa et al. (2013) and Madeira et al. (2014).

3.2.9. Intramuscular fat content and fatty acid composition

Intramuscular fat was extracted from lyophilized samples according to the method of Folch et al. (1957), using dichloromethane-methanol (2:1, v/v) as described by Carlson (1985), and measured gravimetrically after solvent evaporation. Then, intramuscular lipids were transesterified into FAME using a combined basic and acid catalysis, as described by Madeira, Costa et al. (2013). FAME were analysed by gas chromatography with flame ionization detector (GC-FID) (HP6890A; Hewlett-Packard) and the same chromatographic conditions as described by Madeira et al. (2014). The quantification of total FAME was done using heneicosaenoic acid (21:0) methyl ester as the internal standard and the conversion of relative peak areas into weight percentages. Results for each fatty acid are expressed as a percentage of the sum of detected FA (% total FA).

3.2.10. Determination of β-carotene, total cholesterol, diterpenes and pigments of meat

The simultaneous quantification of total cholesterol, tocopherols and tocotrienols was performed as previously described by Prates et al. (2006). The method involves a direct saponification of the fresh meat, only one n-hexane extraction and analysis of the extracted compounds by normal-phase HPLC, using fluorescence (tocopherols and tocotrienols) and UV-Vis photodiode array (cholesterol) detections. The contents of total cholesterol, tocopherols and tocotrienols were calculated based on the external standard technique from a standard curve of peak *vs*. compounds concentrations.

The content of chlorophyll *a*, chlorophyll *b*, total carotenoids and total pheophytins was measured according to Teimouri et al. (2013), with slight modifications, as described above for diets.

3.2.11. Statistical analysis

All data were checked for normal distribution and variance homogeneity. Data were analysed using the PROC MIXED of SAS software package (version 9.4; SAS Institute Inc.). The model considered the dietary treatment as the single effect. When significant effects of treatments were detected, least-squares means were compared using the PDIFF with the Tukey–Kramer adjustment options of SAS. Results are presented as mean ± SEM and were considered significantly different when *p-value* was below 0.05.

3.3. Results

3.3.1. Intake, growth performance and gastrointestinal tract variables of piglets

Data on intake, growth performance, faecal consistency, TTAD and gastrointestinal tract variables of piglets are presented in Table 3.3. The experimental diets led to significant differences in piglet weight at the end of the trial ($p < 0.01$). The control group had higher final weight compared to groups fed with Spirulina, including those supplemented with the exogenous enzymes, with an average difference in weight of 2.8 kg (9.1% of final weight). ADFI was the same for all groups ($p > 0.05$). ADG was significantly higher for the control group, where piglets grew 96 g/day more than the Spirulina-fed groups. With the same ingestion and lower ADG, the piglets fed with Spirulina diets had higher FCR (1.62, 1.62 and 1.69 for SP,

SP+R, SP+L diets respectively) by comparison to the control group (1.48). In addition, the incorporation of exogenous enzymes in diets had no influence on growth, as no significant differences between SP+R and SP+L groups were observed when compared to the SP group ($p > 0.05$). Also, faecal scores were similar for all groups ($p > 0.05$).

The incorporation of Spirulina with or without enzyme supplementation affected all TTAD nutritional fractions, with the exception of NDF. TTAD of DM was significantly higher in the control and SP+L groups, with an average difference of 2.1%, by comparison to the other groups. Regarding TTAD of OM, no significant differences were found between the control and the SP+L groups ($p < 0.05$). Moreover, TTAD of OM for SP+L group was significantly higher than that of the SP+R group. Dietary incorporation of Spirulina, alone or combined with exogenous enzymes, decreased TTAD of CP in 6.6% ($p = 0.0001$), compared to the control group. TTAD of CF was higher for SP+L group (62.8%), followed by SP+R (60.4%), SP (57.8%) and control (55.6%) groups. TTAD of ADF was significantly different between control and SP+L groups, where the SP+L group was 14.3% higher. Finally, TTDA of energy was significantly higher in control and SP+L groups.

Although digesta viscosity of both duodenum plus jejunum ($p = 0.005$) and ileum ($p = 0.023$) was higher in Spirulina-fed animals, only the relative length of small intestine in SP+L group was higher ($p < 0.05$) by comparison to the control and SP groups that had similar values. In particular, piglets fed on SP+L, by comparison to the control group, had a higher increase in viscosity of the duodenum plus jejunum (93%), the ileum (47%) and the relative length of small intestine (16%).

Table 3.3. Effect of diets on feed intake, growth performance, consistency of faeces, total tract apparent digestibility (TTAD) of nutrients and gastrointestinal tract variables of piglets.

	Diets				SEM	*p- value*
	Control	SP	SP+R	SP+L		
Live performance						
Initial weight (kg)	12.1	11.7	12.1	11.9	0.15	0.808
Final weight (kg)	31.0^{a}	28.3^{b}	28.4^{b}	27.8^{b}	0.40	0.009
ADFI (g)[1]	997	960	943	960	12.8	0.521
ADG (g)[2]	677^{a}	593^{b}	582^{b}	567^{b}	12.4	0.001
FCR[3]	1.48^{a}	1.62^{b}	1.62^{b}	1.69^{b}	0.023	<0.001
Faecal score[4]	0.070	0.223	0.145	0.198	0.032	0.355
TTAD (%)						
DM	79.6^{a}	77.6^{b}	77.3^{b}	79.5^{a}	0.35	0.014
OM	83.1^{a}	81.3^{bc}	81.1^{c}	82.9^{ab}	0.32	0.031
CP	80.6^{a}	73.2^{b}	73.4^{b}	75.4^{b}	0.81	<0.001
CF	55.6^{a}	57.8^{ab}	60.4^{bc}	62.8^{c}	0.78	<0.001
NDF	39.8	39.0	37.5	45.4	1.17	0.071
ADF	23.0^{a}	28.9^{ab}	31.1^{ab}	37.3^{b}	1.86	0.039
Energy	79.9^{a}	78.0^{b}	77.5^{b}	79.9^{a}	0.37	0.012
Relative length of gastrointestinal tract (m/kg)						
Small intestine	0.466^{a}	0.487^{ab}	0.532^{bc}	0.541^{c}	0.010	0.007
Large intestine	0.110	0.122	0.128	0.212	0.003	0.154
Content viscosity (cP)						
Duodenum + jejunum	3.16^{a}	4.96^{b}	5.32^{b}	6.11^{b}	0.320	0.005
Ileum	5.88^{a}	8.97^{b}	7.77^{ab}	8.63^{b}	0.403	0.023

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L - Spirulina diet supplemented with lysozyme.
[1]ADFI—average daily feed intake.
[2]ADG—average daily weight gain.
[3]FCR—feed conversion ratio.
[4]Faecal scores—0 (normal), 1 (soft faeces) or 2 (diarrhoea).
[a,b,c]Values within a row with different superscripts differ significantly at $p < 0.05$.

3.3.2. pH, colour and lipid oxidation of meat

Meat quality traits assessed in *longissimus lumborum* muscle of piglets are shown in Table 3.4. Diets had no effect on 24-h pH values ($p > 0.05$). Dietary supplementation of exogenous enzymes in Spirulina diets increased meat colour parameters, L* in SP+R ($p = 0.022$), *a** in SP+L ($p = 0.008$) and *b** in both SP+R and SP+L ($p = 0.001$). In contrast, only a small effect was observed on TBARS values of *longissimus lumborum* muscle at three days of storage at 4°C (Table 3.4). The incorporation of Spirulina in diet without enzyme supplementation (SP) increased TBARS when compared to the control diet.

Table 3.4. Effect of diets on 24-h pH, CIE colour parameters (*L, *a** and *b**) and lipid oxidation evaluated by the concentration of TBARS (mg malonaldehyde/kg of meat) after 0, 3 and 7 days of cold storage of *longissimus lumborum* muscle.**

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
pH 24 h	5.55	5.50	5.52	5.55	0.062	0.902
Colour						
L*	50.4^{a}	51.5^{ab}	53.3^{b}	51.9^{ab}	0.700	0.022
a*	7.08^{a}	7.68^{ab}	6.99^{ab}	8.17^{b}	0.263	0.008
b*	0.927^{a}	1.95^{ab}	2.29^{b}	2.38^{b}	0.264	0.001
TBARS[1]						
Day 0	0.233	0.233	0.224	0.245	0.011	0.588
Day 3	0.350^{a}	0.814^{b}	0.363^{ab}	0.546^{ab}	0.124	0.041
Day 7	0.590	1.97	1.09	2.09	0.429	0.078

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L - Spirulina diet supplemented with lysozyme.
[1]TBARS—Thiobarbituric acid reactive substances.
^{a,b}Values within a row with different superscripts differ significantly at $p < 0.05$.

3.3.3. Cooking loss, shear force and sensory panel scores of meat

The influence of diets on cooking loss, shear force and trained sensory panel scores of *longissimus lumborum* muscle is depicted in Table 3.5. Neither cooking loss nor shear force was affected by dietary treatments. However, meat from animals fed on SP+L had higher tenderness ($p < 0.001$) than that from control piglets (4.6 *vs*. 3.8 respectively). Additionally, when compared to the control group, meat from SP and SP+L groups had higher flavour scores ($p < 0.05$).

Table 3.5. Effect of diets on cooking loss, shear force and sensory panel scores of *longissimus lumborum* muscle of piglets.

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
Cooking loss (%)	33.2	32.9	33.0	32.9	0.890	0.996
Shear force (kg)	3.79	3.82	3.84	4.10	0.499	0.970
Sensory panel scores						
Tenderness	3.76[a]	3.83[a]	4.12[ab]	4.63[b]	0.159	<0.001
Juiciness	4.26	4.27	4.26	4.67	0.128	0.050
Flavour	4.08[a]	4.55[b]	4.45[ab]	4.49[b]	0.110	0.012
Off-flavour	0.309	0.344	0.277	0.479	0.106	0.529
Overall acceptability	4.15	4.25	4.36	4.63	0.136	0.069

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L - Spirulina diet supplemented with lysozyme.
[a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

3.3.4. Intramuscular fat, total cholesterol and fatty acid composition of meat

Intramuscular fat content and fatty acid profile of *longissimus lumborum* muscle are presented in Table 3.6. Diets had no impact neither on intramuscular fat and total cholesterol contents nor on major individual FA (18:1*c*9, 23%–26% of total FA; 16:0 and 18:2*n*-6, 20%–21%; and 18:0, 11%), as well as on partial sums and ratios of FA ($p > 0.05$). Dietary treatment affected only a small number of minor FA, namely 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:3*n*-3, 22:0 and 22:1*n*-9. The incorporation of Spirulina plus enzyme supplementation in the piglet diets increased the percentage of 18:3*n*-6, but decreased the relative proportion of 20:2*n*-6 (both with $p < 0.001$). When compared to the control diet, the percentages of 20:3*n*-6 were significantly higher in SP groups but more markedly in the groups fed with Spirulina plus enzymes ($p = 0.016$). Moreover, piglets fed Spirulina diets had higher percentages of 20:3*n*-3 (SP, $p = 0.001$), 22:0 (SP+R, $p = 0.027$) and 22:1*n*-9 (SP+L, $p = 0.037$) than those fed the control diet.

Table 3.6. Effect of diets on intramuscular fat content (g/100 g muscle), total cholesterol (mg/100 g muscle) and fatty acid (FA) composition (% of total FA) of *longissimus lumborum* muscle of piglets.

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
Intramuscular fat	1.07	1.11	1.11	1.16	0.077	0.880
Total cholesterol	64.7	64.9	61.2	62.6	2.37	0.664
FA composition						
10:0	0.066	0.061	0.068	0.060	0.007	0.649
12:0	0.052	0.055	0.066	0.054	0.005	0.139
14:0	0.732	0.788	0.787	0.777	0.055	0.874
15:0	0.099	0.111	0.103	0.099	0.008	0.688
16:0	20.0	21.1	21.1	21.2	0.36	0.089
16:1*c*7	0.375	0.312	0.388	0.371	0.021	0.067
16:1*c*9	2.00	2.22	2.20	2.03	0.141	0.583
17:0	0.515	0.554	0.546	0.516	0.037	0.821
17:1*c*9	0.213	0.219	0.232	0.177	0.017	0.108
18:0	10.6	10.7	10.8	11.0	0.13	0.131
18:1*c*9	25.6	25.3	24.9	23.3	1.19	0.510
18:1*c*11	3.32	3.27	3.23	3.11	0.059	0.090
18:2*n*-6	20.8	19.8	20.1	21.3	0.89	0.600
18:3*n*-6	0.183^{a}	0.339^{b}	0.339^{b}	0.349^{b}	0.018	<0.001
18:3*n*-3	0.875	0.797	0.833	0.881	0.028	0.133
20:0	0.115	0.137	0.108	0.108	0.012	0.263
20:1*c*11	0.411	0.404	0.377	0.361	0.025	0.450
20:2*n*-6	0.524^{a}	0.441^{b}	0.447^{b}	0.470^{b}	0.011	<0.001
20:3*n*-6	0.485^{a}	0.589^{ab}	0.619^{b}	0.630^{b}	0.034	0.016
20:4*n*-6	4.03	4.05	3.84	4.00	0.359	0.973
20:3*n*-3	0.162^{a}	0.130^{b}	0.140^{ab}	0.153^{a}	0.006	0.001
20:5*n*-3	0.230	0.210	0.201	0.202	0.023	0.780
22:0	0.059^{ab}	0.057^{ab}	0.092^{b}	0.032^{a}	0.014	0.027
22:1*n*-9	0.128^{a}	0.144^{ab}	0.170^{ab}	0.205^{b}	0.019	0.037
22:5*n*-3	0.578	0.583	0.572	0.526	0.067	0.920
22:6*n*-3	0.324	0.301	0.289	0.311	0.037	0.917

Others	7.37	7.26	7.41	7.76	0.452	0.865
Partial sums of FA						
∑SFA[1]	32.3	33.6	33.7	33.8	0.43	0.060
∑MUFA[2]	32.1	31.9	31.5	29.6	1.40	0.547
∑PUFA[3]	28.2	27.3	27.3	28.8	1.37	0.813
∑*n*-3 PUFA[4]	2.17	2.02	2.04	2.07	0.113	0.788
∑*n*-6 PUFA[5]	26.1	25.3	25.3	26.8	1.26	0.797
Ratios of FA						
PUFA:SFA	0.877	0.817	0.816	0.859	0.506	0.771
n-6:*n*-3	12.1	12.6	12.5	12.9	0.28	0.302

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L- Spirulina diet supplemented with lysozyme.
Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.
[1]Sum of 10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 22:0.
[2]Sum of 16:1c7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 20:1*c*11 and 22:1*n*-9.
[3]Sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3.
[4]Sum of 18:3*n*-3, 20:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3.
[5]Sum of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6 and 20:4*n*-6.
[a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

3.3.5. Total carotenoids, chlorophylls and vitamin E contents of meat

The effect of diets on total pigments and diterpene profile of *longissimus lumborum* muscle is presented in Table 3.7. Regarding total pigments, meat of the SP+L group had higher values of chlorophyll *a*, chlorophyll *b*, total chlorophylls, total carotenoids and total pheophytins, comparatively to the control group ($p < 0.05$). The incorporation of Spirulina in the diets, with or without added enzymes, increased total meat carotenoids by comparison to the control group ($p = 0.013$). The diterpene profile of meat was similar for all dietary treatments.

Table 3.7. Effect of diets on total pigments (µg/g) and diterpene profile (µg/g) of *longissimus lumborum* muscle of piglets.

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
Pigments						
β-Carotene	0.081	0.081	0.077	0.082	0.003	0.668
Chlorophyll *a*[1]	0.221[a]	0.346[ab]	0.291[ab]	0.397[b]	0.045	0.045
Chlorophyll *b*[2]	0.328[a]	0.508[ab]	0.475[ab]	0.653[b]	0.076	0.035
Total chlorophylls[3]	0.549[a]	0.853[ab]	0.766[ab]	1.05[b]	0.120	0.037
Total carotenoids[4]	0.092[a]	0.146[b]	0.150[b]	0.153[b]	0.014	0.013
Total chlorophylls and total carotenoids[5]	0.641[a]	0.999[ab]	0.916[ab]	1.20[b]	0.132	0.033
Diterpene profile						
α-Tocopherol	0.498	0.389	0.504	0.366	0.048	0.090
γ-Tocopherol	0.030	0.030	0.031	0.027	0.002	0.522

Dietary treatments: SP - Spirulina diet; SP+R - Spirulina diet supplemented with Rovabio® Excel AP; SP+L - Spirulina diet supplemented with lysozyme.
[1]Chlorophyll *a* = 11.24 × A662 nm − 2.04 × A645 nm.
[2]Chlorophyll *b* = 20.13 × A645 nm − 4.19 × A662 nm.
[3]Total chlorophylls (Ca + b) = 7.05 × A662 nm + 18.09 × A645 nm.
[4]Total carotenoids (Cx + c) = (1,000 × A470 nm − 1.90 × Ca − 63.14 × Cb)/214.
[5]Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).
[a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

3.4. Discussion

The incorporation of microalga Spirulina as a feedstuff and the supplementation with two exogenous enzymes, Rovabio® Excel AP and lysozyme, was the focus of this study. There are several studies about the incorporation of Spirulina in piglet feeding as a supplement (Grinstead et al. 2000; Nedeva et al. 2014; Furbeyre et al. 2017), but none using it as an ingredient.

The incorporation of 10% Spirulina affected negatively growth performance, with a 9.1% decrease on final weight, a 14.2% decrease on ADG and a 11.0% increase on FCR, relatively to the control group. All diets used in this study provide required CP and total essential amino acids levels according to the specific requirements of the species (NRC, 2012). Therefore, no deficit in these nutrients was expected, so as to justify the lower growth performance of piglets receiving Spirulina diets. As a supplement, Nedeva et al. (2014) tested the inclusion of 0.15% and 0.2% of Spirulina to piglet diets (from 12.2–12.5 to 30.9–33.9 kg LW) and found a significant increase on growth, while FCR decreased. Conversely, Grinstead

et al. (2000) studied the zootechnical parameters in weaned piglets fed 0.2%, 0.5% and 2% of Spirulina for 28 days and the results were inconsistent, with minimal improvement on growth performance. This was possibly due to the circumstance that these two studies considered the incorporation of Spirulina only as a supplement, in incorporation levels not high enough to cause a negative effect on growth performance of piglets, as observed in our study. However, Simkus et al. (2013) reported that ADG in fattening pigs fed Spirulina may increase up to 15%–26%, with no effect on back-fat thickness. The supplementation with the exogenous enzymes did not improve growth performance because no differences were detected between SP, SP+R and SP+L dietary groups.

The results of TTAD for CP herein reported clearly indicate that microalgae proteins, at this incorporation level, were not extensively and adequately digested by the piglets. These results, and the lower TTAD for energy on diets SP and SP+L, are in agreement with the piglet's growth performance as the obtained values were higher for the control group, by comparison to the others. It is noticeable that the SP+L group had comparable (DM and energy TTAD) or higher (CF and ADF TTAD) values than the ones observed in the control group. Comparing the SP and SP+L groups, the improvement of TTAD of DM in the SP+L group is justified by the increase in TTAD of CF, ADF and energy. This indicates that lysozyme was effective in degrading the Spirulina cell wall, thus facilitating the access of digestive enzymes to the cell content. However, Spirulina's protein fraction seems to be resistant to the action of piglet endogenous peptidases, thus justifying the lower TTAD of CP to SP, SP+R and SP+L groups. Moreover, the fermentation of Spirulina cell wall in the hindgut may increase the excretion of bacterial protein in faeces and reduce the TTAD of CP (Schulze et al. 1995).

Lower protein digestibility is associated with higher digesta viscosity, which limits the access of the endogenous enzymes to their target substrates. In turn, the higher digesta viscosity observed in piglets receiving Spirulina led to a compensatory small intestine enlargement. In fact, results indicate that the increase in digesta viscosity is not a consequence of the presence of soluble polysaccharides, such as arabinoxylans and β-glucans, since the presence of xylanases and β-glucanases in the SP+R group had no effect on viscosity. Thus, the high increase in digesta viscosity of piglets fed with microalga is likely a result of gelation of the low-digestible Spirulina proteins, as suggested by Evans et al. (2015). In fact, these authors suggested that when Spirulina is incorporated in poultry diets at high levels (>10%), it is observed a gelation of its proteins, which contributes to increased digesta viscosity and reduced amino acid digestibility. The degradation of Spirulina cell wall by lysozyme, in piglets of the SP+L group, led to a higher release of microalga proteins, increasing digesta viscosity, but also to a higher ADF degradation, decreasing digesta viscosity. Overall, data suggest that a high proportion of Spirulina proteins are resistant to the proteolytic action of piglet endogenous peptidases.

Regarding meat colour parameters, groups fed with Spirulina and exogenous enzymes had a lighter, redder and yellower meat than the meat of the group fed with the control diet. In addition, meat from SP and SP+L groups had higher scores of tenderness and flavour, indicating that Spirulina incorporation had no negative effect on meat flavour.

TBARS values of *longissimus lumborum* muscle at 3 days of storage at 4°C were higher for SP group, by 32.6%, in comparison with the control group. The results on the oxidative stability of meat did not reflect the antioxidant activity of Spirulina, reported by some authors (Hoseini et al. 2013; Gutiérrez-Salmeán et al. 2015), because no protective effect against lipid oxidation was observed in pork during the 7 days of storage. However, the higher content of antioxidant pigments in diets incorporated with Spirulina, like β-carotene, would lead us to predict a noticeable effect on meat antioxidants. In fact, this content of β-carotene was not significantly different in meat (Table 3.7). Moreover, the major individual FA were not affected by the diets. The higher concentrations of 18:3*n*-6 observed in meat of Spirulina groups can putatively be explained by its predominance in Spirulina, as previously suggested elsewhere (Gutiérrez-Salmeán et al. 2015).

Concerning pigment contents, the meat of SP+L group had an increase of 79.6%, 99.1%, 91.4%, 87.8% and 107% regarding chlorophyll *a*, chlorophyll *b*, total chlorophylls, total carotenoids and total pheophytins, respectively, and by comparison to the control group. SP+L diet had the higher pigment contents when compared to the other diets, which could possibly explain such an increase observed in meat. By comparison to the control group, meat of the other groups had an average increase of 62.7% in total carotenoids. This is related to the fact that these diets had five times more carotenoids than the control diet.

Simkus et al. (2013) reported that crossbreds of Landrace and Yorkshire fattening pigs fed daily 2 g of 75% humidity fresh Spirulina had no effect on pork traits, such as colour, pH, cooking loss and tenderness. Similarly, in our experiment most of the pork traits were also not affected by the Spirulina incorporation, indicating that meat of pigs fed with Spirulina has similar properties to those of animals fed on standard diets, such as the control used in this study.

3.5. Conclusions

The results in this study clearly show, for the first time, the feasibility of the use of Spirulina as an alternative feedstuff in piglet feeding. Nevertheless, there are clear losses in production parameters by comparison to diets including standard proteinaceous feedstuffs. Indeed, the study indicates that growth performance of *post*-weaning piglets was diminished by the incorporation of 10% Spirulina in diets. Such decrease in animal performance was due to the low digestibility and gelation of Spirulina proteins in the intestine, as a direct consequence of their proteolytic resistance to the piglet endogenous peptidases. In addition,

the use of carbohydrases in the feed does not improve the digestive utilization of this microalga by piglets, in our experimental conditions. However, the effectiveness of lysozyme in the degradation of Spirulina cell wall in the piglet's intestine, with the consequent liberation of nutritional compounds, is an important outcome of this work. This finding warrants further studies that may ultimately contribute to a better digestibility of dietary Spirulina. In general, meat quality traits are not negatively affected by the addition of Spirulina, alone and combined with enzymes, to the piglet's feeding.

Further research should be conducted to assess the effect of exogenous peptidases (E.C. 3.4), most likely from marine organisms, to improve the digestibility of proteins of this microalga. In line with this, *in vitro* studies will be performed in the near future to find the best endopeptidase candidate to degrade Spirulina proteins and avoid their gelation. Later on, the supplementation of piglet diets with the selected endopeptidase, combined or not with lysozyme, will be tested in order to make effective the use of Spirulina in swine nutrition.

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Chapter 4 – DIETARY *ARTHROSPIRA PLATENSIS* IMPROVES SYSTEMIC ANTIOXIDANT POTENTIAL AND CHANGES PLASMA LIPIDS WITHOUT AFFECTING RELATED HEPATIC METABOLIC PATHWAYS IN *POST*-WEANED PIGLETS

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Contribution of Cátia Martins to this paper:
Cátia Martins performed the animal trial and sampling. Furthermore, collaborated in the interpretation of results.

Abstract

The ability of a high level of dietary *Arthrospira platensis*, individually or in combination with two exogenous carbohydrate-degrading enzymes (lysozyme and Rovabio® Excel AP), to improve systemic antioxidant potential and hepatic lipid metabolism was tested in piglets. Forty male *post*-weaned piglets, sons of Large White × Landrace sows crossed with Pietrain boars, were allocated into 4 groups (n = 10) and fed during 28 days one of the following diets: 1) a control basal diet (cereal and soybean meal); 2) a basal diet with 10% of *A. platensis* (SP); 3) the SP diet supplemented with 0.005% of Rovabio® Excel AP (SP+R); 4) the AP diet supplemented with 0.01% of lysozyme (SP+L). *Arthrospira platensis* decreased BW gain of piglets, regardless the addition of feed enzymes. The majority of plasma metabolites were affected by diets. *A. platensis* increased total lipids, total cholesterol and low-density lipoprotein (LDL)-cholesterol, without changing hepatic fatty acid content or modulating, in an expressive manner, the transcriptional profile of lipid sensitive mediators. The antioxidant potential in general, and total carotenoids in particular, were improved by the microalga, regardless lysozyme or Rovabio® Excel AP. Summing up, *A. platensis*, individually and combined with feed enzymes, impacts negatively on piglets' growth but improves the systemic antioxidant potential and changes plasma lipids with a minor modulation on related hepatic metabolic pathways.

Keywords: *Arthrospira platensis*, enzymes, antioxidant potential, hepatic lipid metabolism, piglets

4.1. Introduction

In the pig industry, feed is a paramount topic. In the past two decades, there has been a major investment on the development of pig nutrition and on the improvement of meat quality to satisfy consumers' demands. Cereal grains and soybean are the main energy and protein sources, respectively, for pig diets (FAO, 2004). The high economic and environmental costs associated with production and transport of these ingredients over large distances and their direct competition with human consumption have important implications for the sustainability of feed and animal production (Manceron et al. 2014). Therefore, alternative sources of protein and well-balanced amino acids are urgently needed, as are sources of essential *n*-3 LC-PUFA, vitamins, minerals, carotenoids and bioactive compounds in animal feeding (Florou-Paneri et al. 2014).

The use of microalgae in feed and food represents a promising strategy to solve this problem because microalgae are a natural resource with recognized beneficial health implications for both animals and humans (Calder, 2012). Marine autotrophic microalgae bear attractive properties for sustainable animal production (Lum et al. 2013). Although the nutritional profiles of microalgae differ substantially with the species, the majority is characterized by protein, carbohydrate, and lipid contents that are comparable, if not superior, to conventional feedstuffs (Madeira et al. 2017). In line with this, *Arthrospira* is a genus of these microalgae, characterized by cylindrical, multicellular trichomes in an open left-hand helix (reviewed by Madeira et al. 2017). *Arthrospira platensis* in particular, formerly known as Spirulina, is a rich source of organic nutrients with balanced content of vitamins, minerals, amino acids (Simkus et al. 2013) and essential PUFA (Peiretti and Meineri 2011), as well as carotenoids and chlorophyll pigments with known antioxidants activity (Madhava et al. 2000). However, the microalga cell wall is recalcitrant, with a limited digestion and use by monogastrics (Tibbetts, 2018).

Besides being poorly understood, the microalga cell wall has rigid components embedded within a plastic polymeric matrix, containing cellulose and, in some species, an additional tri-laminar sheath with algaenan, which is a compound that confers resistance to enzymatic degradation (Popper and Tuohy 2010; Gerken et al. 2013). In this respect, CAZymes that lyse the complex polysaccharides of the cell wall may be advantageous in the feed industry to improve nutrient utilization of microalgae (Sander and Murthy 2009). In this respect, lysozyme is an enzyme that cleaves the peptidoglycan of prokaryote cell walls (Oliver and Wells 2015), thus promoting a better exposure of proteins and pigments to the endogenous repertoire of digestive enzymes (Al-Zuhair et al. 2016). Also, a commercial mixture of carbohydrate-degrading enzymes, like Rovabio® Excel AP, can improve the profitableutilization of feed ingredients (Gunawardana et al. 2009).

In spite of being sustainable alternatives to conventional ingredients for animal feeding, the effect of microalgae on the hepatic metabolism and redox status of monogastric species is currently too limited. In particular, the information available on the pattern of genes encoding for key lipogenic and lipolytic enzymes and associated transcription factors is urgently needed because these factors determine the rates of de novo fatty acid biosynthesis, fat uptake from blood and transport of FA and lipid degradation (Zhao et al. 2010). This knowledge could help to improve the feeding strategies of pigs to address the swine industry needs and consumers' demands. In line with this, we hypothesized that high levels of *A. platensis* incorporation in the diet, likely in association with exogenous CAZymes (lysozyme or Rovabio® Excel AP), improve the antioxidant potential and change lipid metabolism in pigs, through the modulation of hepatic related metabolic pathways.

4.2. Material and Methods

4.2.1. Animals and experimental diets

All the procedures used were reviewed by the Ethics Commission of Instituto Superior de Agronomia (ISA) and approved by the Animal Care Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal), following the European Union legislation (2010/63/EU Directive).

The experimental trial was conducted at the facilities of ISA, Universidade de Lisboa. Forty male *post*-weaned piglets from Large White × Landrace sows crossed with Pietrain boars, weaned at 28 days of age and with an initial live weight of 12.0 ± 0.89 kg were obtained with consent from a commercial farm. After an adaptation period of two days, piglets were evenly distributed into four homogeneous groups of 10 piglets each (calculation of sample size by power analysis) and randomly individually allocated in pens (1.9 × 1.1 m), equipped with one stainless steel bowl drinker with nipple, one creep feeder and a modular plastic slatted floor. The room was environmentally controlled with air ventilation, as described by Correia et al. (2017). Piglets had *ad libitum* access to feed and water. Throughout the experiment, the supplied feed was recorded daily, whereas refusals and piglets were weighed weekly, just before feeding, in order to calculate ADFI, ADG and FCR. Each group received one of the four experimental diets: 1) cereal and soybean meal based diet (control); 2) basal diet with 10% of *A. platensis* (SP); 3) basal diet with 10% of *A. platensis* supplemented with 0.005% of Rovabio® Excel AP (Adisseo, Antony, France) (SP+R); 4) basal diet with 10% of *A. platensis* supplemented with 0.01% of lysozyme (62,971, Sigma-Aldrich Ltd., St. Louis, MO, USA) (SP+L). Freeze-dried *A. platensis* powder was obtained from Sopropeche (Wimille, France). Rovabio® Excel AP was composed by endo–1,4-β-xilanase 22,000 viscosity units/ g and endo-

1,3(4)-β-glucanase 30,000 viscosity units/g. The ingredients and feed additives of the experimental diets are described in Table 4.1.

Diets were analysed for DM, ash and CP (automated Kjeldahl method), CF, NDF and ADF contents, following AOAC (2000) methods. FAME of the experimental diets were analysed by one-step extraction and transesterification, using heneicosaenoic acid (21:0) methyl ester as the internal standard (Sukhija and Palmquist, 1988). The pigments of diets were measured according to Teimouri et al. (2013), with slight modifications. Briefly, the samples were extracted with acetone and stored under agitation overnight, then centrifuged at 4000 rpm for 5 min and measured by UV-Vis spectrophotometry (Ultrospec 3100 pro, Amersham Biosciences, Little Chalfont, UK). The pigment content was quantified according to Hynstova et al. (2018). The quantification of tocopherols and tocotrienols in the diets involved a direct saponification, a single n-hexane extraction and analysis of the extracted compounds by normal-phase HPLC using fluorescence detection, as described by Prates et al. (2006). The chemical composition, FA and pigments contents of the experimental diets are shown in Table 4.1.

Table 4.1. Ingredients and detailed chemical composition of the experimental diets.

	Diets			
	Control	**SP**	**SP+R**	**SP+L**
Ingredients (g/kg, as fed basis)				
Wheat	439	460	460	460
Corn	150	170	170	170
Soybean meal 48	250	110	110	110
Whey powder	100	100	100	100
Sunflower oil	30	30	30	30
Spirulina	0	100	100	100
Rovabio® Excel AP	–	–	0.050	–
Lysozyme	–	–	–	0.100
L-lysine	5	6	6	6
DL-methionine	1	1	1	1
L-threonine	1	–	–	–
Calcium carbonate	5	6	6	6
Dicalcium phosphate	13	12	12	12
Sodium chloride	3	2	2	2
Vitamin-mineral complex[1]	3	3	3	3

Metabolizable energy (kcal/kg DM)[2]	3,738	3,809	3,789	3,818
Chemical composition (g/100 g, as fed basis)				
DM	89.8	90.0	90.0	90.0
CP	17.9	18.1	17.9	17.8
NDF	14.6	11.9	11.9	11.8
ADF	4.21	3.97	4.06	3.94
Ash	5.11	4.76	4.89	4.75
Crude fat	5.28	5.62	5.87	5.80
Fatty acid composition (% total FA)				
12:0	0.123	0.122	0.146	0.140
14:0	0.396	0.479	0.520	0.531
16:0	13.5	17.8	18.3	19.3
16:1*c*9	0.140	0.817	0.875	0.836
18:0	3.17	3.19	3.32	3.62
18:1*c*9	24.1	21.1	20.5	20.1
18:1*c*11	1.30	1.23	1.28	1.27
18:2*n*-6	48.6	43.1	42.0	39.1
18:3*n*-3	4.55	4.17	4.31	4.22
20:0	0.364	0.325	0.324	0.323
20:1*c*11	0.298	0.572	0.559	0.820
22:0	0.365	0.365	0.365	0.421
Pigments (µg/g)				
Chlorophyll *a*[3]	2.70	108	112	132
Chlorophyll *b*[4]	4.97	14.6	13.0	17.5
Total chlorophylls[5]	7.67	122	125	149
Total carotenoids[6]	2.41	11.6	12.6	13.0
Total chlorophylls and total carotenoids[7]	10.1	134	138	162
Diterpene profile (µg/g)				
β-Carotene	0.160	3.57	3.14	2.15
α-Tocopherol	7.41	12.0	12.3	12.9
β-Tocopherol	0.676	0.254	0.215	0.213
γ-Tocopherol	1.05	0.997	1.05	0.925
α-Tocotrienol	1.09	0.504	0.923	0.994

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* -SP; basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP - SP+R; basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme - SP+L.
[1]Premix provided per kg of complete diet: vitamin A, 6500 UI; vitamin D3, 1500 UI; vitamin E, 15 mg; vitamin K3, 1 mg; vitamin B1, 1 mg; vitamin B2, 3 mg; vitamin B6, 2 mg; vitamin B12, 0.02 mg; pantothenic acid, 10mg; nicotinic acid, 15 mg; folic acid, 0.5 mg, biotin, 0.03 mg; betaine, 115 mg; vitamin C, 20 mg; Copper, 100 mg; iron, 100 mg; iodine, 0.5 mg; manganese 50 mg; selenium, 0.15 mg; zinc, 100 mg; butylated hydroxytoluene, 3 mg.
[2]Metabolizable energy (kcal/kg DM) = 4412–11,06 × Ash (g/kg DM) + 3,37 × Crude Fat (g/kg DM) − 5,18 × ADF (g/kg DM).
[3]Chlorophyll *a* = 11.24 × A662 nm - 2.04 × A645 nm.
[4]Chlorophyll *b* = 20.13 × A645 nm - 4.19 × A662 nm.
[5]Total chlorophylls (Ca + b) = 7.05 × A662 nm + 18.09 × A645 nm.
[6]Total carotenoids (Cx + c) = (1000 × A470 nm - 1.90 × Ca - 63.14 × Cb)/214.
[7]Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

4.2.2. Slaughter and sampling

After an experimental period of 28 days, during which no sick or dead animals were recorded, piglets were slaughtered using electrical stunning followed by exsanguination, according to commercial abattoirs standard procedures. Blood was collected from the jugular vein and centrifuged at 1500 g for 15 min to obtain plasma. Samples for gene expression analysis were collected from the middle lobe of liver, rinsed with sterile RNAse-free cold saline solution, cut into small pieces, stabilized in RNA Later® solution (Qiagen, Hilden, Germany) and stored at − 80 °C. For fatty acid composition and pigments, liver samples were vacuum packed and stored at − 20 °C, until analysis.

4.2.3. Plasma metabolites

Total cholesterol, high-density lipoprotein (HDL)-cholesterol, LDL-cholesterol, triacylglycerols (TAG), phospholipids, total protein, urea, creatinine and glucose concentrations, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT) were analysed in a Modular Hitachi Analytical System (Roche Diagnostics, Mannheim, Germany), through diagnostic kits (Roche Diagnostics). Very Low-Density Lipoproteins (VLDL)-cholesterol and total lipids were calculated, according to Friedewald et al. (1972) and Covaci et al. (2006) formulas, respectively. The immunoglobulins profile (IgA, IgG and IgM) was determined by immunoturbidimetry. Total antioxidant capacity (TAC) was determined using the Quanti-ChromTM Antioxidant Assay Kit (DTAC-100, Bioassay Systems, Hayward, CA, USA). Glutathione peroxidase (GPX) activity was determined using the EnzyChromTM Glutathione Peroxidase Assay Kit (EGPX-100, Bioassay Systems). One unit of GPX is the amount of GPX that produces 1 µmol of GS-SG per min at pH = 7.6 and room temperature.

4.2.4. Hepatic lipid extraction and fatty acid composition

After liver samples lyophilisation (− 60 °C and 2.0 hPa), total lipids were extracted in duplicate and gravimetrically measured by the Folch et al. (1957) method, using dichloromethane and methanol (2:1 v/v), as reported by Carlson (1985). Fatty acids were converted to FAME by a combined transesterification procedure using NaOH in anhydrous methanol (0.5 M), followed by HCl:methanol (1:1 v/v), at 50 °C during 30 and 10 min, respectively, in accordance to Raes et al. (2001). FAME were determined using a gas chromatograph HP6890A (Hewlett–Packard, PA, USA), with a flame ionization detector (FID) and a CP-Sil 88 capillary column (100 m, 0.25mm i.d., 0.20 µm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), using the conditions described in Alves and Bessa (2009). The quantification of total FAME was carried out using heneicosaenoic acid (21:0) as internal standard and on the conversion of relative peak areas into weight percentages. Fatty acids were identified according to their retention times, corresponding to their FAME standards from Supelco Inc. (Bellefonte, PA, USA) and expressed as g/100 g of total FA.

4.2.5. Determination of total cholesterol and diterpenes in the liver

The simultaneous analysis of total cholesterol and tocopherols in liver samples (0.75 g) was performed, according to Prates et al. (2006). After the direct saponification of samples, an aliquot of the n-hexane layer was filtered and injected into an HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, U.S.A.), using a normal-phase silica column (Zorbax RX-Sil, 250mm×4.6mm i.d., 5 µm particle size, Agilent Technologies Inc., Palo Alto, CA, U.S.A.), with fluorescence detection of tocopherols (excitation wavelength of 295 nm and emission wavelength of 325 nm) and UV–Vis photodiode array detection of cholesterol (202 nm). Total cholesterol and tocopherols contents were calculated in duplicate, based on the external standard technique from a standard curve of peak area *vs*. concentration.

4.2.6. Determination of pigments in the liver

The contents of chlorophyll *a*, chlorophyll *b* and total carotenoids were measured following the procedure of Teimouri et al. (2013), with minor modifications. For the pigment determination, 10 mL of acetone (Merck KGaA, Darmstadt, Germany) was added to 1 g of fresh liver or 0.5 g of feed, then incubated at room temperature and shaken in the dark overnight. After extraction, the samples were centrifuged at 1500 g for 5 min and measured using a UV-Vis spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, Little Chalfont, UK). All procedures associated with pigments extraction and analyses were carried

out in dim light because pigments are photosensitive. The pigment content was calculated, according to Hynstova et al. (2018).

4.2.7. Hepatic RNA isolation and complementary DNA synthesis

Total hepatic RNA was extracted and purified using Trizol (Invitrogen, CA, USA) and RNeasy mini kit (Qiagen), respectively, from small pieces of liver tissue (thickness of about 0.3 cm). Before running the RT-PCR, RNA samples were subjected to DNAse I (Qiagen) treatment. All procedures followed the manufacturer's instructions, according to Madeira, Pires et al. (2013). The quantification of RNA was carried out using a spectrophotometer (Nanodrop ND-2000c, NanoDrop, Thermo Fisher Scientific, Willmington, DE, USA). The A260/280 ratios ranged between 1.9 and 2.1. Ethidium bromide staining of 18S and 28S ribosomal bands was used to verify the sample integrity.The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was applied for reverse transcription. Each 20 μL RT reaction included 1 μg of DNase-treated total RNA template, 50 nM random RT Primer, 1 × RT buffer, 0.25 mM of each dNTP, 3.33 U μL-1 multiscribe reverse transcriptase and 0.25 U μL-1 RNase inhibitor, during 10 min for 25 °C, 120 min for 37 °C and 5 min for 85 °C. The cDNA obtained was separated into several aliquots and kept at −20 °C, until analysis.

4.2.8. Real-time quantitative PCR of hepatic genes

Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/) and Primer Express Software v. 2.0 (Applied Biosystems) based on *Sus scrofa* sequences (www.ncbi.nlm.nih.gov) were used for gene specific intron-spanning primers design, as described by Madeira, Pires et al. (2013). The selected primers were acquired from NZYTech (Lisbon, Portugal) and matched only the sequence to which they were constructed. To guarantee maximum DNA polymerization efficiency, the amplicon length ranged between 67 and 190 bp. Prior qPCR experiments, a conventional PCR was performed for all genes to confirm the amplified fragments. To corroborate the amplification, the products of PCR were sequenced and homology searches were checked with Blast (www.ncbi.nlm.nih.gov/blast). GeNorm30 and NormFinder31 software packages were applied for the analysis of the expression level stability of housekeeping genes. The most stable pair internal controls for normalization were RPLP0 and RPL27 genes. The gene specific primer sequences used for RT-qPCR are shown in Table 4.2. The efficiency of PCR for each amplicon was calculated with the StepOnePlus PCR System software (Applied Biosystems), by amplifying 5× serial dilutions of pooled cDNA and run 3×. All primer sets exhibited an efficiency ranging from 90 to 110% and correlation coefficients

were over 0.99. qPCR reactions were carried out using the MicroAmp Optical 96-well plates (Applied Biosystems) in a StepOnePlus thermocycler (Applied Biosystems) in standard cycling conditions. The 12.5 µL PCR reaction mixture included 6.25 µL of 2 × Power SYBR Green PCR Master Mix (Applied Biosystems), 160 nM of forward and reverse primers, and 2 µL of diluted cDNA as template. No transcription and no template samples were applied as controls. The primer specificity and the formation of primer-dimers were verified by melt curve analysis and agarose gel electrophoresis. All analyses were performed in duplicate, and the relative amounts for each target gene were calculated using the geometric mean of RPLP0/RPL27 as normaliser. The relative gene expression levels were calculated using the Livak and Schmittgen (2001) method, corrected for variation in amplification efficiency, as proposed by Fleige et al. (2006).

Table 4.2. Gene specific primer sequences used for RT-qPCR.

Gene symbol	Full gene name	GenBank accession number	Forward primer	Reverse primer	Product size (bp)
ACACA	Acetyl-CoA carboxylase alpha	NM_001114269.1	ggccatcaaggacttcaacc	acgatgtaagcgccgaactt	120
APOA5	Apolipoprotein A-V	NM_001159308.1	agggaaaggcttctgggacta	tgtctttcagtctcgtgggctc	107
CAT	Catalase	NM_214301.2	agaggaaacgcctgtgtgag	ttgtccagaagagcctgaatg	133
CEBPA	CCAAT/enhancer binding protein (C/EBP) alpha	XM_003127015.2	ggccagcacacacacattaga	cccccaaagaagagaaccaag	71
ChREBP	MLX interacting protein-like	XM_003481002.2	tgacatgatccagcctgacc	gggggctcagagaagtttga	126
CPT1A	Carnitine palmitoyltransferase 1A	NM_001129805.1	cgattatccaccagccagac	caccccataaccatcgtcag	120
CRAT	Carnitine O-acetyltransferase	NM_001113047.1	ggcccaccgagcctacac	atggcgatggcgtaggag	138
DGAT	Diacylglycerol acyltransferase	NM_214051.1	caactaccgtggcatcctga	tagaaacagccgtgcattgc	67
FABP1	Fatty acid binding protein 1	NM_001004046.1	aacttctccggcaaataccaa	attctgcacgatttccgatg	129
FADS1	Fatty acid desaturase 1	NM_001113041.1	gtgggtggacttggcctg	gatgtgcatgggatgtggt	166
FADS2	Fatty acid desaturase 2	NM_001171750.1	gccttacaaccaccagcatga	aggccaagtccaccccagtc	122
FASN	Fatty acid synthase	NM_001099930.1	acaccttcgtgctggcctac	atgtcggtgaactgctgcac	112

GPX1	Glutathione peroxidase 1	NM_214201.1	ggagatcctgaattgcctca	gataaacttggggtcggtca	181
GSR	Glutathione-disulfide reductase	XM_003483635.4	ggtgtgtgccaacaaagagg	aaccctgcagcagcatttcatca	77
HSL	Hormone sensitive lipase	397,583	tcgtggctcaactccttcct	gggtgtcctgtgtctcgg	190
LPIN1	Lipin 1	NM_001130734.1	aagtcgccgccctgtatttc	ttgtcgctggcctgtttttgt	67
NOS2	Nitric oxide synthase 2	NM_001143690.1	cctggtgccctgcttttgt	ctgccagaaactgcggaag	118
NOS3	Nitric oxide synthase 3	NM_214295.1	ggctgcatgacattgagagc	ctcgtcgcggtagagatggt	98
PFKL	Phosphofructokinase liver	100,621,757	gctcaaggaggacaccgact	cgccagcatcttcagcat	85
PLIN2	Perilipin 2	NM_214200.2	catgtccggtgctctcccta	cccagtcacagcccctttag	160
PPARA	Peroxisome proliferator-activated receptor alpha	NM_001044526.1	tttccctctttgtggctgct	ggggtggttggtctgcaag	128
SCD	Stearoyl-CoA desaturase	NM_213781.1	agccgagaagctggtgatgt	gaagaaaggtggcgacgaac	140
SOD1	Superoxide dismutase 1	NM_001190422.1	gctgtaccagrgcaggtcctc	cacagtggccacaccatctt	125
SOD2	Superoxide dismutase 2	NM_214127.2	gtggaggccacatcaatcat	ccgacagatacagcggtcaa	148
SOD3	Superoxide dismutase 3	NM_001078688.1	accagttcggggacctgag	ggcgaagttgccgaagtct	104
SREBF1	Sterol regulatory element binding transcription factor 1	NM_214157.1	gtgctggcggaggtctatgt	aggaagaagcgggtcagaaag	86
Housekeeping genes					
RPL90	Ribosomal phosphoprotein large P0 subunit	NM_001098598.1	tccaggctttaggcatcacc	ggctcccactttgtctccag	95
RPL27	Ribosomal protein L27	NM_001097479.1	gtactccgtggatattg	aacttgaccttggcct	102

4.2.9. Statistical analysis

Data were checked for normal distribution by Shapiro-Wilk test and for variance homogeneity by *Chi-Square* test. Data were analysed using the Generalized Linear Mixed (GLM) model of SAS program (SAS Institute Inc., Cary, NC) (1989) considering the piglet as experimental unit. Significant multiple comparisons test was carried out using the PDIFF option adjusted with Tukey-Kramer to determine statistical differences among dietary treatments. The level of significance was set at $p < 0.05$. A principal component analysis (PCA) was performed with individual plasma metabolites, hepatic markers and immunoglobulins from piglets. The PRIN COMP procedure was applied to a data set of 40 samples and 18 variables to reduce the dimensionality of the data set and to describe the variability of data into two dimensions. After data normalization, the principal components were considered significant if they contributed more than 5% for the total variance.

4.3. Results

4.3.1. Growth performance parameters

Data on piglets' growth performance are shown in Table 4.3. Piglets fed *A. platensis* had lower final body weight ($p = 0.009$) and ADG ($p = 0.01$) than piglets fed the control diet, but a higher FCR ($p < 0.001$). The ADFI was not affected by dietary treatments ($p > 0.05$).

Table 4.3. Effect of *Arthrospira platensis*, individually or combined with exogenous CAZymes, on growth performance parameters of piglets.

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
Initial weight (kg)	12.1	11.7	12.1	11.9	0.15	0.808
Final weight (kg)	31.0[b]	28.3[a]	28.4[a]	27.8[a]	0.40	0.009
ADFI (g)[1]	997	960	943	960	12.8	0.521
ADG (g)[2]	677[a]	593[b]	582[b]	567[b]	12.4	0.001
FCR[3]	1.48[a]	1.62[b]	1.62[b]	1.69[b]	0.023	< 0.001

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* -SP; basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP - SP+R; basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme - SP+L.
[1]ADFI - average daily feed intake.
[2]ADG - average daily weight gain.
[3]FCR - feed conversion ratio.
[a,b]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$)

4.3.2. Plasma biochemical profile

Plasma metabolites of piglets fed *A. platensis*, alone or combined with feed enzymes, are presented in Table 4.4. Total lipids ($p = 0.011$), total cholesterol ($p < 0.001$) and LDL-cholesterol ($p < 0.001$) were increased in piglets fed *A. platensis* individually. Piglets fed SP+R had higher HDL-cholesterol levels ($p < 0.001$) than the ones fed SP+L and control diets. These changes resulted in a lower total cholesterol: HDL-cholesterol ratio in the SP+R group in relation to SP ($p = 0.033$). SP diet increased TAG ($p < 0.001$) when compared to SP+R. Total protein was lower ($p < 0.001$) in piglets fed *A. platensis* individually when compared to the other diets. SP+L increased the contents of glucose ($p < 0.001$) and creatinine ($p < 0.001$) relative to the other diets. Regarding the hepatic markers, *A. platensis* individually and combined with exogenous enzymes increased ALT ($p < 0.001$), while AST ($p < 0.001$) and ALP ($p < 0.001$) were increased in piglets fed the exogenous enzymes. GGT was decreased ($p < 0.001$) by *A. platensis* individually and combined with feed enzymes. Concerning the immunoglobulins, SP diet increased IgM ($p < 0.001$), whereas SP+L diet decreased IgG ($p < 0.001$) levels. In addition, SP+R diet decreased IgM concentrations ($p < 0.001$) when compared to the control diet.

Table 4.4. Effect of *Arthrospira platensis*, individually or combined with exogenous CAZymes, on plasma metabolites of piglets.

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
Plasma metabolites						
Total lipids (mg/L)[1]	3227[a]	3557[b]	3352[ab]	3466[ab]	69.0	0.011
TAG (mg/L)	433[ab]	497[b]	352[a]	524[b]	27.0	< 0.001
Total cholesterol (mg/L)	647[a]	780[b]	750[ab]	721[ab]	28.9	< 0.001
HDL-cholesterol (mg/L)	291[a]	338[bc]	359[c]	315[ab]	9.98	< 0.001
LDL-cholesterol (mg/L)	376[a]	467[b]	393[a]	417[ab]	18.4	0.009
VLDL-cholesterol (mg/L)[2]	86.7[ab]	99.3[b]	70.4[a]	104.8[b]	5.40	< 0.001
Total cholesterol/HDL-C	2.24[ab]	2.31[b]	2.08[a]	2.29[ab]	0.059	0.033
Glucose (mg/L)	1228[a]	1358[b]	1278[ab]	1525[c]	298	< 0.001
Urea (mg/L)	153	168	164	182	7.32	0.058
Creatinine (mg/L)	8.77[ab]	8.52[a]	9.41[c]	9.08[bc]	0.100	< 0.001
Total protein (g/L)	50.8[b]	44.0[a]	48.9[b]	50.1[b]	5.03	< 0.001
Plasma hepatic markers						
ALT (U/L)	36.0[a]	47.2[b]	58.9[c]	47.0[b]	1.21	< 0.001
AST (U/L)	40.4[a]	59.2[ab]	63.9[bc]	81.8[c]	5.08	< 0.001
ALP (U/L)	195[a]	213[a]	241[b]	267[b]	7.19	< 0.001
GGT (U/L)	42.2[b]	24.2[a]	23.4[a]	27.9[a]	1.47	< 0.001
Immunoglobulins						
IgA (mg/L)	23.3	26.7	24.4	25.0	2.23	0.761
IgG (mg/L)	1899[b]	2003[b]	1921[b]	1400[a]	95.4	< 0.001
IgM (mg/L)	484[b]	579[c]	337[a]	458[b]	14.6	< 0.001

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* -SP; basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP - SP+R; basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme - SP+L.

ALT, alanine aminotransferase (E.C. 2.6.1.2); AST, aspartate aminotransferase (E.C. 2.6.1.1); ALP, alkaline phosphatase (E.C. 3.1.3.1); GGT, gamma-glutamyltransferase (E.C. 2.3.2.13).

[1]Total lipids = [total cholesterol] × 1.12 + [TAG] × 1.33 + 148.

[2]VLDL-cholesterol = 1/5 [TAG].

[a,b,c]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).

4.3.3. Plasma antioxidant potential

The variations on plasma TAC and GPX activity from piglets fed *A. platensis* with or without CAZymes are presented in Figure 4.1. *A. platensis* individually and combined with exogenous enzymes increased TAC levels ($p < 0.001$) when compared to the control diet. GPX remained unchanged by dietary treatments ($p = 0.112$).

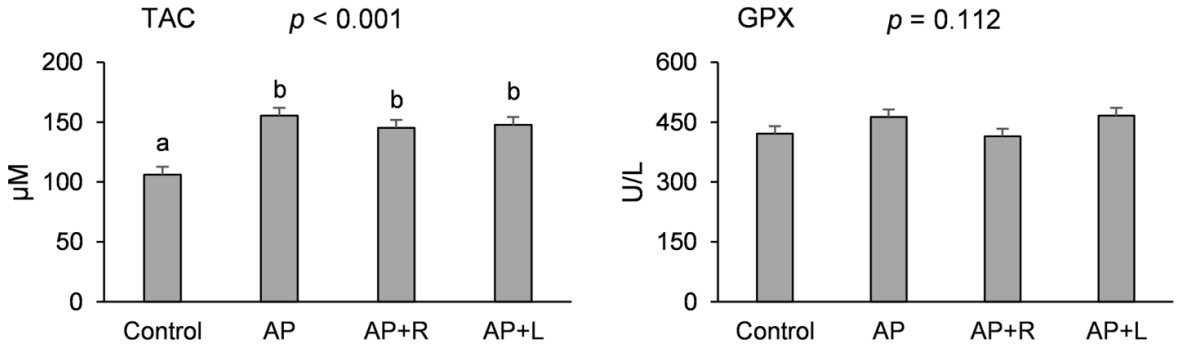

Figure 4.1. Effect of *Arthrospira platensis*, individually or combined with exogenous CAZymes, on plasma total antioxidant capacity (TAC) and glutathione peroxidase (GPX) activity.
One unit of GPX is the amount of GPX that produces 1 µmol of GS-SG per min at pH = 7.6 and room temperature. Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* (AP = SP); basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP (AP+R = SP+R); basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme (AP+L = SP+L).
[a,b]Mean values with unlike letters are significantly different ($p < 0.05$).

4.3.4. Hepatic total lipids and fatty acid composition

Hepatic lipid content and fatty acid composition of piglets fed *A. platensis*, individually or in combination with exogenous CAZymes, are presented in Table 4.5. Total lipid content ($p = 0.977$) and cholesterol ($p = 0.737$) were not affected by dietary treatments. The predominant FA found in liver were: 18:0 (29.1–31.1%), 18:2*n*-6 (16.5–17.8%), 16:0 (13.5–15.3%), 20:4*n*-6 (13.2–14.9%) and 18:1*c*9 (10–11.7% of total FAME). The dietary treatments affected 8 out of 27 FA identified. The proportion of 10:0 ($p = 0.027$) was higher in piglets fed SP+L diet when compared to the control diet. SP+R diet increased 16:0 ($p = 0.021$) when compared to the control diet. Also, 17:0 ($p = 0.016$) increased with SP and SP+L diets when compared to the control diet. SP+R and SP+L diets increased 18:3*n*-6 ($p = 0.001$) relative to the control diet. In contrast, piglets fed SP and SP+R diets had lower 20:2n-6 ($p = 0.006$) and 22:6*n*-3 ($p = 0.005$) when compared to piglets fed the control diet. The proportion of 20:5*n*-3 ($p = 0.002$) decreased in piglets fed *A. platensis* individually and combined with feed enzymes. Regarding the fatty acid sums and ratios, *A. platensis* alone and in combination with feed CAZymes decreased *n*-3 PUFA ($p = 0.004$) amd increased *n*-6:*n*-3 ratio ($p = 0.006$) (Table 4.5).

Table 4.5. Effect of *Arthrospira platensis*, individually or combined with exogenous CAZymes, on total lipids (g/100 g liver), cholesterol (mg/g), fatty acid composition (% total FA), partial sums of FA and related ratios in piglets' liver.

	Diets				SEM	*p-value*
	Control	SP	SP+R	SP+L		
Total lipids	2.37	2.37	2.41	2.42	0.104	0.977
Cholesterol	1.61	1.53	1.51	1.61	0.074	0.737
Fatty acid composition						
10:0	0.012^{a}	0.014^{ab}	0.017^{ab}	0.025^{b}	0.003	0.027
12:0	0.009	0.013	0.014	0.015	0.002	0.057
14:0	0.172	0.200	0.261	0.235	0.024	0.064
15:0	0.143	0.155	0.141	0.162	0.013	0.595
16:0	13.5^{a}	13.9^{ab}	15.3^{b}	14.9^{ab}	0.429	0.021
16:1*c*7	0.344	0.397	0.429	0.376	0.026	0.147
16:1*c*9	0.446	0.429	0.643	0.488	0.064	0.096
17:0	1.33^{a}	1.70^{b}	1.48^{ab}	1.67^{b}	0.088	0.016
17:1*c*9	0.153	0.140	0.166	0.136	0.012	0.300
18:0	29.1	31.2	29.7	30.7	0.945	0.415
18:1*c*9	10.2	10.0	11.7	10.1	0.516	0.093
18:1*c*11	1.52	1.47	1.63	1.59	0.050	0.121
18:2*n*-6	17.8	16.6	16.6	16.7	0.454	0.163
18:3*n*-6	0.162^{a}	0.232^{ab}	0.265^{b}	0.253^{b}	0.018	0.002
18:3*n*-3	0.410	0.362	0.428	0.472	0.038	0.224
18:4*n*-3	0.020	0.020	0.019	0.025	0.002	0.215
20:0	0.051	0.059	0.051	0.054	0.004	0.596
20:1*c*11	0.149	0.116	0.126	0.124	0.011	0.161
20:2*n*-6	0.614^{b}	0.471^{a}	0.454^{a}	0.505^{ab}	0.032	0.006
20:3*n*-6	0.715	0.771	0.809	0.741	0.055	0.654
20:4*n*-6	15.0	14.7	13.2	13.7	0.758	0.333
20:3*n*-3	0.167	0.158	0.153	0.178	0.014	0.588
20:5*n*-3	0.517^{b}	0.362^{a}	0.332^{a}	0.353^{a}	0.035	0.002
22:0	0.033	0.044	0.037	0.033	0.003	0.110
22:1*n*-9	0.326	0.489	0.502	0.544	0.075	0.189
22:5*n*-3	1.84^{b}	1.49^{ab}	1.36^{ab}	1.30^{a}	0.138	0.034
22:6*n*-3	1.75^{b}	1.16^{a}	1.02^{a}	1.33^{ab}	0.140	0.005

Others	2.39	2.10	1.97	1.99	0.165	0.260
Fatty acid partial sums						
SFA[1]	44.4	47.3	47.0	47.8	1.038	0.131
MUFA[2]	13.1	13.1	15.2	13.4	0.642	0.083
PUFA[3]	39.0	36.3	34.7	35.6	1.352	0.147
n-3 PUFA[4]	4.71[b]	3.56[a]	3.30[a]	3.65[a]	0.267	0.004
n-6 PUFA[5]	34.3	32.8	31.3	31.9	1.111	0.283
Fatty acid ratios						
PUFA:SFA	0.884	0.777	0.745	0.753	0.044	0.114
n-6:*n*-3	7.41[a]	9.48[b]	9.77[b]	9.08[ab]	0.476	0.006

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* -SP; basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP - SP+R; basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme - SP+L.
[1]SFA = 10:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0.
[2]MUFA = 16:1*c*7 + 16:1*c*9 + 17:1*c*9 + 18:1*c*9 + 18:1*c*11 + 20:1*c*11 + 22:1*n*-9.
[3]PUFA = 18:2*n*-6 + 18:3*n*-6 + 18:3*n*-3 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3.
[4]*n*-3 PUFA = 18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3.
[5]*n*-6 PUFA = 18:2*n*-6 + 18:3*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6.
[a,b,c]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).

4.3.5. Hepatic tocopherols and pigments

The effect of *A. platensis* individually or combined with exogenous CAZymes on hepatic vitamin E compounds and pigments are presented in Table 4.6. α- and γ-tocopherols were affected by diets, being consistently decreased in piglets fed *A. platensis* with and without exogenous CAZymes ($p < 0.001$ and $p = 0.0003$, respectively). Conversely, piglets fed SP, SP+R and SP+L had higher total carotenoids ($p < 0.001$) than piglets fed the control diet.

Table 4.6. Effect of *Arthrospira platensis*, individually or combined with exogenous CAZymes, on α-tocopherol (µg/g), γ-tocopherol (µg/g) and pigments (µg/g) in piglets' liver.

	Diets				**SEM**	***p-value***
	Control	**SP**	**SP+R**	**SP+L**		
α-Tocopherol	1.64[b]	1.02[a]	1.16[a]	1.07[a]	0.086	< 0.001
γ-Tocopherol	0.073[b]	0.054[a]	0.050[a]	0.052[a]	0.004	0.0003
Chlorophyll *a*[1]	0.360	0.780	0.469	0.580	0.202	0.510
Chlorophyll *b*[2]	1.21	1.52	1.18	1.33	0.506	0.964
Total chlorophylls[3]	1.57	2.30	1.65	1.91	0.703	0.884
Total carotenoids[4]	0.660[a]	1.23[b]	1.28[b]	1.19[b]	0.083	< 0.001
Total chlorophylls and total carotenoids[5]	2.23	3.52	2.93	3.10	0.741	0.666

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* -SP; basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP - SP+R; basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme - SP+L.
[1]Chlorophyll *a* = 11.24 × A662 nm - 2.04 × A645 nm.
[2]Chlorophyll *b* = 20.13 × A645 nm - 4.19 × A662 nm.
[3]Total chlorophylls (Ca + b) = 7.05 × A662 nm + 18.09 × A645 nm.
[4]Total carotenoids (Cx + c) = (1000 × A470 nm - 1.90 × Ca - 63.14 × Cb) /214.
[5]Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).
[a,b]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).

4.3.6. Gene expression levels of antioxidant enzymes and lipid metabolism players in the liver

The expression level of 8 genes controlling redox balance and 18 genes regulating lipid metabolism in piglets' liver upon dependence of *A. platensis*, with or without feed enzymes, are presented in Table 4.7. For the antioxidant potential, only the transcriptional profile of nitric oxide synthase 2 (NOS2) ($p = 0.048$) was affected by diet, with higher mRNA levels found in piglets fed SP+R diet when compared to piglets fed SP diet. In turn, the dietary treatments affected 4 out of 18 key lipogenic enzymes and associated transcription factors. The SP+R and SP+L diets down-regulated the relative expression level of acetyl-CoA carboxylase α (ACACA) ($p = 0.044$) when compared to the control diet. SP+L diet upregulated the relative expression level of carnitine palmitoyl-transferase 1A (CPT1A) ($p = 0.037$) when compared to SP diet, and down-regulated the relative expression level of fatty acid desaturase 2 (FADS2) ($p = 0.028$) when compared to the control diet. Also, SP+R decreased mRNA levels of fatty acid binding protein 1 (FABP1) ($p = 0.049$) relative to the control diet.

Table 4.7. Effect of *Arthrospira platensis*, individually or combined with exogenous CAZymes, on gene expression levels (relative mRNA level) in piglets' liver.

	Diets				SEM	*p-value*
	Control	**SP**	**SP+R**	**SP+L**		
Antioxidant potential						
CAT	37.9	38.0	29.4	30.9	3.139	0.116
GPX1	2.58	1.42	1.96	2.21	0.436	0.305
GSR	0.213	0.206	0.190	0.220	0.021	0.779
SOD1	7.05	6.36	5.54	5.83	0.439	0.097
SOD2	0.258	0.222	0.223	0.202	0.025	0.433
SOD3	0.073	0.073	0.091	0.094	0.011	0.389
Vasodilation						
NOS2	0.001^{ab}	0.0007^{a}	0.0024^{b}	0.0012^{ab}	0.0004	0.048
NOS3	0.287	0.405	0.287	0.299	0.082	0.693
Lipid metabolism						
ACACA	0.414^{b}	0.354^{ab}	0.261^{a}	0.229^{a}	0.050	0.044
APOA5	4.46	3.58	4.00	4.18	0.782	0.879
CEBPA	0.024	0.030	0.028	0.033	0.003	0.215
CHREBP	1.07	1.11	1.37	1.35	0.142	0.318
CPT1A	0.384^{ab}	0.369^{a}	0.421^{ab}	0.564^{b}	0.052	0.037
CRAT	0.970	0.804	0.921	0.821	0.091	0.511
DGAT	0.277	0.286	0295	0.279	0.018	0.907
FABP1	19.7^{b}	13.4^{ab}	10.5^{a}	14.1^{ab}	2.323	0.049
FADS1	4.86	4.98	3.39	2.71	0.850	0.164
FADS2	4.64^{b}	3.92^{ab}	2.97^{ab}	2.51^{a}	0.524	0.028
FASN	0.513	0.438	0.545	0.622	0.134	0.795
HSL	0.018	0.018	0.021	0.025	0.003	0.338
LPIN1	0.036	0.036	0.037	0.033	0.006	0.969
PFKL	0.254	0.243	0.273	0.269	0.020	0.712
PLIN2	0.038	0.034	0.049	0.032	0.009	0.609
PPARA	2.01	2.21	1.95	2.38	0.258	0.620
SCD	11.2	6.90	4.86	4.49	2.605	0.252
SREBF1	6.44	6.47	6.03	4.77	1.233	0.718

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of *Arthrospira platensis* -SP; basal diet with 10% of *Arthrospira platensis* supplemented with 0.005% of Rovabio® Excel AP - SP+R; basal diet with 10% of *Arthrospira platensis* supplemented with 0.01% of lysozyme - SP+L.

[a,b]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).

4.3.7. Principal component analysis

A PCA was performed with all data. It was verified that fatty acid composition, cholesterol, α- and γ-tocopherols, total pigments and gene expression levels in the liver had no relationship using this discriminant analysis. As so, a PCA is presented using only the plasma metabolites to describe the variability of the pooled data into two dimensions (Figure 4.2 (a)). The score plot of the first two PC explained 44.3% of the total variability, with 27.1% for PC1 and 17.2% for PC2 (Table 4.8). The PC1 was characterized by variables with positive loadings, such as GGT and IgG, and by variables with negative loadings, such as total lipids, total cholesterol, TAC, LDL-cholesterol, HDL-cholesterol, AST, VLDL-cholesterol, TAG, ALT, glucose, ALP, GPX, urea, total protein, creatinine, IgA and IgM (Table 4.8). Concerning the PC2, all variables had small contributions with loadings varying between − 0.10 and 0.08.

The score plot depicted in Figure 4.2 (b) showed the location of the four experimental groups, control, SP, SP+R and SP+L, in the multivariate space of the first two PC. These scores were notably arranged into two clusters, corresponding to control and SP diets. The control diet was located in quadrant c, while SP diet was located in quadrant d. SP+L diet was confined to quadrant a. The SP+R diet was dispersed across quadrants a and b.

Table 4.8. Loadings for the first two principal components (PC).

Variables	PC1	PC2
Total lipids	−0.71	−0.04
TAG	−0.40	−0.07
Total Cholesterol	−0.64	−0.01
HDL-cholesterol	−0.51	0.02
LDL-cholesterol	−0.58	−0.04
VLDL-cholesterol	−0.40	−0.07
Glucose	−0.36	0.01
Urea	−0.30	0.03
Creatinine	−0.27	0.07
Total protein	0.29	0.04
ALT	−0.38	0.08
AST	−0.49	0.04
ALP	−0.34	0.08
GGT	0.53	−0.05
IgA	−0.21	−0.01
IgG	0.21	−0.04
IgM	−0.13	−0.10
TAC	−0.61	0.01
GPX	−0.33	−0.03

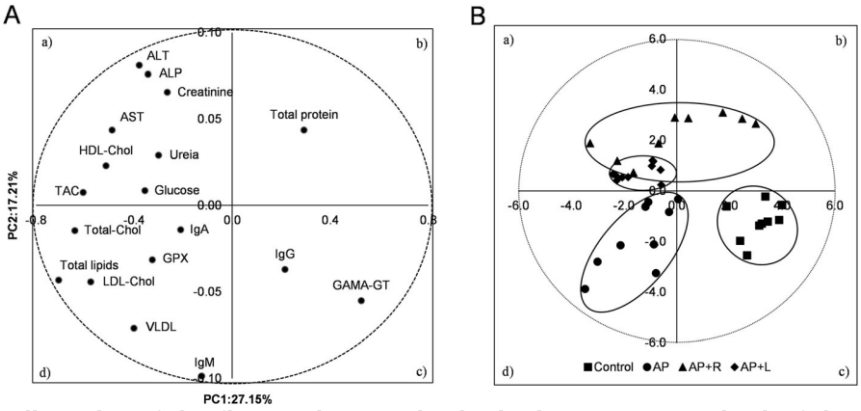

Figure 4.2. Loading plot of the first and second principal components (PC) of the pooled data a and component score vectors b using plasma metabolites from piglets fed *Arthrospira platensis*, individually or combined with exogenous CAZymes.

Dietary treatments: cereal and soybean meal-based diet - Control; basal diet with 10% of Arthrospira platensis (AP = SP); basal diet with 10% of Arthrospira platensis supplemented with 0.005% of Rovabio® Excel AP (AP+R = SP+R); basal diet with 10% of Arthrospira platensis supplemented with 0.01% of lysozyme (AP+L = SP+L).

4.4. Discussion

Herein we assessed, for the first time, the molecular mechanisms of hepatic lipid metabolism and antioxidant potential under the influence of *A. platensis* as feed ingredient, individually and combined with two exogenous CAZymes (lysozyme and a commercial mixture of carbohydrate-degrading enzymes, named Rovabio® Excel AP). In fact, several studies report the use of Spirulina as supplement in piglets feeding (Grinstead et al. 2000; Nedeva et al. 2014; Furbeyre et al. 2017), but not as an ingredient (> 1% in the diet).

Piglets fed diets with 10% of *A. platensis*, had lower ADG but higher FCR than piglets fed a control diet, regardless the addition of feed enzymes. These findings partially agree with the literature. In a general literature overview, the inclusion of *A. platensis* as a dietary supplement increases ADG but negatively affects FCR (reviewed by Madeira et al. 2017). However, ADFI was here unaffected by dietary treatments. TTAD of CP was higher in the control group than in *A. platensis* fed groups Martins, Pestana, Ribeiro et al. (2021). Lower protein digestibility is associated with higher digesta viscosity, which limits the access of endogenous enzymes to their target substrates. The decrease observed in piglets' performance was due to the low digestibility and gelation of *A. platensis* proteins in the intestine, as a direct consequence of their proteolytic resistance to the piglet endogenous peptidases (Martins, Pestana, Ribeiro et al. 2021). Digestible energy reached higher values on piglets fed control and *A. platensis* combined with Rovabio® diets, which are in agreement with piglets' growth performance, as the obtained values were also higher in the control group (Martins, Pestana, Ribeiro et al. 2021). In addition, CF digestibility increased in piglets fed *A. platensis* combined with Rovabio® Excel AP and lysozyme, when compared to the control group, which indicates that enzymes were effective in degrading *A. platensis* cell wall, thus facilitating the access of digestive enzymes to the cell content (Martins, Pestana, Ribeiro et al. 2021).

A. platensis has also been exploited for therapeutic purposes of various conditions (Ovando et al. 2018), such as anaemia, hepatotoxicity, reduction of cholesterol and prevention of cardiovascular diseases, and hyperglycaemia (Belay 2002; Oliveira et al. 2013). Although the plasma lipid profile was largely affected by diet, our data are not in line with the former reports. Total lipids, total cholesterol and LDL-cholesterol were higher in piglets fed *A. platensis*. In fact, total cholesterol exceeded the reference values (Jackson and Cockcroft 2002) in piglets from all dietary treatments. *A. platensis* is known for positive effects on cholesterol metabolism by increasing HDL, which can lead to healthy cardiovascular functions (De Caire et al. 1995; Cheong et al. 2010). This effect was confirmed by our data only when this microalga was combined with commercial Rovabio® Excel AP. The increment of "bad cholesterol" promoted by *A. platensis* was countering by reverse cholesterol transport of HDL,

decreasing the ratio total cholesterol: HDL-cholesterol and thus mitigating cardiovascular risk factors (Rodrigues et al. 2014). Additional discrepancies between our results and literature might be explained by the use of distinct dietary levels and experimental animal models, such as rodents and rabbits.

For hepatic markers, ALT activity was higher with *A. platensis*, and even more with supplementation of both exogenous CAZymes. In line with this, AST and ALP were also higher in piglets fed *A. platensis* combined with Rovabio® Excel AP and lysozyme. Contrarily, the GGT activity was lower in piglets fed *A. platensis*, with and without exogenous CAZymes. All in all, these variations on the hepatic function are devoid of clinical relevance because the levels of enzymatic activity found are still within the reference figures for pigs (31–58 for ALT, 32–84 for AST and 10–52 U/L for GGT, respectively (Jackson and Cockcroft 2002)). If urea variations reflect unaffected renal function, creatinine reached the highest values with lysozyme and Rovabio® Excel AP. Glucose was found increased with *A. platensis* incorporation, alone and combined with lysozyme, but this increase was apparently mitigated by Rovabio® Excel AP, suggesting a positive effect of the commercial mixture of carbohydrate-degrading enzymes on glycemia homeostasis.

It has been reported that *A. platensis* improves the immune system (Spruijt et al. 2016) and exhibits anti-inflammatory properties (Romay et al. 1998; Karkos et al. 2011). While individually *A. platensis* increased IgM levels, its combination with lysozyme decreased IgG concentrations, reinforcing *A. platensis* ability for modulating some immune responses.

TAC is a marker of global antioxidant defence, used as an accurate assessment of redox status *in vivo* (McMichael 2007). *A. platensis*, with and without commercial enzymes, increased TAC in plasma, which is consistent with hepatic total carotenoids increase, rather than with non-variations of GPX activity. *A. platensis* contains a variety of natural carotene and xanthophyll phytopigments, which turns this microalga into a good nutritional supplement for human and animal feed (Farag et al. 2016). GPX plays an important role in protecting haemoglobin, red blood cell enzyme activity and biological cell membranes against oxidative damage (Waggiallah and Alzohairy 2011) and its activity reaches the highest values in the liver and erythrocytes (Behne and Wolters 1983). Herein, the enzymatic activity of GPX measured in plasma had no changes across dietary treatments, which is consistent with similar transcriptional profile found in the liver. The gene expression levels were higher for catalase (CAT), superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPX1), in this particular order. However, none of these genes was affected by dietary treatments in the liver. Some studies have shown that weaning systematically decreases the antioxidant potential and increases the generation of free radicals in tissues and blood (Nieto et al. 2000; Burke et al. 2009). SOD and CAT enzymes constitute the first line of antioxidant defence in the body (Matés et al. 1999), being the values found for their relative gene expression, in accordance

with the literature. Other important antioxidants, some of them with extracellular origin, in particular vitamin E, might have contributed to improve redox status in piglets fed *A. platensis*. Curiously, data on α- and γ-tocopherol contents were observed in the opposite direction. The values found for vitamin E tocopherols in the liver were lower in piglets fed the microalga and the microalga plus exogenous CAZymes and do not match the original amounts on diets formulation, suggesting that feeding *A. platensis* at this high level of incorporation reduces vitamin E, through mechanisms that warrant further elucidation. Nitric oxide, a free radical that acts as a biological mediator in several processes, including neurotransmission as well as antimicrobial and antitumoral activities, is catalysed by the conversion of L-arginine to nitric oxide by nitric oxide synthase (NOS). Only NOS2, a vasodilator marker, was affected by diets, being its gene upregulated by *A. platensis* in combination with the commercial Rovabio® Excel AP. Neuronal NOS, endothelial NOS and inducible NOS (Du et al. 2017) are expressed in the liver and activated by a combination of lipopolysaccharide (LPS) and certain cytokines, mostly common associated with the weaning process. Early weaning predisposes the pig intestine to structural and functional alterations, due to the increase in Escherichia coli populations. These bacteria use the LPS derived from their cell wall as an important pathogenic factor (Amador et al. 2007).

Liver is the principal site of cholesterol synthesis and fatty acid oxidation, whereas de novo lipogenesis occurs essentially in both liver and adipose tissue (Nafikov and Beitz 2007). The majority of individual FA quantified in the liver were not affected by the microalga nor by the exogenous CAZymes. This result aligns well with the low FA content of *A. platensis* (Madeira et al. 2017). Nevertheless, the sum of *n*-3 PUFA decreased in piglets fed diets containing the microalga and exogenous CAZymes and, consequently, *n*-6:*n*-3 ratio increased. For lipid metabolism, higher gene expression levels were found for: apolipoprotein A-V (APOA5) > fatty acid binding protein 1 (FABP1) > fatty acid desaturase 1 (FADS1) > FADS2 > peroxisome proliferator-activated receptor alpha (PPARA) > stearoyl-CoA desaturase (SCD) > sterol regulatory element binding transcription factor 1 (SREBF1). *A. platensis* with exogenous CAZymes down-regulated ACACA, a key lipogenic enzyme for fatty acid biosynthesis, together with fatty acid synthase (FASN), and SCD or delta9 desaturase (Burke et al. 2009) that remained unchanged across dietary treatments, therefore validating the similar values of total lipids observed in the liver. FADS1, encoding for Δ5 desaturase, and FADS2, encoding for Δ6 desaturase, are membrane-bound enzymes that catalyse the synthesis of PUFA (Martins, Pestana, Ribeiro et al. 2017). The mRNA levels of FADS2 were decreased in piglets fed *A. platensis* with lysozyme, not accompanied by a decrease in PUFA content (Nakamura and Nara 2004). FADS1 was not affected by dietary treatments although it showed identical gene expression magnitude as FADS2, which might be explained by the fact that FADS2 is sensitive to lysozyme. FABP1 prevents lipotoxicity of free FA and regulates fatty acid

trafficking and partition (Guzman et al. 2013). Its relative gene expression level was decreased by *A. platensis* combined with Rovabio® Excel AP. This finding requires further investigation. The mRNA levels of carnitine O-acetyltransferase (CRAT), one of the enzymes responsible for fatty acid β oxidation (Van der Leij et al. 2000), as well as PPARA, a major inducer of fatty acid oxidation that suppresses fat synthesis (Poulsen et al. 2012), were kept unchanged by both *A. platensis* and exogenous CAZymes.

4.5. Conclusions

Under the experimental conditions tested in this study, *A. platensis* incorporated as feedstuff, supplemented or not with two exogenous CAZymes (lysozyme and commercial Rovabio® Excel AP), impacted negatively on piglets' growth and increased systemic lipemia, without changing the hepatic fatty acid content. In fact, dietary treatments had a minor effect on fatty acid composition and transcriptional profile of lipid sensitive mediators in the liver. By contrast, and validating our initial hypothesis, the addition of this microalga benefited the systemic redox balance, regardless the presence of lysozyme or Rovabio® Excel AP, as shown by the clear discrimination between the control diet and *A. platensis* diet in the multidimensional space of the PCA analysis. However, this positive variation was not followed by up-regulation of the first line of antioxidant defence, CAT, SOD and GPX enzymes, or the level of vitamin E compounds in piglets' liver. In contrast, these results are supported by total carotenoids increase, which are compounds known to counterbalance oxidative stress. In view of these resuls, further studies are encouraged to incorporate lower percentages of this microalga in pigs feed before final conclusions could be drawn.

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Chapter 5 – INFLUENCE OF *CHLORELLA VULGARIS* ON GROWTH, DIGESTIBILITY AND GUT MORPHOLOGY AND MICROBIOTA OF WEANED PIGLET

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Contribution of Cátia Martins to this paper:
Cátia Martins performed the animal trial and sampling. In addition, performed the laboratory analysis of the digestibility study with proximal analysis for diets and faeces. Finally, Cátia Martins collaborated in the results interpretation and wrote the draft manuscript.

Abstract

The purpose of this study was to evaluate the impact of *Chlorella vulgaris* (5% in the diet), supplemented or not with two exogenous carbohydrase mixtures on piglets' performance, nutrient digestibility and gut morphology, fermentation products and microbiota. Forty-four male piglets weaned at 28 days of age, with 11.2 ± 0.46 kg of live weight, were used and assigned to 1 of 4 dietary treatments: cereal and soybean meal based-diet (control, *n* = 11), control diet with 5% of *C. vulgaris* (CH, *n* = 10), CH diet supplemented with 0.005% of Rovabio® Excel AP (CH+R, *n* = 10) and CH diet supplemented with 0.01% of a recombinant 4-carbohydrase mixture (CH+M, *n* = 11). Growth performance was not changed by the of *C. vulgaris* inclusion during 21 days of trial. However, total tract apparent digestibility of nutritional fractions was negatively impacted by the inclusion. In addition, the viscosity of duodenum plus jejunum contents slightly increased in all groups fed with the microalga. In contrast, dietary microalga increased duodenum villus height and promoted a healthier gut microbiota, with higher abundance of some specific bacterial taxa (*Colidextribacter*, *Oscillospira* and *Lactobacillus*). This study indicates that the dietary inclusion of 5% *C. vulgaris* improves piglets' gut health without impairing performance. Data also indicate that *C. vulgaris* reduces nutrient digestibility but promotes compensatory developments of gut mucosa and prebiotic effects. Dietary supplementation with exogenous carbohydrases does not seem to be necessary for this inclusion level. Therefore, the incorporation of CH as a sustainable feed ingredient in piglets' nutrition is a viable alternative approach.

Keywords: *Chlorella vulgaris*, weaned piglets, digestibility, gut morphology, microbiota

5.1. Introduction

The *post*-weaning period is one of the most critical periods in swine production. It is associated to social, environmental and nutritional changes. In addition, piglets' immune system is not yet fully developed and, there- fore, animals are more susceptible to several digestive and respiratory pathologies. Also, recently weaned pigs experience strong structural and physiological changes in the intestine (Campbell et al. 2013). Furthermore, the use of antibiotics for preventive or therapeutic purposes has been associated with an increased occurrence of antimicrobial resistant microorganisms, showing that strategies to reduce or prevent their utilization are necessary.

Among these strategies, several feed-based solutions are considered interesting alternatives. Innovative com- pounds and feedstuffs, like microalgae, are of interest for their prebiotic properties in order to cope with *post*-weaning stress (Caporgno and Mathys 2018). In addition, they are considered sustainable feedstuffs that do not compete for land and other resources necessary to produce food for human consumption (Valente et al. 2021). Furthermore, they have the potential to fixate carbon dioxide from the atmosphere, thus contributing to mitigate global warming. Furbeyre et al. (2017) observed that a 1% dietary inclusion of Spirulina and *Chlorella vulgaris* as an alternative to antibiotics improved the intestinal health in weaned piglets. As mentioned in the literature, small inclusion levels of different microalgae in piglet diets increase gut health, albeit more research is needed in order to understand, establish and validate their effect on the intestinal microflora (Valente et al. 2021).

Although microalgae have a high nutritive value and are an interesting sustainable alternative to cereals and soybean in swine diets, the particular characteristics of their recalcitrant cell wall make them rather indigestible for monogastric animals (Madeira et al. 2017). However, enzymes that degrade the cell wall, like CAZymes, might improve their utilization with positive effects on nutrient bioavailability, in addition to promote the prebiotic properties of the insoluble polysaccharides typical of these matrices. Accordingly, Coelho, Lopes et al. (2020) described a 4-CAZyme mixture able to degrade *in vitro* the *C. vulgaris* cell wall. Furthermore, Martins, Pestana, Ribeiro et al. (2021) recently studied a higher level of dietary Spirulina incorporation (10% dietary inclusion), either individually and in combination with 2 commercial carbohydrases, in *post*-weaned piglets' diets. Authors showed that lysozyme is efficient in the degradation of this microalga cell wall in the piglet's gut.

Accordingly, the aim of this work was to evaluate the impact of 5% *C. vulgaris* in the diet, combined with 2 exogenous carbohydrase mixtures (Rovabio® Excel AP and the 4-carbohydrase mixture tested by Coelho, Lopes et al. (2020), on piglets' performance, nutrient digestibility and gut morphology, fermentation products and microbiota profile.

5.2. Material and Methods

5.2.1. Experimental design, diets and animal performance

Following the principles and specific guide- lines of the European Union legislation (2010/63/EU Directive), as well as the ARRIVE guidelines 2.0 (https:// arriveguidelines.org/arrive-guidelines), all the procedures used in this animal experiment were revised by the Ethics Commission of ISA and accepted by the Animal Care Committee of the National Veterinary Authority (Process Number 0421/2017, Direção Geral de Alimentação e Veterinária, Portugal).

Forty-four *post*-weaned piglets (50% Pietrain × 25% Large White × 25% Landrace), weaned at 28 d of age and with an initial live weight of 11.2 ± 0.46 kg (mean ± SD) were obtained from a commercial farm. Each animal was allocated to a crate equipped with a feeder, a stainless-steel nipple, a heating lamp and plates for separation of faeces and urines. The environmental conditions of the room were the same as described previously (Ribeiro, Pinho et al. 2013). Animals had 2 days for environmental adaptation and stabilization of stress and digestive condition. After this period, 2 animals failed to this adaption and were withdrawn from the study. Each animal had access to one of the 4 experimental diets: (1) cereal and soybean meal-based diet (control, *n* = 11); (2) control diet with 5% of *C. vulgaris* (CH, *n* = 10); (3) control diet with 5% of CH supplemented with 0.005% of Rovabio® Excel AP (Adisseo, Antony, France) (CH+R, *n* = 10); and (4) control diet with 5% CH supplemented with 0.01% of the 4-carbo- hydrase mixture described by Coelho, Pestana et al. (2020) and Coelho, Lopes et al.(2020), (CH+M, *n* = 11). The microalga was supplied by the company Allmicroalgae—Natural Products SA, Pataias, Portugal as freeze-dried powder and included as supplied in the diets. Its chemical composition was previously described by our team in Coelho, Pestana et al. (2020). The detailed description of the experimental diets is presented in Supplementary Material 1 - Table S1.

Piglets were fed daily, with the same amount of feed provided per animal. To calculate ADFI, feed refusals were recorded daily. Animals had *ad libitum* access to water. Moreover, the individual body weight was recorded weekly in order to calculate ADG and FCR. The faeces were collected for 2 periods of 6 d in order to calculate TTAD. The following equation: $TTAD = ((N_{in} - N_{out})/N_{in}) \times 100$ was used. N_{in} represents the total intake of a specific nutrient in the feed and N_{out} represents the total faecal output for the same nutrient. In addition, the consistency of the faeces was recorded daily, according to the following scale: 0 (normal), 1 (soft faeces) or 2 (diarrhoea).

5.2.2. Slaughtering and sampling

After 21-d experimental time all animals were slaughtered using electrical stunning followed by exsanguination. The gastrointestinal tract was removed to measure the length of the small and large intestines. Also, the contents of stomach, duodenum plus jejunum, ileum, caecum and colon were collected, immediately analysed (pH and viscosity determinations) or stored at – 20 °C for VFA (volatile fatty acids) determination. For histological analysis, 3 segments of the small intestine were collected: duodenum (10 cm below pylorus), jejunum (5.5 m below pylorus) and ileum (60 cm above ileum-caecal valve). These tissue samples were fixed into 10% buffered formalin solution and then processed for paraffin embedding. For microbiome analysis, faecal samples were collected and stored at − 80 °C until DNA extraction.

5.2.3. Chemical analysis of diets and faeces

All the methods used for diets and faeces analysis were previously described (Martins, Pestana, Ribeiro et al. 2021). Briefly, faecal samples were dried at 60 °C for 72 h in an oven with ventilation. Diets and dried faecal samples were ground in 1 mm diameter mesh mill and analysed, in duplicate, for DM, ash, CP and CF contents, following the methods described by AOAC (2000). NDF, ADF and acid detergent lignin (ADL) were per- formed sequentially using crucibles system by Van Soest et al. (1991). Hemicellulose and cellulose were calculated as NDF-ADF and ADF-ADL, respectively.

Determination of amino acids, FAME, diterpene profile, pigments and mineral composition were performed in the microalga and diets. The amino acids, except tryptophan, were measured as described in Commission Regulation (EC) No 152/2009 (2009). Briefly, cysteine and methionine were oxidised to cysteic acid and methionine sulphone, respectively, prior to hydrolysis. All the other amino acids, except tryptophan, were determined in hydrolysates of unoxidized samples. The determination of tryptophan in samples was performed according to la Cour et al. (2019). All amino acids were analysed by HPLC (Agilent 1100, Agilent Technologies, Avondale, PA, USA), combined with automated pre-column derivatisation using o-phthaldialdehyde and 9-fluorenylmethyl chloroformate, as reported by Henderson et al. (2000). FAME were analysed by extraction and acid transesterification, using fatty acid 21:0 as the internal standard (Sukhija and Palmquist, 1988). Diterpene profile was conducted by a single n-hexane extraction succeeded by HPLC (Prates et al. 2006). The determination of pigments was performed according to Teimouri et al. (2013), with small modifications. After overnight extraction with acetone, obtained solutions were centrifuged at 2000×g for 5 min and analysed by UV–Vis spectrophotometry measuring the absorbance at different wavelengths (Ultrospec 3100 pro, Amersham Biosciences, Little Chalfont, UK).

Pigment contents were calculated using the equations described by Hynstova et al. (2018). The mineral composition was performed following the previously described protocol (Ribeiro et al. 2020).

The detailed chemical composition of the microalga and experimental diets is shown in Table 5.1.

Table 5.1. Chemical composition of *Chlorella vulgaris* microalga and experimental diets.

	Microalga powder	Diets			
		Control	CH	CH+R	CH+M
Proximate composition, g/100 g (as fed basis)					
DM	93.1	90.5	90.8	90.8	90.9
OM	81.3	85.1	85.2	85.3	85.3
Ash	11.8	5.43	5.65	5.47	5.60
CP	42.8	19.3	19.2	19.5	19.4
NDF	32.2	12.9	11.9	12.9	10.4
ADF	19.4	2.76	2.45	2.58	2.54
CF	8.75	5.29	5.39	5.39	5.63
Limiting amino acids, g/100 g (as fed basis)					
Cysteine	0.297	0.275	0.242	0.225	0.227
Lysine	3.87	1.32	1.59	1.59	1.58
Methionine	0.819	0.318	0.356	0.335	0.322
Threonine	2.23	0.95	0.88	0.90	0.97
Tryptophan	0.895	0.353	0.357	0.337	0.350
Fatty acid composition, % total FA					
14:0	1.13	0.351	0.380	0.380	0.361
16:0	17.2	10.6	11.0	10.9	11.1
16:1*c*9	3.90	0.158	0.903	0.900	0.677
17:0	0.234	0.095	0.104	0.103	0.104
17:1*c*9	0.610	0.040	0.583	0.643	0.828
18:0	3.00	3.35	3.33	3.38	3.26
18:1*c*9	11.7	24.8	24.5	24.5	24.6
18:1*c*11	–	0.909	1.16	1.16	1.10
18:2*n*-6	11.2	55.8	53.3	53.2	52.9
18:3*n*-3	10.1	1.55	2.18	2.23	2.52

20:0	0.174	0.306	0.286	0.286	0.283
20:1*c*11	0.127	0.227	0.224	0.197	0.215
22:0	0.060	0.566	0.531	0.542	0.521
22:1*n*-9	–	0.105	0.108	0.104	0.085
Pigments, µg/g					
β-Carotene	198	–	13.3	13.7	14.5
Chlorophyll *a*[a]	906	3.38	109	130	135
Chlorophyll *b*[b]	171	6.05	31.9	42.6	39.8
Total chlorophylls[c]	1077	9.43	141	172	174
Total carotenoids[d]	228	2.67	36.9	44.5	52.9
Total chlorophylls and total carotenoids[e]	1305	12.1	178	217	227
Diterpene profile, µg/g					
α-Tocopherol	19.2	28.6	19.9	22.1	24.2
β-Tocopherol	0.339	1.11	1.10	1.00	1.12
γ-Tocopherol	0.521	2.52	2.00	2.21	2.11
δ-Tocopherol	0.371	0.502	0.334	0.387	0.396
α-Tocotrienol	–	3.43	3.73	3.58	3.53
γ-Tocotrienol	0.560	1.38	1.51	1.69	1.46
Macrominerals, g/100 g (as feed basis)					
Calcium	0.703	0.666	0.837	0.785	0.824
Phosphorus	2.04	5.19	6.42	5.94	5.36
Potassium	2.92	1.25	1.33	1.29	1.16
Sodium	0.382	1.53	1.84	1.55	2.04

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
DM dry matter, OM organic matter, CP crude protein, NDF neutral detergent fibre, ADF acid detergent fibre, CF crude fat.
[a]Chlorophyll *a* (Ca) = 11.24 × A662 nm − 2.04 × A645 nm.
[b]Chlorophyll *b* (Cb) = 20.13 × A645 nm − 4.19 × A662 nm.
[c]Total chlorophylls (Ca + Cb) = 7.05 × A662 nm + 18.09 × A645 nm.
[d]Total carotenoids (Cx + Cc) = (1000 × A470nm − 1.90 × Ca − 63.14 × Cb)/214.
[e]Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

5.2.4. Gut content analysis

The pH measurement of the contents of stomach, duodenum plus jejunum, ileum, caecum and colon were immediately determined, using a glass electrode pH meter (Metrohm 744, Metrohm AG, Herisau, Switzerland). The viscosity of small intestine contents was measured as previously described (Martins, Pestana, Ribeiro et al. 2021).

Caecum and colon contents (5 g) were collected in a 5% (v/v) o-phosphoric acid until the quantification of the following VFA: acetic acid (C2), propionic acid (C3), butyric acid (C4), valeric acid (C5) and isovaleric acid (iC5). These compounds were quantified by gas chromatography (GC) as previously described (Jouany 1982), on the supernatant of thawed samples centrifuged at 8000×g for 10 min. The 4-Methyl valeric acid was used as internal standard.

5.2.5. Gut histological analysis

Microscopic examination and measurement of villi heights and widths and crypt depths were performed in 7 µm thick tissue sections, stained with haematoxylin–eosin. An Olympus BX 51 microscope equipped with 4×and 10× lenses was used. Images were digitally captured with an Olympus DP 21 camera. The height and width of the villi and the depth of the crypts were measured using the Olympus DP- Soft software. Ten intact and correctly oriented villi and crypts from each intestinal region were selected for each piglet.

5.2.6. Gut microbiota analysis

Total bacterial DNA was isolated and extracted with QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and purity (absorbance ratio 260/280 and 260/230) of the isolated DNA were checked by spectrophotometry on the NanoDrop (Fisher Scientific, 13 Schwerte, Germany).

For the microbiological analysis of faecal samples, the V4 region of the 16S rRNA gene (~ 380 bp) was amplified 515f: 5′-GTGYCAGCMGCCGCGGTAA-3′; 806r: 5′-GGACTACNVGGGTWTCTAAT-3′ and sequenced using the Illumina 2 × 250 bp MiSeq platform (Illumina Inc., San Diego, CA, USA) (Walters et al. 2015).

The bioinformatic analysis was performed using DADA2 1.14.0 (Callahan et al. 2016) running on R 4.0.2. For the taxonomic assignment, the SILVA database release 138 was used as reference (Quast et al. 2013).

5.2.7. Statistical analysis

. Data homogeneity and normality were verified. Growth performance, nutrient digestibility, intestinal morphology and VFA data were analysed using the PROC MIXED of SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA). The consideration of the model was the dietary treatment as single effect and the piglet as experimental unit. When

significant effects of treatments were detected, least square means were compared using the PDIFF with Tukey–Kramer adjustment options of SAS. TTAD data were analysed using the PROC MIXED, considering repeated measures over time to test the effect of diet, period and their interaction. Results were considered significantly different when the *p-value* < 0.05.

Regarding the statistical analysis of the microbiome, data on alpha diversity, beta diversity and taxonomic composition were carried in R 4.0.2 using phyloseq (McMurdie and Holmes 2013), Vegan (Dixon 2003) and DESeq2 (Love et al. 2014) packages. To test the differences between the groups for the alpha diversity, a Multifactorial Analysis of variance (ANOVA) (MANOVA) model was fitted, considering sequencing depth and group as factors. For the beta diversity, the Euclidian distance was calculated, and the differences between groups were tested using a non-parametric PERMANOVA (Adonis) model, with 999 permutations, pair-wise contrast were made using the pairwise Adonis function provided by the pairwise Adonis R package (Arbizu 2020). In addition, to tests the homogeneity of dispersion among them a PERMDISP test was used (Anderson 2001). Samples abundances were normalized using variance stabilizing transformation provided by DESeq2 package. Differences for the taxonomic composition between treatments were tested using Linear discriminant analysis (LDA) effect size (LEfSe) aggregating the data at Genus level, LDA score cut-off of 3 was used to discriminate bacterial taxa (Segata et al. 2011). The *p-values* were adjusted for multiple comparison using the False Discovery Rate (FDR) method. Significance was declared if *p-value* < 0.05 and a trend was considered when 0.05 < *p-value* < 0.10.

5.3. Results

5.3.1. Growth performance

The effect of experimental diets on growth performance of piglets are presented in Table 5.2. The piglets' weight was similar among experimental groups at the beginning and end of the trial ($p > 0.05$), with mean values of 11.2 and 23.1 kg, respectively. However, the control group had a lower ADFI, with 87 g/day lower feed intake by comparison with the CH-fed groups. In contrast, ADG and FCR were similar for all experimental groups ($p > 0.05$).

Table 5.2. Effect of diets on feed intake and growth performance of piglets.

	Diets				SEM	*p-value*
	Control	CH	CH+R	CH+M		
Initial weight, kg	11.1	11.1	11.3	11.2	0.105	0.851
Final weight, kg	22.3	23.3	23.5	23.1	0.247	0.349
ADFI, g	768[a]	852[b]	857[b]	856[b]	11.6	0.008
ADG, g	535	581	579	569	8.5	0.188
FCR	1.44	1.47	1.48	1.51	0.013	0.282

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
ADFI average daily feed intake, ADG average daily gain, FCR feed conversion ratio.
[a,b] Values within a row with different superscripts differ significantly at $p < 0.05$.

5.3.2. Digestibility of nutrients

The effect of diets on TTAD of nutrients and consistency of faeces are shown in Table 5.3. The experimental treatments affected all TTAD nutritional fractions, with the exception of CF. TTAD of DM and OM were significantly higher in control group, with an average difference of 2.5 and 2.1 percentage points, respectively, compared with the other groups. For these parameters, the CH and CH+M groups had the lowest values, while the CH+R group had the intermediate value, by comparison with the control group. Groups fed with microalga, supplemented or nor with exogenous enzymes, had a decrease in TTAD of CP in about 4.5 percentage points ($p < 0.0001$) in comparison with the control group. Regarding this last-mentioned parameter, the CH group had the lowest value compared with the control and CH+R groups.

TTAD values of NDF, ADF and hemicellulose were significantly different between all experimental groups, with the control group showing the highest values, followed by the CH+R group, whereas the CH+M group had the lowest value of NDF and hemicellulose, and the CH group of ADF. TTAD of cellulose was higher in the CH+M group, albeit with no significant differences in comparison with the CH+R group. It showed, however, significant differences when compared with the CH group (6.3 per- centage points increase). When we look at the results considering the 2 different collection periods, there were significant differences for TTAD for all nutrients, except for CF and ADF. For the second period, the TTAD results were lower than those of the first period.

Concerning faecal scores, no significant differences between the experimental groups were detected, neither considering the effect of diet nor the effect of collecting period (p = 0.1849 and 0.1165, respectively).

Table 5.3. Effect of diets on total tract apparent digestibility (TTAD) of nutrients and consistency of faeces.

	Diets				Period		SEM	*p-value**	
	Control	CH	CH+R	CH+M	1st	2nd		Diet	Period
TTAD, %									
DM	89.8[a]	86.8[c]	88.1[b]	86.9[c]	88.2	87.6	0.25	< 0.0001	0.0019
OM	90.0[a]	87.4[c]	88.5[b]	87.7[c]	88.7	88.1	0.22	< 0.0001	0.0029
CP	86.4[a]	81.3[c]	82.9[b]	81.6[bc]	83.4	82.6	0.46	< 0.0001	0.0193
CF	78.8	77.1	78.7	77.9	78.4	77.8	0.38	0.2091	0.0827
NDF	75.9[a]	68.0[c]	72.0[b]	63.9[d]	70.7	69.2	0.79	< 0.0001	0.0165
ADF	50.8[a]	32.3[d]	39.3[b]	35.0[c]	40.1	38.6	1.42	< 0.0001	0.2506
Hemicellulose	82.7[a]	77.1[c]	80.3[b]	73.5[d]	79.0	77.8	0.63	< 0.0001	0.0060
Cellulose	35.4[ab]	34.1[b]	39.6[a]	40.4[a]	38.7	36.1	1.16	0.0483	0.0467
Faecal score[1]	0.560	0.950	1.00	1.13	0.85	0.97	0.074	0.1849	0.1165

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
DM dry matter, OM organic matter, CP crude protein, CF crude fat, NDF neutral detergent fibre, ADF acid detergent fibre.
[1]Faecal score: 0 (normal), 1 (soft faeces) or 2 (diarrhoea).
*The interaction of the 2 factors (diet × period) was not significant for all the variables.
[a,b,c,d] Values within a row with different superscripts differ significantly at $p < 0.05$.

5.3.3. Gut length, content viscosity, pH and histology

In Table 5.4 we present the effect of diets on gastrointestinal tract variables, relative length, content viscosity and pH and intestinal morphology traits. Diets had no effect on the relative length of small and large intestines ($p > 0.05$). Digesta viscosity of duodenum plus jejunum was 28% higher in groups fed with the microalga in comparison with the control group. Regarding digesta viscosity, no effect was observed for the ileum content of all dietary treatments ($p = 0.6762$). The stomach, caecum and colon pH values were similar for all dietary treatments ($p > 0.05$). In contrast, diet had a significant effect on pH of duodenum plus jejunum and ileum contents ($p = 0.0028$ and $p = 0.0383$, respectively). Regarding the pH of duodenum plus jejunum content, no significant differences were found between the CH and CH+R groups. Additionally, the duodenum plus jejunum content pH for the control group was significantly higher that of the CH group. Groups fed with CH had a 7.1% reduction in the ileum content pH by comparison with the control group.

The control group had lower duodenum villus heights when compared with the other groups fed with the microalga. The incorporation of microalga caused an 18% increase by comparison with the control group. Consequently, the villus height to crypt depth ratio were

higher for groups fed with microalga (p = 0.0088). The villus height for jejunum and ileum were similar for all experimental treatments. Additionally, for the other 2 variables measured, villus width and crypt depth, at 3 different gut locations, no significant differences were detected (p > 0.05).

Table 5.4. Effect of diets on gastrointestinal tract variables and intestinal morphology of piglets.

	Diets				SEM	*p-value*
	Control	**CH**	**CH+R**	**CH+M**		
Relative length of gastrointestinal tract, m/kg						
Small intestine	0.733	0.657	0.656	0.710	0.010	0.0882
Large intestine	0.145	0.139	0.142	0.151	0.003	0.6056
Content viscosity, cP						
Duodenum + jejunum	2.64^{a}	3.64^{b}	3.63^{b}	3.70^{b}	0.15	0.0202
Ileum	5.00	5.64	4.73	5.42	0.28	0.6762
pH						
Stomach	3.95	4.10	4.07	3.86	0.063	0.4475
Duodenum + jejunum	5.67^{ab}	5.42^{c}	5.57^{bc}	5.73^{a}	0.033	0.0028
Ileum	6.37^{a}	5.93^{b}	5.87^{b}	5.96^{b}	0.069	0.0383
Caecum	5.67	5.71	5.65	5.92	0.055	0.3120
Colon	6.19	6.25	6.19	6.22	0.036	0.9260
Villus height, µm						
Duodenum	339^{a}	424^{b}	414^{b}	402^{b}	10.8	0.0160
Jejunum	376	428	399	399	17.7	0.7893
Ileum	327	370	376	321	9.7	0.0978
Villus width, µm						
Duodenum	187	175	177	192	2.9	0.1293
Jejunum	152	146	159	163	3.6	0.4021
Ileum	195	182	176	186	3.9	0.3696
Crypt depth, µm						
Duodenum	503	469	489	431	11.2	0.1130
Jejunum	345	343	367	349	7.0	0.6332
Ileum	313	299	272	267	7.7	0.0902

Villus height/crypt depth						
Duodenum	0.686[a]	0.911[b]	0.859[b]	0.967[b]	0.03	0.0088
Jejunum	1.10	1.26	1.10	1.14	0.05	0.6784
Ileum	1.07	1.26	1.44	1.24	0.05	0.0775

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
[a,b,c]Values within a row with different superscripts differ significantly at $p < 0.05$.

5.3.4. Gut volatile fatty acids

The effect of diets on VFA concentration of piglets' caecum and colon contents is shown in Table 5.5. For VFA concentration in caecum, with the exception of iC5, the CH+M group had lower values than those of the other groups, with a reduction of 26%, 30%, 39%, 53% and 31% for C2, C3, C4, C5 and total concentration, respectively. No differences were observed between control, CH and CH+R groups regarding VFA concentration in caecum content.

For VFA concentration in the colon, there was a significant influence of dietary treatments. By comparison with the control group, microalga-fed experimental groups had a significant decrease of 30%, 24% and 40%, respectively, for the concentration of C2. For the C3 concentration, the same comparisons led a decrease of 24%, 25% and 37%, respectively. For the C4 and C5 concentrations, only the CH+M group had a significant decrease of 45% and 57%, when compared with the control group. For iC5 and total VFA concentrations, comparatively with the control group, all microalga-fed groups had a significant reduction. Regarding the CH *vs*. CH+R groups' comparison, similar values were observed for all VFA concentrations in the colon. When the CH group was compared with the CH+M, the only recorded significant difference concerned the C3 concentration that was 16% decrease in the CH+M group. When comparing the 2 groups supplemented with enzymes regarding C3 concentration, there was significant 16% decrease in the CH+M group.

Table 5.5. Effect of diets on volatile fatty acids (VFA) concentration in caecum and colon of piglets.

Item[1]	Diets				SEM	*p-value*
	Control	CH	CH+R	CH+M		
VFA concentration in caecum, mmol/L						
C2	20.2[a]	19.5[a]	21.4[a]	15.1[b]	0.910	0.0079
C3	13.0[a]	11.0[a]	12.5[a]	8.54[b]	0.624	0.0042
C4	6.17[a]	6.64[a]	7.70[a]	4.18[b]	0.452	0.0114
iC5	0.845	0.144	0.184	0.069	0.177	0.3678
C5	2.05[a]	1.95[a]	1.86[a]	0.920[b]	0.176	0.0444
Total	42.3[a]	39.3[a]	43.7[a]	28.8[b]	1.920	0.0005
VFA concentration in colon, mmol/L						
C2	21.2[a]	14.9[bc]	16.2[b]	12.8[c]	0.785	< 0.0001
C3	10.6[a]	8.03[b]	7.98[b]	6.73[c]	0.470	0.0082
C4	5.78[a]	4.32[ab]	4.47[ab]	3.17[b]	0.299	0.0187
iC5	0.777[a]	0.413[b]	0.499[b]	0.326[b]	0.054	0.0030
C5	2.05[a]	1.39[ab]	1.43[ab]	0.880[b]	0.136	0.0100
Total	40.4[a]	29.1[b]	30.6[b]	23.9[b]	1.611	0.0003

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
[1]C2, C3, C4, C5 and iC5 are acetic, propionic, butyric, valeric and isovaleric acids, respectively.
[a,b,c]Values within a row with different superscripts differ significantly at $p < 0.05$.

5.3.5. Gut microbiota

As a sanity check for the sequencing procedure the rarefaction curve was plotted (please see detail in Supplementary Material 1 - Figure S1). Overall, all samples reached the plateau point suggesting a good sequencing efficiency and indicating that the sequencing procedure has captured all the taxonomic variability present in that specific ecosystem.

All the reads that were maintained in every step of the bioinformatic analysis are presented in Supplementary Material 1 - Table S2. At the end, all samples had a high number of reads (53.6 in average), that resulted in a total of 1684 different Amplicon Sequence Variants (ASVs). Of these, 99.4% were assigned at Phylum level to 22 different Phyla, with Bacteroidota (Bacteroidetes) 48.6% and Firmicutes 32.3% comprising the majority of Phyla. At family level, a total of 81 families were identified (*Prevotellaceae* 38.7%, *Oscillospiraceae* 7.31% and *Rikenellaceae* 5.52%), and at Genus level 176 genera (*Prevotella* 21.4%, Rikenellaceae_RC9_gut_group 4.95% and *Alloprevotell*a 4.30%). Composition plots showing

the relative abundance of the top 10 taxa for Phylum, Family and Genus are reported in Supplementary Material 1 - Figure S2.

For the alpha diversity, Chao1, Shannon and InvSimpson indices were calculated. None of the treatment influenced the alpha diversity measures (Figure 5.1A).

For the beta diversity, meaning the differences in microbial composition between samples, a PCoA plot using a Euclidian distance matrix (calculated based on normalized row counts with the variance stabilization function of DESeq2—Table 5.6), was created (Figure 5.1B). The plot does not show a clear separation of the samples based on treatment, but the results of the Adonis test indicate that diet significantly influenced the microbial composition ($R^2 = 0.09$, $p < 0.05$). Although, the pair-wise Adonis test does not evidence any significant results for each of the possible comparisons. However, when the results for all CH-fed groups are combined and compared against the control group, there is a significant effect ($R^2 = 0.04$, $p < 0.05$). In addition, the PERMDISP test was not significant, confirming the results of the Adonis test.

In order to identify which taxa contributes to those differences, we used the LEfSe on the data aggregated at Genus level. The differential expressed Taxa are reported in Figure 5.2.

As shown in Figure 5.2, animals from the CH group had a higher abundance of a single bacterial taxa from genus *Colidextribacter*. This genus is constituted by a single species *Colidextribacter massiliensis*, which was isolated from human gut microbiota (Ricaboni et al. 2017). Overall, the relative abundance of this taxa is quite low (0.19 ± 0.14%). CH+R group had a higher abundance of genera *Oscillospira* and *Lactobacillus*. Whereas CH+M supplementation increased the abundance of bacteria from genus Helicobacter and horsej-a03 (family *Oligosphaeraceae*). Finally, animals from the control group were characterized by having bacteria belonging to *Ruminococcus*, *Mitsuokella*, *Catenibacterium* and some non-characterized bacteria belonging to *Oscillospirales* and *Clostridia*.

Table 5.6. DESeq2 output and contrast.

Contrast	ASVs[a]	baseMean[b]	log_2FC	lfcSE[c]	*p-value*	Genus
CH+R *vs*. control	ASV537	6.84	5.69	1.34	0.00	*Rhodopseudomonas*
	ASV688	3.94	5.23	1.48	0.00	*Prosthecomicrobium*
	ASV149	90.0	2.13	0.54	0.00	*Colidextribacter*
	ASV8	1515	1.04	0.34	0.00	*Lactobacillus*
	ASV191	251	– 2.30	0.63	0.00	*Ruminococcus*
	ASV25	2136	– 2.65	0.76	0.00	*Treponema*
	ASV124	102	– 3.46	0.66	0.00	*Helicobacter*
	ASV446	14.7	– 4.46	1.51	0.00	*Asteroleplasma*
CH+M *vs*. control	ASV4	1631	6.87	1.44	0.00	*Escherichia/Shigella*
	ASV23	2322	2.04	0.66	0.00	*Alloprevotella*
	ASV108	330	1.86	0.63	0.00	*Sutterella*
	ASV149	90.0	1.75	0.53	0.00	*Colidextribacter*
	ASV199	51.6	– 1.26	0.42	0.00	*Solobacterium*
	ASV151	60.8	– 2.91	0.63	0.00	*Catenibacterium*
	ASV99	108	– 3.61	1.26	0.00	*Streptococcus*
	ASV446	14.7	– 4.48	1.48	0.00	*Asteroleplasma*
	ASV239	68.2	– 4.67	1.36	0.00	*Mitsuokella*
CH *vs*. control	ASV8	1515	1.17	0.34	0.00	*Lactobacillus*
	ASV108	330	2.56	0.64	0.00	*Sutterella*
	ASV149	90.0	2.06	0.54	0.00	*Colidextribacter*
	ASV537	6.84	6.50	1.33	0.00	*Rhodopseudomonas*
	ASV688	3.94	5.17	1.48	0.00	*Prosthecomicrobium*
CH *vs*. CH+R	ASV124	102	3.72	0.68	0.00	*Helicobacter*
CH+M *vs*. CH+R	ASV4	1631	– 7.67	1.48	0.00	*Escherichia/Shigella*
All (CH, CH+R, CH+M) *vs*. control	ASV8	1515	1.04	0.34	0.00	*Lactobacillus*
	ASV25	2136	– 2.65	0.76	0.00	*Treponema*
	ASV124	102	– 3.46	0.66	0.00	*Helicobacter*
	ASV149	90.0	2.13	0.54	0.00	*Colidextribacter*
	ASV191	251	– 2.30	0.63	0.00	*Ruminococcus*

	ASV446	14.7	– 4.46	1.51	0.00	*Asteroleplasma*
	ASV537	6.84	5.69	1.34	0.00	*Rhodopseudomonas*
	ASV688	3.94	5.23	1.48	0.00	*Prosthecomicrobium*

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
[a]ASVs: abundant amplicon sequence variants.
[b]baseMean: mean of normalized counts of all samples.
[c]lfcSE: standard error estimated for the log2 fold change.

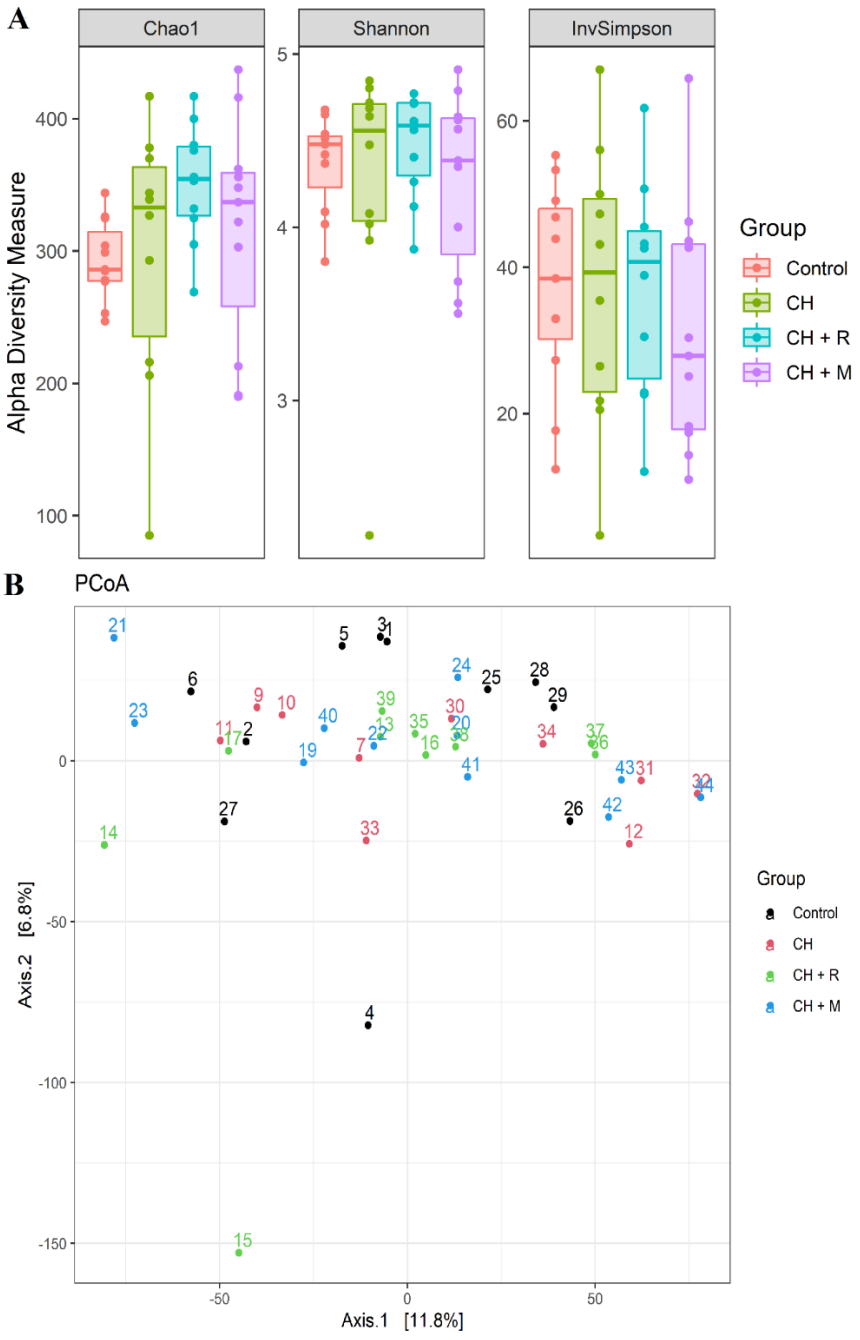

Figure 5.1. A) Boxplots showing alpha diversity for Chao1, Shannon, InvSimpson indices. (B) PCoA plot using a Euclidian distance matrix.
Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris diet* supplemented with a pre-selected enzymatic mixture.

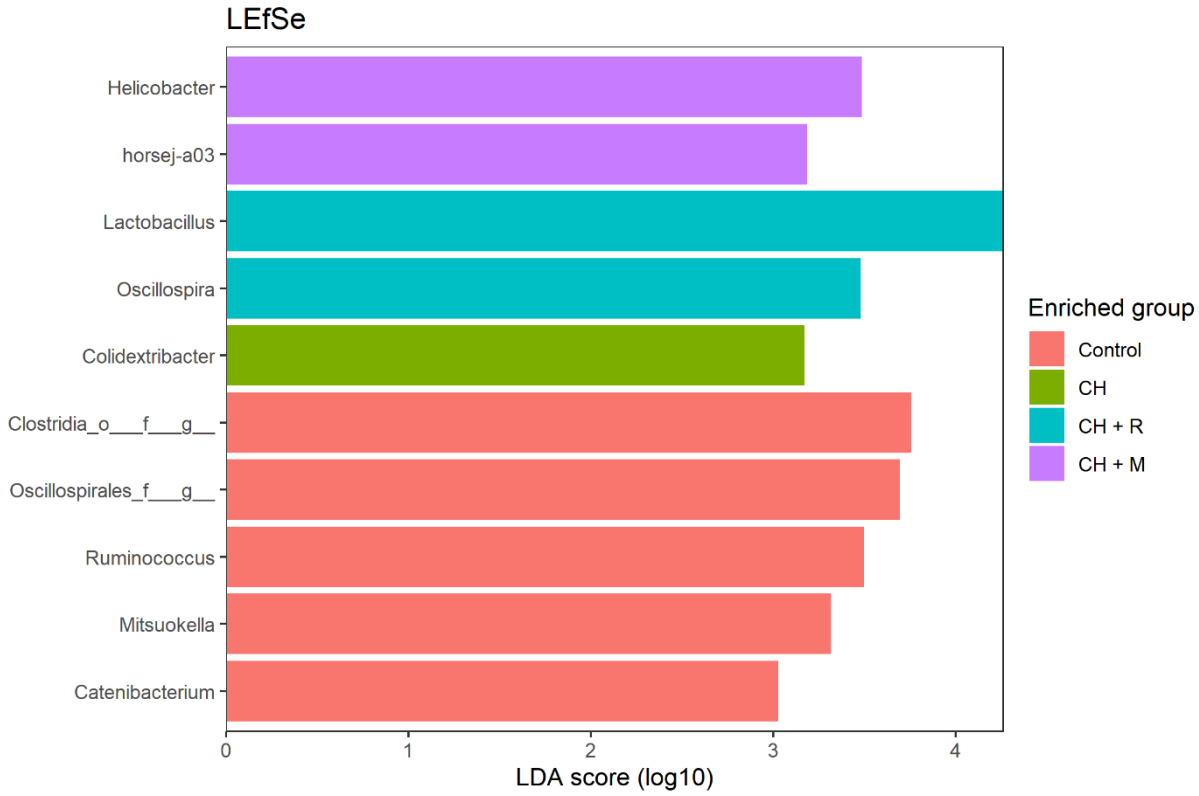

Figure 5.2. Barplot of Linear discriminant analysis (LDA) effect size (LEfSe).
Horizontal bars represent the effect size for each taxon. The length of the bar represents the LDA score. LDA threshold score for discriminative features was set to 3.0.
Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.

5.4. Discussion

This study assessed the effect of dietary inclusion of 5% *C. vulgaris*, alone and in combination with 2 exogenous carbohydrase formulations, on recently weaned piglet performance, nutrient digestibility and intestine morphology, fermentation and microbiota. To the best of our knowledge, this is the first time that the subject was assessed in such detail in the newly weaned piglet. We established that the dietary inclusion of *C. vulgaris* as a feedstuff had no impact on growth performance of piglets, although ADFI was significantly higher in the groups fed with microalga. Such higher ADFI was not enough to significantly increase the growth rate of the animals. In the future, there is interest in confirming these results through a growth performance trial that involving a larger number of animals and *ad libitum* access to experimental diets. In addition, the supplementation of *C. vulgaris*- based diets with exogenous enzymes (Rovabio® Excel AP and the pre-selected 4-carbohydrase mixture) did not exert a particular influence on animal performance parameters of the piglets. Such results are in accordance with a performance study on growing-finishing pigs (59.1 to 101.9 kg), where the

dietary inclusion of *C. vulgaris* and exogenous enzymes did not influence animal productive parameters (Coelho, Pestana et al. 2020). Nevertheless, there are several studies on the subject that use *C. vulgaris* as a supplement (≤ 1% in diet) in piglet feeding to have a prebiotic effect (Furbeyre et al. 2017; Madeira et al. 2017). For instance, Furbeyre et al. (2017) used 1% *C. vulgaris* in piglets' diets to mitigate the *post*-weaning stress. These authors found no significant effects on ADFI, ADG and FCR. In addition, the same authors performed a trial with *C. vulgaris* via drinking water (385 mg/kg LW) in weaning piglets with the same aim and also found no significant differences for ADFI, ADG and FCR (Furbeyre et al. 2018).

The TTAD of nutritional fractions was negatively affected by *C. vulgaris* incorporation, particularly the fibre fractions. This indicates a low efficiency of carbohydrase formulations in the *C. vulgaris* cell wall degradation in the intestine. However, the CH+R group had values closer to those of the control group than to those of the CH+M animals. Therefore, it could be speculated that the Rovabio® Excel AP has a higher *C. vulgaris* cell wall degradation ability than the 4-carbohydrase mixture. As Rovabio® Excel AP contains predominantly β-xylanases and β-glucanases, such hydrolytic activity was likely due to the degradation of the small amount of xylans and β-glucans in the *C. vulgaris* cell wall (Safi et al. 2014). After all, it is noteworthy to mention that dietary treatment influenced the fibre profile that reached piglets' large intestine. Furthermore, the TTAD of nutrients was significantly different for the 2 collection periods, with worse results for the second period. This indicates a difficulty of the piglets to adapt and digest diets containing high levels of *C. vulgaris*. Although no effects of diet and collection period on faecal consistency were observed, the higher value associated to animals fed with microalga in the second period seems to agree with the lower TTAD values determined.

The viscosity of duodenum plus jejunum contents slightly increased in the groups fed with microalga by comparison with the control group. Thus, it could be suggested that this increase in digesta viscosity did not result from the presence of soluble polysaccharides, such as arabinoxylans and β-glucans, as commonly observed in wheat or barley-based diets, since the presence of xylanases and β-glucanases in the CH+R group had no effect on viscosity. It is well known that higher digesta viscosity limits the access of endogenous enzymes to their target substrates (Martins, Pestana, Ribeiro et al. 2021). However, this effect disappeared in the ileum content, where no differences in viscosity were found between experimental groups, and a compensatory small intestine enlargement was not found. Regarding the intestinal morphology, the incorporation of microalga in piglets' diets affected only the duodenum villus height. This effect indicates the development of intestinal tissue in order to increase nutrients absorption. Similarly, Furbeyre et al. (2017) detected an increase in villus height in the jejunum, highlighting the positive effect of *C. vulgaris* supplementation on mucosal restoration or development after weaning. In our study, the increase of duodenum villus height seems to be

able to compensate, at least in part, the higher digesta viscosity promoted by the *C. vulgaris* feed incorporation.

Regarding VFA concentration in the caecum, the CH+M group showed a significantly lower quantity of total VFA compared with all other groups. The lower degradation rate reported in this group may be associated with the lower quantity of cell wall material that reach the caecum. This aspect suggests a possible effect of the enzymatic mixture on the degradation of microalga cell walls at the level of small intestine. In the colon, the concentration of total VFA was decreased for all animals fed with the microalga. This decrease indicates a low level of fermentable carbohydrates in the colon of piglets fed with *C. vulgaris*, with the consequent change in the microbial fermentation profile. The recalcitrant cell wall may justify the presence of less fermentable cell wall constituents in this digestive compartment. Several studies have associated insoluble dietary fibre content of diets to the effect on fermentation, generation and absorption of VFA at the level of the large intestine (Wang et al. 2018). Thus, the lower TTAD of fibre fractions of the CH+M group could also explain the lower VFA values. Additionally, Montoya et al. (2016) refer the importance of not extrapolating the results because the type of dietary fibre influences the quantity of VFA produced by fermentation. Information on physical characteristics, molecular structure and chemical composition of the fibre of *C. vulgaris* microalga is scarce and, therefore, further research has still to be conducted on this aspect.

Microbiome results suggest that the use of *C. vulgaris* in piglet feeding, in combination or not with enzymes, significantly affects the faecal bacterial structure of piglets, as previously observed in humans (Jin et al. 2000). In addition, *C. vulgaris* incorporation supplemented with 0.005% Rovabio® Excel AP increased the relative abundance of *Lactobacillu*s and *Oscillospira*. *Lactobacillus* which is one of the most represented genera. This bacterial taxon usually constitutes the core member of a healthy pig microbiota (Valeriano et al. 2017), preventing intestinal colonization of enteric pathogens (Huang et al. 2004). *Oscillospira* is an anaerobic bacterial genus from the Clostridial cluster, that is widely studied in human research due to its role in preventing specific diseases, such as obesity-related metabolic diseases (Konikoff and Gophna 2016). In addition, due to its ability to produce short-chain fatty acids (SCFAs) such as butyrate, it has been proposed as a potential probiotic (Yang et al. 2021). On the other end, the contrast highlighted that the CH+M group was characterised by *Helicobacter* genus, compared to the other groups. The highest prevalence of Helicobacter, together with the highest pH, in the upper part of the small intestine should be ascribed to the effect of enzyme mixture on the degradation of microalgae cell wall, thus providing a substrate for proteolytic bacteria. It thus seems that the efficacy of this enzyme mixture was site dependent. Indeed, the TTAD is lower, compared with that of the other CH groups, especially for the fibrous fractions. In accordance, VFA concentrations are lower in the CH+M group than in the other groups, particularly in the cecum. This suggests a lower efficiency of the 4-enzyme mixture to

degrade microalga cell wall. In addition, *C. vulgaris* supplementation reduced the abundance of *Ruminicoccus*, a bacterial taxon known for its fibrinolytic activity and the production of VFA, especially butyrate (La Reau and Suen 2018), which can also explain the lower VFA and TTAD of fibrous fractions.

5.5. Conclusions

In this study, we showed that the inclusion of 5% *C. vulgaris* in the diet improves piglets' gut health without compromising animal performance. Data indicates that although nutrients digestibility, mainly for fibre fractions, decreases by the incorporation of microalga in the diet, production performance of piglets is not impaired. This is likely explained by two compensatory mechanisms, the gut mucosa development and the probiotic properties of some specific bacterial taxa in the intestine (*Colidextribacter, Oscillospira* and *Lactobacillus*).

Moreover, the dietary supplementation with exogenous carbohydrases does not seem to be necessary for feeding piglets with *C. vulgaris*-based diets at this level of incorporation.

Considering that weaning is a critical period for piglets' health, the inclusion of *C. vulgaris* as a prebiotic and sustainable feed ingredient in the diet is an interesting strategy for swine production, particularly for the recently weaned piglet. However, its cost-effective utilization for this purpose warrants further investigation.

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Chapter 6 – EFFECTS OF *CHLORELLA VULGARIS* AS A FEED INGREDIENT ON THE QUALITY AND NUTRITIONAL VALUE OF WEANED PIGLETS' MEAT

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Contribution of Cátia Martins to this paper:
Cátia Martins performed the animal trial and sampling. Additionally, performed laboratory analysis for the determination of meat quality traits (namely, fatty acid composition and meat lipid oxidation). Finally, Cátia Martins collaborated in results interpretation and wrote the draft manuscript.

Abstract

Chlorella vulgaris is usually considered a feed supplement in pig nutrition, and its use as an ingredient is poorly studied. Among many interesting characteristics, this microalga has high protein levels and can be a putative alternative for soybean meal. Our aim was to study the effect of a 5% *Chlorella vulgaris* (CH) incorporation in the diet, individually or combined with two carbohydrases, on meat quality traits and nutritional value. Forty-four *post*-weaned male piglets individually housed, with an initial live weight of 11.2 ± 0.46 kg, were randomly distributed into four experimental groups: control (*n* = 11, without CH) and three groups fed with 5% CH incorporation, plain (*n* = 10), with 0.005% Rovabio® Excel AP (*n* = 10), and with 0.01% of a pre-selected four-CAZyme mixture *(n* = 11). After two weeks of trial, piglets were slaughtered and *longissimus lumborum* collected. CH had no effect on piglets' growth performance. In turn, incorporation of CH improved the nutritional value of meat by increasing total carotenoids and *n*-3 PUFA content, thus contributing to a more positive *n*-6:*n*-3 fatty acid ratio. The supplementation with Rovabio® Excel AP benefited tenderness and increased overall acceptability of pork. Our results show beyond doubt the viability of the utilization of this microalga as a feed ingredient for swine production.

Keywords: *Chlorella vulgaris*, feed enzymes, weaned piglets, meat quality, nutritional quality

6.1. Introduction

The global population is expected to grow close to approximately 10 billion by 2050, increasing agricultural demand by 50 percent when compared with 2013 (FAO 2017). In addition, the growth in income per capita in low- and medium-income countries, and the consequent higher consumption of meat, fruits, and vegetables, will lead to an increase in the land used for agriculture and animal production, with the consequent pressure on natural resources and ecosystems (FAO 2017). Among meats, pork is consumed worldwide (36% of total), with a tendency to increase (FAO 2007). The sustainability of monogastric production systems depends, therefore, on the suitability of substitute ingredients to corn and soybean. These crops are considered as the basis of monogastric (poultry and swine) feeding. Indeed, there are numerous issues regarding the sustainability of the feedstuffs, given the fact that they are mostly produced in North and South America and transported to consumer markets, with high economic and environmental costs. Furthermore, they are in direct competition with human nutrition (FAO 2011).

Microalgae have been studied for several economic applications, including animal feeding (Madeira et al. 2017). Microalgae can be produced in non-agricultural lands. They are photosynthetic organisms able to efficiently transform atmospheric carbon dioxide into high-value products, including carbohydrates, lipids, proteins, and pigments. Therefore, they have promising applications in the food and feed industries (Garcia-Vaquero 2020). Large-scale cultivation systems and new technologies are currently being developed to turn microalgae cultivation economically feasible (Pinto et al. 2020). In addition to this challenge, the microalgae cell wall is indigestible by monogastrics. The use of feed enzymes—namely, CAZymes that lysate their recalcitrant cell walls—has been demonstrated to be very efficient in improving the nutrient utilization of microalgae by monogastrics (Sander and Murthy 2009). Rovabio® Excel AP is a commercially available CAZyme mixture containing mainly xylanases and β-glucanases for cereal-based diets. This CAZyme mixture has also been used for microalgae- containing diets (Coelho, Pestana et al. 2020; Martins, Pestana, Ribeiro et al. 2021). Moreover, a four-CAZyme mixture, consisting of alginate lyase, exo-β-glucosaminidase, lysozyme, and peptidoglycan N-acetylmuramic acid deacetylase, has been shown to partially disrupt the *C. vulgaris* cell wall *in vitro* (Coelho, Lopes et al. 2020).

In piglets, weaning is a stressful event derived from social, environmental, and nutritional transitions. In order to decrease the use of antibiotics used to mitigate the piglet *post*-weaning stress, prebiotics can be a solution. The prebiotic properties of microalgae, in particular the *n*-3 PUFA content, have been studied by different authors (Grinstead et al. 2000; Furbeyre et al. 2017; Furbeyre et al. 2018). For instance, *n*-3 PUFA of microalgae improve the

fatty acid composition of animal edible tissues, with recognized beneficial health consequences for both humans and animals.

In addition, spit-roasted piglet is a meat that is consumed worldwide, very popular in Mediterranean Europe, Latin America, Louisiana (USA), China, and several islands of Indonesia and the Pacific. It is particularly consumed on special occasions and at family celebrations, such as Christmas. In Mediterranean Europe, it is a highly valued gourmet food, often considered as a regional specialty. For instance, in Portugal the most popular specialities are *Leitão da Bairrada* and *Leitão de Negrais*, whereas in Spain the *Cochinillo Asado* is a reputed specialty of the Castilla-León region. Finally, body composition at the end of *post*-weaning determines production performance at the growing-finishing period and body composition when pigs achieve 100 kg of body weight (Fix et al. 2010; Collins et al. 2017).

This work aimed to study the dietary incorporation of 5% of *C. vulgaris*, with or without exogenous enzymes, on meat quality characteristics and nutritional significance of piglets. We assessed pH, colour, lipid oxidation, sensorial qualities, fatty acid composition, and pigment profile. We hypothesized that *C. vulgaris* can be a viable ingredient in piglet feeding by improving the digestibility of valuable microalga nutrients without negatively affecting animal performance and meat traits.

6.2. Material and Methods

6.2.1. Animals and experimental diets

The animal trial was performed at ISA (University of Lisbon, Lisbon, Portugal) facilities. All the procedures were reviewed by the Ethics Commission of ISA and accepted by the Animal Care Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Lisbon, Portugal), in accordance with the European Union legislation (2010/63/EU Directive). We selected forty-four castrated male piglets from Large White Landrace sows crossed with Pietrain boars; they were weaned at 28 days of age and had an initial body weight of 11.2 ± 0.46 kg (mean ± SD). The piglets were single housed in metabolic cages (1000 x 500 x 480 mm). During the adaptation period of two days, to minimize stress and stabilize all metabolic conditions, two animals failed to adapt and were not considered in the experiment. The remaining piglets were arbitrarily distributed in 4 experimental groups: Control (*n* = 11, cereal and soybean meal-based diet), CH (*n* = 10, control diet with 5% *C. vulgaris*), CH+R (*n* = 10, control diet with 5% *C. vulgaris* and 0.005% Rovabio® Excel AP from Adisseo (Antony, France)), and CH+M (*n* = 11, control diet with 5% *C. vulgaris* supplemented and 0.01% of a pre-selected four-CAZyme mixture (previously described by Coelho, Lopes et al. (2020))). *C. vulgaris* was produced as described in detail by Coelho, Pestana et al. (2020). Then, this

microalga was supplied as freeze-dried powder (Allmicroalgae—Natural Products SA, Pataias, Portugal) and incorporated in the diets. Rovabio® Excel AP was incorporated in the diet at a 0.005% level following the manufacturer's recommendation.

Diets were dried at 103 ºC to constant weight to assess DM. Crude protein of diets was determined following the method 954.01 (AOAC 2000) utilizing the factor 6.25 x nitrogen content (N) calculated by the Kjeldahl procedure. Crude fat of diets was assessed by an automatic Soxhlet extraction with petroleum ether (Gerhardt Analytical Systems, Königswinter, Germany). Ash content of the experimental diets was assessed following the 942.05 (AOAC 2000) method. NDF and ADF were determined by 989.03 (AOAC 2000) method. Metabolizable energy was estimated in accordance with Noblet et al. (1989). Fatty acids were determined by one-step extraction and converted to FAME through acid transesterification and GC having heneicosanoic acid methyl ester as the internal standard (Sukhija and Palmquist, 1988). β-Carotene and tocopherols of diets were determined by direct saponification, with a single n-hexane extraction followed by HPLC, based on the external standard technique from a standard curve of peak area *vs*. concentration, as previously reported (Prates et al. 2006). The determination of pigments in diets was carried out in accordance with Teimouri et al. (2013), with minor alterations. In brief, diets (0.5 g) were incubated at room temperature with acetone overnight under agitation and in the dark. Following on extraction, samples were subjected to centrifugation at 4000 rpm during 5 min and analyzed by UV-Vis spectrophotometry (Ultrospec 3100; Amersham Biosciences, Little Chalfont, UK). The concentration of pigments was assessed using methodologies described by Hynstova et al. (2018) equations. All diets were formulated to have 3440 kcal ME/kg of energy and 19.5% of CP, as fed basis. The ingredients and chemical composition of diets are shown in Table 6.1. In all dietary formulations, a vitamin-mineral complex, as previously described by Martins, Pestana, Ribeiro et al. 2021, was incorporated at a rate of 3%. The detailed chemical composition of *C. vulgaris* was previously described (Coelho, Pestana et al. 2020).

Table 6.1. Ingredients and chemical composition of experimental diets.

Item	Control	CH	CH+R	CH+M
Ingredients (% as fed basis)				
Wheat	43.9	44.0	44.0	44.0
Corn	15.0	15.0	15.0	15.0
Soybean meal 48	25.0	20.0	20.0	20.0
Whey powder	10.0	10.0	10.0	10.0
Sunflower oil	3.00	3.00	3.00	3.00
Chlorella vulgaris	0	5.00	5.00	5.00
Rovabio® Excel AP	0	0	0.005	0
Four-CAZyme mixture	0	0	0	0.010
Metabolizable energy (kcal ME[1]/kg as fed basis)	3428	3436	3449	3449
Proximate composition (% as fed basis)				
Dry matter	90.5	90.8	90.8	90.9
Crude protein	19.3	19.2	19.5	19.4
Crude fat	5.29	5.39	5.39	5.63
Ash	5.43	5.65	5.47	5.60
NDF	12.9	11.9	12.9	10.4
ADF	2.76	2.45	2.58	2.54
Fatty acid composition (% total FA)				
14:0	0.351	0.380	0.380	0.361
16:0	10.6	11.0	10.9	11.1
16:1*n*-7	0.158	0.903	0.900	0.677
17:0	0.095	0.104	0.103	0.104
17:1*n*-8	0.040	0.583	0.643	0.828
18:0	3.35	3.33	3.38	3.26
18:*1n*-9	24.8	24.5	24.5	24.6
18:1*n*-7	0.909	1.16	1.16	1.10
18:2*n*-6	55.8	53.3	53.2	52.9
18:3*n*-3	1.55	2.18	2.23	2.52
β-Carotene and tocopherol profile (µg/g)				
β-Carotene	n.d.	13.3	13.7	14.5
α-Tocopherol	28.6	19.9	22.1	24.2
β-Tocopherol	1.11	1.10	1.00	1.12

γ-Tocopherol	2.52	2.00	2.21	2.11
δ-Tocopherol	0.502	0.334	0.387	0.396
α-Tocotrienol	3.43	3.73	3.58	3.53
γ-Tocotrienol	1.38	1.51	1.69	1.46
Pigments (µg/g)				
Chlorophyll *a*[2]	3.38	109	130	135
Chlorophyll *b*[3]	6.05	31.9	42.6	39.8
Total chlorophylls[4]	9.43	141	172	174
Total carotenoids[5]	2.67	36.9	44.5	52.9
Total chlorophylls and total carotenoids[6]	12.1	178	217	227

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with 0.005% of Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with 0.01% of four-CAZyme mixture.
ADF, acid detergent fibre; NDF, neutral detergent fibre; n.d., not detected.
[1]Metabolizable energy (kcal/kg DM) = 4412 - 11.06 x ash (g/kg DM) + 3.37 x crude fat (g/kg DM) - 5.18 x ADF (g/kg DM).
[2]$Ca = 11.24\ A_{662} - 2.04\ A_{645}$.
[3]$Cb = 20.13\ A_{645} - 4.19\ A_{662}$.
[4]$Ca + b = 7.05\ A_{662} + 18.09\ A_{645}$.
[5]$Cx + c = (1000\ A_{470} - 1.90\ Ca - 63.14\ Cb)/214$.
[6](Ca + b) + (Cx + c).

6.2.2. Productive parameters

Throughout the animal trial, feed and refusals were recorded daily. Animals were weighed once a week before feeding to calculate ADFI, ADG, and FCR. After 15 days of the experiment, piglets were slaughtered at a body weight of 23.1 ± 2.56 kg, through electrical stunning followed by exsanguination, in accordance with standard protocols applied in commercial abattoirs. *Longissimus lumborum* muscle samples were extracted from both sides of the carcass, between the third and fifth lumbar vertebrae. Muscle samples from the left carcass side were collected, minced, vacuum packed, and stored at - 20 ºC for intramuscular fat and fatty acid profile and for total pigments and tocopherol profile determinations. For TBARS analysis, muscles samples were stored at - 80 ºC. Muscle samples from the right carcass side were stored at 4 ºC during 24 h for colour and pH determinations. Then, the samples were vacuum packed and frozen at - 20 ºC until cooking loss, shear force, and sensory analyses.

6.2.3. Determination of meat quality traits

The pH of *longissimus lumborum* at 24 h *post-mortem* was measured using a pH meter with a glass penetrating electrode from Hanna Instruments (Woonsocket, RI, USA) and was determined as an average of 3 replicates. Meat colour variables, such as L*, a*, and b* were measured 24 h *post-mortem* on 3 spots of cut surface of the *longissimus lumborum* samples using a colorimeter (Minolta CR-300; Konica Minolta, Tokyo, Japan) after 1 h at 4 ºC. Lipid oxidation of meat was assessed by TBARS at days 0 and 8, stored at 4 ºC, following the procedure of Grau et al. (2000). TBARS were calculated in duplicate from a standard curve of 1,1,3,3-tetraethoxypropane (Fluka, Neu Ulm, Germany) and expressed as mg of malondialdehyde/kg of muscle.

6.2.4. Determination of cooking loss and shear force

Meat samples were thawed at 4 ºC overnight and cooked using a water bath at 80 ºC until reaching 78 ºC of internal temperature, monitored by a thermocouple (Lufft C120; Lufft, München, Germany). After 2 h cooling at room temperature, samples were longitudinally cut toward the fibres with a 1 cm^2 cross-section for cooking loss and shear force. Before and after cooking, meat samples were weighed to determine cooking loss. Meat shear force was determined using a Warner-Bratzler blade coupled to a texture analyzer (TA-XT Plus texture analyzer; Stable Micro Systems, Surrey, UK) and is expressed as the mean of the peak value of a minimum of 4 replicate measurements.

6.2.5. Trained sensory panel analysis

Trained sensory analysis was carried out in muscle samples, trimmed of external connective tissue, cut into cubes with approximately 1 cm^3, and cooked in a water bath, as previously mentioned for cooking loss. Samples were arbitrarily allocated across 5 panel sessions, with 8 random samples per session. The attributes were tenderness, juiciness, flavour, off-flavour, and overall acceptability in a numeric scale from 1 to 8, in which 1 was the low/negative score and 8 was the high/positive score. For off-flavour, the scale applied was from 0 (absence) to 8 (maximum). The sensory panel consisted of thirteen panelists, selected after intensive training, according to Cross et al. (1979).

6.2.6. Determination of intramuscular fat and fatty acid profile

Intramuscular fat from lyophilized *longissimus lumborum* samples was extracted according to Folch et al. (1957), utilizing dichloromethane–methanol (2:1, v/v) as reported by Carlson (1985), and measured gravimetrically by weighing the fatty residue after solvent evaporation. Fatty acids were converted to FAME through a combined alkaline and acid sequential transesterification, in accordance with Raes et al. (2001). The fatty acid composition was analyzed by GC (HP6890A; Hewlett-Packard, Avondale, PA, USA), equipped with a flame ionization detector, as described (Coelho, Pestana et al. 2020). The identification of FAME was achieved using a reference standard (FAME mixture of 37 compounds, Supelco Inc., Bellefonte, PA, USA) corroborated by GC along with mass spectrometry using a GC-MS QP2010-Plus (Shimadzu, Kyoto, Japan). FAME calculation was based on the internal standard technique with heneicosanoic acid. Fatty acids are expressed as a percentage of the sum of identified FA.

6.2.7. Determination of total pigments, cholesterol, and tocopherols

Chlorophyll *a*, chlorophyll *b*, and total carotenoids contents were quantified in meat, in accordance with Teimouri et al. (2013). Samples were subjected to incubation overnight with acetone (Merck KGaA, Darmstadt, Germany) and agitation at room temperature in the dark. Following on centrifugation, the absorbance was read at a UV-Vis spectrophotometer (Ultrospec 3100 pro; Amersham Biosciences, Little Chalfont, UK) and results were determined in accordance with Hynstova et al. (2018). The parallel quantification of total cholesterol, β-Carotene, and tocopherols, in duplicate, in meat samples was carried out, according to Prates et al. (2006).

6.2.8. Statistical analysis

All data were analyzed with the PROC GLM of SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA). Data were checked for normal distribution and variance homogeneity. The statistical model assumed the dietary treatment as the single effect and the piglet as the experimental unit. When significant effects of dietary treatments were observed, least-squares means for multiple comparisons were generated using the PDIFF option adjusted with Tukey–Kramer method. Results were considered significantly different at $p < 0.05$.

6.3. Results

6.3.1. Zootechnical parameters

Table 6.2 shows results on growth performance parameters and feed intake of piglets. Diets had no significant effect on growth performance variables, such as final live weight, ADG, and FCR ($p > 0.05$). The reference group had lower ADFI than groups fed with *C. vulgaris* ($p = 0.008$), although this difference had no impact on piglets' growth.

Table 6.2. Effect of experimental diets on feed intake and growth performance of piglets.

	Diets				SEM	*p-value*
	Control	CH	CH+R	CH+M		
Initial live weight (kg)	11.1	11.1	11.3	11.2	0.105	0.851
Final live weight (kg)	22.3	23.3	23.5	23.1	0.247	0.349
ADFI (g)[1]	768[a]	852[b]	857[b]	856[b]	11.6	0.008
ADG (g)[2]	535	581	579	569	8.50	0.189
FCR[3]	1.44	1.47	1.48	1.51	0.013	0.282

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with 0.005% of Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with 0.01% of four-CAZyme mixture.
[1]ADFI, average daily feed intake.
[2]ADG, average daily weight gain.
[3]FCR, feed conversion ratio.
[a,b]Values with different superscript letters in the same row are significantly different ($p < 0.05$).

6.3.2. Meat quality traits

6.3.2.1. pH, colour, and susceptibility to lipid oxidation

The impact of experimental diets on meat quality traits from piglets is shown in Table 6.3. Diets did not affect pH 24 h *post-mortem* and colour parameters ($p > 0.05$). Although TBARS were not detected in meat at day 0, their levels were diminished in the reference group relative to CH+M (0.151 *vs*. 0.805 mg of malondialdehyde/kg of muscle, respectively) after 8 days under refrigeration ($p = 0.019$).

Table 6.3. Effect of experimental diets on pH 24 h, CIE colour parameters (L*, a*, b*) and TBARS levels (mg malondialdehyde/kg muscle) after 0 and 8 days under refrigeration in *longissimus lumborum* muscle.

	Diets				SEM	*p-value*
	Control	**CH**	**CH+R**	**CH+M**		
pH 24 h	5.61	5.54	5.57	5.61	0.033	0.401
Colour						
L*	48.6	48.6	48.5	47.3	0.810	0.582
a*	6.20	6.50	6.79	7.26	0.321	0.112
b*	−0.528	−0.157	−0.391	−0.821	0.2458	0.275
TBARS[1]						
Day 0	n.d.	n.d.	n.d.	n.d.	-	-
Day 8	0.151[a]	0.752[ab]	0.621[ab]	0.805[b]	0.161	0.019

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with 0.005% of Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with 0.01% of four-CAZyme mixture.
[1]TBARS-thiobarbituric acid reactive substances; n.d., not detected (<0.020 mg malondialdehyde/kg muscle).
[a,b]Values with different superscript letters in the same row are significantly different ($p < 0.05$).

6.3.2.2. Cooking loss, shear force, and sensory panel analysis

Table 6.4 shows the impact of experimental diets on cooking loss, shear force, and sensory panel analysis of meat. Cooking loss had a statistically higher value in the control group compared with the CH+M group ($p = 0.011$). Shear force was unaffected by diets ($p > 0.05$). Juiciness, flavour, and off-flavour presented no significant differences among diets ($p > 0.05$). However, for tenderness, the CH+R group showed the tenderest meat ($p < 0.001$). In line with this finding, the overall acceptability was higher in the CH+R muscle compared with the other diets ($p = 0.001$).

Table 6.4. Effect of experimental diets on cooking loss (%), shear force (kg) and sensory panel analysis in *longissimus lumborum*.

	Diets				SEM	*p-value*
	Control	**CH**	**CH+R**	**CH+M**		
Cooking loss	28.5^{b}	27.5^{ab}	25.5^{ab}	24.7^{a}	0.881	0.011
Shear force	3.99	4.04	3.68	4.29	0.269	0.466
Sensory panel scores						
Tenderness	4.95^{a}	5.10^{a}	5.69^{b}	5.04^{a}	0.121	<0.001
Juiciness	5.08	5.34	5.48	5.21	0.109	0.056
Flavour	4.90	4.92	4.96	4.96	0.111	0.965
Off-flavour	0.276	0.362	0.298	0.378	0.0764	0.714
Overall acceptability	4.91^{a}	5.09^{a}	5.54^{b}	5.03^{a}	0.112	0.001

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with 0.005% of Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with 0.01% of four-CAZyme mixture.
^{a,b}Values with different superscript letters in the same row are significantly different ($p < 0.05$).

6.3.3. Intramuscular fat, total cholesterol, and fatty acid profile of meat

The impact of experimental diets on intramuscular fat, total cholesterol, and fatty acid profile of *longissimus lumborum* muscle samples is shown in Table 6.5. Intramuscular fat and cholesterol contents were unaffected by diets ($p > 0.05$). Dietary treatments influenced only a few FA, specifically 15:0, 17:0, 17:1*n*-8, 18:0, 20:1*n*-9, 20:2*n*-6, 22:1*n*-9, 22:5*n*-3, and 22:6*n*-3. Compared with *Chlorella*-fed piglets, the control group had a higher percentage of 15:0 ($p < 0.0001$), 17:0 ($p < 0.001$), and 17:1*n*-8 ($p < 0.001$). Interestingly, stearic acid (18:0) was lower in control and CH+M groups in comparison with the other groups ($p = 0.023$). In contrast, the proportion of 20:2*n*-6 increased in piglets fed control and CH+M diets ($p = 0.004$). Additionally, the reference group had a higher proportion of 20:1*n*-9 ($p = 0.009$) but a lower proportion of

22:1*n*-9 (p = 0.004) than piglets fed CH. Conversely, the proportions of 22:5*n*-3 (DPA, docosapentaenoic acid) (p < 0.001) and 22:6*n*-3 (DHA) (p = 0.001) were enhanced in piglets fed CH and CH+M. Indeed, both DPA and DHA increased at least 1.79-fold and 2.35-fold in CH and CH+M groups, respectively.

Concerning the partial sums and ratios of FA, only the *n*-3 PUFA sum was enhanced in CH and CH+M groups (p < 0.001) compared with the other groups. The remaining partial sums of FA, as well as the PUFA/SFA ratio, were similar across all dietary treatments (p > 0.05). Nevertheless, the *n*-6:*n*-3 ratio was reduced in microalga-fed groups in comparison with the control group (p < 0.001).

Table 6.5. Effect of experimental diets on intramuscular fat content (g/100 g muscle), total cholesterol (mg/100 g muscle), and fatty acid (FA) composition (% of total FA) in *longissimus lumborum*.

	Diets				SEM	*p- value*
	Control	**CH**	**CH+R**	**CH+M**		
Intramuscular fat	1.36	1.14	1.31	1.35	0.078	0.159
Total cholesterol	54.8	53.4	44.4	48.4	0.029	0.058
Fatty acid composition						
10:0	0.024	0.028	0.032	0.019	0.005	0.204
12:0	0.046	0.049	0.047	0.054	0.005	0.660
14:0	0.839	0.826	0.943	0.948	0.091	0.661
14:1*n*-5	0.015	0.015	0.015	0.012	0.003	0.924
15:0	0.310^{b}	0.149^{a}	0.128^{a}	0.135^{a}	0.028	<0.001
DMA-16:0	0.128	0.131	0.122	0.136	0.022	0.974
16:0	21.7	23.8	24.5	23.7	1.03	0.244
16:1*n*-9	2.11	1.95	2.51	2.24	0.170	0.139
16:1*n*-7	0.442	0.510	0.468	0.487	0.017	0.053
17:0	1.25^{b}	0.730^{a}	0.620^{a}	0.710^{a}	0.122	0.001
17:1*n*-8	0.864^{b}	0.500^{a}	0.451^{a}	0.467^{a}	0.080	0.001
DMA-18:0	0.030	0.039	0.027	0.043	0.006	0.210
DMA-18:1	0.029	0.053	0.036	0.047	0.009	0.203
18:0	12.7^{a}	14.3^{b}	14.3^{b}	13.8^{ab}	0.427	0.023
18:1*n*-9	28.8	26.8	30.1	29.0	1.26	0.314
18:1*n*-7	3.35	3.31	3.25	3.26	0.073	0.717
18:2*n*-6	19.3	17.9	15.9	16.7	1.85	0.558
18:2*t*9*t*12	0.074	0.070	0.074	0.073	0.007	0.979

18:3*n*-6	0.131	0.149	0.106	0.113	0.020	0.431
18:3*n*-3	0.382	0.358	0.378	0.424	0.050	0.809
20:0	0.187	0.210	0.187	0.190	0.010	0.333
20:1*n*-9	0.586^{b}	0.463^{a}	0.543^{ab}	0.514^{ab}	0.025	0.009
20:2*n*-6	0.720^{b}	0.487^{a}	0.465^{a}	0.514^{ab}	0.053	0.004
20:3*n*-6	0.369	0.358	0.251	0.295	0.054	0.363
20:4*n*-6	2.69	3.07	1.64	2.11	0.462	0.150
20:3*n*-3	0.056	0.051	0.057	0.051	0.007	0.868
20:5*n*-3	0.053	0.082	0.060	0.065	0.009	0.139
22:0	0.083	0.110	0.092	0.085	0.009	0.171
22:1*n*-9	0.050^{a}	0.093^{b}	0.069^{ab}	0.065^{ab}	0.008	0.004
22:2*n*-6	0.040	0.040	0.035	0.032	0.007	0.766
22:5*n*-3	0.275^{a}	0.595^{b}	0.231^{a}	0.491^{b}	0.056	<0.001
22:6*n*-3	0.305^{a}	0.889^{b}	0.621^{ab}	0.716^{b}	0.096	0.001
23:0	0.155	0.254	0.181	0.202	0.026	0.062
Others	1.89	1.63	1.56	2.35	0.299	0.229
Partial sums of FA						
∑ SFA[1]	37.3	40.4	41.0	39.8	1.29	0.173
∑ MUFA[2]	36.2	33.6	37.4	36.0	1.40	0.288
∑ PUFA[3]	24.4	24.1	19.8	21.6	2.36	0.458
∑ *n*-3 PUFA[4]	1.07^{a}	1.97^{b}	1.35^{a}	1.75^{b}	0.101	<0.001
∑ *n*-6 PUFA[5]	23.3	22.0	18.4	19.8	2.38	0.453
Ratios of FA						
PUFA/SFA	0.661	0.632	0.491	0.573	0.075	0.386
n-6:*n*-3	21.9^{b}	11.7^{a}	13.5^{a}	12.7^{a}	1.51	<0.001

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with 0.005% of Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with 0.01% of four-CAZyme mixture.
SEM, standard error of the mean; FA, fatty acids; DMA, dimethylacetal; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[1]10:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 23:0.
[2]14:1*n*-5 + 16:1*n*-9 + 16:1*n*-7 + 17:1*n*-8 + 18:1*n*-9 + 18:1*n*-7 + 20:1*n*-9 + 22:1*n*-9.
[3]18:2*n*-6 + 18:3*n*-6 + 18:3*n*-3 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6 + 20:3*n*-3 + 20:5*n*-3 + 22:2*n*-6 + 22:5*n*-3 + 22:6*n*-3.
[4]18:3*n*-3 + 20:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3.
[5]18:2*n*-6 + 18:3*n*-6 + 20:2*n*-6 + 20:3*n*-6 + 20:4*n*-6.
[a,b]Values with different superscript letters in the same row are significantly different ($p < 0.05$).

6.3.4. Total pigments and tocopherol profile of meat

Total carotenoids, chlorophylls, and tocopherols of *longissimus lumborum* samples are shown in Table 6.6. The pigment contents and tocopherol profile were identical for all dietary treatments ($p > 0.05$), expect for total carotenoids. Meat from *Chlorella*-fed piglets had values of total carotenoids 2 times higher than the reference group ($p = 0.002$). β-Carotene was undetected in any of the groups.

Table 6.6. Effect of experimental diets on total pigments (µg/100 g) and tocopherol profile (µg/g) in *longissimus lumborum*.

	Diets				SEM	*p-value*
	Control	**CH**	**CH+R**	**CH+M**		
Pigments (µg/100 g)						
β-Carotene	n.d	n.d	n.d	n.d	-	-
Chlorophyll *a*[1]	6.87	14.1	14.1	16.4	2.92	0.107
Chlorophyll *b*[2]	13.3	22.5	21.9	25.0	5.44	0.420
Total chlorophylls[3]	20.2	36.5	36.0	41.4	8.30	0.273
Total carotenoids[4]	3.75[a]	7.14[b]	7.99[b]	7.51[b]	0.819	0.002
Total chlorophylls and total carotenoids[5]	23.9	43.7	44.1	48.9	8.49	0.154
Tocopherols (µg/g)						
α-Tocopherol	1.13	1.08	0.947	1.03	0.066	0.257
γ-Tocopherol	0.025	0.024	0.026	0.027	0.001	0.217

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with 0.005% of Rovabio®Excel AP; CH+M - *C. vulgaris* diet supplemented with 0.01% of four-CAZyme mixture.
[1]Ca = 11.24 A662 - 2.04 A645.
[2]Cb = 20.13 A645 - 4.19 A662.
[3]Ca + b = 7.05 A662 + 18.09 A645.
[4]Cx + c = (1000 A470 - 1.90 Ca - 63.14Cb)/214.
[5](Ca + b) + (Cx + c); n.d., not detected.
[a,b]Values with different superscript letters in the same row are significantly different ($p < 0.05$).

6.4. Discussion

To the best of our knowledge, this is the first study ever to use *C. vulgaris* microalga as a feedstuff in piglets' diet, supplemented or not with exogenous enzyme cocktails, such as the Rovabio® Excel AP and the preselected four-CAZyme mixture (Coelho, Lopes et al. 2020). In this work, a zootechnical trial was performed along with the determination of pork quality and nutritional traits. The dietary incorporation of 5% of *C. vulgaris* had no impact on growth performance of piglets. In agreement, Furbeyre et al. (2017) using Spirulina and *C. vulgaris*, both at a supplement level of 1%, showed no effects over ADFI and ADG in weaned piglets (9.1 to 20 kg LW). The authors studied the administration of the same microalgae via drinking water (385 mg/kg LW) and found no effect on growth performance in suckling (4.9 kg LW) and weaned piglets (9.04 kg LW) (Furbeyre et al. 2017). Like other studies using microalgae as a dietary supplement, Yan et al. (2012) described that 0.1 and 0.2% dietary incorporation of fermented *C. vulgaris* in pigs' diets (26.6 to 53.0 kg LW) promoted an increase in the ADG of 31 g/d relative to the reference diet. For the first time, Martins, Pestana, Ribeiro et al. (2021) used Spirulina as an ingredient (10% of dietary inclusion) and described that the growth performance of piglets was reduced, thus highlighting the need of feed enzymes to enhance the digestive utilization of this microalga. In our study, no significant effects on the growth performance of piglets were found, revealing that the dietary level of 5% *C. vulgaris* did not compromise the productive variables. The exogenous carbohydrases applied had no consequences to the point of a higher level of supplementation being necessary, as advanced by Martins, Pestana, Ribeiro et al. (2021).

Regarding meat quality traits, the level of 5% *C. vulgaris* incorporation, when combined with the pre-selected four-CAZyme mixture, only affected the oxidative stability of l*ongissimus lumborum* at day 8 *post-mortem* (storage at 4 ºC). After 8 days under refrigeration, the increased TBARS reflect a higher instability of meat from microalga-fed piglets with the four-CAZyme mixture in comparison with the control group. This is likely due to poor radical-scavenging activity of the intrinsic antioxidants for mitigating the lipid oxidation promoted by enhanced *n*-3 PUFA content. TBARS over 0.5 mg malondialdehyde/kg of fresh meat are recognized as crucial since, at this level of lipid oxidation, the rancid off-flavours are easily perceived by the consumers (Wood et al. 2008). In the current study, only at day 8 of storage, TBARS were above this threshold value. Moreover, TBARS values for the four-CAZyme mixture diet-fed animals were lower than 0.9 mg malondialdehyde/kg of meat, proposed by Jayasingh and Cornforth (2003) for ground and cooked pork. Martins, Pestana, Ribeiro et al. (2021) found that in comparison with the reference diet, the incorporation of 10% of Spirulina in piglets' diet, without enzyme supplementation, increased TBARS at three days of storage

under refrigeration. Likewise, data on the oxidative stability of meat did not match the antioxidant power of Spirulina, as in the present case of *C. vulgaris*.

An existing relationship between cooking loss and juiciness in pork was described by Aaslyng et al. (2003). The higher value in cooking loss found in the reference group influenced the lower value of juiciness for the same diet. Sensory attributes such as tenderness and overall acceptability were increased by Rovabio® Excel AP commercial supplementation relative to the other diets, suggesting that overall consumer acceptability is mostly determined by tenderness. Furthermore, and according to our trained sensory panel, *C. vulgaris* had no negative effect on meat flavour, thus contributing to consumer's acceptance of this meat.

Feeding piglets with 5% of *C. vulgaris*, individually or combined with the four-CAZyme mixture, increased DPA and DHA, showing a positive correspondence between *n*- 3 PUFA in the diet and *n*-3 PUFA deposited in *longissimus lumborum* muscle. *n*-3 long-chain PUFA display health beneficial effects (Mason 2019). In fact, several animal and epidemiological reports have proven the advantages of *n*-3 PUFA on cardiovascular disease outcomes (Endo and Arita 2016; Kris-Etherton et al. 2019). Furthermore, the FAO, the WHO, and the American Heart Association recommended EPA (20:5*n*-3; eicosapentaenoic acid) plus DHA daily intake from 140 to 600 mg/d, depending on the authority guidelines (Molendi-Coste et al. 2011; Aranceta and Pérez-Rodrigo 2012). However, most Western populations consume an average below 500 mg/day of *n*-3 long-chain PUFA (Martins et al. 2013). For instance, piglets' diet receiving 5% of *C. vulgaris* combined with the four-CAZyme mixture could be a valuable source of these protective FA to both animals and humans. Consistent with our findings, the dietary *C. vulgaris* at this level of incorporation also produced an increment in *n*-3 PUFA amount in finishing pigs (Coelho, Pestana et al. 2020). The enhancement of *n*-3 PUFA content subsequently resulted in a positive decline in *n*-6:*n*-3 ratio in muscle with incorporation of *C. vulgaris* in piglets' diet. Although the *n*-6:*n*-3 ratios were considerably elevated, our data indicate that meat from piglets fed this microalga complies more (around 12.6) with the advised *n*-6:*n*-3 ratio (below 4), thus promoting health-protecting cardiovascular effects for consumers and improving meat quality (HMSO 1994).

A significant increase of total carotenoids in *longissimus lumborum* muscle was observed in piglets fed *C. vulgaris*, which reflects diet composition. In fact, the incorporation of this microalga led to higher content of pigments in the diets, in particular about 17 times more total carotenoids if compared with the reference diet. As highlighted by Coelho, Pestana et al (2020), the transfer of carotenoids from the microalga to the meat adds extra nutritional value to pork. Our data are in accordance with these authors, who also found 2 times higher total carotenoid contents in meat from finishing pigs fed with 5% of *C. vulgaris*. Similar to the study by Coelho, Pestana et al. (2020), β-Carotene (pro-vitamin A) was undetected in meat, possibly

indicating that this pigment was rapidly metabolized into vitamin A because pigs are unable to synthesize carotenoids.

6.5. Conclusions

The incorporation of *C. vulgaris* at a level of 5% in the diet does not impair growth performance of piglets or their meat quality traits. In contrast, at this level of dietary inclusion, it seems that an improvement in the nutritional value of pork occurs, in particular through the increment of total carotenoids and *n*-3 PUFA content, which promotes a beneficial *n*-6:*n*-3 PUFA ratio for the consumers. Additionally, the supplementation with exogenous enzymes, both the commercial Rovabio® Excel AP formulation and the pre-selected four-CAZyme mixture, seems to have a minor impact on the multiple parameters assessed. One exception is the increased score for tenderness and overall acceptability of pork from piglets fed *C. vulgaris* combined with Rovabio® Excel AP. In view of these findings, further research is warranted, focusing in particular on higher levels of *C. vulgaris* incorporation, individually or supplemented with feed enzymes, in order to ascertain whether *C. vulgaris* is a cost-effective alternative feedstock for livestock production.

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Chapter 7 – IMPACT OF DIETARY *CHLORELLA VULGARIS* AND FEED ENZYMES ON HEALTH STATUS, IMMUNE RESPONSE AND LIVER METABOLITES IN WEANED PIGLETS

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Contribution of Cátia Martins to this paper:
Cátia Martins performed the animal trial and sampling. Additionally, performed the HPLC methodology to quantify the hepatic fatty acid and the sample preparation to metabolomic analysis. Finally, Cátia Martins collaborated in results interpretation and wrote the draft manuscript.

Abstract

In this study, we analysed the impact of dietary inclusion of *Chlorella vulgaris* and carbohydrases on general health, redox status, immune response, liver lipids and metabolites in weaned piglets. Forty-four male piglets were allocated into four diets: control (n = 11), CH (control diet with 5% CH, n = 10), CH+R (control diet with 5% CH plus 0.005% Rovabio® Excel AP, n = 10), and CH+M (control diet with 5% CH plus 0.01% of a pre-selected four-CAZyme mixture, n = 11). After 15 days of trial, animals were slaughtered and samples of blood and liver collected. Spectrophotometry methods and commercial kits were used to determine blood parameters and gas and liquid chromatography for hepatic fatty acid and chlorophylls profiles, respectively. While total, LDL- and VLDL-cholesterol were increased by CH, the opposite was recorded for HDL-cholesterol ($p < 0.001$). Piglets fed CH-based diets presented an increase of IgG and a decrease of IgM ($p < 0.001$) which along with lymphocytes exacerbation contributed for piglets' survival after weaning. $n-6$ PUFA were reduced in piglets fed CH and the opposite occurred for $n-3$ PUFA ($p < 0.001$), thus benefiting $n-6/n-3$ ratio in the liver. Chlorophylls amount was not changed by the use of Rovabio® Excel AP or enzymatic mixture. The discriminant analysis applied to hepatic parameters revealed a clear separation between control and CH-based diets but failed to discriminate feed enzymes. Our findings indicate health promoting effects of CH as feed ingredient in piglets' nutrition at weaning, without negatively impacting on animals' performance.

Keywords: *Chlorella vulgaris*, weaned piglets, health, immune, hepatic metabolites

7.1. Introduction

The *post*-weaning phase is one of the most critical periods in swine production (Barba-Vidal et al. 2018). Indeed, animals have to face several adverse factors: complex social changes, related to the separation from their mothers and littermates, changes in feeding and environment, and an immature immune system (Pluske et al. 1997; Weary et al. 2008). Therefore, at the weaning phase, piglets are particularly susceptible to digestive and respiratory pathologies resulting from the imbalance between animals' immunity and environmental stress (Pluske et al. 1997; Weary et al. 2008). The use of antibiotics for preventive or therapeutic purposes of these pathologies is not strongly recommended, thus it is crucial to apply different strategies to reduce or prevent their use. A nutritional strategy that has been received increased attention is the use of prebiotics. Specifically, Liu et al. (2018), focused on the influence of prebiotics on gut health in pigs, highlighted the positive modification in intestinal microbiota and the decrease in enteric diseases in pigs. These authors also suggested that prebiotics impact the immune system but argued that more research is needed to prove these effects (Liu et al. 2018). Microalgae are known for their prebiotic properties as recently reviewed (Patel et al. 2021). Indeed, microalgae prebiotics effects should not be restricted to their polysaccharides and lignin, but should be extended to their monosaccharides, enzymes, polyunsaturated fatty acids (PUFA), peptides, polyphenols and alcohols (De Jesus Raposo et al. 2016).

The use of whole microalgae in animal diets has additionally been studied as an alternative in monogastric feeding, mostly as a supplement (Meadus et al. 2011; Simkus et al. 2013; Yan and Kim 2013; Park et al. 2018) but, in recent years, also as an ingredient (Kalbe et al. 2019; Pestana et al. 2020; Alfaia et al. 2021; Martins, Pestana, Ribeiro et al. 2021) approved by the European Union regulation. *Chlorella vulgaris* (CH) is one of the widely used microalgae, expanding its biomass use for animal feeding, among other purposes. It is characterized by relevant contents of CP, CF and carbohydrates, with respectively, 50-60%, 13-21% and 18-28% of DM (Madeira et al. 2017). In fact, the enriched concentrations of *n*-3 LC-PUFA, vitamins, minerals, carotenoids, other pigments and bioactive compounds by microalgae represent a potential resource with well-known beneficial health implications for both animals and humans (Calder 2012). However, microalgae have recalcitrant cell walls, making them indigestible by the monogastrics. In accordance, the development of new technologies to improve microalgae nutrient utilization is absolutely needed in order to foster the cost-effective use of microalgae for the feed industry (Austic et al. 2013; Lum et al. 2013). CAZymes have been investigated, in several *in vitro* nutritional studies, as being able to degrade the recalcitrant cell wall of microalgae, improving their nutritional value for monogastrics feeding and allowing their use at higher incorporation levels in pig and poultry

diets. Recently, Coelho and colleagues (2020) demonstrated the potential of a novel four-CAZyme mixture to disrupt the recalcitrant cell wall of CH. It is possible that the combination of microalgae and enzymes, in addition to improve nutrient digestibility, could also contribute to increase the prebiotic effect, as suggested by several authors (De Jesus Raposo et al. 2016; Liu et al. 2018). This might be an interesting alternative to the use of antibiotics at the weaning phase of piglets due to the formation of protective prebiotics in the intestine, which is in line with EU recommendations and policies on antibiotic resistance and use in animal production (De Jesus Raposo et al. 2016).

Currently, the knowledge about the effects of microalgae on the general health status and hepatic metabolism of piglets is practically non-existent. Nuclear Magnetic Resonance (NMR) techniques have proven to be important tools to a comprehensive overview on animal physiology and production (Palma et al. 2018) and may be useful in the identification of metabolites associated with hepatic metabolism. As mentioned in Madeira et al. (2021), there is a pressing need to have such information. Thus, we hypothesized that microalgae, mainly in combination with CAZymes, would contribute to improve the health status and metabolic condition of piglets during the weaning period. Therefore, the aim of our study was to assess the effect of 5% of dietary CH, individually or combined with two feed enzymes (the commercially available Rovabio® Excel AP and the four-CAZyme mixture pre-selected by Coelho, Lopes et al. (2020)) on blood biochemical markers, immune function (leucocytes and immunoglobulins), oxidative status (serum antioxidant markers and liver antioxidant diterpenes and carotenoids), and hepatic lipids and metabolomics in weaned piglets.

7.2. Material and Methods

7.2.1. Design trial and experimental treatments

All the procedures used in this animal experiment were revised by the Ethics Commission of ISA and approved by the Animal Care Committee of the National Veterinary Authority (Process Number 0421/2017, Direção Geral de Alimentação e Veterinária, Portugal). All methods were carried out in accordance with the European Union legislation (2010/63/EU Directive) and are reported following the ARRIVE guidelines 2.0 (https:// arriv eguid elines. org/ arrive- guide lines).

Forty-four castrated male piglets from Large White x Landrace sows crossed with Pietrain boars weaned at 28 days of age and with initial live weight of 11.2 ± 0.46 kg were selected. Details on the animal experiment were previously described by Martins, Pestana, Alfaia et al. (2021). Briefly, animals were housed individually in metabolic cages and had *ad libitum* access to water and restricted access to diets (to perform a digestibility study; data not

shown). Following a two-day adaptation period, two animals were excluded from the trial. The piglets were randomly distributed into one of 4 experimental groups: control (corn and soybean meal-based diet, *n* = 11), CH (control diet with 5% CH, *n* = 10), CH+R (control diet with 5% CH plus 0.005% Rovabio® Excel AP, *n* = 10), and CH+M (control diet with 5% CH plus 0.01% of a four-CAZyme mixture, *n* = 11). CH microalga was purchased from Allmicroalgae - Natural Products SA (Pataias, Portugal) and incorporated as freeze-dried powder into the diets. CH chemical details were previously described by Coelho, Pestana et al. (2020). The level of CH incorporation (5%) followed this previous study, maintaining the main objective of our line of investigation (which is to test microalgae as ingredient). Rovabio® Excel AP was incorporated into the diets at 0.005%, as recommended by the manufacturer. The four-CAZyme mixture composed by an exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme was pre-selected and tested *in vitro* for efficient degradation of CH cell walls (Coelho, Lopes et al. 2020). The homogenous distribution of enzymes was guaranteed by a pre-mixture with a feedstuff excipient and the microingredients. Table 7.1 shows diets composition. The chemical composition of diets was described by Martins, Pestana, Alfaia et al. (2021). To determine ADFI, ADG and FCR, feed supplied and refusals were weighed daily and piglets were weighed weekly.

Table 7.1. Ingredients and feed additives of the experimental diets (g/kg).

Ingredients	Control	CH	CH+R	CH+M
Wheat	439	440	440	440
Corn	150	150	150	150
Soybean meal 48	250	200	200	200
Whey powder	100	100	100	100
Sunflower oil	30	30	30	30
Chlorella vulgaris	0	50	50	50
Rovabio® Excel AP	0	0	0.050	0
Four-CAZyme mixture[1]	0	0	0	0.100
L-Lysine	5	5	5	5
DL-Methionine	1	1	1	1
L-Threonine	1	1	1	1
Calcium carbonate	5	6	6	6
Dicalcium phosphate	13	12	12	12
Sodium chloride	3	2	2	2
Vitamin-mineral complex[2]	3	3	3	3

Dietary treatments: Control - control diet; CH - 5% *Chlorella vulgaris* diet; CH+R - *Chlorella vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M - *Chlorella vulgaris* diet supplemented with 0.01% enzymatic mixture.

[1]exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme (CPE1314)

[2]Premix provided per kg of complete diet: vitamin A, 6500 UI; vitamin D3, 1500 UI; vitamin E, 15 mg; vitamin K3, 1 mg; vitamin B1, 1 mg; vitamin B2, 3 mg; vitamin B6, 2 mg; vitamin B12, 0.02 mg; pantothenic acid, 10 mg; nicotinic acid, 15 mg; folic acid, 0.5 mg, biotin, 0.03 mg; betaine, 115 mg; vitamin C, 20 mg; Copper, 100 mg; iron, 100 mg; iodine, 0.5 mg; manganese 50 mg; selenium, 0.15 mg; zinc, 100 mg; butylated hydroxytoluene, 3 mg.

7.2.2. Slaughter and sampling

After an experimental period of 15 days, with a live weight of 23.1 ± 2.56 kg, all animals were slaughtered, following the standard procedures of commercial abattoirs, using electrical stunning, followed by exsanguination. Blood samples were collected with anticoagulant Ethylenediaminetetraacetic acid and analysed for haematology on the same day; for all the other parameters, blood samples were centrifuged at 1500 *g* for 15 min to obtain serum, and stored at -20 ºC, until analysis. Liver samples were collected, vacuum packed and stored at -20 ºC for fatty acid composition and pigment analysis. Samples used for metabolomics were snap-frozen in liquid nitrogen and stored at -80ºC until further analysis.

7.2.3. Determination of blood parameters

As previously described by Madeira et al. (2021), red blood cells, white blood cells and thrombocytes counts were performed using Sysmex XN-10 (Sysmex Corporation, Kobe, Japan) analysers. The red blood cells count was measured using the impedance variation method after hydrodynamic focusing. For white blood cells differential counting (%), the blood smears were discoloured with the May-Grünwald-Giemsa technique. The haemoglobin concentration was measured by photometry, at 522 nm, with sodium lauryl sulphate as reagent.

The determination of total cholesterol, HDL-cholesterol, LDL-cholesterol, TAG, phospholipids, total protein, urea, creatinine and glucose concentrations, AST, ALT, ALP and GGT was performed in a Modular Hitachi Analytical System (Roche Diagnostics, Mannheim, Germany), through commercial kits (Roche Diagnostics, Basel, Switzerland). For VLDL-cholesterol and total lipids, Friedewald et al. (1972) and Covaci et al. (2006) formulas were applied, respectively. The concentration of insulin was determined in serum using the Porcine Insulin RIA kit (PI-12 K; Linco Research, Millipore, Billerica, MA, USA). The degree of insulin resistance was calculated by the homeostasis model assessment using the formula described by Matthews et al. (1985): insulin resistance index (HOMA-IR) is equally to fasting serum glucose (mmol/L) multiplied by fasting serum insulin (mU/L) and divided by 22.5. The immunoglobulin profile (IgA, IgG and IgM) was defined by immunoturbidimetry.

The TAC was measured in serum through the QuantiChrom Antioxidant Assay Kit (https://bioassaysys.com/datsheet/DTAC.pdf, Bioassay Systems, Hayward, CA, USA). The GPX activity was assessed in serum by the EnzyChrom Glutathione Peroxidase Assay Kit (https:// www.bioassaysys.com/datasheet/EGPX.pdf, Bioassay Systems). One unit of GPX is the amount of GPX that produces 1 µmol of glutathione disulphide (GS-SG) per min at pH = 7.6 and room temperature.

7.2.4. Hepatic total fat content and fatty acid profile

After liver samples freeze drying (at -60 °C and 2.0 hPa, Edwards Modulyo freeze drier, Crawley, UK), total lipids were gravimetrically quantified in duplicate, following Folch et al. (1985) method, using dichloromethane and methanol, as reported by Carlson (1985). Subsequently, the fat residue was resuspended in dry toluene and subjected to successive alkaline and acid transesterification reactions to convert FA into FAME (Sukhija and Palmquist 1988). FAME separation was performed by GC-FID (HP7890A Hewlett-Packard, Avondale, PA, USA), as previously described by Madeira et al. (2021). Fatty acids were expressed as percentage of total FA, after identification by their retention times and quantification, using

heneicosanoic acid as internal standard and by converting the relative peak areas into weight percentages.

7.2.5. Hepatic cholesterol, diterpene profile and pigments determination

Total cholesterol and diterpene profile were determined in duplicate in liver samples, as previously described by Prates et al. (2006). After a direct saponification of samples, one aliquot of the *n*-hexane layer was filtered before run into an HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA). Total cholesterol and β-carotene were detected using UV-Vis photodiode array detector (λ = 202 nm and λ = 450 nm, respectively), and tocopherols and tocotrienols using fluorescence detector (excitation at λ = 295 nm and emission at λ = 325 nm). The concentration of total cholesterol, β-carotene and vitamin E homologues in hepatic samples was quantified using a standard curve of peak area *vs*. concentration.

The quantification of pigments in hepatic samples was performed using Teimouri et al. (2013) protocol with slight adjustments. Briefly, hepatic samples were incubated at room temperature with acetone overnight, under agitation, and in the dark. After extraction, samples were centrifuged at 1500 g for 5 min and analysed by UV-Vis spectrophotometry (Ultrospec 3100; Amersham Biosciences, Little Chalfont, UK) UK), at 662 nm for chlorophyll *a*, at 645 nm for chlorophyll *b*, and at 470 nm for total carotenoids. The pigment contents were calculated using Hynstova et al. (2018) equations.

7.2.6. Hepatic NMR-metabolomics analysis

Liver tissue was powdered without thawing in liquid nitrogen. The extraction of the aqueous metabolites from the liver ground powder was performed following the chloroform/methanol method, as previously described by Palma et al. (2016). Then, the aqueous fraction samples were resuspended in phosphate buffer (1.75 M K_2HPO_4 (anhydrous); 1.24 mM sodium formate; 5.0 M 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP); pD 7.40; in ^2H_2O) and 99.8% ^2H2O. Proton-decoupled ^1H NMR spectra were obtained using a Varian VNMRS 600 MHz (Agilent, Santa Clara, CA, USA) spectrometer equipped with a 3 mm $^1H(X)$-PFG inverse configuration probe. A ^1H-Presat pulse sequence was acquired for each sample (spectral width 7 kHz; acquisition time 4 s; saturation delay 3 s; relaxation delay 4 s; 6 scans; at 298 K). All spectra were processed in the ACD/NMR Processor Academic Edition from ACD\Labs 12.0 software (Advanced Chemistry Development, Inc.) applying: zero-filling to 65 k, line broadening of 0.2 Hz, phasing, baseline correction and the chemical shifts were referenced to the TSP peak at 0 ppm (or any other internal standard).

Spectral binning was performed in ACD/NMR Processor Academic Edition using uniform binning with a 0.04 ppm width from −0.5 to 10 ppm. Regions for water (4.705.15 ppm) and TSP (−0.5 to 0.25 ppm) were excluded.

The multivariate analysis was performed with the bin values, using MetaboAnalyst 4.0 software (https://www.metaboanalyst.ca) for PCA and Partial Least Squares analysis (PLS). For the PLS analysis, Q^2 (predictive ability of the model), R^2 (goodness of the fit), and the *p-value* of the permutation test (1000 permutations) were considered as the quality parameters for each model. PLS models were accepted as valid for Q^2 above 0.5 and *p-value* < 0.05 (Xia and Wishart 2016). For both PCA and PLS models, the ellipses in the score plots were drawn using a 95% confidence level

7.2.7. Statistical analysis

Using SAS software package (version 9.4, SAS Institute Inc., Cary, NC, USA), all data were analysed by one-way ANOVA selecting the GLM procedure. Normal distribution and variance homogeneity were verified for all data through the Shapiro-Wilk and Levene tests, respectively. The statistical model considered the piglet as the experimental unit and the dietary treatment as the single effect. To determine the significant effects of dietary treatments, least-squares means for multiple comparisons were generated by the PDIFF option and adjusted with the Tukey-Kramer method. The results were considered significantly different when $p \leq 0.05$. The PCAs were performed with blood parameters and all hepatic variables using the SPSS Statistics for Windows (IBM Corp. released 2020, version 27.0, Armonk, NY, USA).

7.3. Results

7.3.1. Impact of *Chlorella vulgaris* in piglets´ zootechnical performance

Figure 7.1 shows the influence of experimental diets on production performance of piglets. Final body weight (Fig. 7.1 a), ADG (Fig. 7.1 c) and FCR (Fig. 7.1 d) were unaltered by diets. In turn, ADFI (Fig. 7.1 b) was increased by CH with or without feed enzymes (p < 0.05) during 15 days of experimental trial.

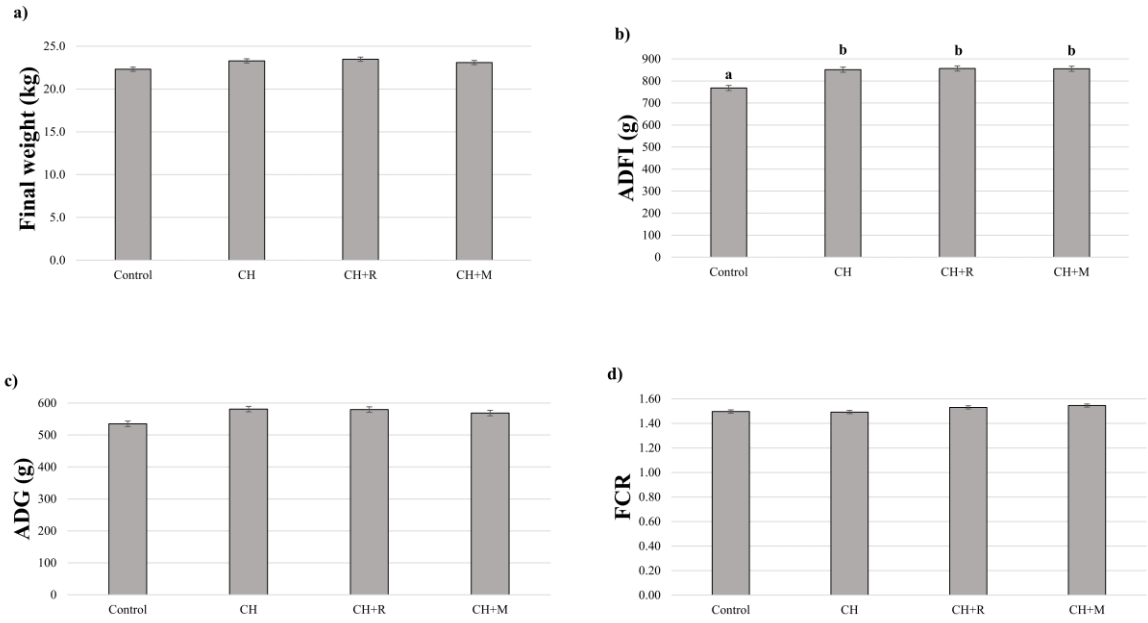

Figure 7.1. Influence of experimental diets on growth performance variables of piglets. a) final body weight (kg), b) ADFI - average daily feed intake (g), c) ADG - average daily gain (g) and d) feed conversion ratio.

Dietary treatments: Control - control diet; CH - 5% *C. vulgaris* diet; CH+R - 5% *C. vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M - 5% *C. vulgaris* diet supplemented with 0.01% enzymatic mixture.

[a,b]Values with different superscripts differ significantly at p < 0.05.

7.3.2. Influence of experimental diets on blood parameters

Table 7.2 presents data on blood metabolites of piglets fed on CH with or without feed enzymes. Major variations were observed across haematology, serum biochemical markers, immunoglobulins and redox status. White blood cells count was higher in piglets fed the combination of CH and exogenous enzymes, when compared to the control animals (p = 0.004). Concerning the leucogram, lymphocytes (p < 0.001) and thrombocytes (p < 0.001) were increased in piglets fed the combination of CH and the enzymatic mixture relative to other experimental groups. The opposite effect was observed for granulocytes (p < 0.001), which were decreased in piglets fed the combination of CH and the enzymatic mixture relative to the

other experimental groups. Monocytes were unchanged by diets. Red blood cells ($p = 0.001$) and haemoglobin ($p < 0.001$) reached the highest values in piglets fed the combination of CH and feed enzymes in relation to the control group and CH alone. Total lipids ($p < 0.001$), TAG ($p < 0.001$), total cholesterol ($p < 0.001$), LDL-cholesterol ($p < 0.001$), VLDL-cholesterol ($p < 0.001$), urea ($p < 0.001$) and creatinine ($p < 0.001$) were increased by CH combined with the enzymatic mixture relative to the other experimental groups. Conversely, the combination of CH and Rovabio® Excel AP increased HDL-cholesterol ($p < 0.001$) relative to the other experimental groups. Total protein did not change across diets. While glucose remained unchanged by diets, insulin reached the lowest value in piglets fed CH and the enzymatic mixture relative to the control ($p = 0.006$). The HOMA-IR followed the same trend ($p = 0.016$). ALT was increased in piglets fed CH and the enzymatic mixture relative to the control and CH alone ($p < 0.001$) whereas AST ($p < 0.001$), ALP ($p < 0.001$) and GGT ($p < 0.001$) were increased in piglets fed Rovabio® Excel AP combined with CH compared to the other experimental groups.

For immunoglobulins, IgA was kept unchanged across diets. However, IgG was increased by CH feeding, when combined with the enzyme mixture, in comparison to the control animals ($p < 0.001$). IgM reached the lowest values in animals fed the CH diet or combined with the enzymatic mixture ($p < 0.001$) when compared to the control and Rovabio® Excel AP combined with CH dietary groups.

For the evaluation of serum redox status, while TAC was decreased by CH feeding, with or without feed enzymes ($p < 0.001$) relative to control, the opposite was observed for GPX activity ($p = 0.043$).

Table 7.2. Influence of experimental diets on blood parameters of piglets.

	Diets				SEM	*p-value*
	Control	**CH**	**CH+R**	**CH+M**		
Haematology						
White blood cells ($\times 10^9$/L)	15.2^{a}	17.4^{ab}	19.7^{b}	20.7^{b}	1.14	0.004
Leucogram (% white blood cells)						
Granulocytes	47.9^{a}	42.9^{a}	44.0^{a}	36.4^{b}	1.46	<0.001
Lymphocytes	47.4^{a}	53.5^{b}	53.0^{ab}	59.9^{c}	1.61	<0.001
Monocytes	4.71	3.63	3.00	3.75	0.706	0.364
Red blood cells ($\times 10^{12}$/L)	6.28^{a}	6.43^{a}	7.09^{ab}	7.39^{b}	0.217	0.001
Haemoglobin (g/L)	107^{ab}	106^{a}	116^{bc}	120^{c}	2.72	<0.001
Thrombocytes ($\times 10^9$/L)	264^{a}	305^{a}	392^{b}	472^{c}	18.0	<0.001

Serum metabolites						
Total lipids[1] (g/L)	2.47^{a}	2.66^{b}	2.79^{b}	3.63^{c}	0.036	<0.001
TAG[2] (mg/L)	322^{a}	399^{ab}	447^{b}	995^{c}	22.4	<0.001
Total cholesterol (mg/L)	505^{a}	578^{b}	637^{b}	741^{c}	16.3	<0.001
HDL-cholesterol[3] (mg/L)	148^{a}	166^{a}	267^{c}	224^{b}	10.0	<0.001
LDL-cholesterol[4] (mg/L)	336^{b}	372^{c}	304^{a}	401^{c}	7.82	<0.001
VLDL-cholesterol[5] (mg/L)	64.4^{a}	79.8^{ab}	89.4^{b}	199^{c}	4.49	<0.001
Glucose (mg/L)	1193	1234	1201	1092	39.5	0.068
Insulin (mU/L)	1.45^{a}	1.18^{ab}	1.18^{ab}	0.683^{b}	0.162	0.006
HOMA-IR[6] (mmol/L×μU/mL)	0.801^{a}	0.665^{ab}	0.616^{ab}	0.339^{b}	0.098	0.016
Urea (mg/L)	248^{a}	255^{a}	248^{a}	428^{b}	12.9	<0.001
Creatinine (mg/L)	7.19^{a}	7.57^{a}	7.47^{a}	10.2^{b}	0.311	<0.001
Total protein (g/L)	62.4	60.4	61.3	60.5	0.658	0.110
Serum hepatic markers (U/L)						
ALT[7]	23.5^{a}	25.2^{a}	27.4^{ab}	30.7^{b}	1.09	0.001
AST[8]	23.8^{a}	23.0^{a}	31.5^{b}	19.7^{a}	1.23	<0.001
ALP[9]	185^{a}	175^{a}	257^{b}	185^{a}	6.20	<0.001
GGT[10]	15.1^{a}	16.5^{a}	23.1^{b}	16.4^{a}	0.980	<0.001
Serum immunoglobulins						
IgA[11] (mg/L)	30.6	28.6	36.0	32.2	2.33	0.159
IgG[12] (g/L)	0.507^{a}	0.658^{b}	0.750^{bc}	0.776^{c}	0.026	<0.001
IgM[13] (g/L)	0.766^{a}	0.496^{b}	0.696^{a}	0.579^{b}	0.024	<0.001
Serum antioxidant potential						
TAC[14] (µM)	101^{c}	64.3^{a}	66.7^{ab}	75.8^{b}	3.76	<0.001
GPX[15] (U/L)	623^{a}	813^{b}	782^{b}	745^{ab}	52.2	0.043

Dietary treatments: Control - control diet; CH - 5% *Chlorella vulgaris* diet; CH+R - 5% *Chlorella vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M - 5% *Chlorella vulgaris* diet supplemented with 0.01% enzymatic mixture.

[1]Total lipids = [total cholesterol] × 1.12 + [TAG] × 1.33 + 148;

[2]TAG - triacylglycerols;

[3]HDL - high-density lipoproteins;

[4]LDL - low-density lipoproteins;

[5]VLDL -very low-density lipoproteins = 1/5 [TAG];

[6]HOMA-IR, insulin resistance index = [fasting plasma glucose] × [fasting plasma insulin] / 22.5.

[7]ALT - alanine aminotransferase (E.C. 2.6.1.2);

[8]AST - aspartate aminotransferase (E.C. 2.6.1.1);

[9]ALP - alkaline phosphatase (E.C. 3.1.3.1);

[10]GGT - gamma-glutamyltransferase (E.C. 2.3.2.13);

[11]IgA - immunoglobulin A;

[12]IgG - immunoglobulin G;

[13]IgM - immunoglobulin M;

[14]TAC - total antioxidant capacity;

[15]GPX - glutathione peroxidase activity. One unit of GPX is the amount of GPX that produces 1 µmol of GS-SG per min at pH = 7.6 and room temperature.

[a,b,c]Values within a row with different superscripts differ significantly at $p < 0.05$.

7.3.3. Influence of experimental diets on hepatic lipids and fatty acid composition of piglets

Table 7.3 shows total lipids, total cholesterol and the detailed fatty acid composition in the liver of piglets fed on CH with or without feed enzymes. Total lipids ($p = 0.014$) decreased in piglets fed CH and the enzymatic mixture, whereas cholesterol was increased in the CH group ($p = 0.003$). The sum of SFA was increased in CH, regardless of feed enzyme use ($p < 0.001$), mostly due to variations in predominant fatty acids, such as 14:0 ($p < 0.001$), 16:0 ($p < 0.001$) and 18:0 ($p < 0.001$), but not 15:0 ($p < 0.001$), 17:0 ($p < 0.001$) and 20:0 ($p < 0.001$). MUFA were reduced by feeding CH in conjugation with both Rovabio® Excel AP and the enzymatic mixture by comparison to the control animals ($p = 0.009$). This finding was supported by the variations in 16:1*c*7 ($p < 0.001$), 18:1*c*11 ($p < 0.001$) and 20:1*c*11 ($p < 0.001$), but not by 16:1*c*9 ($p = 0.016$). Interestingly, the prevalent 18:1*c*9 was not affected by diets ($p > 0.05$). The total PUFA ($p < 0.001$), including *n*-6 PUFA ($p < 0.001$) were reduced by CH feeding with or without feed enzymes, mostly due to the 18:2*n*-6 ($p < 0.001$), 18:3*n*-6 ($p < 0.001$) and 20:2*n*-6 ($p < 0.001$) results. The opposite was observed for *n*-3 PUFA ($p < 0.001$), thus contributing to a decrease in *n*-6:*n*-3 ($p < 0.001$) and PUFA /SFA ($p < 0.001$) ratios in piglets fed CH with or without feed enzymes. 20:4*n*-6 followed a decreasing trend across dietary groups with the lowest percentage in piglets fed CH with the enzymatic mixture ($p < 0.001$). Moreover, 18:3*n*-3 did not vary across diets ($p > 0.05$), but 22:5*n*-3 ($p = 0.010$) and 22:6*n*-3 ($p < 0.001$) increased in the CH groups.

Table 7.3. Influence of experimental diets on total lipids, cholesterol and fatty acid composition in the liver of piglets.

Item	Diets				SEM	*p-value*
	Control	CH	CH+R	CH+M		
Total lipids (g/100 g)	2.12^{b}	1.88^{b}	1.87^{b}	1.84^{a}	0.065	0.014
Total cholesterol (g/100 g)	0.149^{a}	0.176^{b}	0.177^{b}	0.178^{b}	0.006	0.003
Fatty acid composition (g/100 g FA)						
14:0	0.191^{a}	0.254^{b}	0.248^{b}	0.301^{c}	0.012	<0.001
15:0	0.841^{b}	0.341^{a}	0.336^{a}	0.332^{a}	0.058	<0.001
16:0	15.4^{a}	21.3^{b}	21.9^{b}	20.6^{b}	0.614	<0.001
16:1*c*7	0.547^{b}	0.389^{a}	0.388^{a}	0.443^{a}	0.028	<0.001
16:1*c*9	0.570^{a}	0.805^{b}	0.831^{b}	0.707^{ab}	0.062	0.016
17:0	6.33^{b}	2.61^{a}	2.75^{a}	2.66^{a}	0.314	<0.001
17:1c9	0.814^{b}	0.458^{a}	0.470^{a}	0.422^{a}	0.060	<0.001

18:0	33.0[a]	37.7[b]	38.6[b]	40.0[b]	1.125	<0.001
18:1*c*9	15.3	15.0	14.6	14.4	0.412	0.463
18:1*c*11	2.72[b]	2.03[a]	2.05[a]	2.05[a]	0.058	<0.001
18:2*n*-6	12.4[b]	8.53[a]	8.10[a]	8.31[a]	0.370	<0.001
18:2*t*9*t*12	0.123[b]	0.067[a]	0.071[a]	0.060[a]	0.008	<0.001
18:3*n*-6	0.111[b]	0.070[a]	0.067[a]	0.071[a]	0.007	<0.001
18:3*n*-3	0.058	0.066	0.067	0.064	0.004	0.263
20:0	0.172[b]	0.097[a]	0.103[a]	0.103[a]	0.009	<0.001
20:1*c*11	0.364[b]	0.155[a]	0.146[a]	0.182[a]	0.017	<0.001
20:2*n*-6	0.758[b]	0.261[a]	0.231[a]	0.271[a]	0.036	<0.001
20:3*n*-6	0.150[a]	0.212[b]	0.203[b]	0.191[b]	0.009	<0.001
20:4*n*-6	5.83[b]	5.14[a]	4.63[ac]	4.40[c]	0.181	<0.001
20:5*n*-3	0.364[b]	0.155[a]	0.148[a]	0.182[a]	0.017	<0.001
22:0	0.128	0.167	0.162	0.193	0.028	0.413
22:1*n*-9	0.145	0.134	0.131	0.144	0.008	0.436
22:5*n*-3	0.223[a]	0.278[ab]	0.275[ab]	0.299[b]	0.016	0.010
22:6*n*-3	0.519[a]	0.710[b]	0.655[b]	0.649[b]	0.023	<0.001
23:0	0.160[a]	0.181[ab]	0.168[a]	0.197[b]	0.007	<0.001
Other	2.97	2.81	2.50	2.67	0.238	0.528
Partial sums of FA (g/100 g FA)						
SFA[1]	56.3[a]	62.6[b]	64.3[b]	64.4[b]	0.793	<0.001
MUFA[2]	20.4[b]	18.9[ab]	18.6[a]	18.4[a]	0.453	0.009
PUFA[3]	20.3[b]	15.6[a]	14.6[a]	14.6[a]	0.533	<0.001
n-3 PUFA[4]	0.979[a]	1.32[b]	1.28[b]	1.28[b]	0.030	<0.001
n-6 PUFA[5]	19.2[b]	14.2[a]	13.2[a]	13.2[a]	0.527	<0.001
FA ratios						
n-6:*n*-3	19.7[b]	10.8[a]	10.4[a]	10.4[a]	0.623	<0.001
PUFA:SFA	0.365[b]	0.250[a]	0.227[a]	0.227[a]	0.013	<0.001

Dietary treatments: Control - control diet; CH - 5% *Chlorella vulgaris* diet; CH+R - 5% *Chlorella vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M - 5% *Chlorella vulgaris* diet supplemented with 0.01% enzymatic mixture

FA - fatty acids; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids.

[1]Sum (12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0).

[2]Sum (14:1*c*9, 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 20:1*c*11 and 22:1*n*-9).

[3]Sum (18:2*n*-6, 18:3*n*-6, 18:2*t*9*t*12, 18:3*n*-3, 18:4*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3).

[4]Sum (18:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3).

[5]Sum (18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6 and 20:4*n*-6).

[a,b]Values within a row with different superscripts differ significantly at $p < 0.05$.

7.3.4. Influence of experimental diets on hepatic diterpenes and pigment contents

Table 7.4 shows data on diterpene profile and pigments in the liver from piglets fed on CH with or without feed enzymes. Concerning the vitamin E compounds, both α- and γ-tocopherol remained unchanged by diets ($p > 0.05$). Even if chlorophyll *b* did not vary ($p > 0.05$) across dietary treatments, chlorophyll *a* reached the highest levels in piglets fed on CH with feed enzymes by comparison to the control animals ($p < 0.001$). Total chlorophylls were higher in piglets fed the combination of CH with Rovabio® Excel AP when compared to control animals ($p = 0.009$). Total carotenoids were increased by CH, regardless the addition of feed enzymes ($p < 0.001$). The sum of total chlorophylls and total carotenoids followed a similar pattern ($p < 0.001$).

Table 7.4. Influence of experimental diets on diterpene profile and pigments in the liver of piglets.

Item	Diets				SEM	*p-value*
	Control	CH	CH+R	CH+M		
Diterpene profile (µg/100 g)						
α-Tocopherol	266	257	256	246	10.1	0.543
γ-Tocopherol	5.12	5.57	6.39	5.93	0.004	0.101
Pigments (µg/100g)						
Chlorophyll *a*[1]	43.9[a]	76.9[ab]	104[b]	83.5[b]	9.00	<0.001
Chlorophyll *b*[2]	87.7	123	151	125	16.6	0.066
Total chlorophylls[3]	132[a]	199[ab]	255[b]	208[ab]	23.9	0.009
Total carotenoids[4]	128[a]	218[b]	219[b]	239[b]	12.5	<0.001
Total chlorophylls and total carotenoids[5]	260[a]	417[b]	475[b]	447[b]	30.9	<0.001

Dietary treatments: Control - control diet; CH - 5% *Chlorella vulgaris* diet; CH+R - 5% *Chlorella vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M - 5% *Chlorella vulgaris* diet supplemented with 0.01% enzymatic mixture.
[1]C*a* = 11.24 A_{662} - 2.04 A_{645}.
[2]C*b* = 20.13 A_{645} - 4.19 A_{662}.
[3]C*a+b* = 7.05 A662 + 18.09 A645.
[4]Cx+c = (1000 A_{470} - 1.90 Ca - 63.14 Cb) / 214.
[5](Ca+b) + (Cx+c).
[a, b]Values within a row with different superscripts differ significantly at $p < 0.05$.

7.3.5. Principal component analyses using blood parameters and hepatic lipids and related lipid-compounds

PCA was performed with blood parameters and did not reveal a clear clustering between experimental groups (data not shown). Figure 7.2. illustrates the PCA output applied to a data set of 42 animal samples and 32 variables in the liver of piglets used in this trial. The first and second principal components were responsible for 53.4% of the total variance, being 41.9% for component 1 and 11.5% for component 2, respectively. As total variance explained by the first two principal components is higher than 50%, the projection of piglets' liver samples in the plane defined by these components is shown in Figure 7.2. The PCA model revealed a good separation between the control group and the three CH-based diets (Fig. 7.2). The control group was confined to quadrants b and d being clearly discriminated from the other three. Microalga-based dietary groups supplemented or not with exogenous enzymes were more dispersed in quadrants a and c with no possible discrimination on the addition of feed enzymes (that is, CH, CH+R and CH+M dietary groups).

Table 7.5 shows the loadings for the first two principal components. Overall, component 1 was mainly characterized by positive loadings data, in particular 20:2*n*-6 (0.933), 18:2*n*-6 (0.919), 17:0 (0.889), 20:1*c*11 (0.862), 18:1*c*11 (0.856), 15:0 (0.846), 18:2*t*9*t*12 (0.834), 16:0 (-0.810) and total carotenoids (-0.805), while the component 2 was mainly characterized by negative loadings data, in particular 18:0 (-0.624), 22:0 (-0.624). 16:1*c*7 (-0.499) and 20:3*n*-6 (0.486).

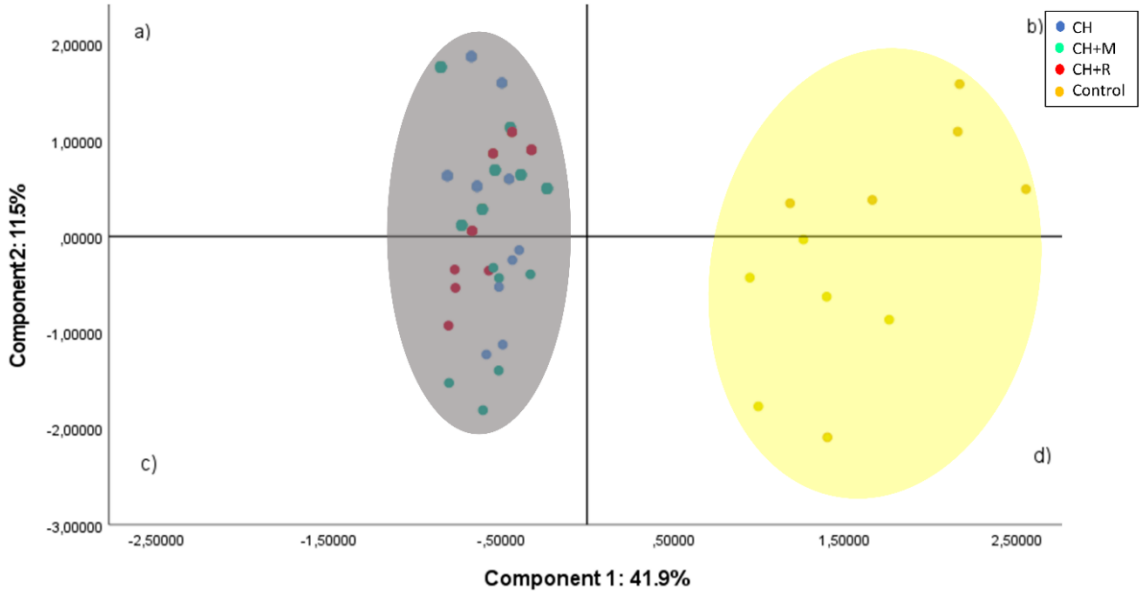

Figure 7.2. Principal component analysis (PCA) score plot using total lipids, cholesterol, fatty acid composition, diterpene profile and pigments in the liver of piglets.
Dietary treatments: Control - control diet; CH - 5% *C. vulgaris* diet; CH+R - 5% *C. vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M - 5% *C. vulgaris* diet supplemented with 0.01% enzymatic mixture.

Table 7.5. Loadings for the first two principal components.

Variables	Component 1	Component 2
Total lipids	0.486	-0.341
Total cholesterol	-0.558	-0.366
14:0	-0.624	0.370
15:0	0.846	0.269
16:0	-0.810	0.363
16:1*c*7	0.575	-0.499
16:1*c*9	-0.370	0.697
17:0	0.889	0.156
17:1*c*9	0.710	0.377
18:0	-0.689	-0.624
18:1*c*9	0.251	0.391
18:1*c*11	0.856	-0.226
18:2*n*-6	0.919	0.133
18:3*n*-6	0.769	0.257
18:2*t*9*t*12	0.834	0.200
18:3*n*-3	-0.302	0.354
20:0	0.787	-0.146
20:1*c*11	0.862	-0.238
20:2*n*-6	0.933	-0.099
20:3*n*-6	-0.615	0.486
20:4*n*-6	0.729	0.290
20:5*n*-3	-0.788	-0.133
22:0	-0.297	-0.624
22:1*n*-9	0.145	-0.367
23:0	-0.445	-0.069
22:5*n*-3	-0.377	0.442
22:6*n*-3	-0.673	0.058
α-Tocopherol	0.109	-0.084
γ-Tocopherol	-0.245	0.370
Chlorophyll *a*	-0.556	-0.080
Chlorophyll *b*	-0.370	-0.076
Total carotenoids	-0.805	-0.184

7.3.6. Influence of experimental diets on the hepatic metabolome

Figure 7.3 shows a representative spectrum of the liver aqueous fraction from piglets with the main metabolites identified. In total, we have identified 28 metabolites that included for instance creatine, betaine and lactate. The PCA score plot computed with the bin values (Supplementary Material 2 - Figure S1 A) showed a complete superimposition of the groups without a clear separation between them. This result is clearly indicative of a similar final metabolome profiles (general composition and concentration) between all experimental groups at the end of the experimental trial. The PLS model (Supplementary Material 2 - Figure S1 B), although revealed some group separation between the experimental groups, was not validated by the quality parameters ($Q^2 < 0$, 1000 permutations; $p = 0.665$). Since the permutation testing did not validate the PLS model, it is not possible to analyse the loadings plot and the variables important in projection values of the model.

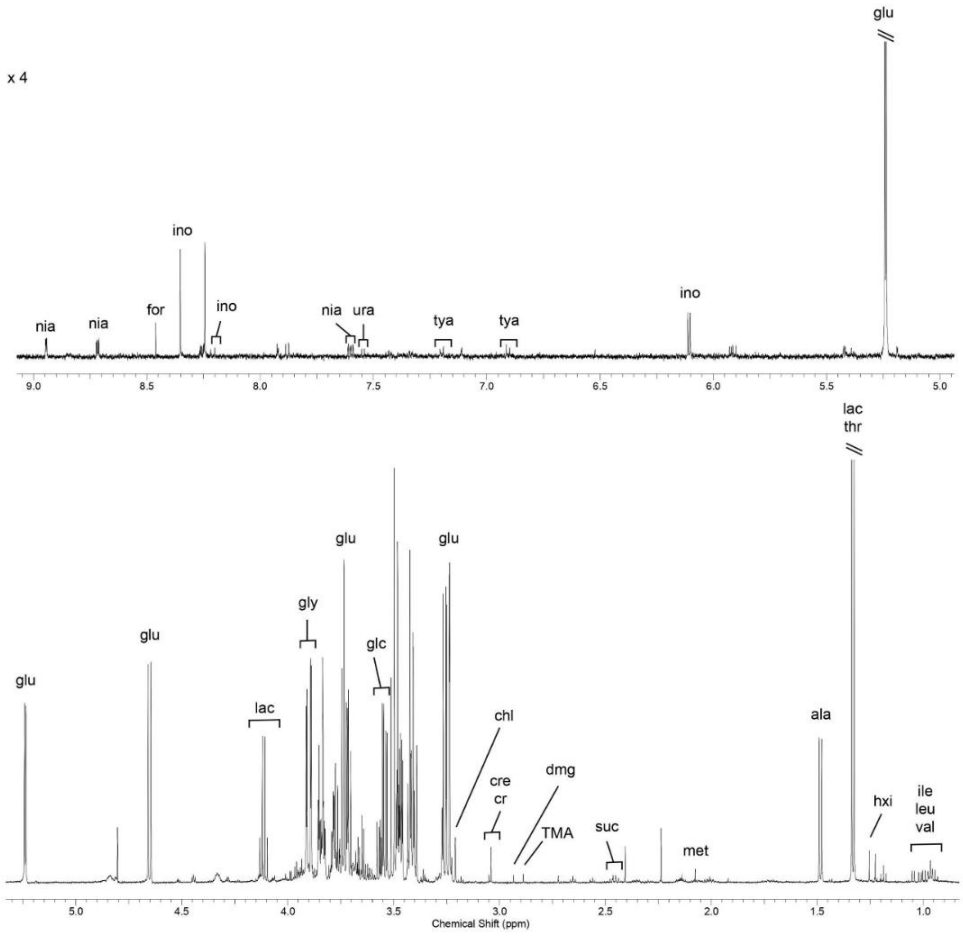

Figure 7.3. Representative NMR spectrum (1H 1D Presat) of liver aqueous fraction from piglets.
Key: val: valine; leu: leucine; ile: isoleucine; hxi: 3-hydroxyisovalerate; lac: lactate; thr: threonine; ala: alanine; met: methionine; suc: succinate; TMA: trimethylamine; dmg: dimethylglycine; cre: creatine/creatine-P; cr: creatinine; chl: choline; glu: glucose; glc: glycerol; gly: glycine; ino: inosine; tya: tyramine; ura: uracil; nia: niacinamide/nicotinurate; for: formate.

7.4. Discussion

The use of microalgae is a relatively novel field in animal nutrition in general, and in the swine industry in particular. Thus, the literature available on this topic is scarce. Very recently, Madeira et al. (2021) studied the effects of a dietary inclusion of 10% of *Arthrospira platensis* in combination with exogenous CAZymes (commercial Rovabio® Excel AP and lysozyme) supplementation in weaned piglets. Despite the negative impact on piglet´s growth performance, in terms of systemic antioxidant potential and hepatic lipid metabolism, this study reported a consistent increase of total lipids, total cholesterol and LDL-cholesterol along with an improvement on antioxidant potential without variations on hepatic fatty acid content by *Arthrospira platensis*, regardless of the inclusion of the exogenous enzymes (Madeira et al. 2021).

In line with these findings, we hypothesized that the dietary effect of 5% CH, alone or in combination with two exogenous enzymes (Rovabio® Excel AP and a four-CAZyme mixture), might improve the immune status, antioxidant potential and change the lipid metabolism in piglets, by assessing blood biochemical parameters and hepatic FA and related lipid-compounds. Dietary treatments with CH had no effect on growth performance, with no significant differences between experimental groups for live weight, ADG and FCR. Accordingly, several authors found no impact on growth parameters with 0.1-1% of CH dietary incorporation in pigs (26.6 to 53.0 kg LW) and weaned piglets' diets (9.1 to 20 kg LW) (Yan et al. 2012; Furbeyre et al. 2017). Contrarily to these studies, ADFI was increased with the incorporation of CH, regardless the addition of feed enzymes, not affecting piglets' growth performance. In the future, there is interest in confirming these results through a growth performance trial involving a large number of animals and ad libitum access to experimental diets. In our last study, we assessed the total tract apparent digestibility of nutrients and concluded that the inclusion of CH decreased the nutrients utilization by animals, with protein and fibre fractions being the most affected nutrients.

Weaning is a stressful event for piglets (Hwang et al. 2016). Changing nutrition from a milk-based diet to a cereal-based diet affects heavily the intestinal immune status and the intestinal microflora (Hampson et al. 1985; Barnett et al. 1989). Furthermore, changes in facilities and grouping animals of different litters can have negative consequences on physical, nutritional, immunological and behavioural status of piglets (Stanton and Mueller, 1976; Blecha et al. 1985; Pajor et al. 1991). In terms of the immune function, while IgA did not vary among experimental groups, IgG increased in piglets fed CH-based diets and the opposite occurred for IgM, supporting their fundamental role in protecting piglets' health. IgA, IgG and IgM are the first line of defence of the organism against infections (Reyneyeld et al, 2020). In particular, IgG and IgM antibodies act together in immediate and long-term protection against infections,

in a concerted way (Stiehm and Fudenberg 1966). Upon infection, the IgM level will rise for a short time and then it will begin to drop as the IgG levels increase, protecting the organism in the long-term (Stiehm and Fudenberg 1966). In fact, CH polysaccharides have already shown strong immunomodulatory activities and recent evidence demonstrated the prebiotic effect of CH powder in treated rats (Hyrslova et al. 2021). The main action of prebiotics is to stimulate growth and/or activate metabolism of protective bacteria in the intestinal tract, thus benefiting intestinal microbiome, and ultimately, piglets' health. In line with this, lymphocytes were also increased in piglets fed CH-based diets and even more when combined with the enzymatic mixture. Taken together, these positive variations reflect a boost on the immune response stimulated by CH that likely assures piglets' survival at the critical period of weaning.

Blood parameters have been increasingly used as body condition indicators as they provide valuable information on the physiological condition of the animal (Muriel et al. 2013). Herein, the lipid profile of piglets was largely influenced by dietary treatments. Cholesterol is partially obtained through the diet, through the consumption of animal-derived products, and from *de novo* biosynthesis in the liver (O'Hea and Leveille 1969). Even if a pattern of increase was promoted by CH for total cholesterol, LDL-cholesterol and VLDL-cholesterol, not in agreement with previous studies (Hyrslova et al. 2021), these variations were positively counterbalanced by a rise in HDL-cholesterol in piglets fed the exogenous enzymes, putatively leading to healthy cardiovascular functions (De Caire et al. 1995; Cheong et al. 2010). The reverse cholesterol transport is the mechanism by which the organism removes excess cholesterol from peripheral tissues and delivers it to the liver, where it will be redistributed to other tissues or removed from the organism being HDL-cholesterol, the main lipoprotein responsible for this process. Total lipids and TAG reached also higher values in piglets fed CH diets with or without feed enzymes. Notwithstanding, it should be underlined that the values found for systemic lipemia were not very far from the ones obtained previously by our research team (Madeira et al. 2021). These variations do not seem to promote, in the long-term, fatty liver, which is a serious pathophysiological condition associated with several human metabolic disorders, in particular obesity, diabetes and hyperlipidaemia (Kirpich et al. 2015; Lim et al. 2015).

Despite the variations observed for aminotransferase activities, it is worth noticing that the levels found are close to the reference values for pigs, which are 31-58 U/L for ALT, 32-84 U/L for AST and 10-52 U/L for GGT (Jackson and Cockcroft 2002). In view of these results, there is no clear evidence of CH toxicity. Urea and creatinine reached the highest levels in piglets fed the combination of CH with the enzymatic mixture, in agreement with the same range of levels variations found for creatinine by Madeira et al. (2021) with *Arthrospira platensis* and lysozyme. Glucose was unaffected by CH-based diets, but insulin decreased with the enzymatic mixture pointing towards a positive effect on glycemia homeostasis by degrading

enzymes, in virtue of a tendency for glucose decrease (albeit with no statistical significance) in this same experimental group. Insulin is a well-known stimulator of lipogenesis (Osborne 2000) and stimulates fatty acid synthesis in the liver with formation and storage of triacylglycerols (Wilcox 2005). Nevertheless, the values found for HOMA-IR were within the normal physiological range, below 2.4 (Blat et al. 2012).

The accurate assessment of redox status *in vivo* of the organism can only be determined by the measurement of TAC (McMichael 2007). Although the concentration of serum antioxidant components can be measured individually, these measurements may be time- and cost-consuming as well as labour intensive (Suresh et al. 2009). In addition, it may not accurately reflect the total antioxidant status (Wayner et al. 1987). CH with or without exogenous enzymes decreased TAC in serum, which is not consistent with the increase on hepatic total carotenoids and total chlorophylls. Carotenoids and chlorophylls are natural lipophilic pigments with antioxidant behaviour and free radical-scavenging properties, especially for chlorophylls, that are present in the diet (Pérez-Gálvez et al. 2020). In the present study, the decrease of TAC variation in serum was accompanied by a consistent increase in GPX activity in piglets fed CH-based diets suggesting a compensatory mechanism to avoid imbalance of oxidative stress homeostasis. GPX, an important antioxidant enzyme plays a key role in protecting haemoglobin, red blood cell enzyme activity and biological cell membranes against oxidative damage (Waggiallah and Alzohairy 2011) with the highest activity found in the liver and red blood cells (Behne and Wolters 1983).

In pigs, fatty acid composition of skeletal muscle, subcutaneous fat and liver is much more modulated by the pig genotype than by the dietary protein level (Madeira, Pires et al. 2013). The SFA sum was increased by CH, whereas MUFA sum was reduced by the microalga in conjugation with either Rovabio® Excel AP or the enzymatic mixture, but not by the microalga itself. On the positive side, *n*-6 PUFA were reduced by CH with or without feed enzymes, most at the expenses of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6 and 20:4*n*-6, this last fatty acid being responsible for overproduction of prothrombotic and pro-inflammatory eicosanoids, thromboxanes and leukotrienes (Martins et al. 2014). The inverse was observed for *n*-3 PUFA, in particular for the valuable DPA and DHA fatty acids (Calder 2012), impacting positively on *n*-6:*n*-3 ratio. *n*-3 fatty acids are substances of particular interest in animal feeding due to their anti-microbial and antioxidant action, as well as their biofortification ability of animal products (Kouba and Mourot 2011). Moreover, the enrichment in *n*-3 PUFA in the liver has been linked to positive events, such as downregulation of PUFA oxidation-associated genes expression, diminished lipid peroxidation and enhanced antioxidant properties (Tao et al. 2018).

The impact of CH dietary incorporation, with or without feed enzymes, on hepatic levels of tocopherols and pigments was also determined. Vitamin E is known as the major free radical chain terminator in the lipophilic environment (Brigelius-Flohé et al. 2022). Among the vitamin

E compounds, α-tocopherol was the major vitamin E homologue in all dietary groups, whereas γ-tocopherol was the minor, which strongly agree with Madeira et al. (2021). Contrarily to what was demonstrated for *Arthrospira platensis* (Madeira et al. 2021), there was no negative impact of CH or carbohydrases on vitamin E compounds. On the contrary, pigments were overall increased by CH, with and without feed enzymes. The rise of total chlorophylls and total carotenoids contents in the liver is thought to be a key indicator of their respective dietary bioavailability. Chlorophylls and carotenoids are powerful dietary antioxidants (Pérez-Gálvez et al. 2020), which are extremely important for human and piglets health (Nabi et al. 2020).

The discriminant analysis herein presented, a PCA based on the relationship among all hepatic variables, showed a clear separation of dietary treatments with or without CH. Interestingly, for the control group, and in contrast with CH-based diets, a higher dispersion pattern of animal cases was observed. It remains to be elucidated what might be the cause for such observation.

Finally, and concerning the metabolomics analysis, the PCA applied to the liver aqueous metabolites showed a complete superimposition of the four experimental groups. Moreover, the PLS model, although presented some group clustering, was not validated by the quality parameters. These results clearly indicate the existence of very similar metabolite profiles for the four experimental groups. Such metabolite profiles suggest that the inclusion of CH in the diet, supplemented or not with exogenous enzymes, had a minimal effect on the overall hepatic intermediate metabolism. These results are similar to other studies on the effects of diet in the metabolite profiles of several swine tissues (Zabek et al. 2017; Tremblay-Franco et al. 2020; Parenti et al. 2021) where only minor differences were noticeable in the hepatic metabolome. This could be considered expectable given the fact that these animals, from a physiological standpoint, were in similar physiological stages, at a young age and still growing. On the contrary, when the liver metabolome of piglets with limited growth is compared to that of control growing piglets (Li et al. 2018), the number of affected metabolites and metabolic pathways are increased. Nevertheless, it is noteworthy to mention that the NMR-based metabolomics approach was a useful tool to, not only complement datasets obtained from the other techniques, but also to evaluate the overall dietary influence on the liver metabolome.

7.5. Conclusions

The dietary inclusion of 5% CH supplemented or not with feed enzymes, the commercial Rovabio® Excel AP and the pre-selected four-enzyme mixture, had no impact on the growth performance of piglets, although systemic antioxidant potential and hepatic lipid metabolism were affected. The first line of antioxidant defence through GPX activity and hepatic *n*-3 PUFA contents, in particular the beneficial DHA, were increased by the microalga inclusion in the feed. In piglets fed CH-based diets, the interaction observed between IgG increase and IgM decrease, along with lymphocytes exacerbation, reflected a boost on the immune response promoted by CH that likely assures piglets' survival at the critical *post*-weaning phase.

Considering the findings obtained in this study, particularly those concerning long-term immune reinforcement, our data indicate health benefits of CH used as feed ingredient in piglets' nutrition, without negatively impacting animals' performance. Nevertheless, further research with higher incorporation levels of CH in piglets' diets are suggested, in order to maximize both the sustainability of swine diets and the health promoting effects of dietary CH incorporation. The dietary supplementation with exogenous carbohydrases does not seem to be necessary for feeding piglets with CH-based diets at this level of incorporation. Although, testing higher levels of microalgae incorporation can be interesting to verify the effect of this supplementation with feed enzymes, and also to ascertain the cost-effective to their use.

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Chapter 8 – GENERAL DISCUSSION

During the weaning phase, piglets are particularly susceptible to digestive and respiratory pathologies resulting from the imbalance between the animals' immunity and environmental infection (Pluske et al. 1997; Weary et al. 2008). Antibiotics are an effective tool to control and treat infectious diseases associated to this critical stage. Currently, different strategies to reduce or prevent their use are being adapted, especially due to their past overuse being considered one of the main sources of antibiotic resistance in both animals and humans. In addition, with the rise in global population and *per capita* meat consumption, it is estimated that the demand for animal-derived products will double by 2050 (FAO 2007). Such demand will be relevant for the monogastric production systems and, consequently, for the corn and soybean crops, which are the basis of their feeding (FAO 2011). The lack of sustainability of these crops due to droughts, drastic climate changes and competition with the human nutrition are the main reasons for the need to find alternatives to these feedstuffs.

Arthrospira platensis (Spirulina) and *Chlorella vulgaris* are the microalgae that have deserved the most attention from the animal science community due to their potential application in livestock diets (Madeira et al. 2017). Taxonomically different, Spirulina is a prokaryotic cyanobacterium microalga and *C. vulgaris* is a photoautotrophic eukaryotic green microalga, which confers them different cellular and chemical compositions. *C. vulgaris* has a more recalcitrant cell wall than Spirulina (Safi et al. 2014). This is important for adapting the higher or lower complexity of the technologies developed to improve microalgal nutrient bioavailability for animals (Austic et al. 2013; Lum et al. 2013). Despite the well-known variations in chemical composition associated with growth conditions, Spirulina has a higher protein and lower fat contents when compared to *C. vulgaris* (Madeira et al. 2017). In a context of an increasing protein and energy demands, these microalgae are dietary sources of macro and micronutrients that should be considered for industrial use.

Several studies explored the incorporation of Spirulina in pig diets as supplement (2% or lower in diet) (Grienstead et al. 2000; Simkus et al. 2013; Nedeva et al. 2014; Furbeyre et al. 2017; Altmann et al. 2019). Only a single study in the literature involved a high inclusion level of Spirulina (13 and 21%) in diets for piglets and growing pigs, focused on nitrogen balance by measuring parameters of apparent nitrogen digestibility, complex dietary protein quality and individual amino acid efficiency, being the productive performance parameters neglected (Neumann et al. 2018). Several studies have also been conducted with *C. vulgaris* as a supplement (1% or lower in diet) in pig diets (Baňoch et al. 2012; Yan et al. 2012; Furbeyre et al. 2017; Furbeyre et al. 2018). At high dietary levels, only one very early study used sewage-grown *C. vulgaris* (5 and 10%), which is currently not considered a safe ingredient for

pigs (Hintz and Heitman, 1967). Recently, Coelho, Pestana et al. (2020) used 5% of *C. vulgaris* in finishing pig diets. The recalcitrant cell walls of microalgae decrease its digestibility by pigs, which is prominent when the microalgae are included at higher dietary levels. CAZymes have been *in vitro* studied, showing positive results in cell wall disruption of Spirulina and *C. vulgaris* (Coelho et al. 2019; Coelho, Lopes et al. 2020). The latter authors in a previously mentioned study tested CAZymes *in vivo* as feed supplement in a diet with 5% *C. vulgaris* for finishing pigs (Coelho, Pestana et al. 2020).

The two *in vivo* experiments with weaned piglets reported in this Thesis aimed to evaluate the effects of a high dietary incorporation level of Spirulina and *C. vulgaris* and the supplementation with a pre-selected CAZyme mixture indicated by our research team in a previously *in vitro* study for disruption of Spirulina (Coelho et al. 2019) and *C. vulgaris* cell walls (Coelho, Lopes et al. 2020).

The first part of this study consisted in a growth experiment, where piglets had *ad libitum* access to the diets studied, to evaluate the effects of a high incorporation level of Spirulina, individually or in combination with two commercial carbohydrases. Forty *post*-weaned male piglets from Large White x Landrace sows crossed with Pietrain boars with an initial live weight of 12.0 ± 0.89 kg were used. The parameters analysed were: growth performance, total tract apparent digestibility using an external marker, meat quality traits (colour, pH, cooking loss and shear force determinations), sensorial analysis, oxidative stability of pork and chemical composition of *longissimus lumborum*, namely lipid content, total cholesterol, fatty acid profile, diterpene profile and pigments content. The healthy status of the piglets was assessed through analysis of haematology, plasma metabolites, plasma hepatic markers, immunoglobulins profile, plasma antioxidant potential, and also hepatic metabolism, such as lipid content, fatty acid profile, diterpenes, pigments content and different gene expression levels. The control diet used was a cereal-soybean meal diet with 44, 15 and 25% (as fed basis) of wheat, corn and soybean meal, respectively. The microalga Spirulina was added to the other three diets in a partial replacement of soybean meal, which remained with 46, 17 and 11% (as fed basis) of wheat, corn and soybean meal, respectively. The three diets with Spirulina incorporation were: only Spirulina incorporation; one was supplemented with 0.005% of Rovabio® Excel AP, a commercial mixture of CAZymes with xylanase and β-glucanase often used in cereal-based diets for monogastrics; and, one was supplemented with 0.01% of lysozyme, commercially available and *in vitro* tested as efficient in the disruption of Spirulina cell walls.

The second part of this study consisted in a digestibility trial, where piglets were individually allocated in metabolic cages and had equal access to diets, to evaluate the effects of 5% *C. vulgaris*, individually or in combination with a commercial carbohydrase mixture or a mixture of pre-selected CAZymes developed by our research team (Coelho, Lopes et al. 2020).

Forty-four *post*-weaned male piglets from Large White x Landrace sows crossed with Pietrain boars with an initial live weight of 11.2 ± 0.46 kg were used. The parameters analysed were: growth performance, total tract apparent digestibility, meat quality traits (colour, pH, cooking loss and shear force determinations), sensorial analysis, oxidative stability of pork and chemical composition of *longissimus lumborum*, namely lipid content, total cholesterol, fatty acid profile, diterpene profile and pigments content. The healthy status of piglets was assessed through analysis of haematology, plasma metabolites, plasma hepatic markers, immunoglobulins profile, plasma antioxidant potential, and also regarding hepatic metabolism, such as lipid content, fatty acid profile, diterpenes, pigments and metabolites content. The control diet used was a cereal-soybean meal diet with 44, 15 and 25% (as fed basis) of wheat, corn and soybean meal, respectively. The microalga *C. vulgaris* was added to the other three diets in direct replacement of soybean meal. The three diets with *C. vulgaris* incorporation were: only *C. vulgaris* incorporation; one was supplemented with 0.005% of Rovabio® Excel AP, a commercial mixture of CAZymes with xylanase and β-glucanase often used in cereal-based diets for monogastrics; and, one was supplemented with 0.01% of four-CAZyme mixture developed previously by our research team (Coelho, Lopes et al. 2020) and *in vitro* tested as efficient in the disruption of *C. vulgaris* cell walls.

Table 8.1 presents an overview of the major outcomes obtained in the two trials carried out in this work.

Table 8.1. Major findings of the trials with dietary incorporation of Spirulina and *C. vulgaris* and supplementation with enzymes in piglets. The results are compared with the respective control group.

Item	Spirulina			*Chlorella vulgaris*		
	SP	**SP+R**	**SP+L**	**CH**	**CH+R**	**CH+M**
Growth performance	↓ Final weight ↓ ADG ↓ FCR	↓ Final weight ↓ ADG ↓ FCR	↓ Final weight ↓ ADG ↓ FCR	= All parameters, except ↑ ADFI	= All parameters, except ↑ ADFI	= All parameters, except ↑ ADFI
Nutrient digestibility	↓ DM ↓ OM ↓ CP ↓ Energy	↓ DM ↓ OM ↓ CP ↑ CF ↓ Energy	↓ CP ↑ CF ↑ ADF	↓ All nutritional fractions, except CF and cellulose	↓ All nutritional fractions, except CF	↓ All nutritional fractions, except CF
Gastrointestinal tract variables	↑ viscosity duodenum+ jejunum and ileum	↑ viscosity duodenum+ jejunum ↑ length small intestine	↑ viscosity duodenum+ jejunum and ileum ↑ length small intestine	↑ viscosity, pH duodenum+ jejunum ↑ pH ileum ↑ villus height	↑ viscosity duodenum+ jejunum ↑ pH ileum ↑ villus height	↑ viscosity duodenum+ jejunum ↑ pH ileum ↑ villus height
Gut volatile fatty acids	-	-	-	↓ C2 ↓ C3 ↓ iC5 ↓ Total [in colon]	↓ C2 ↓ C3 ↓ iC5 ↓ Total [in colon]	↓ C2 ↓ C3 ↓ C4 ↓ iC5 ↓ C5 ↓ Total [in caecum and colon]
Gut microbiota	-	-	-	↑ *Colidextribacter*	↑ *Lactobacillus* ↑ *Oscillospira*	↑ *Helicobacter* ↑ *horsej-a03*
Meat quality and sensory attributes	↑ Flavour	↑ L* ↑ b*	↑ a* ↑ b* ↑ Tenderness ↑ Flavour	= All parameters	↑ Tenderness ↑ Overall acceptability	↓ Cooking loss

Meat fatty acid profile	↓ 20:2*n*-6 ↓ 20:3*n*-3	↓ 20:2*n*-6 ↑ 20:3*n*-6 ↑22:0	↓ 20:2*n*-6 ↑ 20:3*n*-6 ↑ 22:1*n*-9	↓ 15:0 ↓17:0 ↓ 17:1*n*-8 ↑18:0 ↓20:1*n*-9 ↓20:2*n*-6 ↑22:1*n*-9 ↑22:5*n*-3 ↑ 22:6*n*-3 ↑ *n*-3 PUFA ↓ *n*-6:*n*-3	↓ 15:0 ↓17:0 ↓ 17:1*n*-8 ↑18:0 ↓20:2*n*-6 ↓ *n*-6:*n*-3	↓ 15:0 ↓17:0 ↓ 17:1*n*-8 ↑22:5*n*-3 ↑ 22:6*n*-3 ↑ *n*-3 PUFA ↓ *n*-6:*n*-3
Meat oxidative stability	↑ TBARS [at day 3]	= TBARS	= TBARS	= TBARS	= TBARS	↑ TBARS [at day 8]
Meat diterpene profile and pigment content	↑ Total carotenoids = Diterpene profile	↑ Total carotenoids = Diterpene profile	↑ All pigments = Diterpene profile	↑ Total carotenoids = Diterpene profile	↑ Total carotenoids = Diterpene profile	↑ Total carotenoids = Diterpene profile
Plasma biochemical profile	↑ Total lipids ↑TAG ↑ Total cholesterol ↑ HDL-cholesterol ↑ LDL-cholesterol ↑ Glucose ↓ Total protein ↑ ALT ↓ GGT ↑ IgM	↑ HDL-cholesterol ↑ Creatinine ↑ ALT ↑ AST ↑ ALP ↓ GGT ↓ IgM	↑TAG ↑ Glucose ↑ ALT ↑ AST ↑ ALP ↓ GGT ↓ IgG	↑ Lymphocytes ↑ Total lipids ↑ Total cholesterol ↑ LDL-cholesterol ↑ IgG ↓ IgM	↑ White blood cells ↑ Thrombocytes ↑ Total lipids ↑TAG ↑ Total cholesterol ↑ HDL-cholesterol ↓ LDL-cholesterol ↑ VLDL-cholesterol ↑ AST ↑ ALP ↑ GGT ↑ IgG	↑ White blood cells ↓ Granulocytes ↑ Lymphocytes ↑ Red blood cells ↑ Haemoglobin ↑ Thrombocytes ↑ Total lipids ↑TAG ↑ Total cholesterol ↑ HDL-cholesterol ↑ LDL-cholesterol ↑ VLDL-cholesterol ↓ Insulin ↓ HOMA-IR ↑ Urea ↑ Creatinine ↑ ALT ↑ IgG ↓ IgM

Plasma antioxidant potential	↑ TAC	↑ TAC	↑ TAC	↓ TAC ↑GPX	↓ TAC ↑GPX	↓ TAC
Hepatic fatty acid profile	↑ 17:0 ↓ 20:2*n*-6 ↓ 20:5*n*-3 ↓ 22:6*n*-3 ↓ *n*-3 PUFA ↑ *n*-6:*n*-3	↑ 16:0 ↑ 18:3*n*-6 ↓ 20:2*n*-6 ↓ 20:5*n*-3 ↓ 22:6*n*-3 ↓ *n*-3 PUFA ↑ *n*-6:*n*-3	↑ 10:0 ↑ 17:0 ↑ 18:3*n*-6 ↓ 20:5*n*-3 ↓22:5*n*-3 ↓ *n*-3 PUFA	↑ Total cholesterol ↑ SFA ↓ PUFA ↑ *n*-3 PUFA ↓ *n*-6 PUFA ↓ *n*-6:*n*-3 ↓ PUFA:SFA	↑ Total cholesterol ↑ SFA ↓ MUFA ↓ PUFA ↑ *n*-3 PUFA ↓ *n*-6 PUFA ↓ *n*-6:*n*-3 ↓ PUFA:SFA	↓ Total lipids ↑ Total cholesterol ↑ SFA ↓ MUFA ↓ PUFA ↑ *n*-3 PUFA ↓ *n*-6 PUFA ↓ *n*-6:*n*-3 ↓ PUFA:SFA
Hepatic diterpene profile and pigment content	↓ α-Tocopherol ↓ γ-Tocopherol ↑ Total carotenoids	↓ α-Tocopherol ↓ γ-Tocopherol ↑ Total carotenoids	↓ α-Tocopherol ↓ γ-Tocopherol ↑ Total carotenoids	↑ Total carotenoids	↑ Chlorophyll *a* ↑ Total carotenoids ↑ Total chlorophylls	↑ Chlorophyll *a* ↑ Total carotenoids

Dietary treatments: SP – 10% Spirulina diet; SP+R – 10% Spirulina diet supplemented with 0.005% Rovabio® Excel AP; SP+L – 10% Spirulina diet supplemented with 0.01% lysozyme; CH - -5% *Chlorella vulgaris* diet; CH+R – 5% *Chlorella vulgaris* diet supplemented with 0.005% Rovabio® Excel AP; CH+M – 5% *Chlorella vulgaris* diet supplemented with 0.01% enzymatic mixture.

↑ increase; ↓ decrease; = not changed; ADFI – average daily feed intake; ADG – average daily weight gain; FCR – feed conversion ratio; DM – dry matter: OM- organic matter; CP – crude protein; CF- crude fat; ADF – acid detergent fibre; VFA – volatile fatty acids; C2, C3, C4, C5 and iC5 are acetic, propionic, butyric, valeric and isovaleric acids, respectively; SFA – Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; TBARS – thiobarbituric acid reactive substances; TAG – triacylglycerols; HDL – High density lipoproteins; LDL – Low density lipoproteins; VLDL- Very low-density lipoproteins; HOMA-IR Homeostasis model assessment using the insulin resistance index; ALT – alanine aminotransferase (E.C. 2.6.1.2); AST- aspartate aminotransferase (E.C. 2.6.1.1); ALP- alkaline phosphatase (E.C. 3.1.3.1); GGT- gamma-glutamyltransferase (E.C. 2.3.2.13); IgG – immunoglobulin G; IgM – Immunoglobulin M; TAC – total antioxidant capacity; GPX- Glutathione peroxidase.

The limiting level of dietary incorporation of microalgae without impairing the productive performance of piglets warrants further investigation, mainly when considering the incorporation as an ingredient because the well-known problem of cell wall digestibility. The dietary incorporation of 10% Spirulina in diets of piglets impaired the growth performance of piglets, which has never been reported in the literature. Several studies indicate that the dietary incorporation of Spirulina has no effect on productive parameters of pigs at supplementation levels (1% or lower in diets) (Grinstead et al. 2000; Simkus et al. 2013; Nedeva et al. 2014; Furbeyre et al. 2017). The incorporation of exougenous enzymes in diets with 10% Spirulina had no influence on growth, as no significant differences between experimental groups were found. In the second part of our work, the dietary incorporation of 5% *C. vulgaris* had no effect on productive performance of piglets, which was also reported in the study with finishing pigs of Coelho, Pestana et al. (2020). Although, in the trial with *C. vulgaris* the aim was to study the digestibility of diets, so the animals were not fed *ad libitum* and these results need confirmation in a growth study. We observed a higher ADFI in all diets with *C. vulgaris*, which was not enough to significantly increase the growth rate of the animal and had no effect on FCR. The animals had the same access to the feed in all groups, which means that the groups fed with *C. vulgaris* refused less amount of feed, thus justifying the higher ADFI compared to the control group. In the future, it would be interesting to confirm these results through a growth performance trial that involved a larger number of animals and *ad libitum* access to experimental diets. At supplementation levels of *C. vulgaris* (1% or lower in diets), several studies in the literature indicate that there are no effects on the productive parameters (Baňoch et al. 2012; Furbeyre et al. 2017; Furbeyre et al. 2018). At higher *C. vulgaris* dietary incorporation levels there are no studies, which can be justified by the higher costs and lower digestibility of this microalgae for pigs. The incorporation of exogenous enzymes in diets with microalgae 5% of *C. vulgaris* had no influence on growth performance of the animals, as no significant differences were observed between experimental groups.

Digestibility results of the two trials, that to the best of your knowledge were explored here for the first time in piglets using diets with microalgae inclusion, confirm in general the difficulty of microalgae digestion by pigs, particularly in piglets. In the first trial, the inclusion of 10% Spirulina decreased TTAD of DM, OM, CP and energy, without affecting the TTAD of CF and NDF. The supplementation of Spirulina diets with Rovabio® Excel AP did not improve any TTAD nutritional fraction. On the other hand, supplementation of Spirulina diets with lysozyme demonstrated an increase in the digestive availability of DM, OM and energy from the microalga, leading to an improved feed efficiency. However, the TTAD of CP did not improve with such supplementation. The highest TTAD values of CF, ADF and energy for the SP+L group indicated that lysozyme was effective in degrading the Spirulina cell walls, leading the digestive enzymes to access the cellular content, which is the known fraction with the highest

digestibility. The low TTAD value of CP showed that the Spirulina proteins seem to be resistant to the piglet´s endogenous peptidases. Another explanation to such high TTAD value of CP could be the greater excretion of bacterial proteins in faeces by high fermentation of cell walls in the hindgut, leading to an underestimation of this calculated parameter. In the second trial, the inclusion of 5% *C. vulgaris* also negatively affected all studied TTAD nutritional fractions (DM, OM, CP, NDF, ADF, hemicellulose and cellulose), with the exception of CF. We also compared the TTAD values of two collection periods and observed worse results for the second period. This confirmed the difficulty of piglets to adapt and digest diets containing 5% of *C. vulgaris*. The supplementation of *C. vulgaris* diets with Rovabio® Excel AP positively affected TTAD compared to the two groups with microalga and with closer values to those of the control group (to DM, OM, CP, NDF, ADF, hemicellulose and cellulose). The presence of β-xylanases and β-glucanases in this CAZyme is usually associated with the degradation of non-starch polysaccharides present in plant-origin feedstuffs. In our study, this Rovabio® Excel AP seemed to be associated with the degradation of *C. vulgaris* cell walls and the release of intracellular nutrients to be digested by pigs, improving the digestibility of this feedstuff and proving to be more effective than the 4-carbohydrase mixture tested.

So far, few studies have focused on the nutritional value of microalgae for pigs. Because it is a novel feedstuff, we consider it important to determine the nutritional value, which is the beginning of all nutrition and feed work. Furbeyre et al. (2017) studied the effect of dietary supplementation with 1% Spirulina and 1% *C. vulgaris* on TTAD of DM, OM, CP, NDF, ADF and energy in weaned piglets from 28 to 42 days of age and concluded that TTAD in pig receiving microalgae was greater for gross energy ($p < 0.05$), and tended to be higher for DM, OM and NDF ($p < 0.10$) compared with control pigs. These results, so different from those observed in our work, can be explained by the lower level of incorporation, admitted by the authors themselves as a low level of inclusion to minimize the potential deleterious effect of Spirulina and *C. vulgaris* on the bioavailability of nutrients. With another microalgae, *Schizochytrium*, Kibria and Kim (2019) studied the TTAD in weaning piglets and disovered that with 0.5 and 1% of dietary inclusion, an increase in the TTAD of DM and nitrogen ocuured. This was justified by the supplementation with the microalga as a *n*-3 fatty acid source in the diet, helping in the maintenance of the function and structure of the small intestine and, consequently, increasing the digestive capacity of gut.

Regarding the digestibility results of our trials, it is interesting to combine them with the intestinal parameters analysed. Thus, in both trials, the incorporation of microalgae in diets of piglets increased digesta viscosity of duodenum plus jejunum. In the first trial, the Spirulina-fed animals also had this parameter higher in the ileum. The highest value of viscosity in absolute terms was found for SP+L group in both analysed intestinal contents, which corroborates with the previously mentioned results that showed a high value of TTDA of DM,

OM and energy for this group in relation to the other microalgae groups. A high viscosity of digesta leads to an increased time in the intestine and thus a more efficient action by endogenous enzymes of piglets, increasing the digestibility of the nutrients. Conversely, the TTAD of CP for this group was similar to the other microalgae groups and lower than the control group, which may be associated with the higher release of proteins from Spirulina and subsequently increases the digesta viscosity that works as a limiting factor of access to the endogenous enzymes to their target substrates. These results suggest that the Spirulina proteins are resistant to the proteolytic action of endogenous peptidase of piglets and require further investigation into the possible use of a protease to help this digestion.

Regarding the first trial, a direct increase in relative length of the small intestine was observed, where the groups with Spirulina plus enzyme supplementation had a greater length compared with control group, mainly with lysozyme supplementation. These results could be seen as a compensatory mechanism associated with the higher digesta viscosity previously mentioned and discussed.

With Spirulina inclusion, no changes were observed in the histological measurements of the small intestine, while with *C. vulgaris* inclusion, piglets had higher duodenum villus heights when compared to the control group. Furbeyre et al. (2017) also found higher villus heights at the jejunum in piglets fed with 1% of *C. vulgaris* compared to the control group. The authors found the same results with 1% of Spirulina and suggested a positive effect of both microalgae supplementation on mucosal restoration or development after weaning, being associated with the increase in nutrient digestibility they observed. In our case, a lower digestibility and a higher digesta viscosity was observed with 5% of *C. vulgaris,* which suggests compensatory development of intestinal tissue in order to increase nutrient absorption. Later, using oral supplementation with *C. vulgaris* or Spirulina (385 mg/kg LW) in piglets, they found that mucosal morphology at the jejunum was not affected by this supplementation, although ileal villus height was lower in piglets fed with microalgae than in control piglets (Furbeyre et al. 2018). In this study the authors reported these results as unexpected and difficult to explain, adding that the viscosity of intestinal contents was not measured and could be associated, raising the hypothesis that the bead-milling process may have has a negative impact on the physical properties of *C. vulgaris* and subsequent negative effects on intestinal health.

The intestinal content pH, VFA and microbiota determinations were not performed in the first trial. In the second trial, the pH results revealed significant differences in terms of duodenum plus jejunum (decrease in CH group) and ileum contents (decrease in all *C. vulgaris* groups), in comparison with control group. Intestinal pH affects the digestion and absorption of nutrients with low intestinal pH in *post*-weaning stage of piglets promoting intestinal health (Shi et al. 2022). Regarding VFA concentration in the caecum, only the CH+M group showed a significantly lower quantity to all VFA analysed. The lower degradation rates associated with

this group may be associated with the lower amount of cell material that reaches the caecum, revealing the effectiveness of this enzymatic mixture to degrade *C. vulgaris* cell wall, although the digestibility values for all nutritional fractions analysed did not corroborate it. In the colon, the concentration of VFA was decreased for all animals fed with the microalga, with the lower quantity of fermentable carbohydrates associated with the recalcitrant cell wall being the main justification to this change in the microbial fermentation profile. Microbiome results revealed that the use of *C. vulgaris* increased the bacteria genera associated with a healthy pig microbiota. Only *C. vulgaris* dietary inclusion increased the relative abundance of *Colidextribacter* and the genus was recently isolated from human gut microbiota (Ricaboni et al. 2017). The supplementation with Rovabio® Excel AP increased the relative abundance of *Lactobacillus* and *Oscillospira*. Janczyk et al. (2009) showed that 0.5% *C. vulgaris* supplementation promotes *Lactobacillus* in the cecum content of laying hens. In a meta-analysis study with 12 trials using piglets, Zhu et al. (2022), reported that porcine duodenal villus height was significantly increased by *Lactobacillus* spp. supplementation when compared to the control animals. In the same trials, no significant correlation between the inclusion of *Lactobacillus* spp. And duodenal crypt depth was observed. In our study, we can infer such an association, because the groups with *C. vulgaris* that had an increased population of *Lactobacillus* also had an increase of duodenal villus height, despite the fact that villus heights for jejunum and ileum were similar in all experimental treatments. *Oscillospira* is a bacterial genus usually studied in weaned piglets for its potential benefits to the gut health, being associated with the production of butyrate with a probiotic effect. Highest prevalence of *Helicobacter* to CH+M group, this genus is commonly associated with intestinal inflammation, which once again corroborates the data obtained for this group in TTAD results and VFA concentrations, demonstrating that the efficiency of the 4-enzyme mixture to degrade *C. vulgaris* cell walls was not confirmed in our *in vivo* study.

Concerning the effect of dietary inclusion of microalgae on meat quality traits and sensorial attributes, we found no differences for pH 24 h *post-mortem* (for both trials) and colour (for the 5% *C. vulgaris* trial). Dietary supplementation of exogenous enzymes in Spirulina diets increased L* and b* with Rovabio® Excel AP and a* and b* with lysozyme. The trained sensory panel scores denoted that meat from animals fed only Spirulina or SP+L had higher flavour scores, when compared to the control group. In addition, meat tenderness from SP+L and CH+R groups was higher than that of control piglets. The latter group had a higher overall acceptability of meat compared to other experimental groups. Shear force and cooking losses were unaffected by the diets, with the exception of the CH+M group, which showed a lower value for the latter parameter when compared to the control group. The low cooking loss value is usually associated with greater juiciness meat, which was not observed specifically in this group, but was noticed in the homologous group (CH+R). All the mentioned differences in

this paragraph for meat were slight and did not appear to affect acceptability by consumers. In the study conducted by our colleagues (Coelho, Pestana et al. 2020), authors found no significance influence on meat quality traits and sensorial attributes from pigs fed diets with the same level of *C. vulgaris*, studying the same enzymatic mixture and parameters. Previously, only Baňoch et al. (2012) used a very low level of *C. vulgaris* (0.0002%) to feed female pigs during 3 months and found no significant effect on colour, pH, cooking loss and drip loss ok pork. In the literature, references to high levels of Spirulina inclusion (6.6-12.5%) in pigs from 25 kg LW until 122 kg LW were associated to stronger odours and astringent aftertaste of Spirulina pork in comparison to the control groups. These negative aspects were not observed in our first trial with the use of 10% of Spirulina, quite the opposite, as meat from animals fed Spirulina was associated with higher scores for flavour and tenderness. Interestingly, to the best of our knowledge, this is the first time that the meat of piglets fed with microalgae has been characterized at these ages and we think that these results are important to be indicators to the next phase, mainly for the growing-finishing phase. Our experimental facilities only allow us to bring animals up to a limited live weight, however, our colleagues carried out an *in vivo* trial with finishing pigs, which we will use as a basis for comparison when discussing of our results obtained for meat (Coelho, Pestana et al. 2020).

C. vulgaris dietary inclusion affected the fatty acid profile of *longissimus lumborum* muscle. It affected the partial sums of fatty acids (increase of *n*-3 PUFA in CH group and decrease of *n*-6:*n*-3 ratio in all experimental groups with microalga), albeit it was slightly influenced by Spirulina, that only influenced some fatty acids (decrease of 20:2*n*-6 to all groups, increase of 18:3*n*-6 to all groups, decrease 20:3*n*-3 to SP group, increase of 22:0 and 22:1*n*-9 to SP+R and SP+L, respectively). Altmann et al. (2019) with Spirulina inclusion at high dietary levels (6.60-12.5%) found higher levels of *n*-6 FA and increased level of ALA and GLA in backfat of pigs from 22 ± 1.6 kg of LW until 110.48 ± 5.1 kg of LW when compared to the control group. Our results in the Spirulina trial corroborate the previously reported results that relate inclusion in the diets with an increase in n-6 FA content in meat, despite having no effect on its partial sum, there was special emphasis on the increase in GLA, on average 46.5% higher in meat from animals fed with Spirulina compared to controls. The differences observed between the two trials can be explained because Spirulina and *C. vulgaris* have different characteristics regarding fatty acid composition. The levels of unsaturated fatty acids in *C. vulgaris* is higher than that in Spirulina, highlighting the high amounts of alpha-linolenic, 18:3n-3 (ALA), in this microalga (Ötleş and Pire 2001). In the analysed FA profile of experimental diets, the *C. vulgaris* diets presented 33% more ALA on average than the control diet. For ALA content of Spirulina diets, no differences were observed in relation to the control diet, and in the single microalga this FA was not detected. Thus, focusing on *C. vulgaris* diets, where EPA and DHA were not detected, but these FA were, they represent the ability of muscle to capture

the ALA precursor present in *C. vulgaris* diets and convert it into these *n*-3 PUFA derivates (Meadus et al. 2013). Additionaly, Coelho, Pestana et al. (2020) detected an increase of EPA and DHA content in *longissimus lumborum* of pigs fed with 5% *C. vulgaris* and a decrease in the *n*-6:*n*-3 ratio. With *Schizochytrium* sp. in pigs diets (0.06-7.00%), several authors have reported an increase in EPA and DHA contents and a decreased in the *n*-6:*n*-3 ratio in muscle, backfat, dry-cured hams and bacon (Sardi et al. 2006; Meadus et al. 2011; Moran et al. 2017; Vossen et al. 2017; Moran, Morlacchini et al. 2018). The recommended *n*-6:*n*-3 ratio to prevent cardiovascular disease is reported to be around 5 (Wood et al. 2004). The meat of piglets fed with *C. vulgaris* showed a *n*-6:*n*-3 ratio of 12.6, on average, which represented a decrease of 42% comparing to controls, being 2.52-fold higher in comparison with the recommended value while the control value was 4.58-fold higher than the recommended value. In association with the higher content of EPA and DHA, we can say that our results demonstrated that 5% of *C. vulgaris* in piglets' diets improved the *n*-3 PUFA content of meat, promoting health-protecting cardiovascular effects for consumers and improving meat quality. In the Spirulina trial, the main individual FA were not affected by the diets. For both trials, the supplementation with CAZyme mixtures had slight changes that did not affect the partial sums of FA, only the group CH+R had a similar value of *n*-3 PUFA to the control group but it was not reflected in the result of *n*-6:*n*-3 ratio of this group.

Meat rich in *n*-3 PUFA is associated with oxidative stability problems by several authors (Meadus et al. 2011; De Tonnac et al. 2017; Vossen et al. 2017). Based on the above-mentioned results, we expected that the increase in *n*-3 PUFA of meat from *C. vulgaris* groups would decrease its oxidative stability. However, the TBARS results demonstrated that only the CH+M group had a higher value after 8 days under refrigeration compared to the control group. Coelho, Pestana et al. (2020) stated that 5% of *C. vulgaris* in the diets of pigs had no negative impact on oxidative stability of pork. Also, with a very low dietary incorporation level of *C. vulgaris* (0.0002%), Baňoch et al. (2012) observed no influence on the oxidative stability of pork. With another microalga, *Schizochytrium* sp. (0.3-1.6%), lipid oxidation of dry-cured ham and bacon was reported, which in turn has been associated with increased *n*-3 PUFA content in the products (Meadus et al. 2011; Vossen et al. 2017). In our trial with Spirulina, only a small effect was observed on TBARS values of *longissimus lumborum* after 3 days under refrigeration for the SP group compared to the control group, but after 8 days no differences were observed between experimental groups. Also, Altmann et al. (2019) found no effects on pork TBARS values with use of Spirulina in diets (6.60-12.5%). Lipid oxidation often contributes to the development of off-favours and off-odours and changes the colour of the meat. The results obtained for both trials through the sensorial pannel and colour measurements did not detect significant differences, not even in the groups referred to as higher TBARS values (SP and CH+M groups). Different studies have established values of 2-2.5 mg MDA/kg of meat as

the allowed limit at which there is no rancidity in the meat (Domínguez et al. 2019). In our study, even after 8 days of refrigerated storage, no meat reach had a TBARS value above this reference range.

The diterpene profile of meat was not influenced by the microalga dietary inclusion (Spirulina and *C. vulgaris*), which was also demonstrated in the results by Coelho, Pestana et al. (2020). Regarding the total pigments in meat, with 5% of *C. vulgaris* only the total carotenoid content was affected. It was 2 times higher than in the control group, while with 10% of Spirulina this parameter was also affected (1.7 times higher than reference group). Additionally, there was a significant increase on all analysed pigments in the Spirulina supplemented with lysozyme group comparatively to the control group. The pigment profile of diets with microalgae revealed higher contents for all pigments in relation to control diet. Indeed, we recorded a several fold increase in the average values for both Spirulina and *C. vulgaris* diets, respectively: 36.9 and 43.5 for chlorophyll *a*, 6.30 and 3.02 for chlorophyll *b*, 17.2 and 17.2 for total chlorophylls, 16.8 and 5.15 for total carotenoids and 17.1 and 14.3 for total pheophytins. These results demonstrated that only the carotenoids were converted in meat and not chlorophylls, with the exception of SP+L group. In addition, Coelho, Pestana et al. (2020) found similar results. These authors attributed them to the difference in polarity of the molecules in question, with chlorophylls being more polar and showing less affinity for pork intramuscular fat. The enrichment of pork with carotenoids by the dietary inclusion of microalgae is rarely reported in the literature, however for poultry this is specifically reported with Spirulina by Venkataraman et al. (1994) and Pestana et al. (2020) and with *C. vulgaris* by Alfaia et al. (2021). This enrichment from the microalgae increased the nutritional value of pork. Animals are not able to synthesize carotenoids, which have to be supplemented in the diet. These pigments upregulated the production of antioxidant enzymes and play an important biological role contributing to therapeutic effects, including anticancer, immunomodulatory, anti-inflammatory, antibacterial, antidiabetic and neuroprotective (Lauretani et al. 2008; Arain et al. 2018). Thus, supplementation with both microalgae, combined or not with CAZyme mixtures, enriched pork in carotenoids that have a beneficial outcome for the health of consumers. The exception observed in SP+L group with the higher contents of all pigments by comparison to the control group may be ascribed to the already mentioned effectiveness of lysozyme in the degradation of Spirulina cell walls with the consequent greater liberation of these pigments that can be more easily converted into meat. β-carotene was undetected in any meat from the *C. vulgaris* trial groups; however, it was detected in meat from all experimental groups in Spirulina trial. In contrast, in diets with *C. vulgaris* this pigment was detected at a higher level than in diets with Spirulina (on average, 13.8 vs. 2.95 µg/g). These results possibly indicate that this carotenoid was rapidly metabolized into vitamin A or is not readily available from the feed, especially from those with *C. vulgaris*, characterized by a more

rigid cell wall than that of Spirulina. To understand this, it would be interesting to determine the vitamin A content of meat. The higher content of carotenoids, with a well-documented antioxidant activity, observed in the meat from animals fed with microalgae, could allow inferences about the higher antioxidant capacity of this meat, with a positive impact on the TBARS values and extension of the shelf life of meat. However, as mentioned previously, no clear effects were detected that could demonstrate the antioxidant capacities of microalgae because the groups with microalgae had meats with TBARS values equal to or higher than those of the control group.

The effect of dietary inclusion of microalgae on health status of piglets was assessed through blood parameters. We confirmed that the values obtained for serum metabolites and hepatic markers to both trials remained within the reference range for pigs and piglets, specifically for urea, creatinine, total protein, ALT, AST, ALP and GGT (Egeli et al. 1998; Jackson and Cockcroft 2002). This indicates no toxic effects of microalgae on liver or kidney. Microalgae have been exploited for therapeutic purposes, mainly cholesterol reduction and prevention of cardiovascular diseases and hyperglycaemia (Belay 2002; Oliveira et al. 2013). Although the plasma lipid profiles found in both trials followed a contrary tendency where the groups fed with microalgae showed equal or higher values of total lipids, TAG, total cholesterol and LDL-cholesterol compared to the control group. However, in line with the reported positive effects of microalgae on cholesterol metabolism (De Caire et al. 1995; Cheong et al. 2010), the inclusion of microalgae had a positive effect on HDL-cholesterol levels with higher values for SP, SP+R, CH+R, CH+M groups or equal for other groups compared with control groups. For hepatic markers, there was a clear effect of dietary Spirulina on increasing ALT and decreasing GGT levels comparatively to the control group. In the trial with *C. vulgaris*, such influence was associated with enzymatic supplementation, as Rovabio® Excel AP was responsible for the increase in AST, ALP and GGT and the 4-CAZyme mixture for the increase in ALT, compared to the other experimental groups. This may be associated with a greater effectiveness of Rovabio® Excel AP in the degradation *C. vulgaris* cell walls, as Coelho et al. (2021) observed a similar effect on liver enzymes (increase in ALP levels) of broilers with 10% *C. vulgaris* and the four-CAZymes mixture tested. Regarding the immunoglobulin results, a clear evidence of involvement was found in the *C. vulgaris* trial, with microalga incorporation leading to the increase in IgG levels. This result supports the idea that the immune function was triggered by the microalga, protecting the organism in the long-term, as we observed a decrease in the IgM level in CH and CH+M groups. Corroborating such results, the haematological analysis revealed that lymphocytes levels were increased in CH and CH+M groups comparing with control group. Kibria and Kim (2019) with weaned piglets and *Schizochytrium* sp. (0.5-1%) also found an improvement in blood lymphocytes count. Thus, we can state that *C. vulgaris* helped to improve the immune system of piglets.

Such a clear set of results was not found in the Spirulina trial, where a decrease in the IgG level in SP+L group and an increae the IgM in the SP group and a decrease in the SP+R group where recorded. Although there are no well-stablished ranges of values to be acceptable at such ages, the results of immunoglobulins found in both trials did not show a negative impact on the health of the piglets of the inclusion of microalgae and studied enzymes, proving to be interesting and enriching results for future reference in this type of study. Contrarily, Coelho et al. (2022) in finishing pigs observed a strong immunosuppressive effect promoted by the microalga *C. vulgaris* at the 5% level of incorporation. The authors justified the increased susceptibility of pigs to infectious diseases to the dose-dependent immunoregulatory properties of *C. vulgaris* polysaccharides.

The measurement of plasma GPX activity and TAC allowed assessing the antioxidant potential. GPX activity remained unchanged by dietary treatments with 10% Spirulina, however with *C. vulgaris* it was increased for CH and CH+R groups. Contrary, Coelho et al. (2022) with 5% of *C. vulgaris* verified that this factor was not significantly altered by diets. TCA levels were increased and decreased, respectively, with Spirulina and *C. vulgaris*, individually and combined with exogenous enzymes, when compared to the control groups. Coelho et al. (2022) found a decrease in TAC levels occurred only with 5% *C. vulgaris* inclusion, without enzyme supplementation. Authors attributed this to two different mechanisms: CAZyme mixtures effective in disrupting cell walls with release of internal carotenoids with antioxidant capacity or alternatively an effect of CAZyme mixtures on the decomposition of alginate releasing alginate oligosaccharides that were absorbed at the gastrointestinal tract and that may have positively affected TAC levels. These results found only a slight increase of TCA level, equalling those obtained in the control group. In our trials, we found higher TCA levels in the group with 10% of Spirulina by comparison to controls, showing the effect of carotenoid content from microalga, another evidence that the cell wall is less rigid, contrasting with the results obtained to *C. vulgaris* that have this pigment in diets at higher level. The contradictory results found in CH and CH+R groups for TAC levels and GPX activity can be explained as being a compensatory mechanism to avoid imbalance of oxidative stress homeostasis. It may also be associated, and it is important to highlight, the fact that individually measurement of the concentration of serum antioxidant components may not accurately reflect the total antioxidant status.

The PCA plot of blood parameters from *C. vulgaris* trial revealed a lack of relation between analysed variables. The same discriminant analysis of blood parameters from Spirulina trial explained 44.3% of the total variation, 27.1% for factor 1 and 17.2% for factor 2, with a clear discrimination between the control group and Spirulina groups and a discrimination between the groups with and without enzymatic supplementation.

In the Spirulina trial, few hepatic FA were affected by the microalgae dietary inclusion. Common to both trials was a decrease of EPA in the liver. In the *C. vulgaris* trial, a decrease in *n*-6 PUFA (decrease in the level of linoleic and GLA levels) and an increase of *n*-3 PUFA in the liver, corroborated our findings on meat analysis. This increase was exclusively related to the increase in DHA and not to that of EPA, which was reduced for all groups with microalga. Additionaly, Coelho et al. (2022) observed an increase in the hepatic content of *n*-3 PUFA, albeit related to the increase of DHA and EPA contents. Enrichment with *n*-3 PUFA in the liver has been linked to positive events, such as a decrease in lipid peroxidation, which we also confirmed with an increase in one of the two evaluated plasma antioxidant potentials.

In the Spirulina trial but not in the *C. vulgaris* trial, we performed hepatic gene expression levels determinations and found an up-regulated vasodilator marker for SP+R group, which is associated with an increase in the *Escherichia coli* population commonly correlated with the weaning process. A more detailed analysis of the microbiota, such as the one carried out in the *C. vulgaris* trial, could confirm such asn association. The main genes associated with lipid metabolism remained unchanged across the dietary treatments, which validates the results of total lipids observed in the liver that were slightly changed by dietary Spirulina. Bearing in mind that it aligns with the fact that this microalga has low fatty acids contents.

Total hepatic carotenoids were increased in all groups with inclusion of both microalgae. In the Spirulina trial, dietary inclusion was also associated with changes in the diterpene profile, with a significant decrease in α- and γ-tocopherol contents, and were not influenced by enzymatic supplementation. Additionaly, Coelho et al. (2021 and 2022) found a significant decrease in hepatic α-tocopherol in pigs fed 5% *C. vulgaris* and a decrease in hepatic γ-tocopherol in broilers fed 10% *C. vulgaris*. The authors refer to this as a fact that needs to be further elucidated, as it is possible that the animals would have a difficulty in fixating diterpene compounds in the liver. In addition, the diterpene profile of meat in the Spirulina study remained unchanged, so that this affectation at the liver level had no repercussions on the tissue with edible value. In the *C. vulgaris* trial, enzymatic supplementation was responsible for the increase in chlorophyll *a,* and specifically Rovabio® Excel AP for the increase in total chlorophylls. Chlorophylls and carotenoids are powerful dietary antioxidants. In our studies, the increase of total hepatic carotenoids with microalgae confirmed the bioavailability of these compounds do be effectively used and deposited in the meat, enriching this product as we saw in the results of increased total carotenoids in meat.

The PCA plot of hepatic fatty acids composition, cholesterol, pigments, diterpene profile and gene expression levels from Spirulina trial revealed no relationship between the analysed variables. The same discriminant analysis for hepatic total lipids, cholesterol and fatty acids composition, pigments and diterpene profile from *C. vulgaris* trial explained 53.4% of the total

variation, 41.9% for factor 1 and 11.5% for factor 2, with a clear discrimination between the control group and the three *C. vulgaris*-based diets. In fact, there is a greater effect on the composition of hepatic fatty acid composition and pigment contents with the dietary inclusion of *C. vulgaris* than with Spirulina. This is mostly related to the *C. vulgaris* high lipid content (on average more 3.8 times higher in total lipids than Spirulina). Despite all this, we performed a metabolomics analysis on the *C. vulgaris* trial that revealed that dietary supplementation with this microalga and enzymatic supplementation tested had a minimal effect on the overall hepatic intermediate metabolism.

Chapter 9 – CONCLUSIONS AND FUTURE PERSPECTIVES

In the first part of our study, a growing trial was performed with *post*-weaned piglets to assess the effects of incorporating 10% Spirulina in diets and its supplementation with two exogenous CAZymes on growth performance, nutrient digestibility, meat quality traits, blood health markers, immune function, oxidative status and hepatic lipids. The results indicate that 10% of Spirulina in piglet diets impaired growth performance because of increased digesta viscosity and lower digestibility. Meat quality traits were not negatively affected by the inclusion of Spirulina alone or combined with enzymes. Studied dietary treatments had a minor effect on fatty acid composition and transcriptional profile of lipid sensitive mediators in the liver, highlighting the improvement of the systemic antioxidant potential. Adding the carbohydrase did not improve the digestive utilization of this microalga by piglets and, despite the effectiveness of lysozyme in cell wall degradation, the animals were not able to digest the released proteins. With these results, it can be suggested that it will be interesting to consider the use of exogenous peptidases combined with lysozyme to improve general digestibility, and specifically that of protein, degrading cell walls and proteins and preventing the latter from gelation.

In the second part of this study, a digestibility trial was carried out with *post*-weaned piglets to evaluate the effects of dietary incorporation of 5% *C. vulgaris* and the supplementation with two exogenous CAZyme mixtures on growth performance, nutrient digestibility, gut morphology, fermentation products, microbiota profile, meat quality traits, blood health markers, immune function, oxidative status and hepatic lipids and metabolites. This trial allowed concluding that the inclusion of *C. vulgaris* did not impair growth performance. However, the digestibility of the studied nutritional fractions was negatively impacted. In addition, this promoted the development of compensatory mechanisms in the gut mucosa (increased villus height) and prebiotics effects (higher abundance of some specific bacterial taxa in the intestine). The meat nutritional value was increased with *C. vulgaris* dietary inclusion, increasing total carotenoids and *n*-3 PUFA contents. Regarding the effect on health status and immune response, our results demonstrated a boost in the immune response by increasing IgG and deceasing IgM, together with lymphocytes exacerbation. Additionally, microalga inclusion increased antioxidant defences and hepatic *n*-3 PUFA contents. At this level of *C. vulgaris* incorporation, supplementation with two exogenous CAZymes did not appear to be necessary, as it had a minor impact on the multiple parameters assessed.

The inclusion of microalgae in piglets' diets allowed the covering of nutritional requirements, with diets balanced in energy and protein, as well as fatty acids and amino acid profiles, although digestibility results showed a loss of bioavailability of nutritional compounds.

This was aggravated in the Spirulina trial with a negative affect on growth performance. For the swine industry, these negative results can in turn represent a negative impact on the financial result and a bad start for a new feedstuff as an ingredient. Thus, another important point is the limiting levels of incorporation of microalgae and, in order to better understand this topic, we find it extremely important to determine the nutritional value of microalgae. Our focus in this study was the combination of two factors: inclusion of microalga in diets at high levels and utilization of enzymatic mixtures previously tested *in vitro*. In future studies, it may be crucial to perform a digestibility trial with increasing levels of microalga and the determination of the digestibility of all nutrients from microalgae. Furthermore, an important consideration is the price of microalgae which is another aspect that limits the use of this product as an ingredient for swine feeding. Just to have an ideia, presently, a metric ton of Spirulina and *C. vulgaris* can cost, on average, respectively, 13 and 21 times more than a metric ton of soybean meal. If prices continue to show such a trend, increasing the percentage of microalgae in pig diets could mean increasing the cost of feeds. To solve this problem the microalgae production technologies must be optimized to be less dependent on fossil fuels for drying and obtaining the final powdered products and a consequent extension of shelf life.

The main aim of incorporation CAZyme mixtures to the diets was to improve the bioavailability of nutritional compounds of the studied microalgae. Based on the digestibility results, we concluded that the effectiveness of their use was not clear. However, due to the results established by previous *in vitro* studies that proved the effectiveness of these enzymatic mixtures, we need to test their effectiveness *in vivo*. This is particularly relevant because using high percentages of microalgae inclusion in the diets would lead to an increase of problems with recalcitrant walls that would furthermore affect nutrient digestibility. In view of the results obtained, it would be interesting to further explore the mixture to fine-tune the substrate affinity and the effective release of nutritional components. One can also consider an *in vitro* study about the resistance of Spirulina proteins revealed by the use of lysozyme, testing the combination of this enzyme with different proteases. The use of CAZymes represents an additional cost to diets. Thus, and in order to achieve cost-effectiveness, it is important to choose one that would be effective *in vivo*.

The results obtained in both trials that underline the development of pork products enriched in *n*-3 PUFA and carotenoids, focused on the benefit for consumers and the acceptance/validation of this type of meat. This is in line with the growing consumer demand for healthy products. However, it also demonstrates that the animal develops adequate conditions to continue in the rearing and finishing phases. We used a commercial breed in both trials and the effects on the fatty acid profiles were detected. This breed is considered a lean breed, so it can be inferred that if microalgae are used as ingredient in the diets of fatty swine breeds (for example Duroc crosses or authochtonous breeds) such an accumulation

may be proportional, and a greater effect will be seen in the increase of *n*-3 PUFA. This meat could possibly be a niche with consumers willing to pay more for this type of product.

In both trials, and albeit the effects on piglet growth and performance, the dietary inclusion of microalgae demonstrated to have a positive impact on the piglet's immune system that can in turn have positive repercusssions on piglet health. This is particularly interesting at this stage when animals are recovering from the critical weaning phase. Even in the trial where piglets' growth was negatively affected, animals showed a profile of plasmatic biochemical indicators within the range considered as acceptable. Immune results showed immunoreactivity effects of microalgae. Moreover, we can say that the microalgae did not compromise animal health and, compared to other studies where microalgae were used as a supplement, the effect was greater and significant in parameters such as immunoglobunlin and lymphocytes levels. Also noteworthy were the beneficial effects on the gut microbiota detected in the *C. vulgaris* trial, which further denote the role of the microalgae as a possible useful tool in restoring the balance of gut microbiota associated to the weaning phase in piglets.

The few existing studies in the literature on the subject and on the evaluated parameters show the innovative nature of our study and the novelty of our results. In addition, we carried out proteomic and metabolomic studies (data under evaluation, not included in this Thesis) which in the future could be of interest to relate to these and will allow us to better understand the molecular mechanisms involved in the nutritional use of these two microalgae.

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SUPPLEMENTARY MATERIAL 1

Table S1 - Ingredients and feed additives of the experimental diets (g/kg).

Ingredients	Control	CH	CH+R	CH+M
Wheat	439	440	440	440
Corn	150	150	150	150
Soybean meal 48	250	200	200	200
Whey powder	100	100	100	100
Sunflower oil	30	30	30	30
Chlorella vulgaris	0	50	50	50
Rovabio® Excel AP	0	0	0.050	0
Mix of 4 carbohydrases	0	0	0	0.100
L-Lysine	5	5	5	5
DL-Methionine	1	1	1	1
L-Threonine	1	1	1	1
Calcium carbonate	5	6	6	6
Dicalcium phosphate	13	12	12	12
Sodium chloride	3	2	2	2
Vitamin-mineral complex[1]	3	3	3	3

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.
[1]Premix provided per kg of complete diet: vitamin A, 6500 UI; vitamin D3, 1500 UI; vitamin E, 15 mg; vitamin K3, 1 mg; vitamin B1, 1 mg; vitamin B2, 3 mg; vitamin B6, 2 mg; vitamin B12, 0.02 mg; pantothenic acid, 10 mg; nicotinic acid, 15 mg; folic acid, 0.5 mg, biotin, 0.03 mg; betaine, 115 mg; vitamin C, 20 mg; Copper, 100 mg; iron, 100 mg; iodine, 0.5 mg; manganese 50 mg; selenium, 0.15 mg; zinc, 100 mg; butylated hydroxytoluene, 3 mg

Table S2 - Number of reads that survived in each step of the bioinformatic analysis from gut microbiota analysis.

Sample ID	input	filtered	denoisedF	denoisedR	merged	nonchim
1	50848	44022	43087	43515	41225	39674
10	59649	50557	49172	49792	46882	46046
11	54338	46761	45581	46068	43394	42824
12	60001	53819	53255	53541	51699	48331
13	63010	55407	53964	54531	50612	46947
14	79119	69775	68500	69055	65852	64759
15	58540	53295	52315	52697	50179	48989
16	75541	68231	67073	67514	64668	62817
17	70356	61212	60121	60598	57592	56771

19	70145	63490	62409	62862	60439	59089
2	62776	50240	48948	49328	46823	45766
20	71330	64336	63187	63739	60209	58467
21	64745	55208	54099	54629	51566	50685
22	68774	62178	60804	61444	57766	54768
23	60410	51123	50070	50432	47989	46714
24	57692	53031	52064	52523	49968	48578
25	64986	55776	54619	55112	52793	50678
26	65860	58001	56923	57422	54532	51721
27	63448	57211	56018	56539	54982	54523
28	71699	64079	62917	63363	60745	58448
29	76462	68512	67572	67931	65255	62802
3	57376	49589	48294	48927	46566	45415
30	62272	55350	54196	54756	52101	50696
31	41433	38958	38239	38631	36844	35699
32	62214	57298	56330	56842	54510	52783
33	70277	64257	62946	63561	60628	59510
34	64152	57583	56445	56982	54147	52590
35	79577	70073	68689	69379	66272	64134
36	75681	67776	66735	67132	64372	61421
37	66783	59357	58458	58724	55520	52612
38	65184	59508	58369	58938	56017	54029
39	80547	72708	71583	72006	69363	68347
4	74356	62280	60853	61332	58943	57708
40	78793	70851	69569	70066	67179	66034
41	72938	65194	64247	64609	62320	61316
42	61677	56720	55894	56333	54091	52312
43	75874	68725	67722	68197	65822	62555
44	69029	61832	60920	61464	59408	58045
5	71892	63332	61870	62715	59275	55676
6	50406	44520	43410	43925	41130	40121
7	67168	60526	59337	59886	57206	55764
9	57000	50624	49313	49936	46869	46023

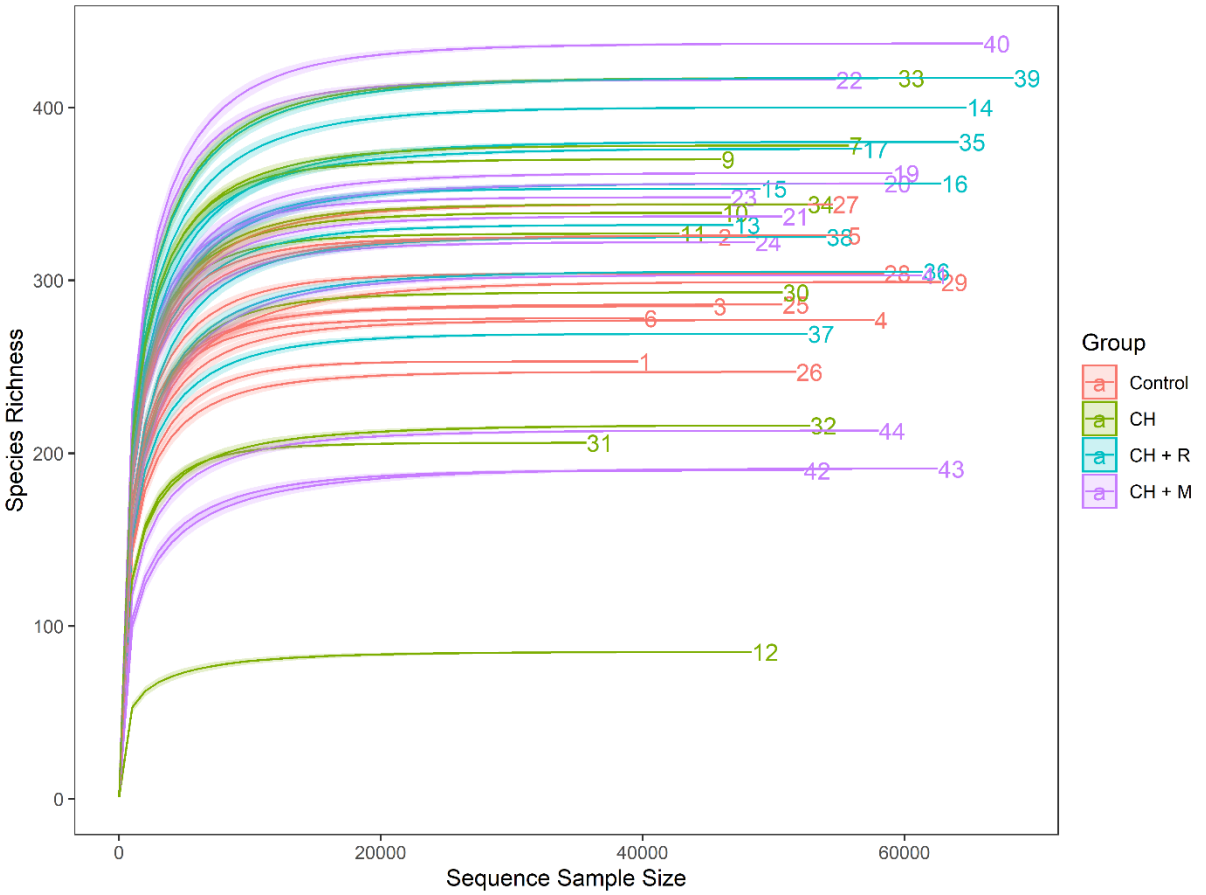

Figure S1 - Rarefaction curve to sanity check for the sequencing procedure.
Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.

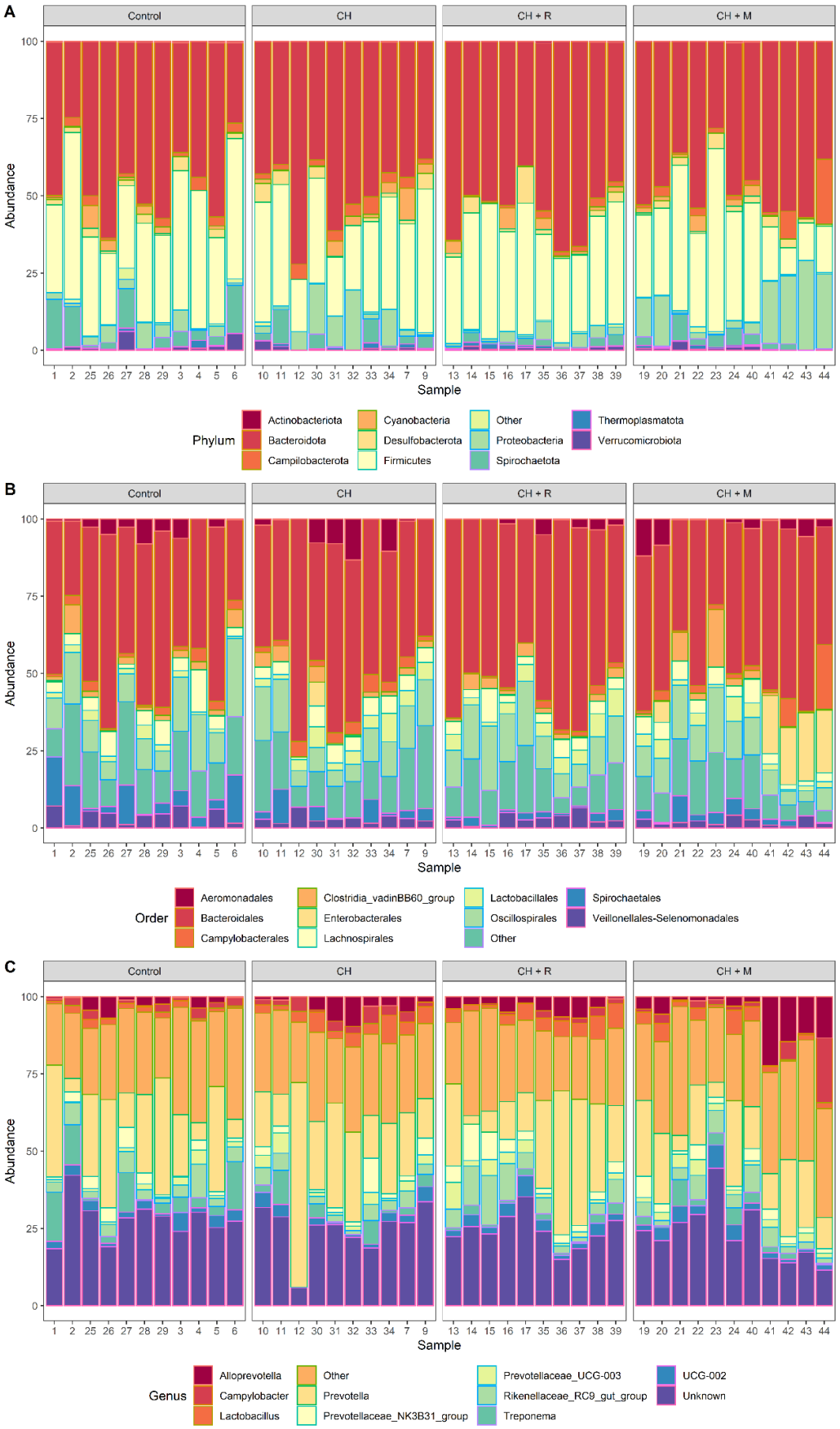

Figure S2 - Bar plots of relative abundance for Phylum (a), Family (b) and Genus (c) level. Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.

SUPPLEMENTARY MATERIAL 2

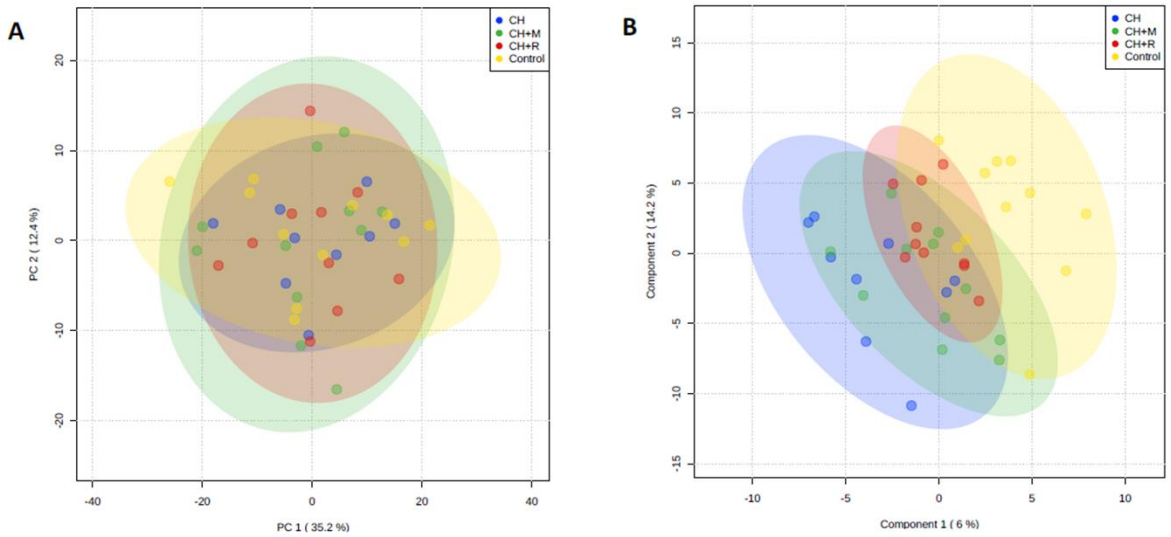

Figure S1 - Principal Component Analysis (PCA) scores plot (A) and Partial Least Squares (PLS) scores plot (Q^2 < 0, 1000 permutations; *p* = 0.665) (B) computed with the bin values of NMR spectra of all liver aqueous fractions.

Dietary treatments: Control - control diet; CH - *C. vulgaris* diet; CH+R - *C. vulgaris* diet supplemented with Rovabio® Excel AP; CH+M - *C. vulgaris* diet supplemented with a pre-selected enzymatic mixture.