UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

IMPROVING THE NUTRITIONAL VALUE OF MICROALGAE FOR FEEDING PIGS THROUGH THE USE OF NOVEL ENZYMES

DIOGO FRANCISCO MAURÍCIO COELHO

Orientadores: Professor Doutor José António Mestre Prates

Professor Doutor Carlos Mendes Godinho Andrade Fontes

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade Ciências Biológicas e Biomédicas

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Aos meus pais e irmã,

À minha família

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RESUMO

Melhoramento do valor nutricional das microalgas na alimentação de suínos através do uso de novas enzimas

Prevê-se que a suinicultura irá enfretar em breve novos desafios, como consequência do aumento da procura por carne de porco, devido ao impacto ambiental negativo que esta acarreta e ao baixo teor dos benéficos ácidos gordos polinsaturados (AGPI) *n*-3 na carne. As microalgas apresentam uma composição nutricional equilibrada, inclusive em AGPI *n*-3, e a sua produção tem baixo impacto ambiental. Assim, as microalgas podem ser uma alternativa adequada aos alimentos correntemente utilizados na suinicultura. *Arthrospira platensis* e *Chlorella vulgaris* são as duas microalgas mais estudadas e com maior expressão comercial. Contudo, estas apresentam paredes celulares recalcitrantes, diminuindo a biodisponibilidade dos seus nutrientes para os suínos. Portanto, na primeira parte deste estudo, desenvolvemos uma mistura de duas- "Carbohydrate-Active Enzymes" (CAZymes) e outra de quatro-CAZymes com capacidade de romper as paredes celulares da *A. platensis* e *C. vulgaris*, respetivamente. Este processo foi realizado através de uma metodologia de alto rendimento, onde as misturas enzimáticas foram selecionadas a partir de uma biblioteca de 178 CAZymes e 22 sulfatases, de acordo com sua aptidão para romper a parede celular das microalgas, avaliada através da libertação de açúcares da parede celular, diminuição da intensidade de fluorescência e libertação de compostos das microalgas. Verificamos que estas misturas de CAZYmes têm capacidade de romper as paredes celulares, podendo integrar uma metodologia para melhorar a biodisponibilidade dos nutrientes de microalgas em dietas de suínos. Na segunda parte deste estudo avaliamos pela primeira vez o efeito de um alto nível de incorporação na dieta de *C. vulgaris* (5%) suplementada ou não com duas misturas de CAZymes, a mistura de quatro CAZYmes desenvolvida na primeira parte e a mistura comercial Rovabio® Excel AP, na performance produtiva, qualidade e composição da carne, estado de saúde e composição do fígado de suínos em acabamento. Apuramos que as dietas com *C. vulgaris* não afetaram a performance produtiva, os parâmetros de qualidade da carne e a sua estabilidade oxidativa, mas promoveram um aumento no teor de carotenoides da carne e no teor de AGPI *n*-3 da carne e fígado. A mistura de quatro CAZymes foi fundamental para a diminuição da lipemia sanguínea. As dietas com *C. vulgaris* também promoveram a diminuição do conteúdo de imunoglobulinas plasmáticas. Conclui-se que a incorporação de um alto nível de *C. vulgaris* melhora o valor nutricional da gordura sem comprometer o desempenho produtivo e o estado de saúde dos suínos, excetuando o efeito imunossupressor promovido pela microalga, que necessita de mais investigação.

Palavras–chave: *Arthrospira platensis*; *Chlorella vulgaris*; "Carbohydrate-Active Enzymes"; porcos em acabamento, ácidos gordos polinsaturados *n*-3

ABSTRACT

Improving the nutritional value of microalgae for feeding pigs through the use of novel enzymes

The pig industry will face new challenges due to the increase demand for pork, concerning the negative environmental impact and the low content in the beneficial *n*-3 polyunsaturated fatty acids (PUFA) of pork. Microalgae exhibit a well-balanced nutritional composition, including in *n*-3 PUFA, and its production has a low environmental impact. Thus, microalgae could be a suitable alternative to traditional feedstuffs of pig industry. *Arthrospira platensis* and *Chlorella vulgaris* are the two most studied microalgae, also with the highest commercial expression. However, these microalgae are endowed by recalcitrant cell walls, which impairs the bioavailability of their compounds for pigs. In line with this, in the first part of this study, we developed a two-Carbohydrate-Active Enzyme (CAZYme) and a four-CAZYme mixtures with the ability to disrupt *A. platensis* and *C. vulgaris* cell walls, respectively. This process was performed via a high-throughput (HTP) approach, where the enzyme mixtures were selected from a 178 CAZymes and 22 sulfatases library, according to its ability to disrupt the microalgae cell wall, which was evaluated through the release of cell wall sugars, decrease of fluorescence intensity and the release of several microalgae compounds. We verified that this two CAZYme formulations are able to degrade these cell walls and may constitute a good approach to improve the bioavailability of these microalgae nutrients for pig diets. In the second part of this study, we assessed for the first time the effect of a high dietary incorporation level of the selected microalga (5% of *C. vulgaris*) supplemented or not with the respetive CAZYme mixture, the four-CAZYme mixture developed in the first part, and the commercially available Rovabio® Excel AP, on productive performance, meat quality and composition, health status and liver composition of finishing pigs. We observed that *C. vulgaris* diets had no effect on productive performance, meat quality traits and pork oxidative stability, but promoted an increase in pork carotenoids content and in *n*-3 PUFA composition of pork and liver. The action of the four-CAZYme mixture was preponderant for the decrease of blood lipemia. The *C. vulgaris* diets also promoted the decrease of plasma immunoglobulins content. It is concluded that the use of *C. vulgaris* in finishing pig diets, at this high incorporation level, improves the nutritional value of pork fat without compromising pig performance and health status of pigs, except the immunosuppressive effect promoted by the microalga, which deserves further investigation.

Key–words: *Arthrospira platensis*; *Chlorella vulgaris*; Carbohydrate-Active Enzymes; finishing pigs, *n*-3 polyunsaturated fatty acids

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS AND SYMBOLS

®	Registered Trademark
µE m^{-2} s^{-1}	Microeinsteins per second per square meter
µg	Microgram
µL	Microliter
a*	Redness (colour dimension)
AA	Auxiliary Activities
ADF	Acid Detergent Fibre
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
AID	Apparent Ileal Digestibility
ALA	α-Linolenic Acid (18:3*n*-3)
AMSA	American Meat Science Association
ANOVA	Analysis of Variance
ANSES	Agence Nationale de Sécurité Sanitaire de L'alimentation, de L'environnement et du Travail
AOAC	Association of Official Analytical Chemists
ATP	Adenosine Triphosphate
ATTD	Apparent Total Tract Digestibility
b*	Yellowness (colour dimension)
BHT	Butylated Hydroxytoluene
bp	Base Pairs
BW	Body Weight
CAZymes	Carbohydrate-Active Enzymes
CBM	Carbohydrate-Binding Modules
CE	Carbohydrate Esterases
CIE	Commission Internationale de l'Éclairage
CIISA	Centro de Investigação Interdisciplinar em Sanidade Animal
cm	Centimetre
d	Day
DAD	Diode array detector
DHA	Docosahexaenoic acid (22:6*n-3*)
DM	Dry Matter
DNA	Deoxyribonucleic acid
DNSA	3,5-Dinitrosalicylic Acid
DPA	Docosapentaenoic acid (22:5*n-3*)

E.C	Enzyme Commission
e.g	*Exempli Gratia*
EC	European Commission
EPA	Eicosapentaenoic acid (20:5*n-3*)
EU	European Union
FA	Fatty acids
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FID	Flame ionization detector
g	G force (acceleration)
g	Gram
G:F	Gain:feed ratio
GC	Gas Chromatography
GH	Glycoside Hydrolases
GLM	Generalized Linear Mixed
GT	GlycosylTransferases
h	Hour
HCl	Hydrochloric Acid
HCW	Hot Carcass Weight
HDL	High Density Lipoproteins
His	Histidine
HOMA-IR	Homeostasis Model Assessment using the Insulin Resistance Index
hPa	Hecto Pascals
HPLC	High Performance Liquid Chromatography
HTP	High-Throughput
i.d.	Inner diameter
IMAC	Immobilised Affinity Chromatography
IMF	Intramuscular fat
INE	Instituto Nacional de Estatística
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
INPI	Instituto Nacional da Propriedade Industrial
IPMA	Instituto Português do Mar e da Atmosfera
kcal	Kilocalorie
Kg	Kilogram
L*	Lightness (colour dimension)
LA	Linoleic Acid (18:2*n*-6)
LCPUFA	Long-Chain Polyunsaturated Fatty Acids

LDL	Low Density Lipoproteins
LSMEANS	Least Squares Means
m	Metre
M	Molar
MDA	Malonaldehyde
ME	Metabolizable Energy
mg	Milligram
min	Minute
mL	Millilitre
Mm	Milimolar
mm	Millimetre
mU	Miliunits of activity
MUFA	Monounsaturated Fatty Acids
n-3	Omega 3
n-6	Omega 6
n-6:n-3	Total omega 3 fatty acids: total omega 6 fatty acids ratio
NDF	Neutral Detergent Fibre
nM	Nanomolar
nm	Nanometre
ºC	Degree Celsius
p	Probability
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
pH	Negative decimal logarithm of the hydrogen ion activity in a solution
pHu	*Post-mortem* ultimate pH
PL	Polysaccharide Lyases
PUFA	Polyunsaturated Fatty Acids
PUFA:SFA	Polyunsaturated fatty acid: saturated fatty acid ratio
rpm	Rotation Per Minute
s	Second
SAS	Statistical Analysis System
SEM	Standard Error of the Mean
SDS–PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SFA	Saturated Fatty Acids
TAG	Triacylglycerols
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic acid

UIPA	Unidade de Investigação em Produção Animal
USDA	United States Department of Agriculture
UV	Ultraviolet
v/v	Volume per volume
VLDL	Very Low-Density Lipoproteins
WBSF	Warner-Bratzler shear force
WHO	World Health Organization
λ	Wavelength

Chapter 1 – INTRODUCTION

Pork is the most preferred meat in the EU and accounts for about 50% of the total meat supply. In fact, the EU is among the top pork producers in the world, coming on the 2nd position after China and being followed by the USA. Moreover, the EU is the largest pork exporter worldwide. The EU enlargement in 2004, 2007 and 2013 has favoured the increase of the number of pigs and farms, production in pig live weight, and in carcass weight equivalent, promoted the extension of free internal market among the 28 member states and also stimulated exports (Popescu 2020). Producing pork requires various resources: the animals themselves, housing facilities, feed, farming machinery, trained farmers and animal caretakers, slaughter facilities, transportation networks, and energy (Zira et al. 2020). To maintain the economic viability, implement innovation and promote the production of high-quality products, the swine industry has to focus on key points such as: providing appropriate facilities, genetics, health programs to the pig and optimal nutrition (Moeller and Crespo 2009). Regarding to pig nutrition, the nutritional requirements and feeding programs in use have been revised over the years in order to maximise the feed efficiency while minimizing production costs (Pomar and Remus 2019). Actually, due to the revolutionary progresses of basic nutrition research, novel methods and techniques have been developed which bring a profound technological revolution to pig production (Wu et al. 2020). Conventionally, mathematical models are used to estimate nutrient requirements for pigs fed in large groups that receive the same feed for extended periods throughout their production cycle (NRC 2012; Pomar and Remus 2019). In the pig production industry, the raw materials used to produce the feedstuffs, ensuring the adequate supply of nutrients to the pigs, are corn and wheat as the main fed energy sources and soybean meal as the main fed protein source (Woyengo et al. 2014).

The high rate of pig production ensured by the swine industry is necessary to suppress the consumer demand, since pork is the most consumed terrestrial animal meat product in Europe as well as globally (Zira et al. 2020). The consumer demand by pork is related to the fact that this meat is generally less expensive and more affordable than beef and sometimes serves as an alternative to beef among red meat consumers (An et al. 2020). Despite pork ranks first among the most consumed meats, pork's image among consumers is not univocally positive since they perceived it as the least healthy and fattest meat compared to poultry (Verbeke et al. 2011). The unhealthy connotation of pork is also due to the unfavourable fatty acid profile, with lower proportions of polyunsaturated fatty acids (PUFA) and lipid-soluble antioxidant vitamins, and higher percentages of saturated fatty acids (SFA) (Morgan et al. 1992; Dugan et al. 2015).

Although the high consumption rate of pork, it is expected the double of the overall demand for this meat by 2050 driven via the rise in global population, the increase in income as well as the dietary transition towards more animal protein *per capita*. Consequently, it is expected that pig production will increase which will bring new challenges to the pork industry (Madeira et al. 2017; Lassaletta et al. 2019). Thus, this challenge will be at the level of feeding sustainability and the need to improve the nutritional value of pork. The increase in livestock production will result in the need to intensify the production of feeding raw materials, like corn and soybean food crops which will have a negative pressure in our planet due to arable land degradation, water deprivation, drastic climate changes, direct competition with human food and the unbalance among food, feed and biofuel industries (Madeira et al. 2017; Rauw et al. 2020). Moreover, a large part of the human population does not consume the recommended levels of *n*-3 PUFA by World Health Organization (WHO), which are important in human health maintenance. The enrichment of pork in *n*-3 PUFA it will not only be important in improving the nutritional value of pork to meet the requirements of the most demanding consumers, but it will act as a vehicle to promote the consume of the recommended values of *n*-3 PUFA by the human population due to the increase of pork consumption (Dugan et al. 2015; Ma et al. 2016). However, it is well established that pig diet provides an effective approach for altering the fat composition of pork (Wood and Enser 1997). Therefore, it is imperative to find good sustainable alternatives to conventional feedstuffs with good proportions of *n*-3 PUFA (Florou-Paneri et al. 2014; Ma et al. 2016).

Microalgae, an important aquatic resource, could be an appropriate alternative to conventional feedstuffs for monogastrics (Madeira et al. 2017). Microalgae production has a minimal environmental impact, with a high efficiency of carbon dioxide mitigation, possibility of cultivation in non-arable land and with the use of non-potable water (Gouveia et al. 2010). Furthermore, microalgae presents a well-balanced chemical composition in proteins, with essential amino acids, polysaccharides, monounsaturated and polyunsaturated fatty acids, including the beneficial *n*-3 PUFA, minerals, pigments and fibre, which are comparable, if not superior, to conventional feedstuffs (Becker 2007; Matos et al. 2017).

Arthrospira platensis is a filamentous prokaryote microalga, classified as a blue–green alga (*Cyanophyceae*, also known as cyanobacteria) (Seyidoglu et al. 2017). The cellular organization of *A. platensis* is typical of a prokaryote Gram-negative bacterium, lacking membrane-bound organelles (Sotiroudis and Sotiroudis 2013). *A. platensis* displays the highest protein content of any natural food (55% – 70%) and a good proportion of fatty acids, polysaccharides, fatty acids and carotenoids which makes this microalga attractive to different applications such as in food, feed and pharmaceutical and cosmetic industry (Holman and Malau-Aduli 2013). *Chlorella vulgaris* is a freshwater unicellular eukaryotic green microalga. *C. vulgaris* is known for its high biomass productivity, relative ease of cultivation and a high-

quality nutritional composition, what makes one of the most cultivated microalgae and an attractive alternative for monogastric diets (Liu and Chen 2014; Kotrbáček et al. 2015). In particular, *C. vulgaris* exhibits an interesting content in some *n*-6 PUFA (18:2*n*-6 and 18:3*n*-6) and in the *n*-3 PUFA α-linolenic acid (18:3*n*-3) (Batista et al. 2013). *A. platensis* and *C. vulgaris* has gained significant attention in feed industry due to the ease of culturing in a short time period, high biomass rate production, and high content of the protein/amino acid, lipid/PUFA/sterol and pigments (Raji et al. 2020).

Seeing that microalgae present a well-balanced nutritional composition, with good proportions of key nutrients, such as fatty acids, proteins and carbohydrates and its production has low environmental impact, its use as a micro- or bulk ingredient in formulated animal feeds has been proposed in several studies, including *A. platensis* and *C. vulgaris* (Shields and Lupatsch 2012). It has been found that feed enriched, with supplementation amounts (1% or lower in diet), of microalgal biomass improves the production performance of pigs and further promoted an amelioration of *n*-3 PUFA content in meat and an increase of its quality, in terms of flavour, colour or texture, which has favourably contributed to consumer acceptance (Madeira et al. 2017; Camacho et al. 2019). Furthermore, these studies demonstrated that the incorporation of these microalgae in feeds had a positive effect on animal physiology, by controlling lipemia and glycaemia, improving their immune response, disease resistance, and gut function, besides enhancing antiviral and antibacterial protection, as well as increasing reproductive performance (Lee et al. 2008; Abdelnour et al. 2019; Camacho et al. 2019).

However, the biggest drawback on the use of microalgae as a feed ingredient (>1% in diet) is its recalcitrant cell wall, which makes microalgae poorly digestible by monogastric animals and, consequently, decreases the bioavailability of the microalga valuable nutrients and its absorption rate by animals. Along these lines, there is the impossibility of scale-up the incorporation level in diets (Williams and Laurens 2010; Milledge 2011; Amorim et al. 2021). Despite the molecular structure and composition of *C. vulgaris* cell wall are still unwell defined, it is known that this varies according to growth stage and environmental conditions. Its rigidity increases according to the degree of the cell maturation and is conferred by a an incredibly diverse and complex matrix of cross-linked insoluble carbohydrates (Safi, Zebib, et al. 2014; Baudelet et al. 2017). Although *A. platensis* present a less rigid cell wall when compared to *C. vulgaris* (Williams and Laurens 2010), this species cell wall is constituted by an envelope composed by several layers, mostly of peptidoglycan and lipopolysaccharide nature (Van Eykelenburg et al. 1980; Sotiroudis and Sotiroudis 2013).

The use of feed enzymes is a proposed strategy to overcome the problem of microalgae cell wall recalcitrance (Alhattab et al. 2019; Ahmad et al. 2020). In the field of feed enzymes, are highlighted the Carbohydrate-Active enzymes (CAZymes). CAZymes are able to act on a vast range of glycosidic monomers, oligomers or polymers in a regio-specific or

stereo-specific manner (André et al. 2014). Several *in vitro* studies identified different CAZymes as potentials in the cell wall degradation of the two species of microalgae under study, *A. platensis* and *C. vulgaris* (Zheng et al. 2011; Aikawa et al. 2013; Cho et al. 2013; Gerken et al. 2013). Thus, CAZymes can be a sustainable mild cell wall disruption technique, acting in a specific manner on cell wall compounds under mild temperature and pH conditions and with a low energy impute (Vanthoor-Koopmans et al. 2013; Phong et al. 2018). Amongst biotechnological additives, feed enzymes, including CAZymes, for monogastrics have made the most progress and impact in the past decade (Kiarie et al. 2013). Therefore, exogenous CAZymes, mainly xylanases and beta-glucanases, are now widely used to supplement diets of monogastric livestock species to improve nutrient digestibly and directly impact on animal performance and health (Ravindran and Son 2012). As discussed in the meta-analysis study performed by Torres-Pitarch et al. (2019), the use of CAZymes is currently a cost-effective strategy to improve the nutritional value of cereal-based diets for finishing pigs, although it remains to be established for microalgae biomass.

Overall, this work aims to explore, for the first time, the high incorporation level of microalgae on finishing pig diets as a sustainable alternative to traditional feedstuffs and a mechanism to improve the *n*-3 PUFA content of pork, without impairing the production performance and the meat quality traits. The effect of a high incorporation level of the microalgae on finishing pigs' blood parameters and liver lipid content was also analysed, since it remains unknown. In order to solve the problem of low digestibility of microalgae, this work also aimed to identify two CAZymes mixtures with the ability to degrade the cell wall of *A. platensis* and *C. vulgaris*, respectively. The mixture for *C. vulgaris* was used *a posteriori* in the respective *in vivo* trial.

This Thesis is divided in six chapters. The chapter 1 provides a general introduction. The chapter 2, entitled "Scientific background and objectives", revises our current knowledge on pig industry, namely the current and future trends of pig production and consumption market, pig nutrition and pork quality, specially its fatty acid composition. Then, the new challenges of pig industry are reviewed. The microalgae, mainly the two species under study, *A. platensis* and *C. vulgaris* are characterized and the introduction of microalgae on pig nutrition as well as the biggest drawback of its use and the supplementation with CAZymes to overcome this problem are also explored. At the end of this chapter, the objectives of this work are described. Chapters 3, 4, 5 and 6 are organized in papers based on scientific manuscripts already published or submitted to publication in international peer reviewed journals. The chapters 3 and 4 describe the identification of two CAZyme mixtures with capacity to disrupt *A. platensis* and *C. vulgaris* cell walls, respectively. The effect of a high dietary incorporation level of *C. vulgaris* and the supplementation with the CAZyme mixture developed in chapter 4 on nutritional value of pork fat, meat quality traits and production performance are presented

in chapter 5. Chapter 6 explores the impact of *C. vulgaris* as feed ingredient and the supplementation with the CAZyme mixture developed in chapter 4 on the health status and hepatic fatty acids profile of finishing pigs. Finally, chapter 7 integrates the results obtained in each of the four previous chapters, providing an overall discussion, and chapter 8 presents the conclusions, implications and futures perspectives of this work.

Chapter 2 – SCIENTIFIC BACKGROUND AND OBJECTIVES

2.1. Pork Industry

2.1.1. Pig Production Worldwide

Since domestication around 4900 B.C., the pig was fundamental as a source of food for survival, but it has also important in cultural, religious, and social aspects of humans. Nowadays, the modern pig continues to play a major role in providing food for human consumption (Figure 2.1 A). The pig industry continues to thrive in areas of the world where access to grains and protein sources are plentiful. Pigs are adaptable to various climatic conditions as evidenced by the large number of breeds present throughout the world. The swine industries of the world are very dynamic and continue to evolve and change along with the external forces that shape agriculture world-wide (Moeller and Crespo 2009).

Analysing pig production globally (Figure 2.1 B), according to official statistics, the main swine producer worldwide is China. It accounts about 50% of the world's world production of pig and this higher pig production rate recorded in China was mainly due to an increase in the domestic demand for pork. Moreover, a significant aspect worth highlighting is that in China the swine sector is stimulated by government subsidies (Soare and Chiurciu 2017). Followed by China comes the EU, responsible for the production of about 20% of swine worldwide, and the United States closes the list of the three largest pork producers worldwide with 10% of swine production worldwide (Szűcs and Vida 2017; Eurostat 2020a). It should be noted that this is due to the fact that the combined effect of higher output prices and increased production costs tend to favour production in developing countries, where low input production systems prevail (Szűcs and Vida 2017). Although there are fluctuations in the pork production market over the years, the trend for the future is that there will be a growth in production worldwide (Soare and Chiurciu 2017; Szűcs and Vida 2017).

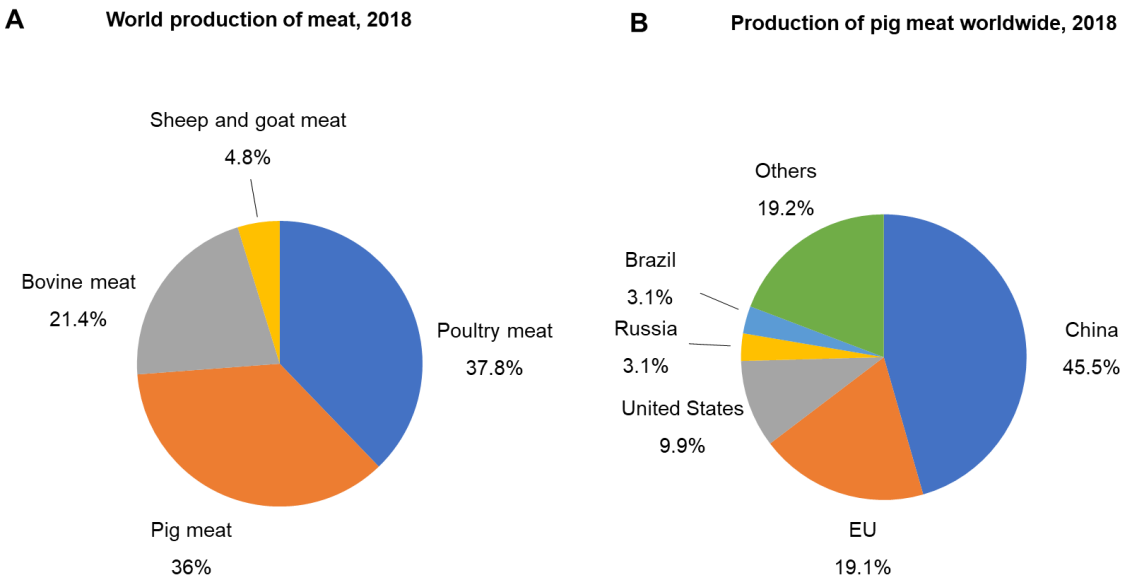

Figure 2.1. A. The world production of meat in 2018. B. The production of pig meat worldwide in 2018. Adapted from Eurostat (2020a).

In the EU, pork is the most widely produced type of meat and accounts for about 50% of the total meat supply (Figure 2.2 A) (González-García et al. 2015; Popescu 2020). The EU enlargement in 2004, 2007 and 2013 was crucial for pig industry since has favoured the increase of the number of pigs and farms, production in pig live weight, and in carcass weight equivalent, promoted the extension of free internal market among the 28-member states and also stimulated exports. At present, the EU is the largest pork exporter worldwide (Popescu 2020). In 2018, the EU presented 148.2 million pigs. The top 10 pig growing countries in the EU are Germany, Spain, France, Poland, Netherlands, Denmark, Italy, Belgium, Romania and Austria, whose pig population represents 86% of the EU swine livestock, and as a consequence these countries have influenced the dynamics of the pig livestock, pork production and trade (Figure 2.2 B) (Popescu 2020). The top pork exporting countries in the EU are: Germany 22.6%, Spain 22.1%, Denmark 14.9% and Netherlands 12.2% and the top pork importing EU countries were Germany 53.1%, Denmark 6.45 and Netherlands 5.9% Analysing the pork market between the years 2007 and 2018 in EU, was observed an increase of pork production by 3.8% from 22,972 thousand tonnes in 2007 to 23,846 thousand tonnes in 2018, an increase of pork exportation by 93.91% and a decrease of pork importations by 30.8% (Popescu 2020). In 2019, the EU produced 43.5 million tonnes of meat, about half of which came from pigs (22.8 million tonnes), representing a slight decline of 0.7% from the previous year. Even so, the pig production in 2019 remained 1.2 million tonnes higher than in 2010 (Eurostat 2020b).

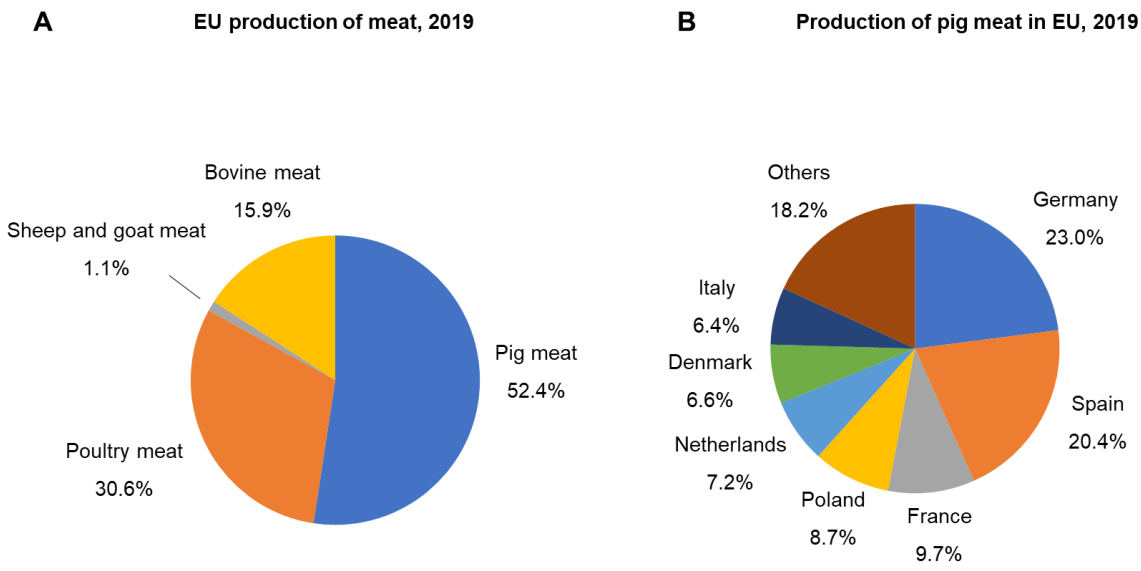

Figure 2.2. A. The EU production of meat in 2019. B. The production of pig meat in EU top producers member states in 2019. Adapted from Eurostat (2020b).

In 2018, Portugal produced 892 thousand tons of meat, being pork the most produced meat (383 thousand tons), which represents about 43% of the total meat production (INE 2019). In 2019, the production of pork was of 366 thousand tons and continues to be the most produced meat in Portugal, representing about 45% of the total national meat production and 1.6% of the total pork production in EU (Eurostat 2020b).

To meet these production levels, the pig production industry requires various resources: the animals themselves, housing facilities, feed, farming machinery, trained farmers and animal caretakers, slaughter facilities, transportation networks, and energy (Zira et al. 2020). In addition, European pig production industries currently face numerous challenges such as globalization, emerging markets, changing consumer requirements, and new governmental regulations related to issues such as environmental pollution and food safety. These challenges require continuous innovation of supply chain network structures, reconsideration of business processes, relocation of logistics infrastructures and renewed allocation of chain activities to these infrastructures in order to achieve sustainable performances (Aramyan et al. 2011).

To maintain the economic viability, implement innovation and promote the production of high-quality products, the swine industry has to focus on key points such as: providing appropriate facilities, genetics, health programs to the pig and optimal nutrition (Moeller and Crespo 2009).

2.1.2. Pig Nutrition

Pigs are omnivore animals with simple, single-chambered stomach, also known as monogastrics, where most of digestion is carried out through endogenous enzymes. Consequently, pigs require high quality feed, with nutrients readily available to the digestive enzymes (Moeller and Crespo 2009; Vukmirović et al. 2017).

The major goal of the feeding in pig production is to produce piglets from breeding animals and subsequently meat from those pigs, with a maximum efficiency and profitability. Feed is the costliest component in the pig industry, representing typically about 60 to 70% of the production costs. Therefore, the nutritional requirements and feeding programs in use have been revised over the years in order to maximize the feed efficiency while minimizing production costs and environmental impact (Dubeau et al. 2011; Van Milgen et al. 2012; Pomar and Remus 2019). The correct formulation and rationing of diets for different phases of growth is critical in order to obtain good results. In line with this, the revolutionary progresses of basic nutrition provided the development of novel methods and techniques, bringing a profound technological revolution to pig production (Wu et al. 2020). Conventionally, mathematical models are used to estimate nutrient requirements for pigs fed in large groups that receive the same feed for extended periods throughout their production cycle (NRC 2012; Pomar and Remus 2019). However, with the recent technological advances, improved knowledge in pig nutrition and productive performance, and based on conventional mathematical models, was possible to develop modelling software tools to access directly to nutritional requirements of pigs (Brossard et al. 2019).

In the formulation of diets for growing pigs is important to ensure that the diet meets the nutritional and energy requirements of animals without excess of nutrients, which would not be cost-effective and would have a high negative environmental impact due to increased excretion of nitrogen, phosphorus and other minerals (Dubeau et al. 2011).

Energy is required for all biological processes in pigs, such as thermoregulation, physical activity, growth, pregnancy, lactation and developing boars and gilts. Moreover, energy is essential for the production of animal products through involvement of adenosine triphosphate (ATP) on the physiological process of synthesis and degradation of body protein, determining protein deposition and lean growth (Van Milgen et al. 2012; Kil et al. 2013). Consequently, an adequate supply of energy in addition to the supply of nutrients is a prerequisite for optimal pig production (Kil et al. 2013). The requirements of energy depend on the growth phase, sex, genotype, physiological state environment and feed strategy (Noblet et al. 1999; Dubeau et al. 2011). The majority of the diet costs are associated with provide the energy requirements of pigs. The production of energy occurs when macronutrients like carbohydrates, lipids and proteins in feeds are metabolised by oxidative processes in body.

The primary energy source is starch present in cereal grains, yielding up its energy after enzymatic digestion in the small intestine, and absorption in the form of glucose (Kyriazakis and Whittemore 2006; Noblet and Van Milgen 2013).

The carbohydrate fraction of pig diets represents a diverse group of compounds, classified according to their degree of polymerization into simple mono- and disaccharides, also known as sugars, to complex organized polysaccharides; the latter consist of starches with different degrees of resistance to completely digestible and non-starch polysaccharides. These molecules make up the vegetable cell walls (Knudsen et al. 2016). Carbohydrates are the single most abundant feed energy in diets for piglets, growing pigs, and sows, making up 60 to 70% of the total energy intake (Knudsen et al. 2012; Knudsen et al. 2016). This energy comes mainly from the digestion of starch and sugars to monosaccharides, such as glucose, in the small intestine which are absorbed and metabolized (Knudsen et al. 2012).

Protein is one of the major components of the pig's body. In terms of dry matter, protein makes up the largest percentage of the pig's body at birth (Kim 2018). Protein, and more specifically its monomers, the amino acids, have major functions on pig metabolism. They perform a central role on build protein in the body, mostly in muscle and to replace protein lost during the process of protein tissue turnover (Kyriazakis and Whittemore 2006). In addition, amino acids are important regulators of key metabolic pathways that are necessary for maintenance, growth, reproduction and immunity in animals (Wu 2013). Pigs also can use dietary amino acids as a source of energy, carbon and hydrogen, to support various body functions or to synthesise body lipid. The feedstuffs for pigs contain approximately twenty amino acids, of which nine are considered essentials. The essential amino acids are lysine, methionine, threonine, tryptophan, histidine, isoleucine, leucine, phenylalanine and valine. They are considered essentials since cannot be synthesised by pigs but are necessary for different physiological functions, and therefore it is mandatory to supply in the pig's diet (Kyriazakis and Whittemore 2006). The biosynthetic pathway of the nonessential amino acids has as precursors carbon skeletons that are synthesized *de novo* by animal cells. However, several studies demonstrated that there has been no compelling evidence for sufficient synthesis of nutritionally nonessential amino acids in animals (Wu 2013). Thus, it is imperative to provide the "ideal dietary protein" quantity to ensure the adequate protein metabolism and the correct balance of amino acids for the various purposes of pig maintenance and production (Whittemore et al. 2001; Kyriazakis and Whittemore 2006).

The lipid fraction of pig diets is comprised of a diverse group of compounds with large differences in chemical structure but a common character of being insoluble in water and soluble in nonpolar reagents. Lipids can be further grouped into two categories: simple lipids and complex lipids. The simple lipids include fatty acids (FA), triacylglycerols (TAG), steroids, prostaglandins, and waxes, and the complex lipids include phosphoglycerides, glycolipids, and

fat-soluble vitamins. Lipids (*e.g.* FA and TG) are the primary form of metabolic energy storage and transport in the animal body. Subcutaneous lipid depots protect the animal from heat loss and mechanical damage. As a component of cell membranes and biologically active materials such as vitamins and hormones, lipids are involved in cell metabolism, cell recognition, and cell immunity. The dietary lipids, usually referred to as dietary fat, are primarily composed of TAG and FA. Due to the innumerable biological processes of the pig where lipids are involved, it is necessary to fulfil the requirements and ensure the adequate and complete supply of lipids to the diet (Lin et al. 2012). The major sources of lipids in pig diets are fats from feedstuffs and oils (Lin et al. 2012; Shurson et al. 2015).

2.1.2.1. Raw Materials in Pig Production

Currently, the raw materials used to produce the feedstuffs for pig production industry are corn, wheat and their by-products as well as oil crop, such as soybean meal and soybean oil (Chen et al. 2019). These raw materials have to provide the adequate supply of the nutrients referred in the previous section to the pig feed (Woyengo et al. 2014).

Cereal grains, such as corn and wheat, are the major sources of carbohydrates on pig diets. The carbohydrates in the feeds are not present as pure chemical entities but as a mix of sugars, oligosaccharides, and polysaccharides, the latter predominantly linked to other biopolymers such as proteins and lignin. Corn presents in its nutritional composition about 680 g/kg dry matter (DM) of starch and 108 g/kg DM of fibre. Wheat presents 647 g/kg DM of starch and 138 g/kg DM of fibre (Knudsen et al. 2012). As mentioned above, starch is directly involved in the metabolic process of energy production by the pig and the dietary fibre, if supplied in the ideal amount and in the correct growth stage, can have a positive impact on the digestive tract and intestinal health of pigs (Noblet and Van Milgen 2013; Jarrett and Ashworth 2018). Thus, corn is the most fundamental feed ingredient for it provides high energy input (Yun et al. 2018).

In 2001, the use of meat and bone meal and its by-products in diets for livestock was banned by the European Commission (EC) (EC Directive 999/2001) to assure consumer safety on animal products. Therefore, soybean meal is the most commonly used protein supplement of plant origin in pig diets, and is generally known as protein source with a high and consistent product quality (Jezierny et al. 2010). The nutritional composition of soybean meal contains 541 g/kg DM of crude protein (CP) and further presents a complete profile of amino acids with good amount of essential and non-essential amino acids (Jezierny et al. 2010). Moreover, soybean meal presents a relatively low crude fibre value which facilitates its digestibility by the pig, increasing the bioavailability of its protein (Yun et al. 2018).

The major sources of lipids in pig diets are plant oils, such as soybean oil. The lipids in the diet can also be a component of the basal ingredients. For example, corn typically contains

3.5–4.0% of lipids (Lin et al. 2012). However, soybean has a higher amount of lipids, of about 21%, which makes soybean oil an ideal source of lipids to suppress the nutritional requirements of pigs (Perkins 1995; Lin et al. 2012). Plant materials fed to pigs, whether grain, oilseed meal, or plant oil, have higher amounts of TAG and the predominant FA in its composition have eighteen-carbon chain length and 1–3 double bonds (Perkins 1995; Lin et al. 2012). The soybean oil is comprised largely of C_{18} PUFAs, containing approximately 16% SFAs (primarily 16:0 and 18:0), 23% monounsaturated fatty acids (MUFAs; primarily18:1*n*-9) and 61% C_{18} PUFAs (primarily 18:2*n*-6) (Trushenski et al. 2013).

2.1.3. Pork Consumption Worldwide

Meat has exerted a crucial role in human evolution and is an important component of a healthy and well-balanced diet due to its nutritional richness. Although some epidemiological data has revealed a possible association between its consumption and increased risk of several forms of cancer, cardiovascular and metabolic diseases, meat continues to play a central role in the human diet (Pereira and Vicente 2013; Macho-González et al. 2020).

Globally, meat consumption is generally influenced by a number of factors, such as food consumption patterns, the standard of living, meat production and animal husbandry conditions and consumer prices (Soare and Chiurciu 2017).

Pork is the most consumed terrestrial meat product worldwide, in spite of an increase in poultry products (Pereira and Vicente 2013; Macho-González et al. 2020; Zira et al. 2020). Macho-González et al. (2020) in its revision analysed the data of meat consumption worldwide and by continent in 2017 (Table 2.1) and reported consumes of 15.7 kg/*per capita*/year of pork *versus* 15.18 kg/*per capita*/year of poultry in world. The global meat consumption in 2017 was: pork 37%; poultry 35.6%; beef 21%; others 6.4%. By continent, pork was the most consumed meat in Asia and Europe with 15.18 and 35.75 kg/*per capita*/year, respectively. In Africa, America and Oceania pork was the second most consumed meat, with poultry the most consumed meat in these continents (Macho-González et al. 2020). In terms of total amounts, the largest consumer of pig meat in 2017 was China (57 million tonnes), followed by EU member states (20.9 million tonnes) and USA (9.5 million tonnes) and are responsible for 74% of the world consumption of pork (Table 2.2) (Szűcs and Vida 2017). In 2018, these values remained practically constant (Table 2.2) (USDA 2021). The data show that pork consumption has increased over the past 30 years in these countries, especially in China (Szűcs and Vida 2017). The increase trend in the consumption of pork that has occurred is mainly due to the increase in population incomes and urbanization (Soare and Chiurciu 2017). However, data already available for 2019 and 2020 indicate a slight slowdown in pork consumption worldwide

(Table 2.2) to a one-off effect due to African swine fever, even so the growth trend is expected to recover (USDA 2021).

The consumption of pork in EU in 2019 was about 20 million tonnes, 0.9% less than in 2015 and 2.3% less than in 2018 (Popescu 2020). In 2020, the consumption decreased slightly to 19.6 million tonnes, minus 2% compared to the previous year (USDA 2021). In the specific case of the EU, the decline in pork consumption is caused by several reasons such as: nutritional considerations of people who will look for more plant-based proteins; the increased preference of the EU consumers for poultry meat which contains high value protein, less cholesterol and it is cheaper than pork; environmental considerations and the ageing of the population in the EU, which determines a lower consumption of food *per capita* (Popescu 2020). At present, the EU population's needs are covered by the internal production, the self-sufficiency rate being 110% (Popescu 2020). The countries from EU with the highest volumes of pork consumption in 2019 were Germany (4.5 Mtonnes), Spain (3 Mtonnes), and Poland (2.4 Mtonnes), with a combined 47% share of total consumption. The countries with the highest levels of pork *per capita* consumption in 2019 were Denmark (115 kg *per capita*), Spain (65 kg *per capita*) and Poland (62 kg *per capita*) (Popescu 2020).

In Portugal, the data for the year 2018 indicate that pork was the most consumed meat with consumes of 44.7 kg *per capita* followed by poultry with 42.8 kg *per capita* (INE 2019).

As reviewed above, pork consumption rates are high all over the world, with a particular incidence on countries like China and EU member states (Zira et al. 2020). In addition to socio-cultural reasons of each country, the consumer demand by pork is related to the fact that this meat is generally less expensive and more affordable than beef and sometimes serves as an alternative to beef among red meat consumers (An et al. 2020).

Pork consumption is closely related with pig production, since the actual pork demand requires high rates of pig production. Consequently, the pig production industry needs to have innovative and optimized production processes where nutrition plays a key role (Zira et al. 2020).

Table 2.1. Meat consumption worldwide and by continent in 2017. Adapted from Macho-González et al. (2020).

Type of meat	Meat Consumption (kg/per capita/year)					
	World	**Africa**	**America**	**Asia**	**Europe**	**Oceania**
Bovine	9.00	5.63	27.8	4.68	14.0	31.2
Mutton and goat	1.86	2.49	0.620	1.93	1.75	10.8
Pork	15.7	1.48	18.7	15.2	35.8	24.2
Poultry	15.2	6.21	42.0	9.71	24.6	44.0
Others	0.840	1.43	0.650	0.550	1.84	2.10
Total	42.6	17.2	89.7	32.1	77.9	112

Table 2.2. Pork consumption values by top pork consumption countries in the latest four years. Adapted from USDA (2021).

Country	Pork Consumption (million tonnes)			
	2017	**2018**	**2019**	**2020**
China	55.8	55.3	44.9	41.5
European Union	20.9	21.3	20.4	19.6
United States	9.54	9.75	10.1	10.0
Russia	3.30	3.20	3.36	3.47
Brazil	2.95	3.04	3.12	2.95
Vietnam	2.74	2.87	2.49	2.69
Japan	2.73	2.77	2.71	2.72
Mexico	1.98	2.12	2.16	2.05
South Korea	1.93	2.00	2.01	1.98
Philippines	1.80	1.88	1.81	1.28
Taiwan	0.919	0.936	0.928	0.914
Others	6.85	7.11	7.05	6.94
Total	111	112	100	96.2

2.1.4. Pig Productive Performance and Pork Quality

The innovative and optimized production processes implemented by pig industry go through optimizing the productive performance of pigs to the maximum in order to increase the yield of meat supply to the supply chain (Maples et al. 2019). The productive performance of pigs depends of several factors, such as sex, breed, rearing systems, environmental conditions and nutrition (Latorre et al.; Bona et al. 2016). Due to the enormous importance that the productive performance of pigs has for the pig industry, over the years, the research into growth programs has intensified. This research has given rise to the development of growth

models for pigs that provide to producers tools to obtain maximum productive yield (Emmans and Kyriazakis 1997; Schinckel et al. 2004; Song et al. 2017; Lee et al. 2019). The average growth rate for commercial bred hybrid pigs for finishing pigs (60 to 100 kg) is about 0.950 kg of daily weight gain and 2.32 kg of feed consumption per day (Magowan et al. 2007).

In addition to the development of production processes to optimize production performance, the livestock industry also needs to implement processes that lead to the production of pork with the quality required by the consumer. Pork quality involves different parameters like carcass traits, pork pH, pork colour, pork sensory attributes and pork nutritional composition (Huff-Lonergan et al. 2002; Čobanović et al. 2020). Pork quality is highly dependent on factors such as genetics, crossbreeding, rearing system, processing conditions and nutrition (Suarez-Belloch et al. 2013; Doti et al. 2014; Tejeda et al. 2020).

The fat reduction was the major aspect of improvement in pork quality in the last years. Thorough breeding selections for lean types and an improved understanding of nutritional requirements, pig carcasses have become leaner, meeting the demand of new consumers demand (Kyriazakis and Whittemore 2006; Kantono et al. 2021).The major criterion of assessment of quality in pig carcasses is the backfat depth, usually at the P2 site in the last rib position, which is the most representative location (Teye et al. 2006; Hoa et al. 2021). Generally, the best pork quality is obtained at levels of fatness associated with P2 backfat depths at 100 kg live weight of between 8 and 14 mm. At below 8 mm, the quality of the lean meat falls, while above 14 mm the meat is excessively fatty (Kyriazakis and Whittemore 2006).The carcass yield is the proportion of the weight of the commercial carcass (without giblets) recovered from the body live weight and is expressed in percentage and is a common practice to grading pig carcasses. At the weights of 100-120 kg values are 80% approximately (Kyriazakis and Whittemore 2006; Marcoux et al. 2007).

One of the most important parameters to assess pork quality is the *post-mortem* ultimate pH (pHu) (Wang et al. 2018). After slaughter, while the carcass is warm, energy metabolism lowers the muscle pH, due to chemical reactions where lactic acid is formed from muscle glycogen. Typically, pH of longissimus muscle declines gradually from 7.4 in living muscle to a pH of about 5.5, 24 hours after slaughter (Kyriazakis and Whittemore 2006; Scheffler et al. 2013). Changes in the pHu are directly linked to physicochemical and sensory changes in pork (Van Laack et al. 2001; Long et al. 2018). Several studies established a relationship between high pHu values and an increased tenderness (Purchas et al. 1999; Van Laack et al. 2001). On the contrary, lower pHu values was associated with lighter-coloured with higher drip loss, less tender and with less pork flavour and more off-flavour in the meat (Boler et al. 2010; Scheffler et al. 2013).

Meat colour plays an important role as a quality consideration attributed to fresh pork and is of major importance for consumer acceptability. The consumer establish a strong

association between the colour of pork and its quality and prefers pork with a high intensity of pink in detriment of excessively dark meat (Hughes et al. 2020). Different factors have the ability to influence the meat colour, such as breed, gender, rearing systems production, nutrition, handling and slaughter conditions, including pH and temperature (Salueña et al. 2019; Hughes et al. 2020). The colour of pork is conferred by the pigment content, the chemical form of the pigments, the oxidative state of the pigments, the conditions of meat conservation and the meat structure. In meat, the pigment responsible of colour is myoglobin (Salueña et al. 2019). Colour can be measured by the International Commission on Illumination CIE (Commission Internationale de l'Éclairage) recommendations and by the three principle colour dimensions: L* (lightness), a* (redness) and b* (yellowness) (CIE 2004; Salueña et al. 2019).

Sensory characteristics of pork are extremely important since they can influence the choice of consumers. The most critical characteristics for eating quality of pork are flavour, tenderness and juiciness. The sensory attributes of pork could be affected by many factors, such as breed, gender, carcass weight, diet, genetic variation and biochemical changes that occur during slaughtering, cooling routines, maturation and cooking methods (Verbeke et al. 1999; Aaslyng et al. 2018). Sensory attributes are defined as appearance, odour/aroma/fragrance, flavour, texture and specific feeling/chemical factors. Pork sensory attributes can be identified using humans either as trained sensory panellists or as untrained consumers. Thus, the sensory evaluation of pork is fundamental to determine the consumer preference or acceptability of this meat (Miller and Prusa 1998).

2.1.4.1. Pork Nutritional Composition

Pork is rich in protein and other essential nutrients such as iron, zinc, and several B vitamins (Pereira and Vicente 2013; An et al. 2020). The nutritional composition of pork is highly variable depending on breeds, origin, feeding system and the meat cut (Pereira and Vicente 2013). As reference, pork loin raw presents per 100 g, 131 kcal of energy value, 22.2 g of protein, 4.7 g of fat of which 1.6 g of saturated fat, 1 µg of vitamin B12, 53 mg of sodium, 221 mg of potassium, 0.6 mg of iron and 1.6 mg of zinc (Table 2.3) (Pereira and Vicente 2013).

Table 2.3. Nutritional composition of several meats. Adapted from Pereira and Vicente (2013).

Meat cut	Energy value (kcal)	Protein (g)	Fat (g)	Saturated fat (g)	Vitamin B12 (µg)	Na (mg)	P (mg)	Fe (mg)	Zn (mg)
Chicken breast, skinless, raw	108	24.1	1.2	0.3	0.37	60	220	0.5	0.8
Beef, loin, raw	114	21	3.3	1.4	2	60	145	1.5	3.6
Pork, loin, raw	131	22.2	4.7	1.6	1	53	221	0.6	1.6
Turkey, breast, skinless, raw	105	23.4	1.3	0.3	1	63	210	0.7	0.6
Duck meat, skinless, raw	133	19.3	6.2	1.6	3	92	202	2.4	1.9
Mutton meat, raw	124	19.7	5	2.2	2	64	220	1.7	3.8

2.1.4.1.1. Pork Fatty Acid Profile

Fatty acids can be classified as SFA, which contain no double bounds, MUFA, which feature one double bound, and polyunsaturated fatty acids (PUFA), which contain multiple double bonds. Fatty acid composition of intramuscular fat (IMF) has a considerable impact on eating quality and human health (Dugan et al. 2015).

The fatty acid content and profile of pork depends on several factors, such as breed, rearing system, nutrition and the meat cut (Mourot and Hermier 2001). However, pork presents high levels of SFA compared to poultry (Table 2.3). In fact, the intramuscular fat of pork is composed by 35 to 40% of SFA (Dugan et al. 2015). The prevalent saturated fatty acids in pork are myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) (Schmid 2011; Dugan et al. 2015). The percentage of MUFA in pork fat is commonly around 40-50% of the fat. Overall, oleic acid (18:1*n*-9) is the most frequently monounsaturated fatty acid found in pork fat. Finally, the PUFA levels in pork are approximately 10-20%. The major PUFA include the linoleic acid (18:2*n*-6), α-linolenic acid (18:3*n*-3) and arachidonic acid (20:4*n*-6) (Table 2.4) (Wood and Enser 1997; Dugan et al. 2015).

For an equilibrate and healthy diet, the ratio of PUFA/SFA recommended for humans and considered beneficial is about 1 to 1.5. Since in meat this ratio is generally unfavourable,

with the SFA far outweigh the PUFA, the PUFA/SFA ratio recommended for pork is above 0.4 (Wood et al. 2004; Schmid 2011; Dugan et al. 2015). This ratio is fundamental since the fatty acid composition, in general, and high amounts of SFA relative to PUFA content, in particular, have been identified as dietary risk factors, related to various cancer and especially coronary heart disease (Pascual et al. 2007; Dugan et al. 2015). Another important parameter of pork PUFA composition is the ratio *n*-6/*n*-3 PUFA, which is generally much higher in pork than in beef or veal. The recommendation for this ratio is less than 4, but some meats have higher values, including pork (Wood et al. 2004; Dugan et al. 2015). The decrease of this ratio is of major importance for human health since the *n*-3 PUFA perform a protective role in several human diseases. Therefore, the imbalance of this ratio has been associated with numerous diseases, from cardiovascular and inflammatory diseases to diabetes, autoimmune disorders and cancer. Moreover, *n*-3 PUFA are involved in several biological processes, including infant brain and intestine development (Dugan et al. 2015; Ma et al. 2016).

The *n*-3 PUFA present the first unsaturated bond on carbon number 3, the methyl carbon is number 1, and these are also called omega-3 PUFA with reference to the carboxyl carbon. As important components of the lipid bilayer membrane of cells, *n*-3 PUFA affect structure, fluidity and permeability, membrane-protein conformation, and membrane protein-mediated responses. The major types of *n*-3 fatty acids with roles in the body include: α-linolenic acid (18:3 *n*-3, ALA, the simplest *n*-3 PUFA), eicosapentaenoic acid (20:5 *n*-3, EPA), docosapentaenoic acid (22:5 *n*-3, DPA), and docosahexaenoic acid (22:6 *n*-3, DHA). Among the *n*-3 PUFA, ALA is essential for humans since it cannot be synthesized *de novo* and must be obtained from the diet. Once obtained, ALA can be converted to EPA, DPA, and DHA, albeit with efficiency rate of less than 5%. In order to adequately meet human requirements, most of these PUFAs must be obtained from the diet (Ma et al. 2016).

Table 2.4. Typical fatty acid composition of *longissimus* muscle in pork from pigs fed a barley/wheat/soybean meal diet. Adapted from Enser et al. (1996) and Dugan et al. (2015).

Fatty acid (mg/100 g tissue)	*Longissimus* muscle	Fatty acid (mg/100 g tissue)	*Longissimus* muscle
14:0	30	22:4*n*-6	1.35
16:0	718	∑*n*-6	249
18:0	378	18:3*n*-3	22.7
∑SFA	1177	20:3*n*-3	3.01
16:1*c*9	101	20:5*n*-3	6.35
18:1*c*9	1148	22:3*n*-3	5.40
18:1*c*11	116	22:5*n*-3	11.0
∑MUFA	1409	22:6*n*-3	5.45
18:2*n*-6	189	∑*n*-3	54.0
18:3*n*-6	8.08	∑PUFA	306
20:2*n*-6	5.54	Total	2922
20:3*n*-6	6.92	*n*-6/*n*-3	4.77
20:4*n*-6	46.0	PUFA/SFA	0.301

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

2.1.5. The New Challenges of Pork Industry

The global population, 7.3 billion today, is expected to surpass 9 billion by 2050 and the Food and Agriculture Organization (FAO) has forecast that in 2050, 70% more food will be needed to fulfil the demand of the growing population (Madeira et al. 2017; Chriki and Hocquette 2020). Even if meat consumption is decreasing in developed countries, its global consumption is increasing because consumers are generally unwilling to reduce their meat consumption, in particular in developing countries such as in China, India, and Russia (Chriki and Hocquette 2020). These populations becoming more middle-class, they are looking for more luxury products, such as meat or other animal products (*e.g.*, cheese, dairy products) (Chriki and Hocquette 2020).

Thus, this increased demand for meat products, including pork, will promote an increase of pressure on the pig industry, requiring an increase of pig production, which will bring new challenges to the pig industry (Madeira et al. 2017; Lassaletta et al. 2019). These challenges will have an environmental dimension and a consumer health concern dimension (Figure 2.3).

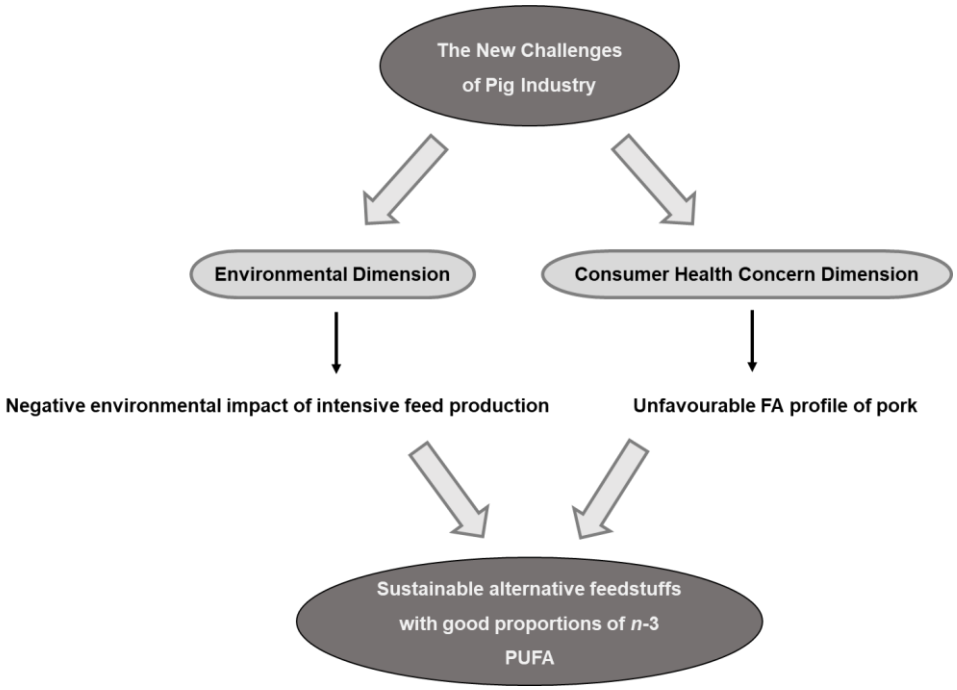

Figure 2.3. Schematic representation of the new challenges of pig industry, the associated problems and a proposed solution to overcome them.

The pig industry exhibits environmental impact at the level of different stages along the pork supply chain, from the feed industry to slaughter, processing and distribution (Aramyan et al. 2011). One of the main concerns about the environmental impact caused by the pig industry is the amount of surplus nutrients in excreta and gaseous losses to the environment. Main nutrients of concern are nitrogen (N), phosphor (P), and heavy metals, such as copper, zinc and cadmium, and main gaseous losses of concern are ammonia, odour, and methane. This compounds promote water and soil contaminations and methane is the most important non-CO_2 greenhouse gas (Aarnink and Verstegen 2007). However, different Life Cycle Assessment (LCA) studies identified the crop and feed production as the most influential stage in the environmental impact mainly due to agricultural activities involved in the production of feed components. A LCA study is a cradle-to-slaughterhouse analysis technique to assess environmental impacts associated with all the stages of pig life cycle (González-García et al. 2015). In fact, the production of feeding raw materials, like corn and soybean food crops is responsible by 60–80% of greenhouse gas emission linked to pig farming (Sporchia et al. 2021). In addition, the production of these raw materials requires 98% of water use for pork meat, involves the use of large tracts of arable land and implies the use of fertilizers and other agrochemicals (Sporchia et al. 2021). Thus, the increase of pig production to meet the needs of the population will result in intensive production of this feeding raw materials, which will have a negative pressure in our planet due to arable land degradation, water deprivation, drastic climate changes, direct competition with human food and the unbalance among food, feed and

biofuel industries (Madeira et al. 2017; Rauw et al. 2020). In line with this, a proposed solution to the problem of the environmental impact of the pig industry is to find good and more sustainable alternatives to conventional feedstuffs (Madeira et al. 2017).

The consumer health concern dimension is directly linked with the new trends in meat consumption. One of the trends identified is the increasing importance of extrinsic cues in consumers' quality perception of meat. This increased interest at the consumer level was linked to two developments: increasing awareness of the link between food and health, and consumers' interest in stories related to the origin and production of their food (Verbeke et al. 2010; Thorslund et al. 2016). Thus, despite pork ranks first among the most consumed meats, pork's image among the new consumers is not univocally positive, since they are more informed about the health concerns and perceived it as the least healthy and fattest meat compared to poultry (Verbeke et al. 2011). The unhealthy connotation of pork is also due to the unfavourable fatty acid profile, with lower proportions of PUFA and lipid-soluble antioxidant vitamins, and higher percentages of SFA, as discussed in the previous section (Morgan et al. 1992; Dugan et al. 2015). Furthermore, the WHO reported that a large part of the human population, including European, does not consume the recommended levels of *n*-3 PUFA (Kanakri et al. 2017; Guesnet et al. 2019; Tocher et al. 2019). The daily recommended values for *n*-3 PUFA vary depending on several factors including geographic region, age, gender, health status and physiological conditions, but they are around 500 mg/day (Kanakri et al. 2017; Guesnet et al. 2019; Tocher et al. 2019). However, a very large global study into consumption of dietary fats including 266 country-specific surveys, showed that the global mean consumption of *n*-3 PUFA was 163 mg/day (Micha et al. 2014). While there was variation in *n*-3 PUFA consumption at both regional and national levels, the mean value was well below the lowest recommended level for intake of around 250 mg/day (Tocher et al. 2019). Currently, the principal source of *n*-3 PUFA for human consumption is fish, but global fish stocks are declining and cannot provide a sustainable source of *n*-3 PUFA. In addition, the presence of chemical contaminants (*e.g*. mercury) in fish can be harmful to consumers (Ma et al. 2016). Hence, taking advantage of the high consumption of pork, the enrichment of this product in *n*-3 PUFA may provide the achievement of the recommended daily intake values of *n*-3 PUFA and improve the connotation of pork for consumers (Dugan et al. 2015; Ma et al. 2016). Besides, pigs are monogastric animals and many dietary components are directly transferred from the feed to the muscle and fat tissues and consequently pig diet provides an effective approach for altering the fat composition and fatty acid profile of pork (Wood and Enser 1997; Bona et al. 2016). However, in some studies where there was the enrichment of meat in *n*-3 PUFA, undesirable flavour and palatability changes have been reported especially with fish oil supplementation (Pereira and Vicente 2013). In addition, the enrichment of pork in *n*-3 PUFA will make meat more susceptible to lipid oxidation (Macho-González et al. 2020)

Therefore, the new challenge of pork industry can be overcome through the use of good sustainable alternatives to conventional feedstuffs with good proportions of *n*-3 PUFA that do not impair the productive performance of the pigs or affect the properties and acceptability characteristics of the meat (Florou-Paneri et al. 2014; Ma et al. 2016).

2.2. Microalgae in Animal Nutrition

Microalgae are microscopic, unicellular or simple multicellular prokaryotic or eukaryotic photosynthetic organism that can produce biomass and oxygen by using sunlight as energy source, CO_2 as carbon source and inorganic salt. The photosynthetic process of microalgae is ten times more efficient than terrestrial plants (Patel et al. 2017; Sathasivam et al. 2019). Some species can grow autotrophically and produce organic molecules while others are heterotrophic in nature, growing in dark on complex organic material for energy and carbon sources. Cynobacteria from *Cynophyceae* are example of prokaryotic microalgae and in similar way green algae from *Chlorophyta* and diatoms from *Bacillariophyta* are example of eukaryotic microalgae (Patel et al. 2017).

Microalgae are ancient organisms, inhabiting the earth for over two billion years (Patel et al. 2017). There are more than 50,000 different types of microalgal species present in oceans and fresh water (lakes, ponds and rivers); among these species, only 30,000 have been studied (Patel et al. 2017). Applied phycology was originated with first microalgae culture establishment of *Chlorella* by Beijerinck in 1890 (Richmond 2004). Since then the study of microalgae has intensified. Many microalgae species were collected from variety of habitats and analysed. Different microalgae collection centres have been established with highly diversified culture collection. For example university of Coimbra from Portugal have more than 4000 strains of fresh water microalgae (Mata et al. 2010).

Microalgae have been used as food by humans for thousands of years (Patel et al. 2017). Currently, microalgae present extensive application potential in the renewable energy, biopharmaceutical, nutraceutical, cosmetic and food and feed industries (Khan et al. 2018). A well balanced nutritional composition, the presence of bioactive substances such as PUFA, antioxidants, pigments, vitamins and polysaccharides and a rich source of carbon compounds allow microalgae to be an excellent resource and with a wide range of applications like food supplements, in animal feed, as pharmaceuticals, cosmetics and for biofuels production (Khan et al. 2018). Moreover, microalgae have environmental applications like wastewater treatment and atmospheric CO_2 mitigation (Khan et al. 2018). Due to the numerous applications of microalgae and their products, the concept of biorefinery was born. The concept of biorefining is similar to the petroleum refineries in which multiple fuels and chemicals are derived using

crude oil as the starting material. Similarly, biorefining is sustainable biomass processing to obtain energy, biofuels and high-value products through processes and equipment for biomass transformation (Trivedi et al. 2015; Zhu 2015; Eppink et al. 2019).Thus, microalgae biotechnology is a relatively new research area that has increased exponentially over the last few years in parallel with the rapid appearance of facilities and microalgae-based products (Garrido-Cardenas et al. 2018).

The commercial production of microalgae is approximately 20,000 tons/year of biomass, corresponding to about 5,000 tons/year of dry matter. This production capacity is not too much in comparison with other biomasses or crops, but it is increasing more than 10% annually (Fernandez et al. 2017; Sathasivam et al. 2019). Microalgae biomass is today produced mainly for high-value applications related to human consumption, including food, nutraceuticals, cosmetics and pharmaceuticals and animal feed applications (Fernández et al. 2019; Sathasivam et al. 2019).

The nutritional and production characteristics allow microalgae to have enormous potential in animal feed and aquaculture (Benemann 2013). In fact, microalgae could be an appropriate alternative to conventional feedstuffs for monogastrics, including pig (Madeira et al. 2017; Camacho et al. 2019). Microalgae present a very complete nutritional composition, rich in carbohydrates, proteins with amino acid profile compared with conventional protein sources, vitamins, carotenoids, minerals and other valuable trace elements (Table 2.5) (Becker 2007; Yaakob et al. 2014; Madeira et al. 2017; Matos et al. 2017). Furthermore, in general microalgae exhibit appreciable amount of lipids with good proportions of *n*-3 PUFA, including the essential ALA, EPA and DHA (Ryckebosch et al. 2012; Batista et al. 2013; Martins et al. 2013). Environmental factors, such as temperature, salinity, illumination, pH-value, mineral content, CO_2 supply, population density, growth phase and physiological status, can greatly modify the microalgae chemical composition (Batista et al. 2013) and these can be manipulated to lead to an increase in the production of a certain microalgae product (Harun et al. 2010). As production characteristics, the microalgae production has a minimal environmental impact compared with traditional feedstuffs for animal feed (Gouveia et al. 2010). Microalgae neither compete for arable land with food crops for human consumption nor interfere with food security concern. They can grow in different environments like fresh and brackish or marine water and also tolerate marginal lands like arid, semi-arid and desert which are not suitable for other agriculture crops (Patel et al. 2017). Microalgae also promote the mitigation of atmospheric CO_2, the principal greenhouse gas (Gouveia et al. 2010; Patel et al. 2017). Thus, microalgae could be a viable alternative in the partial replacement of soybean, especially considering the case of the EU where there is a large production of pigs but there is a great dependence on soybean imports (Altmann et al. 2019).

In spite of microalgae nutritional composition varies with the species, *Tetraselmis* sp., *Chlorella* sp., *Spirulina* sp. (*Arthrospira platensis*), *Nannochloropsis* sp., *Schizochytrium* sp., *Phaeodactylum* sp., *Scenedesmus* sp., and *Tisochrysis lutea* stand out in feed production for animal nutrition (de Medeiros et al. 2021). Several studies reported the incorporation of microalgae in animal feed, including in fish, rabbit, sheep, cow, poultry and pigs, and its effects on productive performance, meat quality and blood parameters. It was observed that the results vary depending on the species of microalgae used and the level of incorporation in the diet. However, it was possible to identify cases in which the microalgae was able to promote benefits to animal growth, improvements in the meat quality and immune system and reduction of blood glycaemia and lipemia (Madeira et al. 2017; Camacho et al. 2019; de Medeiros et al. 2021).

The two most studied microalgae with more commercial expression are *Arthrospira platensis* and *Chlorella vulgaris* and current combined production adds up to 5000 and 2500 tons of biomass, respectively (Garrido-Cardenas et al. 2018; Camacho et al. 2019).

Table 2.5. Comparison between the nutritional composition of two traditional feedstuffs on pig production (corn and soybean) with two microalgae with high commercial expression (*A. platensis* and *C. vulgaris*). Adapted from Asiedu et al. (1993), Perkins (1995), Lin et al. (2012), Batista et al. (2013) and Prasanthi et al. (2017).

Item	Corn	Soybean	*A. platensis*	*C. vulgaris*
Carbohydrates (% DM)	74.3	35	16.6	19.9
Crude protein (% DM)	8.8	37.9	44.9	38.0
Crude fat (% DM)	4.4	17.8	3.6	5.1
Fatty acids (% of total fatty acids)				
16:0	12.3	10.7	42.3	29.1
18:0	3.5	3.9	1.3	0.7
∑SFA	17	15	44.9	35.9
18:1*c*9	20.2	22.8	4.5	12.9
∑MUFA	20.2	23.1	15.8	24.0
18:2*n*-6	60.4	50.8	18.9	8.4
∑*n*-6 PUFA	60.4	50.8	37.0	12.3
18:3*n*-3	1.9	6.8	1.6	18.9
20:5*n*-3	-	-	-	0.5
22:6*n*-3	-	-	-	0.5
∑*n*-3 PUFA	1.9	6.8	2.3	27.8

DM – dry matter; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

2.2.1. *Arthrospira platensis*

Arthrospira platensis is a prokaryote Gram-negative cyanobacteria species, lacking membrane-bound organelles (Figure 2.4 A) (Pignolet et al. 2013). *A. platensis* is characterized by a loosely spiral shaped trichomes arranged in an open helix enclosed in a thin mucilaginous sheath (Figure 2.4 B), usually found in alkaline, brackish and saline waters from tropical and subtropical regions. They usually become predominant species and form massive blooms (Yaakob et al. 2014). *A. platensis* is Earth's oldest living plant approximately 3.6 billion years ago and a first photosynthetic life form that has created our oxygen atmosphere so all life could evolve. *A. platensis* was first described by Spanish Scientist Hernando Cortez and Conquistadors in 1519. Cortez observed that *A. platensis* was eaten at the tables of the Aztecs during his visit in Lake Texcoco in the Valley of Mexico. Lately, Pierre Dangeard and the botanist Jean Leonard discovered the health benefits of *A. platensis*, but today it is known that this was already used for thousands of years by the populations of America and Asia (Patel et al. 2017; Soni et al. 2017).

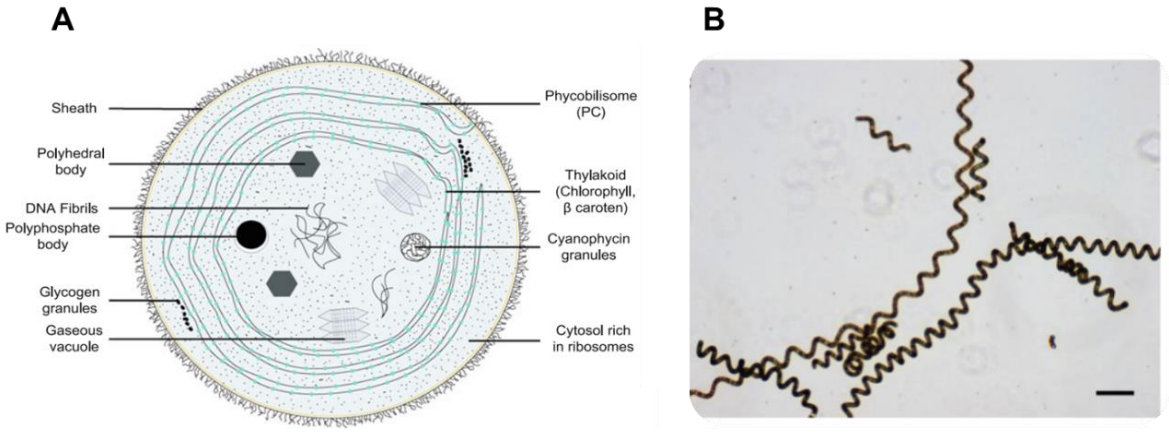

Figure 2.4. A. Ultrastructure of *Arhrospira platensis*. Adapted from Pignolet et al. (2013). B. Optical microscopy observation of *A. platensis* cells forming spiral shape trichomes. Scale bar = 15 µm. Adapted from Wells et al. (2017).

A. platensis is the most nutritious, concentrated food that is known to mankind containing antioxidants, phytonutrients, probiotics, and nutraceuticals. The United Nations world at food conference declared that *A. platensis* as the best food for future, and it is gaining popularity nowadays. WHO has described *A. platensis* as Mankind's best health product according to UNESCO, *A. platensis* is the most ideal food for tomorrow. According to NASA and European Space Agency, it is one of the primary foods that can be cultivated in long-term space missions in space. FDA validated it as “One of the best protein sources”. Intergovernmental institution permitted for the use of microalgae *A. platensis* against

Malnutrition (Soni et al. 2017). This popularity of *A. platensis* among the best food supplements worldwide is due to its nutritional composition. In general, *A. platensis* is composed by 55-70% protein, 6-9% fat, and 5-20% carbohydrate by % of DM (Yaakob et al. 2014). The first outstanding aspect is its high protein content. Moreover, *A. platensis* also have an amino acid profile that compares well with egg, notably containing all of the essential amino acids that humans cannot synthesize and must obtain from foods (Wells et al. 2017). Then, *A. platensis* presents a panoply of compounds with bioactive properties, such as sterols, vitamins, carotenoids, phycobiliproteins and minerals (Soni et al. 2017; Andrade et al. 2018). These bioactive compounds are implicated in different health benefits including diabetes prevention, depression prevention, immune system stimulation, anti-infectious properties, tissue repair, decreases cholesterol levels and helps to lower the risk of cardiovascular disease and anti-inflammatory properties (Soni et al. 2017; Andrade et al. 2018).

Due to its popularity and high demand, large-scale production of *A. platensis* occurs throughout the world and have “GRAS” designations (Generally Recognized As Safe) by FDA (Wells et al. 2017). The processes and technology used in the production of microalgae, including *A. platensis*, have been optimized in order to increase production yield and thus reduce costs associated with production (Soni et al. 2017).

In addition to the wide applicability in human food, *A. platensis* has high potential to be applied in animal feed. For instance, *A. platensis* outyields many other traditional animal feed types, including wheat, corn, barley and soybeans, in protein output per land unit and has appreciable biomass production yields. Approximately half of the total *A. platensis* production being used in livestock and fish feeds (Holman and Malau-Aduli 2013). The use of *A. platensis* in pig nutrition will be reviewed in detail in the section 2.2.3.

2.2.2. *Chlorella vulgaris*

One of the most remarkable microalga is the green eukaryotic microalga *Chlorella vulgaris* (Figure 2.5 A) (Safi, Zebib, et al. 2014). *C. vulgaris* displays a spherical shape with 2–10 µm of diameter (Figure 2.5 B) (Yamamoto et al. 2004) and was first discovered by the Dutch researcher Martinus Willem Beijerinck in 1890 as the first microalga with a well-defined nucleus (Safi, Zebib, et al. 2014).

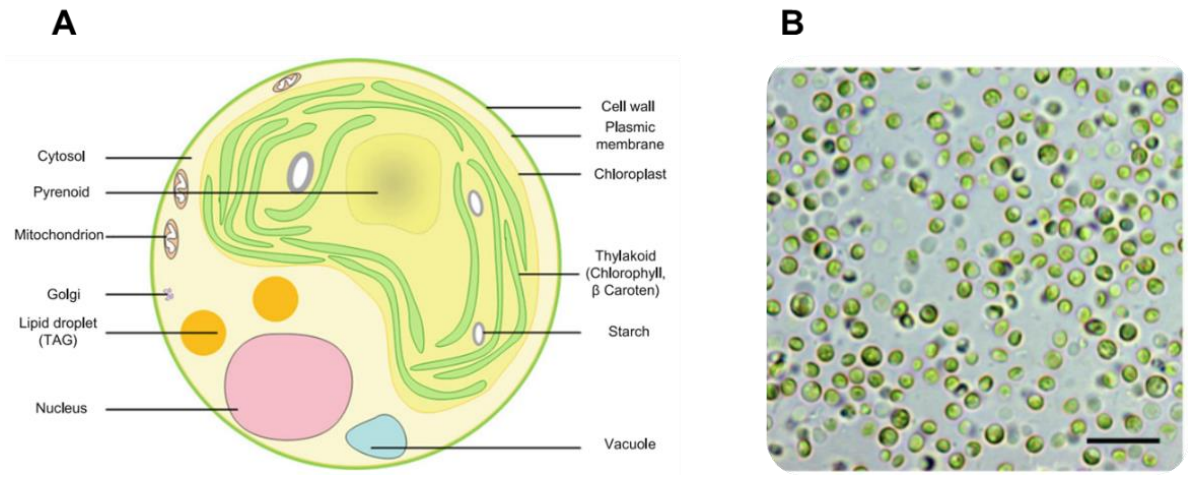

Figure 2.5. A. Ultrastructure of *Chlorella vulgaris*. Adapted from Pignolet et al. (2013). B. Optical microscopy observation of *C. vulgaris* cells. Scale bar = 15 µm. Adapted from Wells et al. (2017).

The name *Chlorella* comes from the Greek word *chloros*, which means green, and the Latin suffix *ella* referring to its microscopic size (Safi, Zebib, et al. 2014; Andrade et al. 2018). It is a unicellular microalga that grows in fresh water and has been present on earth since the pre-Cambrian period 2.5 billion years ago and since then its genetic integrity has remained constant. By the early 1900s, Chlorella protein content attracted the attention of German scientists as an unconventional food source. In the 1950s, the Carnegie Institution of Washington [19] took over the study and managed to grow this microalga on a large scale for CO_2 abatement. Nowadays, Japan is the world leader in consuming *Chlorella* and uses it for medical treatment because it showed to have immune-modulating and anti-cancer properties (Safi, Zebib, et al. 2014).

Compared with *A. platensis*, *C. vulgaris* displays a lower content in protein but a higher content in fat (Table 2.5). Even though, the nutrition composition of *C. vulgaris* is composed by 51–58% protein, 14–22% fat, and 12–17% carbohydrates by % of DM (Becker 2007; Varfolomeev and Wasserman 2011). The amino acid profile of *C. vulgaris*, like *A. platensis*, compares favourably and even better with the standard profile for human nutrition proposed by WHO and FAO, since the cells of *C. vulgaris* synthesise essential and non-essential amino acids (Becker 2007; Safi, Zebib, et al. 2014). Relative to the lipid content, *C. vulgaris* has a very interesting fatty acid profile, with good proportions in some *n*-6 PUFA (18:2*n*-6 and 18:3*n*-6) and, specifically in the *n*-3 PUFA α-linolenic acid (18:3*n*-3) (Batista et al. 2013). However, the culture conditions of *C. vulgaris* can also be changed in order to manipulate the contents of the nutritional composition components. The lipid content of *C. vulgaris* can reach 58% during specific growth conditions (Mata et al. 2010; Stephenson et al. 2010). Nevertheless, the fatty acid profile of *C. vulgaris* can also be manipulated through the growing conditions and make *C. vulgaris* suitable for different applications, such as a higher content in SFA and MUFA

which is indicated for biodiesel production or a higher content in PUFA which is suitable for nutritional uses (Stephenson et al. 2010; Chen et al. 2011; Yeh and Chang 2011).

C. vulgaris is also rich in micronutrients with bioactive properties such as: carotenoids (astaxanthin, cantaxanthin and lutein); minerals (sodium, potassium and phosphor); vitamins (vitamin E, vitamin A and vitamin C) (Safi, Zebib, et al. 2014). These bioactive compounds perform major roles in health maintenance, such as antioxidant activity, protective effect against retina degeneration, regulating blood cholesterol, prevention from chronic diseases and fortifying the immune system (Safi, Zebib, et al. 2014; Andrade et al. 2018; Ru et al. 2020).

In addition to its excellent nutritional composition, the ease of production of *C. vulgaris* makes this microalga very popular compared to others, and profitable to use. This microalga has a rapid growth rate and is ideal for production because it is remarkably resistant against harsh conditions and invaders (Liu and Chen 2014; Safi, Zebib, et al. 2014; Ru et al. 2020). The production of this microalgae can be done through different processes implemented in production plants, which are in constant optimization through new scale-up methods in order to decrease the utilization cost of *C. vulgaris*. These processes combine growth and cultivation, harvesting and dewatering (Ru et al. 2020). Currently, Japan, Germany and China are the main producers of *C. vulgaris* (Saka et al. 2020).

Although *C. vulgaris* be highly appreciated as a food supplement, consumed especially in Germany, China, Japan and US after Industrial Revolution, the interest in the application of this microalgae in other fields has been increasing, including in animal feed as a promising alternative to traditional feedstuffs due to its high biomass productivity, relative ease of cultivation, minor environmental impact of production and a high-quality nutritional composition (Kotrbáček et al. 2015; Chen et al. 2019; Ru et al. 2020). The use of *C. vulgaris* in pig nutrition will be reviewed in detail in the section 2.2.3.

2.2.3. The Microalgae in Pig Nutrition

As microalgae, more specifically *Arthrospira platensis* and *Chlorella vulgaris*, display productive and nutritional characteristics that place them as good sustainable alternatives of traditional feedstuffs on pig production, its use as a micro- or bulk ingredient in formulated pig feeds has been proposed in several studies (Shields and Lupatsch 2012; Raji et al. 2020). Microalgae has been inserted in animal feed mainly through incorporation of the whole microalgae biomass as dried powder, high moisture extruded biomass or microalgae extracts (de Medeiros et al. 2021).

A. platensis has been applied in feeding pigs as feed ingredient (>1% in diet) or as feed supplement (1% or lower in diet) (Madeira et al. 2017; de Medeiros et al. 2021).

In a study conducted by Simkus and colleagues in 2013, the diets of fattening pigs with 30.6 kg were supplemented with 0.2% of *A. platensis* biomass. Pigs were slaughtered at 96.4 kg and was observed an increase of average daily gain (ADG) and feed conversion ratio (FCR). Was also observed no effect on backfat thickness and on meat quality traits. However, the intramuscular fat was decreased (Simkus et al. 2013). Furbeyre et al in 2017 developed a study where 1% of *A. platensis* biomass was incorporated into diets of weaned pigs with 9.1 kg during 14 days of trial. The authors observed no effect on average daily feed intake (ADFI), ADG and FCR (Furbeyre et al. 2017). Lately, Altmann et al in 2019 performed a study replacing 50%, 75% and 100% of soy protein by *A. platensis* biomass, with percentages of incorporation of microalgae up to 12.5% in pigs from 22 kg to 110.48 kg. The authors observed an increase of PUFA content (α-linolenic acid and γ-linolenic acid) and improved the sensory aroma of the meat. However, an increased astringent aftertaste was also observed. Moreover, the diet did not influence growth performance of pigs or the technological quality of the meat (Altmann et al. 2019).

The most studies involving the incorporation of *C. vulgaris* on pig feeding diets have used levels of incorporation as feed supplement (Madeira et al. 2017).

Baňoch et al. (2012) investigated the effect of a very low level (0.0002%) of incorporation of *C. vulgaris* biomass in female pigs, with an initial weight of 30 kg, and found no significant differences in ADG, hot carcass weight (HCW), lean muscle thickness and backfat thickness. The authors also verified no effect on meat quality traits such as, colour, pH, lipid oxidation stability, cooking loss and drip loss (Baňoch et al. 2012). Later, Furbeyre and colleagues in 2017 showed no significant effects on ADG, ADFI, and FCR, by using 1% of *C. vulgaris* biomass in weaned piglet diets, with an initial weight of 9.1 kg, during 14 days. In another study, the same authors assessed the effect of oral supplementation with *C. vulgaris* (385 mg/kg body weight, BW) on growth and digestive health of weaning piglets and also found no significant changes in ADG, ADFI and gain:feed ratio (G:F) (Furbeyre et al. 2018). On contrary, a study conducted in growing pigs, with an initial weight of 26.6 kg and *C. vulgaris* incorporation of 0.1% and 0.2% in the diet, described an increase of ADG with the lower dietary level without significant variations in ADFI and G:F (Yan et al. 2012).

In addition to the effects promoted by *A. platensis* and *C. vulgaris* on productive performance and meat quality of animals, several studies described that these microalgae have direct implications on health status of animals (Camacho et al. 2019). These studies demonstrated that the incorporation of these microalgae in diets had a positive effect on animal physiology, by controlling lipemia and glycaemia (Lee et al. 2008; Abdelnour et al. 2019), improving their immune response (Kang et al. 2013), disease resistance, and gut function, besides enhancing antiviral and antibacterial protection, as well as increasing reproductive performance (Furbeyre et al. 2018; Camacho et al. 2019). Madeira et al. (2021) described an

effect of improvement of the systemic antioxidant potential of weaned pigs fed with *A. platensis* in a 10% level of incorporation during 28 days. In this trial was also observed changes in plasma lipids, immunoglobulins and a minor modulation on related hepatic metabolic pathways in animals fed with *A. platensis* when compared with the control group (Madeira et al. 2021). However, the impact of dietary *C. vulgaris* on plasma metabolites, haematology, immune system and lipid metabolism in pigs, mainly in finishing pigs, needs further research.

2.2.4. The Biggest Drawback on the Use of Microalgae

The biggest drawback on the use of microalgae is their recalcitrant cell wall. The microalgae cell wall plays a protective role against invaders and/or harsh environmental conditions (such as desiccation during growth) (Acton 2013).

The recalcitrance of microalgae cell wall is refractory to breakage and drying, trapping valued nutrients, and therefore restraining their direct use. It makes the disruption process an energy and cost-intensive step. Ideally, an effective pre-treatment is necessary to proceed with the degradation of the microalgae cell wall and access to its trapped compounds that will be later used in food, feed, biofuel, pharmaceutical and cosmetic industries (Lum et al. 2013). An effective pre-treatment should allow degradation of the cell wall components without affecting the bioactive inner nutritional compounds to be recovered, at the lowest possible expense and with the lowest environmental impact (Acton 2013; Austic et al. 2013; Lum et al. 2013). Various mechanical, chemical and enzymatic cell-disruption methods have been developed and compared based on microalgae species and status (wet or dried), scale, energy consumption, efficiency, solvent extraction and harmfulness, and synergistic combinations (Figure 2.6) (Lee et al. 2017; Alhattab et al. 2019). For microalgae species, in opposition to macroalgae, mechanical methods, like hammer mills, are not typically applied (Makkar et al. 2016). In turn, bead milling is used to incorporate microalgae cells as food additives and this constitutes a well-achieved, expanding strategy in the food industry. On the downside, this mechanical process is hard working and expensive with high energy consumption whereupon cells are massively destroyed (Austic et al. 2013; Lum et al. 2013).

In addition to these industrial processes, the introduction of microalgae in animal feed may also be compromised due to the recalcitrant cell wall of microalgae, namely in monogastric animals. The recalcitrant cell wall makes microalgae poorly digestible by monogastric animals and, consequently, decreases the bioavailability of the microalga valuable nutrients and its absorption rate by animals. Along these lines, a high incorporation level of microalgae (>2% in diet) may have negative consequences on the productive performance and health status of monogastric animals, including pigs (Williams and Laurens 2010; Milledge 2011; Amorim et al. 2021).

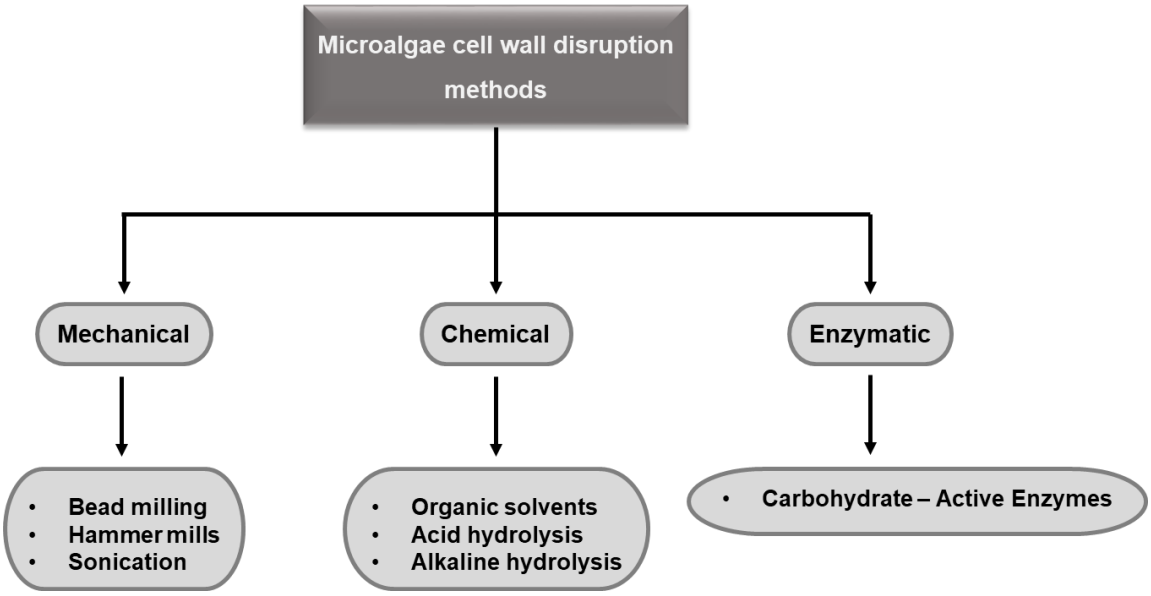

Figure 2.6. The different microalgae cell wall disruption methods. Adapted from Lee et al. (2017).

Unlike other microalgae, *A. platensis* presents a more fragile cell wall (Figure 2.7) (Williams and Laurens 2010; Safi et al. 2013). The cell wall of *A. platensis* presents characteristics of a Gram-negative bacterial cell wall, constituted by a simple envelope composed by several layers, mostly of peptidoglycan and lipopolysaccharide nature, without cellulose (Van Eykelenburg et al. 1980; Sotiroudis and Sotiroudis 2013). Due to these characteristics, *A. platensis* has a digestibility of 86%, and could be easily absorbed by monogatrics when compared with others microalgae (Li and Qi 1997; Lu et al. 2006).

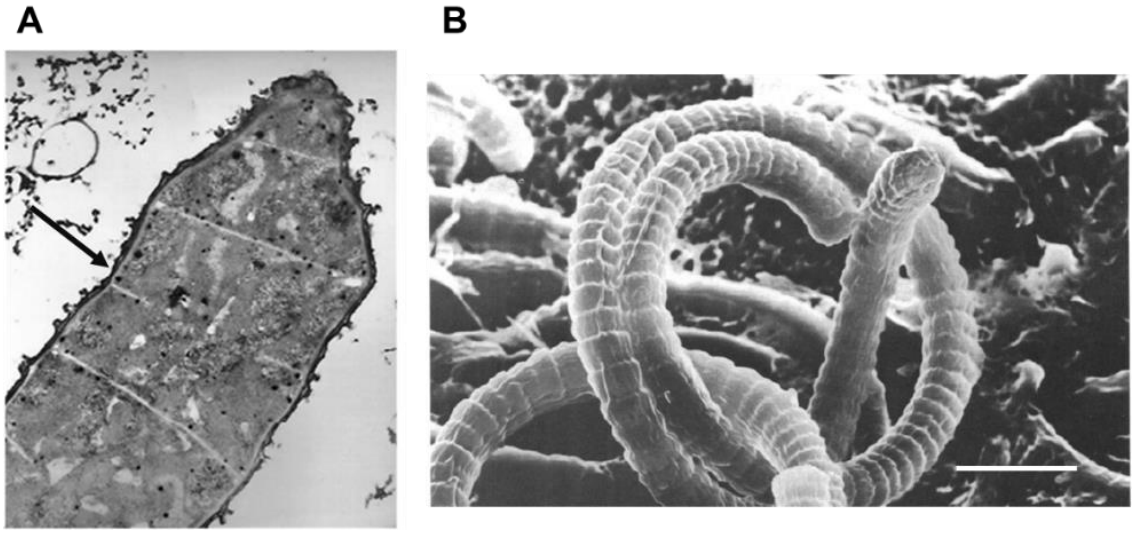

Figure 2.7. A. Electron microscopy observations of a longitudinal section through a trichome formed by *A. platensis* cells. Black arrow pointed to *A. platensis* cell wall. Magnification = 7200 x. Adapted from Vladimirescu (2010). B. Scanning electron micrograph of a portion of a trichome of *A. platensis*. Scale bar = 10 µm. Adapted from Koru (2012).

On the other hand, *C. vulgaris* displays a more rigid cell wall. It is well known that the *C. vulgaris* cell wall varies according to growth stage and environmental conditions and its rigidity increases according to the degree of the cell maturation (Figure 2.8) (Safi, Zebib, et al. 2014). However, the molecular composition and structure of *C. vulgaris* cell wall still generates some controversy. Several authors indicate that the cell wall of *C. vulgaris* is composed by an unilaminar layer without sporopollenin, an extremely resistant polymerised carotenoid found on the cell wall of *Haematococcus pluvialis* and *Chlorella fusca* (Safi, Zebib, et al. 2014). However, a contradictory study conducted on *C. vulgaris* by Martínez et al. 1991 reported the presence of sporopollenin by observing an outer trilaminar layer and by detecting resistant residues after being submitted to acetolysis. The presence of cellulose in the cell wall has also raised some doubts and several studies suggest that the cell wall does not have cellulose in its composition or it does not play a fundamental role in its integrity (Gerken et al. 2013). These doubts about the structure of the cell wall of *C. vulgaris* may be directly linked to the strain-specific composition (Ahmad et al. 2020). However, it is established that *C. vulgaris* cell wall rigidity derive from an extremely diversified and complex matrix of cross-linked insoluble carbohydrates. It reaches 17–21 nm after maturation, where a microfibrillar layer is formed representing a chitosan-like layer composed of glucosamine The main neutral sugars encountered beside glucosamine in the rigid wall are galactose, rhamnose and mannose (Kapaun and Reisser 1995; Safi, Zebib, et al. 2014; Baudelet et al. 2017).

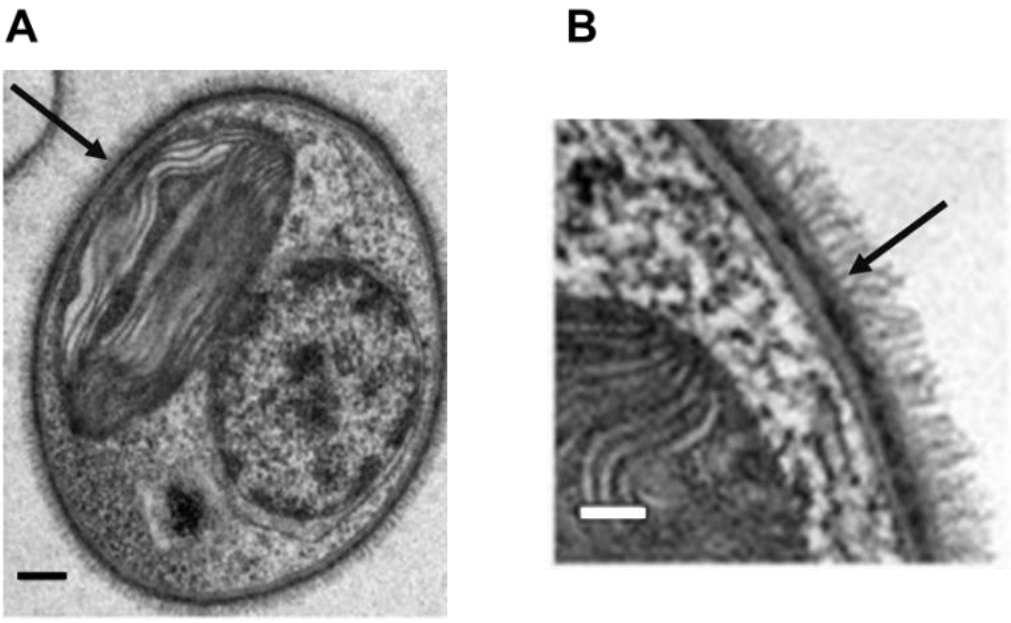

Figure 2.8. A. Transmission electron micrograph of a *C. vulgaris* cell. Black arrow pointed to cell wall. Scale bar = 200 nm. B. *C. vulgaris* cell wall magnification indicated by the black arrow. Scale bar = 100 nm. Adapted from Gerken et al. (2013).

Hence, it becomes imperative for the feed industry to develop adequate technologies to disrupt the cell wall that endowed *C. vulgaris* and improve microalgal nutrient bioavailability in monogastric animals (Madeira et al. 2017).

2.3. Carbohydrate–Active Enzymes in Animal Nutrition

For a successful and profitable use of microalgae, it is necessary that they undergo a cell wall disruption process. This process may involve mechanical, chemical or enzymatic methods as referred in section 2.2.4 (Günerken et al. 2015). In the group of enzymatic methods, the application of exogenous feed enzymes is a proposed strategy to disrupt the microalgae cell wall in the gastrointestinal tract of monogastric animals, thus facilitating their digestion by these animals and consequently increasing the bioavailability of microalga nutrients (Alhattab et al. 2019; Ahmad et al. 2020). In the field of feed enzymes, are highlighted the CAZymes (Contesini et al. 2021).

CAZymes encompasses a large class of enzymes involved in the modification, degradation, or biosynthesis of complex carbohydrates and their derivatives of the cell. Especially, CAZymes capable of degrading polysaccharide fraction of plant biomass into simple sugars, through acting on glycosidic bonds, have proven to be crucial for the significant biotechnological advances within sectors that include bioenergy and biobased (food/feed, materials, and chemicals) industries (Park et al. 2017; Contesini et al. 2021). The concept of CAZymes and their organization into families, based on amino acid sequences, similar structurally, related catalytic or functional domains, was established in the late 1990'ties, and in 1998 Lombard et al. (2014) launched the CAZy database (www.cazy.org). The CAZy database, and associated bioinformatics tools, organize all known CAZymes into the following classes; glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE), and auxiliary activities (AA) (Lombard et al. 2014; Contesini et al. 2021) The CAZy database provides a continuously update of CAZymes list (Park et al. 2017). The GH are responsible for the hydrolysis and/or trans-glycosylation of glycosidic bonds and presently about 47% of the enzymes classified in CAZy. Because of their widespread importance for biotechnological and biomedical applications, GHs constitute so far the best biochemically characterized set of enzymes present in the CAZy database. The GT are responsible for the biosynthesis of glycosidic bonds from phospho-activated sugar donors and represent about 36% of CAZy content. The PL cleave the glycosidic bonds of uronic acid-containing polysaccharides and corresponding to only about 1% of CAZy content. The CE remove ester-based modifications present in mono-, oligo- and polysaccharides and thereby facilitate the action of GHs on complex polysaccharides and represent roughly 5% of CAZy

entries. Finally, the AA are redox enzymes that act in conjunction with CAZymes and comprises 1% of CAZy content. The remaining 10% correspond to Carbohydrate-Binding Modules (CBM) which display a structure with non-catalytic modules responsible for adhesion to carbohydrates (Cantarel et al. 2009; André et al. 2014; CAZypedia Consortium 2018).

Amongst biotechnological additives, feed enzymes, including CAZymes have made the most progress and impact in the past decade (Kiarie et al. 2013). The use of enzymes in animal feed was first reported in 1925 where diet of female leghorns was supplemented by a fungal enzymic material during 20 weeks and resulted in a 22% increase in final BW. (Hervey 1925; Bedford 2018). Since then, the chemistry of target substrates in feed ingredients has been better understood and it has become possible to fine-tune the production of enzymes that are specific for individual substrates. Another development has occurred in the area of biotechnology, specifically in fermentation and microbiological technologies and molecular biology. As a result, it is now possible to produce feed enzymes cheap enough to warrant their use in commercial diet formulations. Other advances include the development of specific enzymes designed to function optimally in the gastrointestinal tract of the animal and production technology to improve enzyme stability during the processing of commercial feeds (Ravindran 2013).

The use of exogenous enzymes in ruminant diets has been limited because of the view that fibrolytic activity within the rumen environment is normally very high and it is assumed that exogenous enzymes would not survive proteolysis in the rumen. However, in aquaculture there is an increasing interest in addition exogenous enzymes to overcome the low nutrient digestibility of alternative feedstuffs to conventional protein sources for fish meal (Ravindran and Son 2012) In monogastric livestock species , such as poultry and pigs, the use of exogenous CAZymes, mainly xylanases and beta-glucanases, are now widely accepted to supplement diets, as a cost-effective strategy in order to improve feed nutritive value of cereal-based diets, increasing its digestibility and directly impact on animal performance and health (Ravindran and Son 2012; Cardoso et al. 2018). The use of CAZymes in pig nutrition will be reviewed in detail in the section 2.3.2.

2.3.1. Carbohydrate – Active Enzymes on Microalgae Cell Wall Disruption

If cell disruption is a critical step concerning the economy and recovery of biotechnological processes from microalgae, enzymatic cell disruption has shown positive results when compared to mechanical or chemical methods (Ho et al. 2013; Lee et al. 2013; Kim et al. 2014). Despite the use of enzymes involve an associated cost in the production process and a huge challenge on finding the ideal formulation of enzymatic cocktails, in

composition and dosages (Demuez et al. 2015), the enzymatic cell disruption methodology encompasses numerous advantages, such as; reduction of energy input, high selectivity of the enzymatic reactions, mild temperature and pH conditions, and the absence of inhibiting by-products. Consequently, it is considered a less energy intensive and more environmentally sustainable approach than the conventional mechanical or chemical methods (Y. Zhang et al. 2018; Córdova et al. 2019). Thus, CAZymes can be a sustainable mild cell wall disruption technique, acting in a specific manner on cell wall compounds (Vanthoor-Koopmans et al. 2013; Phong et al. 2018).

Due to the numerous advantages that the enzymatic lysis displays as a disruption method for microalgae cell wall, highly valued research teams have been working worldwide on this topic looking for economical solutions (Phong et al. 2018; Nagappan et al. 2019). It has been successfully demonstrated that the addition of CAZyme mixtures for disrupting microalgae cell walls improves sugar recovery, solubilization of organic matter and lipid extraction, including in the two species of microalgae under study, *A. platensis* and *C. vulgaris* (Choi et al. 2010; Liang et al. 2012; Aikawa et al. 2013; Mahdy et al. 2014). Zheng and colleagues in 2011 tested the effectiveness of different lysis methods using two CAZymes individually and an enzymatic complex on *C. vulgaris*. The enzymes used individually were a lysozyme and a cellulase, both GH, and the enzymatic complex was snailase, a mixture of different enzymes including the CAZymes cellulase, hemicellulase, pectinase and β-glucuronidase, extracted from the digestive tract of snails. To measure the degree of cell wall degradation after each enzyme action, the lipid extraction yield was quantified and the enzymes individually as well as the snailase were found as effective (Zheng et al. 2011).In a similar study, Cho et al. (2013) evaluated the power of cellulases and β-glucosidases, both from GH family, combined towards the disruption of *C. vulgaris* cell wall. These authors assessed the disruption degree of cell wall through the lipid extraction yield (Cho et al. 2013). In a study conducted by Gerken et al. (2013), the enzymatic cell wall degradation of different microalgae species was tested. To exploit the enzyme activity against microalgae cell wall, the authors applied a growth inhibition assay, in which microalgae were cultivated in the presence of different enzymes, individually or combined. The inhibition of microalgae growth suggests that the enzyme is degrading the cell wall during construction. Then, the authors measured the permeability percentage of microalgae derived from the enzymatic action in the cell wall through a flow cytometer coupled with imaging. The enzymatic action increases the permeability of microalgae leaving DNA into the extracellular space, being detected by flow cytometry. Finally, through electron microscopy, it was possible to identify the extent of cell wall damage promoted by the enzymes. The authors verified that *C. vulgaris* is typically most sensitive to chitinases and lysozymes, both enzymes that degrade polymers containing N-acetylglucosamine belonging to the GH family of CAZymes (Gerken et al. 2013). In *A.*

platensis, Aikawa and colleagues in 2013 observed that the addition of lysozyme for *A. platensis* cell wall disruption allowed an increase in the yield of glycogen extraction from the microalgae, and also increasing the yield of glycogen conversion to ethanol (Aikawa et al. 2013).

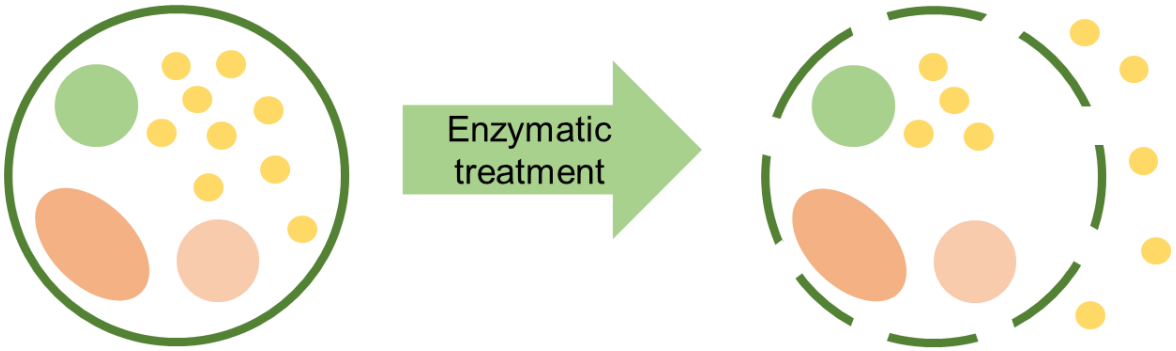

Figure 2.9. Schematic representation of the disruptive action of CAZymes on the microalgae cell wall and the release of their internal compounds.

Although the enzymatic method of cell wall disruption is very promising, it has many disadvantages. One of them is the prohibitive cost of this economically unviable strategy. This is directly linked to the fact that enzymes cannot be generally recovered after being used (Phong et al. 2018). A possible resolution on this problem was introduced by Fu et al. (2010) through the immobilization technology applied to cellulase onto an electrospun polyacrylonitrile nanofibrous membrane. In this sense, in addition to achieve appreciable rates of microalgae cell wall degradation and an improvement on microalgae lipid extraction yield, it was possible to reuse enzymes and to reduce the amount needed.

These results highlight the importance of the application of CAZymes in an enzymatic treatment to improve the product recovery yield from microalgae (Phong et al. 2018).

2.3.2. Carbohydrate–Active Enzymes in Pig Nutrition

Strategies to improve feed efficiency of pigs are of particular interest as a means of increasing environmental as well as economic sustainability (Clark and Tilman 2017). Dietary supplementation of pig diets with exogenous CAZymes has been suggested as a strategy to increase nutrient digestibility and improve feed efficiency in grow-finisher pigs (Torres-Pitarch et al. 2019). Feed enzymes are substrate-specific. They target specific chemical bonds present in the undigestible components of feed ingredients, normally plant materials, converting them into substrates that can be digested by the pig (Adeola and Cowieson 2011). Plant-based diets are rich in non-starch polysaccharides (NSP) that are poorly digested by the pig's endogenous

enzymes. Nevertheless, in-feed supplementation of CAZymes (i.e. xylanase, β-glucanase, β-mannanase, α-galactosidase, cellulase, and amylase) can increase the digestibility of substrates present in the NSP fraction of the diet such as arabinoxylans, glucans, mannans or galactans among others (Bhat 2000; Torres-Pitarch et al. 2019). Thus, CAZymes were used as feed supplements on pig diets to degrade certain cereal or vegetables components, which are anti-nutritional factors (cell wall, NSP), in order to improve the nutritional value of feed (Bhat 2000; Partridge 2001).

However, the *in*-vivo response to dietary enzyme supplementation is inconsistent in grow-finisher pigs (Agyekum and Nyachoti 2017; Torres-Pitarch et al. 2019). Several studies demonstrated that the supplementation of pig diets with CAZymes promoted an increase of nutrient digestibility and growth performance with an improvement of feed efficiency (Woyengo et al. 2008; Emiola et al. 2009; Ndou et al. 2015). Woyengo et al. (2008) observed that the supplementation of growing pig diets with xylanase improved apparent ileal digestibility (AID) of several amino acids. In the study conducted by Emiola et al. (2009) was concluded that the supplementation with a mixture of xylanase, β-glucanase and cellulase improved growth performance and apparent total tract digestibility (ATTD) of, DM, gross energy (GE) and crude fiber in growing pigs and apparent ileal digestibility of nutrients in finishing pigs. Ndou et al. (2015) found that the supplementation of growing pig diets with xylanases was fundamental to improve growth performance. This authors also observed that the successful use of xylanase in improving dietary component utilization and pig growth performance is dependent on its microbial origin and dietary substrate (Ndou et al. 2015). On the contrary, other studies reported no beneficial effect of enzyme supplementation on pig diets (Willamil et al. 2012; Upadhaya et al. 2016). In the study conducted by Willamil et al. (2012) no improvement effects were observed in the parameters of productive performance of growing pigs fed with a corn-based diet supplemented with a mixture of xylanase and β-glucanase. Upadhaya et al. (2016) observed that mannanase supplementation had no influence on growth performance and nutrient digestibility of growing pigs fed with corn–soy bean meal-based diet.

Due to the inconsistent results found in the literature, Torres-Pitarch et al. (2019) performed a systematic review and meta-analysis in order to determine which exogenous CAZymes are most consistent in improving feed efficiency in grow-finisher pigs. It was hypothesized that the type of enzyme supplemented, and the cereal source used in the diet during supplementation would influence the nutrient digestibility, growth and feed efficiency response to in-feed enzyme supplementation. Therefore, enzyme type and dietary cereal source were the main explanatory variables included in the models. The mean difference effects of enzyme supplementation on ADG, ADFI, G:F, AID, ATTD of DM, CP, and GE were calculated for each study and these were used as the effect size estimates in the meta-analysis. Overall, DM and GE AiD, and ATTD were improved by xylanase, xylanase + β-

glucanase and mannanase. Dietary supplementation with xylanase alone improved ADG of corn- and co-product- based diets but had no effect on the G:F of grow-finisher pigs. Dietary supplementation with xylanase + β-glucanase had no effect on ADG, ADFI and G:F. Dietary supplementation with multi-CAZyme complexes (mixture of xylanase, β-glucanase, mannanase, cellulase, α–amylase and α-galactosidase) improved the ADG and G:F of corn-, wheat-, barley- and co-product-based diets. In conclusion, dietary supplementation with multi-CAZyme complexes improved growth and feed efficiency most consistently (Torres-Pitarch et al. 2019). Consequently, is clear that the next generation of enzymes will be those with multiple enzyme activities rather than individual enzymes. These developments will improve the cost effectiveness of enzyme addition under practical situations (Ravindran and Son 2012).

In addition to the effect on the parameters of productive performance, it is recognized that supplementation of pig diets with CAZymes can manipulate the intestinal microbiota and the digestive content characteristics, which can indirectly affect gut mucosa integrity. Thereat, several studies are focused on figuring out whether supplementation with CAZymes can also improve gut health in pigs (Kiarie et al. 2013; Z. Zhang et al. 2018).

To conclude, the dietary supplementation of diets with CAZymes can improve nutrient digestibly and directly impact on animal performance and health, being a cost-effective strategy to improve the nutritional value of cereal-based diets for finishing pigs (Ravindran and Son 2012; Torres-Pitarch et al. 2019).

2.4. Objectives

The overall aim of the current study was to explore, for the first time, the high incorporation level of one of the two microalgae with more commercial expression, *Arthrospira platensis* and *Chlorella vulgaris*, on finishing pig diets as a sustainable alternative to traditional feedstuffs. It is hypothesised that, due to the richness of microalgae in health beneficial compounds, the nutritional value of pork could be improved without impairing the production performance and the meat quality traits. However, due to the biggest drawback on the use of microalgae, its recalcitrant cell wall, mainly in pig nutrition, was necessary to develop a mixture of CAZymes able to disrupt the microalgae cell wall under study, since the effect of CAZymes that are usually added to pig feed remains to be established for microalgae biomass. In line with this, a primary objective of this study was the development of CAZyme mixtures capable of degrading the cell wall of both microalgae. The suggested most efficient microalga for pig nutrition will be selected for further *in vivo* assays. The effect of a high incorporation level of the selected microalga on finishing pigs' blood parameters and liver lipid content was also analysed, since its health and metabolic effects remains unknown.

The specific objectives of this study were as follows:

1. The development of a novel CAZyme mixture composed of as few enzymes as possible, from a large repertoire of 178 CAZymes and 22 sulfatases, with capacity to degrade *A. platensis* cell wall and release its compounds including proteins, fatty acids and pigments (chapter 3).
2. The development of a novel CAZyme mixture composed of as few enzymes as possible, from a large repertoire of 178 CAZymes and 22 sulfatases, with capacity to degrade *C. vulgaris* cell wall and release its compounds including proteins, fatty acids and pigments (chapter 4).
3. Evaluation of the effect of a high dietary incorporation level of the most promising microalga and the supplementation with the respective mixture of CAZymes developed in chapter 3 or 4 on pig production performance, feed ingestion, carcass traits, meat quality, sensory attributes of pork, meat composition in vitamin E, pigments, cholesterol, total lipids and on pork fatty acid profile and pork oxidative stability (chapter 5).
4. Assessment of the impact of the most promising microalga as feed ingredient and the supplementation with the respective CAZyme mixture developed in chapter 3 or 4 on the health and metabolic parameters of finishing pigs (chapter 6).

Chapter 3 – A TWO-ENZYME CONSTITUTED MIXTURE TO IMPROVE THE DEGRADATION OF *ARTHROSPIRA PLATENSIS* MICROALGA CELL WALL FOR MONOGASTRIC DIETS

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Contribution of Diogo Coelho to this paper:
Diogo Coelho collaborated on enzyme library construction and enzymes production. In addition, Diogo Coelho performed the enzymes incubation technique, reducing sugars measurement, HPLC methodology to quantify oligosaccharides, optical and fluorescence microscopy observations, quantification of protein, pigments and fatty acids and thermostability and proteolysis experiments. Finally, Diogo Coelho proceeded to data and statistical analysis and wrote the manuscript.

A TWO-ENZYME CONSTITUTED MIXTURE TO IMPROVE THE DEGRADATION OF *ARTHROSPIRA PLATENSIS* MICROALGA CELL WALL FOR MONOGASTRIC DIETS

Abstract

The main goal of this study was to test a rational combination of pre-selected carbohydrate-active enzymes and sulfatases, individually or in combination, in order to evaluate its capacity to disrupt *Arthrospira platensis* cell wall, allowing the release of its valuable nutritional bioactive compounds. By the end, a two-enzyme constituted mixture (Mix), composed by a lysozyme and a α-amylase, was incubated with *A. platensis* suspension. The microalga cell wall disruption was evaluated through the amount of reducing sugars released from the cell wall complemented with the oligosaccharide profile by HPLC. An increase of the amount of reducing sugars up to 2.42 g/L in microalgae treated with the Mix relative to no treatment ($p < 0.05$), as well as a 7-fold increase of oligosaccharides amount ($p < 0.001$), were obtained. With resort of fluorescence microscopy, a 36% reduction of fluorescence intensity ($p < 0.001$) was observed using Calcofluor White staining. In the supernatant, the Mix caused a 1.34-fold increase in protein content ($p = 0.018$) relative to the control. Similarly, *n*-6 polyunsaturated fatty acids (PUFA) ($p = 0.007$), in particular 18:2*n*-6 ($p = 0.016$), monounsaturated fatty acids (MUFA) ($p = 0.049$) and chlorophyll *a* ($p = 0.025$) contents were higher in the supernatant of microalgae treated with the enzyme mixture in relation to the control. Taken together, these results point towards the disclosure of a novel two-enzyme mixture able to partial degrade *A. platensis* cell wall, improving its nutrients bioavailability for monogastric diets with the cost-effective advantage use of microalgae in animal feed industry.

Key words: *Arthrospira platensis*, carbohydrate-active enzymes, cell wall, fatty acids, reducing sugars, total proteins.

3.1. Introduction

In recent years, the use of microalgae as a source of proteins, lipids, carbohydrates and other bioactive compounds has been the focus of intensive research (Chew et al. 2017), mainly directed to its use for biofuel, nutraceutical and pharmaceutical applications (Baudelet et al. 2017), as well as sustainable animal production (Lum et al. 2013). The nutritional profile of microalgae is species-specific but has, in general, contents of proteins, lipids, carbohydrates, vitamins, pigments and minerals that are comparable, if not superior, to conventional feedstuffs (Liu and Chen 2014). Microalgae are highly rich in beneficial *n*-3 long-chain polyunsaturated fatty acids (*n*-3 LCPUFA) (Madeira et al. 2017), turning microalgae into an untapped natural resource with well-known health benefits for both animals and humans (Calder 2012).

Arthrospira platensis is a filamentous microalga, classified as a blue–green alga (*Cyanophyceae*, also known as cyanobacteria) (Seyidoglu et al. 2017). The cell organization of *A. platensis* is typical of a prokaryote Gram-negative bacterium, lacking membrane-bound organelles. The cell wall constitutes an envelope composed by several layers, mostly of peptidoglycan and lipopolysaccharide nature. *A. platensis* grows naturally in alkaline lakes but is commercially produced in large outdoor or greenhouse ponds under controlled conditions (Van Eykelenburg et al. 1980; Sotiroudis and Sotiroudis 2013).

This specific microalga has been designated as a healthy food by the WHO (Seyidoglu et al. 2017) due to its content in bioactive substances (Ovando et al. 2018), in which stand out the highest protein content of any natural food (60%–70%), essential amino acids, fatty compounds, including the beneficial *n*-3 LCPUFA, and carotenoids. *A. platensis* presents several applications, mainly in food, pharmaceutical, nutraceutical, cosmetics, wastewater treatments and animal feed industries (Holman and Malau-Aduli 2013; Soni et al. 2017; Seyidoglu et al. 2017). In fact, this microalga is responsible solely for 50% of worldwide production as feed supplement (Yamaguchi 1996).

The majority of microalgae exhibit recalcitrant cell walls, largely indigestible by monogastric animals, preventing them from accessing their valuable nutritional compounds, such as proteins and lipids. For microalgae species, unlike macroalgae, the mechanical methods, such as hammer mills, are not commonly applied (Makkar et al. 2016). In turn, bead milling is a successfully, rising process in the food industry used to incorporate microalga cells as food additives. However, this mechanical process is laborious and expensive whereupon cells are massively destroyed. Therefore, it is imperative to find novel technologies, cheaper and under a strictly controlled process, to disrupt *A. platensis* cells to improve microalgal nutrient utilization, as proteins and lipids by monogastric animals (Austic et al. 2013; Lum et al. 2013). Despite *A. platensis* presents a relatively less complex cell wall, it still remains a

barrier in the use of its compounds, whereby its degradation will improve the accessibility to such compounds (Safi, Ursu, et al. 2014). This aspect is particularly relevant if microalgae are included at higher percentages in the diet, that is used as feed ingredient not as feed supplement (Madeira et al. 2017).

CAZymes are largely accepted as a class of feed additives for pigs and poultry diet formulations to surpass the negative effects of anti-nutritional factors, and to improve the digestion of dietary components and, ultimately, animal's performance (Ravindran and Son 2012). These enzymes are produced by micro-organisms and are complex enzymes, in which the catalytic module(s) is (are) appended to one or more non-catalytic CBM (Fontes and Gilbert 2010). According to circumstances, the utilization of CAZymes for microalgae biomass might represent a good strategy to value the nutritional compounds of cereal-based diets for monogastrics.

Taking into account these considerations, we hypothesized that the nutrients bioavailability of *A. platensis* could be greatly improved by using individually or combined CAZymes and sulfatases that can efficiently degrade the microalga cell wall and be used, in the long run, as feed catalysts for monogastric diets. The cell wall disruption was achieved by enzymatic treatment and assessed by optical and fluorescence microscopies, complemented with the amount of reducing sugars released and the oligosaccharide profile. The nutritional bioactive compounds were detailed by measuring proteins and pigments, as well as fatty acid profile in both supernatant and residue fractions, after incubation with the enzymatic mixture treatment.

3.2. Material and Methods

3.2.1. Microalga cultivation

To cultivate *A. platensis* (LB 2342), axenic microalga cultures from the institutes algae banks were inoculated in an adapted Krauss medium (Vonshak 1986) to stimulate *A. platensis* growth: $NaNO_3$ (250 mg/L), KH_2PO_4 (105 mg/L), $MgSO_4$ (75 mg/L), $CaCl_2$ (25 mg/L), NaCl (25 mg/L), K_2HPO_4 (75 mg/L) and 3 ml of trace metal solution: $FeCl_3$ (0.194 g/L), $CoCl_2$ (0.16 g/L), $MnCl_2$ (0.082 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.008 g/L) and $ZnCl_2$ (0.005 g/L). *A. platensis* was first grown in airlift bioreactors with 1 litre capacity and then scaled up to 25 L polyethylene bag bioreactors with bubbling filtered air, without carbon dioxide addition at low incident light conditions (150 µE m^{-2} s^{-1}), and at 34 °C, which is the optimal temperature for *A. platensis*. Once reached the stationary growth phase, the harvesting step was carried out without

flocculation by removing agitation, followed by centrifugation in a continuous centrifuge LPX 40 (Alfa Laval) (25 L). Then, the concentrated biomass slurry was frozen and freeze-dried (Powerdry LL 3000; Thermo), until analysis.

3.2.2. Recombinant enzymes: high-throughput gene synthesis, cloning and protein expression/purification

One hundred and seventy-eight CAZymes theoretically capable of disrupting *A. platensis* cell wall were selected from a vast library, comprising GH, PL and CE (Supplementary Material 1). Twenty-two sulfatases likely involved in microalgae cell wall disruption were selected for screening, as well (Gerken et al. 2013) (Supplementary Material 1). The coding genes for all of these enzymes were synthesized *in vitro* using NZYGene Synthesis kit (Nzytech). The protein sequence of each enzyme is presented as Supplementary Material 1 (Excel). Synthetic genes were codon optimized for expression in *Escherichia coli* using NZYTech´s codon optimization software ATGenium (Sequeira et al. 2017). All genes included the required 16 base pairs (bp) overhangs on both 5′ and 3′ ends for direct cloning into the bacterial expression vector pHTP1 (Nzytech), based on NZYEasy Cloning & Expression kit I (Nzytech) protocol. The generated recombinant plasmids were subjected to inducible T7 promoter control, while encoding the 200 enzymes fused to an N terminal His6 tag to allow purification using immobilized affinity chromatography (IMAC). The two hundred plasmids were sequenced to guarantee no mutations generated during gene synthesis and were used to transform *E. coli* BL21 (DE3) cells. The transformed cells were grown on solid media. The resulting colonies were used to inoculate 5 ml of NZY Auto-Induction LB medium (Nzytech, Portugal) supplemented with kanamycin (50 µg/mL) at 37 °C to early-exponential phase (absorbance λ = 600nm being 1.5–2.0). The recombinant protein was produced following a step of incubation at 25 °C during 16 hr. All steps were performed in 24-deep-well plates (Sequeira et al. 2017). Cells were harvested by centrifugation at 75,000 × *g* at 4 °C during 15 min and lysed using the NZY Bacterial Cell Lysis buffer (NZYTech). The His6-tagged recombinant enzymes were purified from cell-free extracts by IMAC, based on an automated procedure that enables the purification of 96 proteins per day, as previously reported (Saez and Vincentelli 2014). In short, the crude cell lysates were incubated with Sepharose chelating beads (200 µl with bound Ni^{2+}) and transferred to 96-well filter plates (Macherey-Nagel). Then, wells were washed 2 × with buffer A (50 mM NaHepes, pH 7.5, 500 mM NaCl, 10 mM imidazole). The recombinant proteins were eluted from the column resin beads using 200 µL of elution buffer (50 mM NaHepes, pH 7.5, 500 mM NaCl, 300 mM imidazole) into 96-deep-well plates. All steps involved in protein purification were automated on a Tecan robot (Tecan)

that contains a vacuum manifold. The homogeneity of purified proteins and the molecular mass of recombinant enzymes were evaluated by SDS-PAGE in 14% (w/v) acrylamide gels. The protein concentration of enzymes was determined spectrophotometrically by the Bradford method (Bradford 1976) and varied between from 0.5 to 20 g/L.

3.2.3. Preparation of microalga cell suspension

The concentration of *A. platensis* suspension was 20 g/L. The preparation of microalga cell suspension included a pre-wash step with phosphate-buffered saline (PBS), followed by centrifugation and resuspension of the microalgae pellet in PBS, as described by Coelho et al. (2019).

3.2.4. Enzymatic cell wall disruption

In order to disrupt *A. platensis* cell wall, the microalgae suspension was incubated with CAZymes, under strictly controlled conditions. The cell wall disruption assay was performed, according to Coelho et al. (2019).

3.2.5. Reducing sugars measurement

To quantify the amount of reducing sugars released, the 3,5-dinitrosalicylic acid (DNSA) method (Miller 1959) was used, as described by Coelho et al. (2019).

3.2.6. Thermostability and proteolysis experiments

Each enzyme composing the mixture (Mix; Provisional Patent number 20191000008190, INPI) was biochemically characterized, in particular for thermostability and proteolysis resistance. The thermostability analysis was performed, according to Coelho et al. (2019). As the temperature of incubation increased, the amount of protein in the supernatant reduced. This was validated by running 14% SDS-PAGE gels in the supernatants and visualizing the intensity of the band. The resultant images were acquired with Bio-Rad ChemiDoc XRS imaging system (Bio-Rad). To evaluate the proteolysis resistance, each enzyme was incubated with porcine pancreatin (VWR Chemicals), as described by Coelho et al. (2019). The samples were then removed and analysed by 14% SDS-PAGE gels. The proteolysis was confirmed by visualizing fragments with different molecular weights. The

resultant images were once again acquired with Bio-Rad ChemiDoc XRS imaging system (Bio-Rad).

3.2.7. Determination of total oligosaccharides

After control and Mix treatments, the profile of mono- and oligosaccharides from the supernatants of *A. platensis* was analysed and quantified by high-performance liquid chromatography (HPLC), following on a protocol developed by Coelho et al. (2019).

3.2.8. Optical and fluorescence microscopic observations

The residue fractions (pellets) from control and Mix treatments were analysed through optical and fluorescence microscopic observations. On the one hand, the optical microscopy enabled to count the number of cells in the microalgae suspension; on the other hand, the fluorescence microscopy, through fluorochrome Calcofluor White (Sigma-Aldrich) staining that binds to the cell wall (Safi, Ursu, et al. 2014), enabled to quantify fluorescence intensity. The optical and fluorescence microscopic procedures are described in detail by Coelho et al. (2019).

3.2.9. Determination of protein content

After control and Mix treatments, the nitrogen (N) content in lyophilized supernatant and residue fractions from *A. platensis* suspension, was quantified using the Kjeldahl method (984.13) (AOAC 2000). Crude protein was calculated as 6.25 × N.

3.2.10. Pigment analysis

Chlorophyll *a*, chlorophyll *b* and total carotenoids were quantified in supernatant and residue fractions from *A. platensis* suspension, after control and Mix treatments, as reported by Hynstova et al. (2018) with slight modifications as described by Coelho et al. (2019).

3.2.11. Determination of fatty acid content and composition

The fatty acid profile and content of supernatant and residue fractions of *A. platensis* suspension after control and Mix treatments were determined, as described by Coelho et al. (2019).

3.2.12. Statistical analysis

Data were analysed using the generalized linear mixed (GLM) model of the SAS software package (version 9.4; SAS Institute Inc.). All experiments were conducted in triplicate. Results are presented as mean and standard error of the mean (SEM) and considered significantly different when the *p*-value was < 0.05.

3.3. Results

3.3.1. Individual screening of enzymes in *Arthrospira platensis* cell wall disruption

Each one of CAZymes and sulfatases from our vast repertoire was incubated individually with the microalgae suspension to degrade *A. platensis* cell wall. The majority of the enzymes tested were unable to deconstruct the microalgae biomass, except 26 enzymes, as described in Table 3.1. The capacity to disrupt *A. platensis* cell wall was assessed by the amount of reducing sugars released through the DNSA method and applying the following qualitative scale (g/L): −, 0.00 < 0.005; +, 0.05 < 0.200; ++, 0.200 < 0.300; +++,>0.300. Among this set of 26 enzymes, the ones with ID 5, 14, 18, 37 to 42, 60 to 69, 78, 81, 85, to 104 and (2) 72 showed the highest amount of reducing sugars released from the biomass, whereas the others revealed a minimal or moderate capacity to attack the complex polysaccharides.

3.3.2. Composition of a two-enzyme constituted mix based on reducing sugars released

To disclose synergistic actions, the 26 enzymes presented in Table 3.1 were tested in combination for the capacity to release reducing sugars from the microalgae. From that point on, several mixtures were tested, in which enzymes were consecutively removed, according to results from DNSA method. By the end, a mixture (Mix) of two enzymes was found to be the most constrained mixture, showing the highest amount of reducing sugars released. This Mix was composed by a lysozyme (ID 104) and a α-amylase (ID (2) 72) and is presented in Table 3.1. When this mixture was incubated with A. platensis suspension, a value of 2.42 g/L (p < 0.05) of reducing sugars released was obtained, representing a 1.24-fold increase in relation to the highest value observed in the individual screening. The rates for released sugars were

calculated as: for Mix versus control = 407.3%; for Mix *versus* lysozyme = 102%, and for Mix versus α-amylase = 30.2%.

3.3.3. Thermostability and proteolysis assays

We next tested the thermostability of the two enzymes that constitute the Mix treatment, individually. The variation of protein concentration across the temperatures tested is shown in Figure 3.1. For the internal temperature of mammals and poultry which are, respectively, 37 °C and 40 °C, all enzymes maintained their stability. However, the stability of ID 104 decayed from 65 °C upward, while ID (2) 72 remained stable up to 80 °C. Next, the same enzymes were treated with pancreatin at 37 °C to test their capacity to resist to proteolytic attack in the animal gastrointestinal tract. The proteolytic resistance scores of these enzymes are shown in Table 3.2. Enzyme ID (2) 72 displayed partial resistance along the entire assay; in contrast, ID 104 showed a complete degradation after 15 min of incubation (Table 3.2).

Table 3.1. Screening of the selected individual CAZymes – sulphatases and Mix in *Arthrospira platensis* cell wall disruption.

ID	Name	Category	E.C	Main Substrate	Reducing sugars released scale
5	Cellulose 1,4-β-cellobiosidase	Cellobiohydrolases	3.2.1.91	Phosphoric acid-swollen cellulose, Avicel and others forms of insoluble cellulose	+++
10	Laccase	Laccases	1.3.3.5	2,20-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	++
14	Laminarinase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans such as laminarin	+++
16	Chitinase 1	Chitinases & Chitosanases	3.2.1.14	Chitin and chitosan	++
18	Oligoalginate lyase	Alginate lyases	4.2.2.	Low-viscosity alginate	+++
25	β-1,3-1,4-glucanase P2	1,3-1,4-β-Glucanases	3.2.1.73	1,3-1,4-β-glucans	+
33	β-1,3-glucanase / laminarinase	1,3-β-Glucanases	3.2.1.39	Laminarin	++
36	Chitosanase	Chitinases & Chitosanases	3.2.1.132	Chitosan	+
37	Endo-β-2,6-fructanase	Fructanases	3.2.1.65	Levans	+++
38	Cellobiohydrolase	Cellobiohydrolases	3.2.1.91	Amorphous and crystalline cellulose	+++
42	Trans-sialidase B	Sialidases	3.2.1.18	Sialic acids from complex carbohydrates and glycoprotein human alpha-1 (AGP)	+++
50	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	+
60	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	The 1,4-β-glycosidic bond of cellooligosaccharides, also hydrolysis nonreducing end of chitooligosaccharides (Glc-PNP)	+++
66	Alginate lyase	Alginate lyases	4.2.2.3	Polyguluronate and polymannuronate	+++
69	α-1,3-glucanase	α-Glucosidases	3.2.1.59	1,3-α-glucan	+++
73	Exo-β-agarase D	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	+
78	Keratan sulfate hydrolase / keratanase II	Acetylglucosaminidases	3.2.1.103	Cartilage keratan sulfate and cornea keratan sulfate	+++
81	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	Lactose, GlcNAc2, GlcNAc3, cellobiose and cellotriose, as well as colloidal chitin, cellulose, lichenan, laminarin and xylan	+++
82	β-1,3-glucanase B	Laminarinases	3.2.1.39	Insoluble 1,3-β-glucan	+

85	β-galactosidase	β-Galactosidases	3.2.1.23	β-galactosides	+++
86	Lytic transglycosylase	Peptidoglycan lytic exotransglycosylases	4.2.2.n1	1,4-β-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine residues in the cell wall peptidoglycan, producing 1,6-anhydromuropeptides	+++
92	Endo-rhamnogalacturonan lyase	Rhamnogalacturonan lyases	4.2.2.23	Rhamnogalacturonan	+++
93	Peptidoglycan N-acetylmuramic acid deacetylase	Acetylglucosamine deacetylases	3.5.1.104	Peptidoglycan	+++
95	Lysozyme	Lysozymes	3.2.1.17	Peptidoglycans	+++
104	Lysozyme	Lysozymes	3.2.1.17	Peptidoglycans	+++
(2)72	α-amylase	Amylases	3.2.1.1	Endohydrolysis of 1-4-α-D-glucosidic linkages in polysaccharides containing three or more 1-4-α-linked D-glucose units	+++
Mix	**Lysozyme 104 + α-amylase (2) 72**				2.42 g/L

For each enzyme, is presented the ID, the name, the category, the Enzyme Commission (E.C) number, the main substrate and a qualitative scale of reducing sugars released. It is also presented the enzymatic constitution of the Mix as well as the value of the reducing sugars released in g/L. Qualitative scale on the amount of reducing sugars released (g/L): −, <0; +, 0.05 < 0.2; ++, 0.2 < 0.3; +++,>0.3.

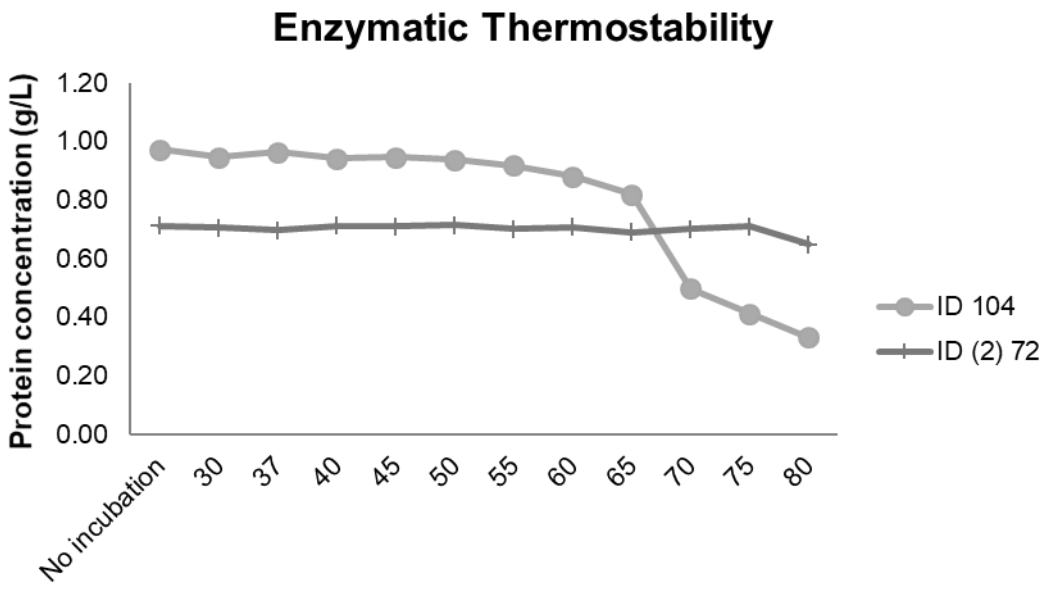

Figure 3.1. Thermostability characterization of the two enzymes constituting the Mix at different temperatures (30–80 °C) and for the control without incubation.

Table 3.2. Proteolysis resistance for each one of the two enzymes that constitute the Mix.

ID	Time				
	15 min	**30 min**	**60 min**	**90 min**	**120 min**
104	-	-	-	-	-
(2) 72	+	+	+	+	+

Each enzyme, at a concentration of 1 g/L, was subjected to the proteolytic action of pancreatin, which was incubated at a final concentration of 2.5 g/L. The reactions were incubated at 37°C, at regular intervals of 15 min for 120 min. Results are presented at periods of 15, 30, 60, 90 and 120 min of incubation for each enzyme. The qualitative scale on proteolysis resistance is based on SDS-PAGE gels visualisation: -, no resistant (only fragmentation bands); +, partially resistant (protein and fragmentation bands).

3.3.4. Effect of mix treatment on *Arthrospira platensis* cell number and cell wall integrity

The number of cells was kept unchanged between control and Mix (Figure 3.2 A, $p > 0.05$) and was approximately 16,000 cells for both treatments (Figure 3.2 B and C). When *A. platensis* was incubated with the Mix (Figure 3.2 F), the fluorescence intensity was diminished by 36% (Figure 3.2 D, $p < 0.001$), relative to the control (Figure 3.2 E).

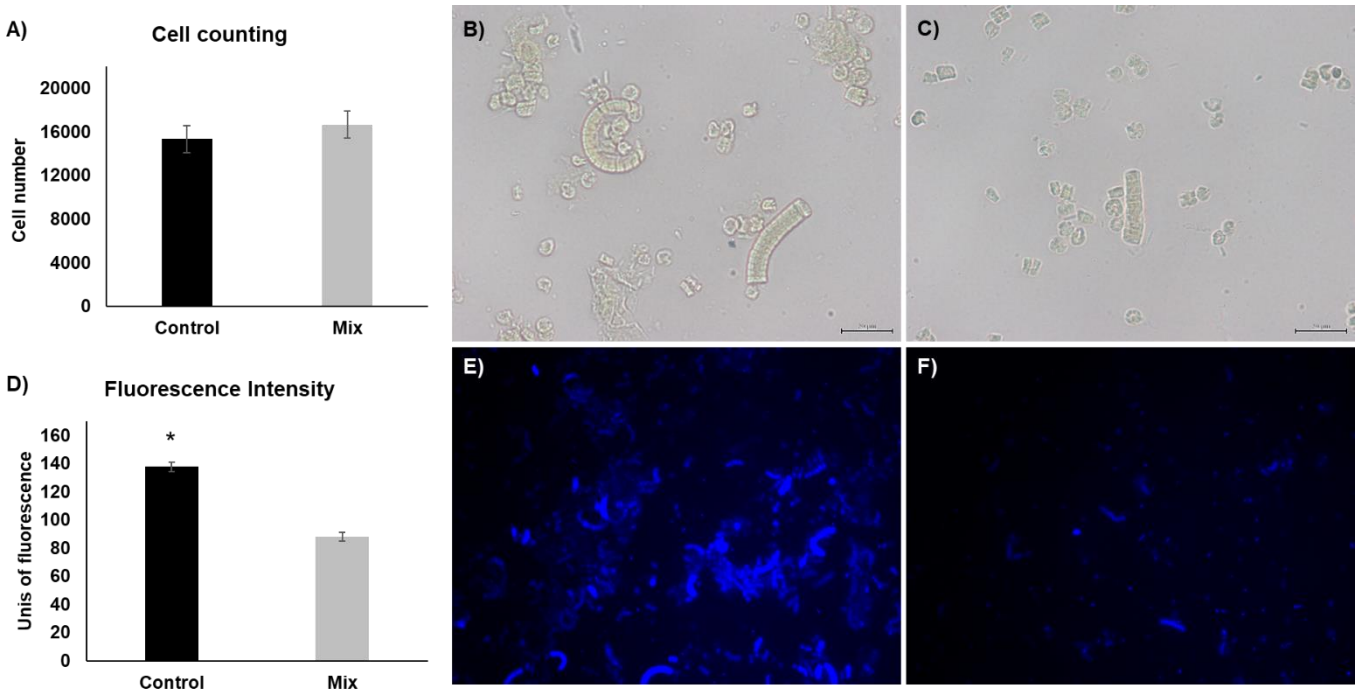

Figure 3.2. A. Cell counting using a Neubauer chamber for control and Mix treatments. B and C. Light microscopy images (×400) of *Arthrospira platensis* suspension for control and Mix treatments, respectively (scale bar: 20 µm). D. Fluorescence intensity derived from Calcofluor White staining for control and Mix treatments. Asterisk denotes statistical difference at $p < 0.001$. E and F. Fluorescence images (×400) of *A. platensis* suspension stained with Calcofluor White for control and Mix treatments, respectively.

3.3.5. Effect of mix treatment on the release of oligosaccharides from *Arthrospira platensis* cell wall

In the oligosaccharides region, three large peaks were detected in the Mix treatment chromatogram (Figure 3.3 B), compared to the control (Figure 3.3 A), which corresponds to a 7-fold increase on the oligosaccharides content ($p < 0.001$; Figure 3.3 C).

3.3.6. Effect of mix treatment on the release of proteins

In order to verify if the enzyme mixture favoured the release of proteins from *A. platensis* cells to the exterior, the amount of protein was quantified in supernatants and residues (Table 3.3). In the supernatant fraction, the Mix treatment led to a 1.34-fold increase in protein content when compared to the control ($p = 0.018$). In the residue fraction, the Mix treatment caused a 1.14-fold reduction when compared to the control ($p = 0.003$).

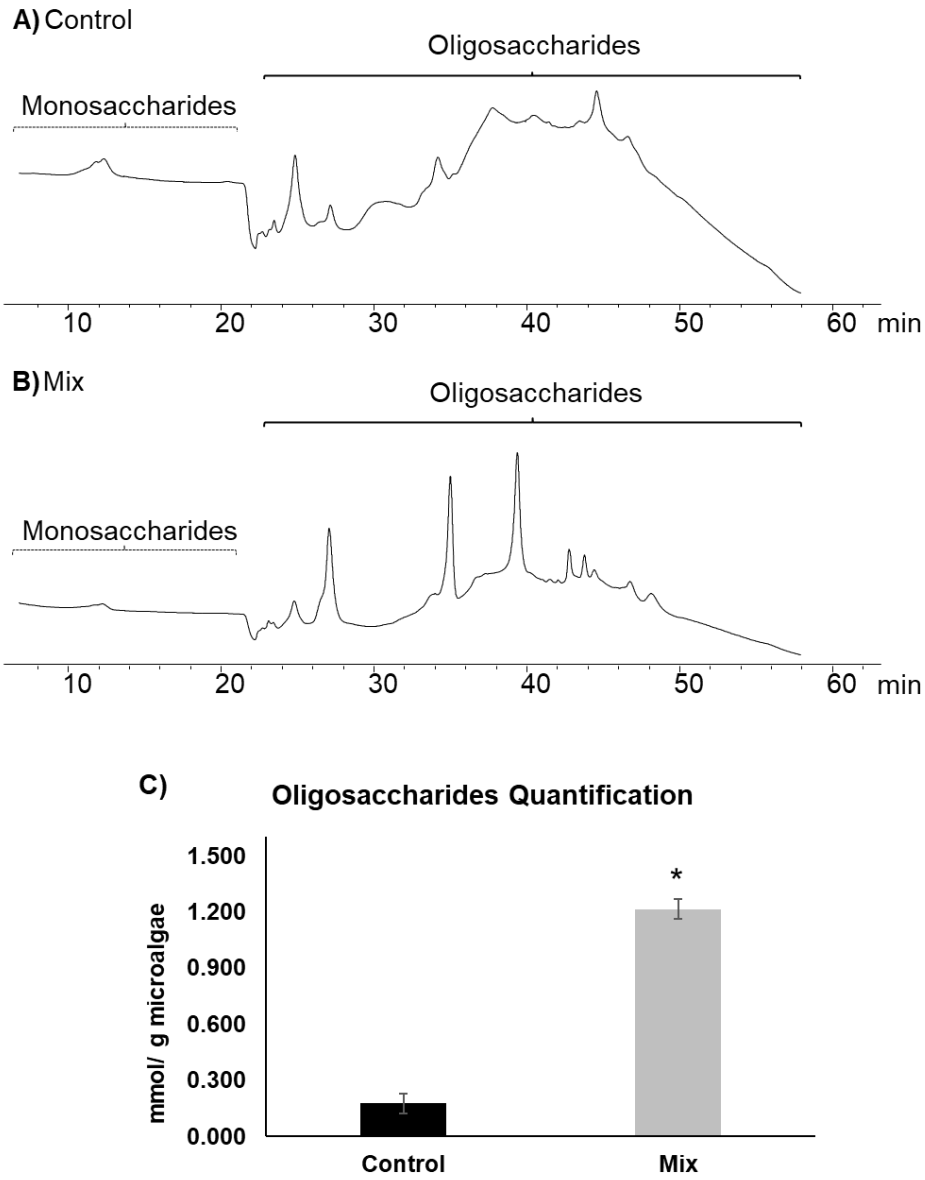

Figure 3.3. Illustrative chromatograms obtained by HPLC analysis of supernatants for the control (A) and the Mix (B) treatments. Monosaccharides and oligosaccharides regions are shown. The quantification of oligosaccharides is graphically displayed in C. Asterisk denotes statistical difference at $p < 0.001$.

3.3.7. Effect of mix treatment on the release of chlorophylls and carotenoids

Applying the same rationale as the previous point, the release of pigments from *A. platensis* cells to the exterior was quantified in supernatants and residues (Table 3.3). Chlorophyll *a* displayed a 1.15-fold increment in the supernatant fraction of the Mix treatment relative to the control ($p = 0.025$), whereas in the residue fraction the Mix treatment led to a 1.24-fold increase relative to the control ($p = 0.017$). Chlorophyll *b*, total chlorophylls and total carotenoids did not vary ($p > 0.05$).

3.3.8. Effect of mix treatment on the release of fatty acids

The fatty acid content and profile after incubation with the enzyme mixture was determined in supernatants and residues to verify if the Mix treatment promoted the beneficial release of fatty acids from *A. platensis* cells to the exterior (Table 3.3). The prevalent fatty acids were saturated SFA > PUFA > *n*-6 PUFA > MUFA > *n*-3 PUFA in both fractions. In the supernatant fraction, 16:0, SFA, total FA content and 17:0 were increased in the control relative to the Mix ($p = 0.002$, $p = 0.011$, $p = 0.016$, $p = 0.030$, respectively). In opposition, PUFA, *n*-6 PUFA, 18:2*n*-6, 18:3*n*-6, 22:2*n*-6 and MUFA increased in the Mix relative to the control ($p = 0.005$, $p = 0.007$, $p = 0.016$, $p = 0.040$, p = 0.040 and $p = 0.049$, respectively). In the residue fraction, the Mix treatment led to higher proportions of 22:2*n*-6, 18:2*n*-6, 18:3*n*-3, *n*-3 PUFA and 14:1*c*9 ($p = 0.003$, $p = 0.013$, $p = 0.032$, $p = 0.032$ and $p = 0.049$, respectively), and to a lower proportion of total FA ($p = 0.009$) in comparison to the control.

Table 3.3. Content of protein, chlorophyll, carotenoids and fatty acids of the supernatant and residue fractions derived from incubation of *Arthrospira platensis* with control and Mix treatments.

	Supernatant				Residue			
	Control	**Mix**	**SEM**	***p*-value**	**Control**	**Mix**	**SEM**	***p*-value**
Total protein (mg/g microalgae)	412	554	26.0	0.018	669	586	8.81	0.003
Chlorophyll *a* (mg/g microalgae)	0.454[1]	0.520[1]	0.016	0.025	6.10[2]	7.60[2]	0.324	0.017
Chlorophyll *b* (mg/g microalgae)	2.02[1]	2.05[1]	0.039	0.645	0.355[2]	0.520[2]	0.062	0.111
Total chlorophylls (mg/g microalgae)	2.48[1]	2.57[1]	0.054	0.274	6.46[2]	8.71[2]	0.725	0.071
Total carotenoids (mg/g microalgae)	0.162[1]	0.209[1]	0.017	0.102	3.04[2]	2.73[2]	0.164	0.218
Total fatty acids (mg/g microalgae)	4.27	3.63	0.136	0.016	46.7	41.8	0.910	0.009

Fatty acid composition (% total fatty acids)								
12:0	0.345	0.605	0.112	0.154	0.089	0.078	0.013	0.560
14:0	1.87	1.31	0.193	0.087	1.27	1.33	0.059	0.526
14:1*c*9	nd	nd	-	-	0.372	0.408	0.010	0.049
15:0	0.340	0.260	0.043	0.235	0.040	0.047	0.010	0.619
16:0	46.0	42.7	0.423	0.002	41.3	41.3	0.216	0.978
16:1*c*7	0.665	0.724	0.051	0.454	1.51	1.51	0.014	0.985
16:1*c*9	2.84	3.16	0.104	0.077	5.23	5.09	0.047	0.074
17:0	1.28	0.983	0.073	0.030	0.341	0.407	0.041	0.294
18:0	21.6	19.9	1.23	0.378	3.10	2.74	0.288	0.413
18:1*c*9	4.35	6.20	0.643	0.089	2.43	2.23	0.138	0.332
18:1*c*11	0.469	0.704	0.163	0.348	0.236	0.265	0.056	0.730
18:2*n*-6	9.06	10.4	0.284	0.016	18.4	18.8	0.086	0.013
18:3*n*-6	7.42	8.31	0.242	0.040	24.7	24.6	0.124	0.751
18:3*n*-3	0.141	0.345	0.062	0.058	0.090	0.106	0.004	0.032
20:0	1.18	1.18	0.087	0.964	0.202	0.224	0.040	0.709
22:0	1.58	1.81	0.105	0.182	0.181	0.209	0.011	0.111
22:2*n*-6	0.866	1.40	0.145	0.040	0.066	0.103	0.005	0.003
Others	0.068	0.062	0.065	0.952	0.436	0.479	0.037	0.439
∑ SFA	74.1	68.7	1.06	0.011	46.5	46.4	0.283	0.669
∑ MUFA	8.33	10.8	0.708	0.049	9.78	9.50	0.185	0.318
∑ PUFA	17.5	20.4	0.484	0.005	43.3	43.7	0.187	0.163
∑ *n*-3 PUFA	0.141	0.345	0.062	0.058	0.090	0.106	0.004	0.032
∑ *n*-6 PUFA	17.3	20.1	0.491	0.007	43.2	43.6	0.188	0.179

Two mL of microalgae suspension was incubated with the two enzymes, which constitute the Mix, at a final concentration of 20 mg/L for each enzyme. The control treatment took the same amount of PBS. Incubations were done overnight at 37 °C and 140 rpm. After incubations, supernatant and residue fractions were separated by centrifugation. Only fatty acids whose percentage was > 0.25% are presented; nd – not detected.

[1]Values measured in phosphate-buffered saline (PBS).

[2]Values measured after extraction with acetone.

3.4. Discussion

In this work, a vast repertoire of 178 CAZymes and 22 sulfatases was created by recombinant expression in *E. coli* cells to assess the hypothesis that nutritional bioactive compounds availability of *A. platensis* may be enhanced after disruption of its cell wall. These 200 enzymes were chosen based on the composition of matrix polysaccharides of microalgae cell walls, which includes pectin, chitin agar, alginates and the aliphatic polymer algenan

(Scholz et al. 2014), in addition to the cyanobacterium peptidoglycan (Palinska and Krumbein 2000; Sotiroudis and Sotiroudis 2013). The chosen enzymes were produced on a high-throughput (HTP) system that includes several steps, from gene synthesis, gene cloning, protein expression to purification. These enzymes were screened, one by one, to disrupt *A. platensis* cell wall, by measuring the amount of reducing sugars released. In the following phase, the 26 recombinant enzymes able to partially or entirely disrupt *A. platensis* cell wall (Table 3.1) were combined and tested to achieve the maximum degradation of *A. platensis* cell wall. A two-enzyme mixture (Mix) was found to be the most confined combination with the highest activity towards the disruption of *A. platensis* cell wall, and applied in subsequent steps. It was constituted by two well characterized recombinant glycosylases, a lysozyme (EC 3.2.1.17) and a α-amylase (EC 3.2.1.1). Lysozyme belongs to the family 24 of GH, according to the CAZy database (Cantarel et al. 2009). The enzyme-coding gene was obtained from *E. coli* (Srividhya and Krishnaswamy 2007) and has peptidoglycan, containing muramic acid δ-lactam, as the main substrate (Srividhya and Krishnaswamy 2007; Babu et al. 2018). α-amylase was firstly characterized by Liebl et al. (1997) and, according to the CAZy database, belongs to the family 13, subfamily 36, of GH (Cantarel et al. 2009). The enzyme-coding gene was obtained from the aquatic hyperthermophilic *Thermotoga maritima*, and its main substrates comprise various α-glucans, such as amylose, amylopectin and glycogen (Liebl et al. 1997).

It has been shown that cell walls of Gram-negative bacteria, containing peptidoglycan, are susceptible to lysozyme, as appears to be the case of *A. platensis* (Van Eykelenburg et al. 1980; Sotiroudis and Sotiroudis 2013). Mehta et al. (2015) developed a complete lysis technique, which included the incorporation of a lysozyme using different cyanobacterial strains. Aikawa et al. (2013) observed that when lysozyme was added to the fermentation medium, the bioethanol production yield reached 86% of the theoretically expected amount, since lysozyme degraded *A. platensis* cell walls. Pyo et al. (2013) performed the extraction of bioethanol from two fresh water Gram-negative cyanobacteria species, *Microcystis aeruginosa* and *Anabaena variabilis* with similar peptidoglycan cell wall layers (Thiel et al. 2014; Sun et al. 2016), which resemble *A. platensis*. In the same study, the authors used an enzyme mixture composed of three enzymes, including a α-amylase to hydrolyse the cyanobacteria (Pyo et al. 2013). This finding was also supported by Carrillo-Reyes et al. (2016). The starch in cyanobacteria is a highly branched α-1,4-polyglucan, denominated as cyanophycean starch (Pulz and Gross 2004), deeply located in the cyanobacterial cell wall with an irregular whitish spherical form (Lang 1968; Pyo et al. 2013). In turn, *A. platensis* has a low content of internal energy storage, as starch due to high activities of α- and β-amylase. The *in vitro* digestibility of *A. platensis* has been reported using an amylase (Usharani et al. 2012; Pyne et al. 2017).

Bearing those former observations in mind, that clearly establish a link between *A. platensis* cell wall composition and the degrading enzymes identified in this study, the enzymatic composition of the Mix described is in line with the cell wall composition of this microalga.

The two enzymes constituting the mixture were characterized individually for thermostability and proteolysis resistance. ID (2) 72 remained stable throughout the temperature range and resistant to the proteolytic attack of pancreatin. The tertiary structure of protein, which provides thermotolerance to enzymes, may provide inherent proteinase resistance, as reported by Fontes et al. (1995). The high thermotolerance, which characterizes this enzyme, is due to the circumstance that it is isolated from *Thermotoga maritima*, one of the most thermophilic bacteria presently known, with maximum growth temperature at 90 °C (Huber et al. 1986; Liebl et al. 1997; Singh et al. 2017). In opposition, ID 104 was sensitive to temperature increase and proteolysis.

The Mix was proven capable at disrupting *A. platensis* cell wall through the increase of 1.24-fold in reducing sugars relative to the highest individual value found, suggesting a synergistic action between these enzymes when combined, as demonstrated by Phong et al. (2018), when degrading diverse carbohydrate mixtures. Pyo et al. (2013) also selected the release of reducing sugars to assess the ability of different methods, including the enzymatic method, to hydrolyse the two species of Gram-negative cyanobacteria above mentioned. Markou et al. (2013) used different acids at different concentrations to hydrolyse *A. platensis* and quantified the outcome through the measurement of reducing sugars released. A higher amount of reducing sugars released corresponds to a higher hydrolysis yield (Markou et al. 2013; Pyo et al. 2013), which is in agreement with results obtained in our study.

The *A. platensis* cell number was not changed by the enzyme mixture. Contrarily, the fluorescence intensity reduced 36% after the Mix treatment, suggesting that the cell wall integrity was affected to a considerable extent. Safi et al. (2014) applied the same fluorochrome when testing various cell wall disruption methods (like, high pressure and ultrasonication). They concluded that after treatment a variation on fluorescence intensity was observed suggesting a clear change in cell wall structure, justifying the use of Calcofluor White staining. This evidence was reinforced by a 7-fold augment of oligosaccharides content after the enzyme mixture treatment, as observed by Heo et al. (2017). These same authors, and contrarily to our findings, reported a large increase on glucose amount in a different species of microalga, *Chlorella vulgaris*, after osmotic shock suggesting the complete disruption of the cell wall. Contrarily, in our study, no complete degradation of carbohydrates from the cell wall was obtained, since a complex mixture of oligosaccharides rather than single sugars, was observed. In addition, Leal et al. (2017) observed that the implementation of an acidic method to hydrolyse microalgae/cyanobacteria cell walls led to a high release of oligosaccharides to obtain prebiotic oligosaccharides from *A. platensis* biomass via phosphoric acid hydrolysis.

The release of cytoplasmic (hydro-) soluble proteins from *A. platensis* cell wall was observed after the enzymatic mixture treatment. This result was naturally followed by a decrease of protein content in the residue, which was expected. These findings concur with Safi, Ursu, et al. (2014), even if different mechanical and chemical cell wall disruption methods were applied. In addition, Lupatini et al. (2017) reported different methods of cell wall disruption, including enzymatic, as capable of promoting the extraction of microalgae proteins from *A. platensis*. The results obtained in our study assume particular relevance due to the high protein content of *A. platensis* (60%–70%) (Soni et al. 2017), re-enforcing its great value as feed ingredient for animal production and human health (Holman and Malau-Aduli 2013; Lupatini et al. 2017).

Besides proteins, the enzymatic mixture treatment released chlorophyll *a* to the supernatant. *A. platensis* contains relevant amounts of chlorophylls, about 1%–1.5% (Jiménez et al. 2003; Leema et al. 2010), which are located within thylakoid bundles circling the peripheral part of the cytoplasm with their associated structures, the phycobilisomes (containing the phycobiliproteins) on the surface of the thylakoids (Safi, Ursu, et al. 2014). In addition to phycocyanin (30%) (Cisneros and Rito-Palomares 2004; Leema et al. 2010), *A. platensis* also displays an appreciable content of other pigments, like carotenoids (0.2%–0.35%), including β-carotene and lutein (Jiménez et al. 2003; Leema et al. 2010). Notwithstanding, no changes were detected for chlorophyll *b* and total carotenoids. Previous studies reported the successful disruption of the cell wall by several chemical and mechanical methods on the release of chlorophylls and carotenoids to the supernatant (Safi et al. 2015), and therefore concur with ours for chlorophyll *a*. We speculate that the cell wall degradation promoted by the Mix treatment favoured the release of chlorophyll *a* to the external aqueous medium due to the more hydrophilic nature of the chlorophyll molecule, which contains a hydrophilic part, compared with the hydrophobicity displayed by carotenoids (Schoefs 2002).

The fatty acid content and detailed profile presented here for *A. platensis* cells are in agreement with other studies (Batista et al. 2013; Bellou et al. 2014), despite the enzymatic treatment. Several authors applied different methodologies to extract and analyse the lipid fraction of *A. platensis* (Andrich et al. 2006; Mendes et al. 2006). In none of the aforementioned studies, the authors considered the presence of the cell wall. In parallel studies with *C. vulgaris* microalga, a considerable enzymatic cell wall degradation was reported using cellulases and β-glucosidases (Cho et al. 2013), or snailase, lysozyme and cellulose (Zheng et al. 2011) combined. In any case, the enzymatic treatment led to successful lipid extraction efficiency. Herein, our goal was not on whether the Mix led to improved lipid extraction efficiency, but instead, on the release of fatty acids from *A. platensis* to the extracellular space, through the partial or complete degradation of microalgae cell wall. Most differences were found for MUFA and some *n*-6 PUFA, particularly 18:2*n*-6 and 18:3*n*-6, when *A. platensis* was incubated with

the enzymatic mixture treatment, justifying a higher proportion of these fatty acids in the corresponding supernatant. The increased release of *n*-6 PUFA, but not of SFA, in both residue and supernatant fractions, when applying this two-enzyme constituted mixture, points out to future work due to their potential application in animal feed industry (Bellou et al. 2014).

3.5. Conclusion

Herein, we report the disclosure of a novel two-CAZyme constituted mixture capable of efficiently degrade *A. platensis* microalga cell wall, thus allowing the release of trapped bioactive compounds with important nutritional value. Our findings set the opportunity to use feed catalysts for monogastric diets incorporated with microalgae, in particular with *A. platensis*, as feed ingredient. In addition, biotechnological applications, like those associated with biofuel, cosmetics and nutraceutical, are also envisaged. Animal trials are currently in progress to assess: (a) how essential really is α-amylase, knowing that this enzyme is endogenously produced by monogastrics; (b) how capable this two-enzyme constituted mixture actually is when using *A. platensis* microalga as feed ingredient (10%–15% of diet weight).

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Chapter 4 – NOVEL COMBINATION OF FEED ENZYMES TO IMPROVE THE DEGRADATION OF *CHLORELLA VULGARIS* RECALCITRANT CELL WALL

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Contribution of Diogo Coelho to this paper:

Diogo Coelho collaborated on enzyme library construction and enzymes production. In addition, Diogo Coelho performed the enzymes incubation technique, reducing sugars measurement, HPLC methodology to quantify oligosaccharides, optical and fluorescence microscopy observations, quantification of protein, pigments and fatty acids and thermostability and proteolysis experiments. Finally, Diogo Coelho proceeded to data and statistical analysis and wrote the manuscript.

NOVEL COMBINATION OF FEED ENZYMES TO IMPROVE THE DEGRADATION OF *CHLORELLA VULGARIS* RECALCITRANT CELL WALL

Abstract

In this study, a rational combination of 200 pre-selected CAZymes and sulfatases were tested, individually or combined, according to their ability to degrade *Chlorella vulgaris* cell wall to access its valuable nutritional compounds. The disruption of microalgae cell walls by a four-CAZYme mixture (Mix) in comparison with the control, enabled to release up to 1.21 g/L of reducing sugars ($p < 0.001$), led to an eight-fold increase in oligosaccharides release ($p < 0.001$), and reduced the fluorescence intensity by 47% after staining with Calcofluor White ($p < 0.001$). The Mix treatment was successful in releasing proteins ($p < 0.001$), some MUFA ($p < 0.05$), and the beneficial 18:3*n*-3 fatty acid ($p < 0.05$). Even if no variation was detected for chlorophylls ($p > 0.05$), total carotenoids were increased in the supernatant ($p < 0.05$) from the Mix treatment, relative to the control. Taken together, these results indicate that this four-CAZYme mixture displays an effective capacity to degrade *C. vulgaris* cell wall. Thus, these enzymes may constitute a good approach to improve the bioavailability of *C. vulgaris* nutrients for monogastric diets, in particular, and to facilitate the cost-effective use of microalgae by the feed industry, in general.

4.1. Introduction

Autotrophic microalgae are currently considered an attractive source of high-value chemicals for biofuel, nutraceutical and pharmaceutical industries (Baudelet et al. 2017), as well as sustainable animal production (Lum et al. 2013). While the nutritional profile of microalgae varies considerably with the species, a large majority are characterised by having high protein, carbohydrate, lipid, vitamin, mineral and pigment contents (Liu and Chen 2014), which are comparable, if not superior, to conventional feedstuffs. These alternative feedstuffs are rich in beneficial *n*-3 LCPUFA (Madeira et al. 2017). The enriched concentration of *n*-3 LCPUFA by microalgae represents a largely untapped natural resource with well-known beneficial health implications for both animals and humans (Calder 2012).

Chlorella vulgaris, a freshwater unicellular eukaryotic microalga, is one of the most cultivated microalgae worldwide. Although it is known for its relative ease of cultivation and high biomass productivity (Kotrbáček et al. 2015), *C. vulgaris*, like the majority of microalgae, is endowed with a recalcitrant cell wall that confers resistance against invaders and harsh environmental conditions such as desiccation during growth, and is therefore refractory to breakage and drying, and hence to product extraction (Acton 2013). These cell walls have shown to contain an incredibly diverse and complex matrix of cross-linked insoluble carbohydrates, which trap valuable nutrients, thus limiting their direct use. The cell wall structure and composition were recently reviewed by Baudelet et al. (2017) for *Chlorellae* genus, and Safi, Zebib, et al. (2014) for *Chlorella* species.

Due to their recalcitrant nature, microalgal cell walls are largely indigestible by monogastric animals. For microalgae species, contrary to macroalgae, mechanical methods, as hammer mills, are not usually applied (Makkar et al. 2016). In turn, bead milling is used to incorporate *Chlorella* cells as food additives and this is a successfully, rising process in the food industry. However, this mechanical process is characterised as being hard working and expensive whereupon cells are massive destroyed. It is therefore imperative to find novel technologies to disrupt *Chlorella vulgaris* cells whereby cell wall disruption would be under a strictly controlled process to improve microalgal nutrient utilization, in particular to gain access to their protein and lipids (Austic et al. 2013; Lum et al. 2013). Another essential prerequisite for the large-scale use of algal biomass as a feed supplement is to achieve a low production cost (Kotrbáček et al. 2015). In addition, *Chlorella* is also considered as a potential source of microalgae oils for biofuel production and recognised as one of the alternatives to current biofuel crops, such as soybean, corn, rapeseed and lignocellulosic feedstock because it does not compete with food and does not require arable lands to grow (Singh et al. 2011). The exploitation of biofuel production by *Chlorella* is thus attracting considerable attention. *Chlorella* has the ability to fix carbon dioxide efficiently and to remove nutrients rich in nitrogen

and phosphorous, making it a good candidate for greenhouse gas biomitigation and wastewater bioremediation (Liu and Chen 2014).

Exogenous CAZymes, mainly xylanases and β-glucanases, are now widely used to supplement diets of monogastric livestock species to improve feed nutritive value and directly impact on animal performance and health (Ravindran and Son 2012). The use of feed enzymes is currently a cost-effective strategy to improve the nutritional value of cereal-based diets for monogastric animals, although it remains to be established for microalgae biomass. In line with this, we hypothesised that the efficiency of *C. vulgaris* microalgae could be fine-tuned using individually or combined CAZymes and sulfatases, due to the degradation of recalcitrant cell wall and subsequent increase in nutrients bioavailability. Herein, cell disruption induced by enzymatic treatment was assessed by optical and fluorescence microscopies, and by measuring the reducing sugars and the oligosaccharides profile. The release of bioactive compounds with nutritional interest was assessed by quantifying proteins and pigments, as well as the fatty acid content and detailed composition, in both supernatant and residue fractions after incubation with the enzymatic treatment.

4.2. Material and Methods

4.2.1. Microalga production

Chlorella vulgaris is an unicellular freshwater microalgae of the genus *Chlorella* characterised by a relative ease of cultivation, high productivity and high content of proteins, lipids and other valuable components (Kotrbáček et al. 2015). It has emerged as a promising alternative feedstock that represents an enormous biodiversity with multiple benefits exceeding the potential of conventional agricultural feedstock (Safi, Zebib, et al. 2014).

C. vulgaris was cultivated through inoculation of axenic microalgal cultures (from the Institutes algal banks) in a medium that stimulates the growth of *C. vulgaris*: $NaNO_3$ (250 mg/L), KH_2PO_4 (105 mg/L), $MgSO_4$ (75 mg/L), $CaCl_2$ (25 mg/L), NaCl (25 mg/L), K_2HPO_4 (75 mg/L), and 3 mL of trace metal solution: $FeCl_3$ (0.194 g/L), $CoCl_2$ (0.16 g/L), $MnCl_2$ (0.082 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.008 g/L) and $ZnCl_2$ (0.005 g/L), using the adapted Krauss medium (Vonshak 1986).

C. vulgaris was first grown in 1 L capacity airlift bioreactors and then scaled-up until 25 L capacity polyethylene bag bioreactors (40 cm diameter) with bubbling filtered air (without CO_2 addition), at low incident light conditions (150 $\mu E.m^{-2}.s^{-1}$), and at the optimal temperature of 25 °C for *C. vulgaris*. The harvesting step was done after reaching the stationary growth phase. Microalgal biomass was harvested without flocculation by simply removing agitation,

followed by centrifugation in a continuous centrifuge LPX 40 (Alfa Laval, Sweden) (25 L). The concentrated biomass slurry was then frozen at −20 °C and freeze dried (Powerdry LL 3000, Thermo, Denmark) for further analysis.

4.2.2. High-throughput gene synthesis, cloning and protein expression/purification of recombinant enzymes

One-hundred and seventy-eight CAZymes with high potential for degradation of microalgae cell wall were selected from a diverse repertoire, including GH, pectate lyases PL and CE (Supplementary Material 1) In addition, twenty-two sulfatases were also selected for screening, as they are also likely involved in microalgae cell wall degradation (Gerken et al. 2013) (Supplementary Material 1). The coding genes for the selected enzymes were synthesised *in vitro* using NZYGene Synthesis kit (Nzytech, Portugal). The protein sequence of each enzyme is presented as Supplementary Material 1 (Excel). Synthetic genes were codon optimised for expression in *E. coli*, using NZYTech′s codon optimization software ATGenium (Sequeira et al. 2017). All genes included the required 16 bp overhangs on both 5′ and 3′-ends for direct cloning into the bacterial expression vector pHTP1 (Nzytech, Portugal), following the procedure described in the NZYEasy Cloning & Expression kit I (Nzytech, Portugal). The generated recombinant plasmids were subjected to inducible T7 promoter control, while encoding the 200 enzymes fused to an N-terminal His6-tag that facilitates purification through IMAC. The two-hundred plasmids were sequenced to ensure that no mutations accumulated during gene synthesis and were used to transform *E. coli* BL21 (DE3) cells. Transformed cells were grown on solid media and resulting colonies were used to inoculate 5 mL of NZY Auto-Induction LB medium (Nzytech, Portugal) supplemented with kanamycin (50 mg/L) at 37 °C to early-exponential phase (absorbance λ = 600nm being 1.5–2.0). Recombinant protein production occurred following a further incubation at 25 °C for 16 hours. All steps were carried out in 24 deep-well plates (Sequeira et al. 2017). Cells were harvested by centrifugation at 75,000 × *g* at 4 °C for 15 min and lysed in NZY Bacterial Cell Lysis Buffer (NZYTech, Portugal). The His6-tagged recombinant enzymes were purified from cell-free extracts by IMAC, based on an automated protocol that allows the purification of 96 proteins by day, as described previously (Saez and Vincentelli 2014). Briefly, the crude cell lysates were incubated with Sepharose chelating beads (200 µL with bound Ni^{2+}) and then transferred into 96-well filter plates (Macherey-Nagel). The wells were washed twice with buffer A (50 mM Na-HEPES, pH 7.5, 500 mM NaCl, 10 mM imidazole). The recombinant fusion proteins were eluted from the resin beads with 200 µL of elution buffer (50 mM Na-HEPES, pH 7.5, 500 mM NaCl, 300 mM imidazole) into 96-deep-well plates. All protein purification steps were automated on a Tecan robot (Tecan, Switzerland) containing a vacuum manifold.

Homogeneity of purified proteins and molecular mass of recombinant enzymes were determined by SDS-PAGE in 14% (w/v) acrylamide gels. Protein concentration of enzymes stock solutions varied between 0.5–20 g/L, as determined spectrophotometrically by the Bradford method (Bradford 1976).

4.2.3. Preparation of microalga cells suspension

Chlorella vulgaris suspension was prepared at 20 g/L, as follows: dry microalgae were weighed, re-suspended in PBS and incubated for 30 min at 37 °C in an orbital incubator shaker at 200 rpm. After incubation, the suspension was centrifuged at 2500 × *g* for 30 min, the supernatant was discarded and the pellet re-suspended in the same amount of PBS.

4.2.4. Enzymatic cell wall disruption

The cell wall disruption assay was performed in a 24 well microplate (VWR Chemicals, West Chester, PA, USA). Two mL of microalgae suspension was added to each well along with the respective enzyme added at a final concentration of 20 mg/L. Control wells took the same amount of PBS. The microplate was then sealed and incubated overnight in an orbital incubator shaker at 37 °C and 140 rpm. Microplate was centrifuged for 15 min at 3210 × *g* and the supernatants and pellets were recovered. To precipitate and remove the enzymes, the supernatant for DNSA and HPLC analyses was boiled for 5 min and centrifuged for 5 min at 10,000 × *g* and the supernatant recovered.

4.2.5. Reducing sugars measurement

The DNSA method (Miller 1959), was employed as a standard protocol to quantify the released amount of reducing sugars. In this method, the aldehyde and ketone groups of the reducing sugars reduce the yellow 3,5-dinitrosalicylic acid to orange 3-amino-5-nitrosalicylic acid. Glucose was used as standard. After mixing 0.6 mL of glucose solutions or supernatants with 0.6 mL of DNSA reagent, samples were heated at 100 °C for 15 min. Then, samples were cooled on ice for 5 min and detected by ultraviolet-visible spectrophotometry at 570 nm.

4.2.6. Thermostability and proteolysis experiments

Each enzyme from the four-enzyme constituted mixture (Mix; Provisional Patent number 20181000067928, INPI, Portugal) was subjected individually to 12 different temperature conditions (without incubation and with incubation at 30 °C, 37 °C and 40 °C to 80 °C at 5 °C intervals) for 30 min. Then, the incubation was cooled on ice for 10 min and centrifuged at 16,100 × *g* for 8 min at 4 °C. The supernatant was recovered and the protein amount was quantified in triplicate using a NanoDrop 2000/2000c (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). To validate results, the supernatants were also analysed by 14% SDS-PAGE gels and the images were acquired with BioRad ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA, USA).

Two-hundred microliters of each enzyme that compose the Mix, at a concentration of 1 g/L, was placed in individual tubes. For each enzyme, there was a control and a treatment: 200 µL of PBS was added for the control; 200 µL of porcine pancreatin (VWR Chemicals, West Chester, PA, USA) at 5 g/L was added for the treated sample. The reactions were incubated at 37 °C, at regular intervals of 15 min until 2 h. The samples were then removed and analysed by 14% SDS-PAGE gels to validate results. The images from gels were acquired with BioRad ChemiDoc XRS imaging system (Bio-Rad). A qualitative scale was created to assess the proteolytic resistance based on the visualisation of protein fragments in SDS-PAGE gels. The qualitative scale was defined as follows: −, no resistant (only fragmentation bands) which means the complete disappearance of the protein band along with visualisation of protein fragments from enzymatic digestion; + , partially resistant (protein and fragmentation bands) meaning that the protein band is visualised associated with protein fragments bands from enzymatic digestion.

4.2.7. Determination of total oligosaccharides

The mono and oligosaccharide profiles from the supernatants of *C. vulgaris* after control and Mix treatments were analysed by HPLC on an Agilent system (Agilent 1200 Series, Agilent Technologies Inc., Palo Alto, CA), equipped with an electrochemical detector (Coulochem III, ESA Dionex Thermo Fisher Scientific Inc, USA). The HPLC analysis was performed using a Dionex CarboPac PA10 column (4 × 250 mm, Thermo Fisher Scientific Inc, USA) fitted to a CarboPac PA10 guard column (4 × 50 mm), following the procedure described by Thermo Fisher Scientific (Thermo Scientific 2009) with slight modifications. The separation of mono and oligosaccharides was achieved using a mobile phase with a flow rate of 1 mL/min for 60 min at 25 °C, as follows: isocratic elution with 18 mM NaOH (eluent A) during 18 min, gradient with 100–0 mM NaOH (eluent B) and 0–75 mM sodium acetate in 100 mM NaOH

(eluent C) from 18–40 min, and re-equilibration to 18 mM NaOH during 20 min. The quantification of total oligosaccharides was based on a standard curve, using a range of concentrations from 0.025 mM to 0.2 mM of glucose. The results were expressed as equivalent moles of glucose released per gram of microalgae.

4.2.8. Optical and fluorescence microscopic observations

The pellets from the enzymatic cell wall disruption assay were re-suspended in 2 mL of PBS. The number of cells in the microalgae suspension was determined using a Neubauer counting chamber by direct observation on a bright-field Olympus CH30 microscope (Olympus, Center Valley, PA, USA) and images were acquired with an Olympus DP21 (Olympus) digital camera. The fluorochrome Calcofluor White (Sigma-Aldrich, St. Louis, Mo, USA) that binds to the cell wall (Safi, Ursu, et al. 2014) was added to the same suspensions used for optical microscopy. Cells were observed with an epifluorescence microscope and images were captured with a Leica DFC-340FX (Leica, Wetzlar, Germany) camera system. When excited at λ = 488 nm, cells were identified as blue coloured. The fluorescence intensity, expressed as arbitrary units, was measured using the Image J software (NIH).

4.2.9. Determination of protein content

The nitrogen (N) content in lyophilised supernatants and residues from *C. vulgaris* suspension after control and Mix treatments was determined by the Kjeldahl method (984.13) (AOAC 2000), assuming that no nitrogen from the media interfere with the assay. The crude protein was calculated as N × 6.25.

4.2.10. Pigment analysis

The content of chlorophyll *a*, chlorophyll *b* and total carotenoids in supernatants and residues from *C. vulgaris* suspension after control and Mix treatments was measured according to Hynstova et al. (2018), with slight modifications. For the pigment determination in the residue fraction, 4 mL of acetone was added to 40 mg of residue and incubated in the dark during 1 h at 45 °C and 200 rpm. After incubation, the samples were analysed using UV–Visible spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, Little Chalfont, UK) and the pigment content was calculated according to equations described by Hynstova et al. (2018). The supernatant fraction was reed directly after treatment using UV–Visible spectrophotometer and the pigment content was calculated as defined above.

4.2.11. Determination of fatty acid composition

Fatty acids from the lyophilised supernatants and pellets of *C. vulgaris* after control and Mix treatments were extracted based on the method of Folch et al. (1957), replacing chloroform:methanol (2:1, v/v) by dichloromethane:methanol (2:1, v/v), according to Carlson (1985). FA were esterified to methyl esters (FAME) by acid catalysis with acetylchloride-methanol solution (1.25 M Sigma-Aldrich, St. Louis, Mo, USA) at 80 °C for 60 min as described by Batista et al. (2013). The analysis of FAME was done using a gas chromatograph HP7890A (Hewlett-Packard) coupled with a flame ionization detector (GC-FID). The separation was performed in a SupelcowaxTM10 capillary column (30 m × 0.20 mm i.d., 0.20 µm film thickness; Supelco Inc., Bellefonte, PA) with helium as a carrier gas at a flow rate of 1.3 mL/min. The injector and detector temperatures were 250 and 280 °C, respectively. The oven temperature program was started at 150 °C and held for 11 min, then increased to 210 °C at a rate of 3 °C/min and maintained for 30 min. The identification of FAME was achieved by comparison with retention times of fatty acids standards (37 Component FAME mixture from Supelco Inc.). The quantification of total FAME was carried out using heneicosanoic acid (21:0) as internal standard. Each fatty acid was expressed as a percentage of the sum of identified fatty acids (% total fatty acids).

4.2.12. Statistical analysis

All experiments were conducted in triplicate, and the mean values are presented. The error bars on figures indicate the standard error. Data were checked for normality and analysed using the GLM model test. *p* value lower than 0.05 was considered to be statistically significant. All statistical analyses were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

4.3. Results

4.3.1. Individual screening of enzymes in *Chlorella vulgaris* cell wall disruption

In order to evaluate which CAZymes and sulfatases of the library created in this work have the capacity to degrade *C. vulgaris* cell wall, each one of the enzymes was individually incubated with a microalgae suspension. Although a great majority of the enzymes were

unable to deconstruct the marine biomass, 29 individual enzymes displayed a measurable capacity to degrade the cell wall of C. vulgaris, as described in Table 4.1. The ability to degrade the microalgae was assessed by the capacity to release reducing sugars as evaluated through the DNSA method. Table 4.1 data is presented in a qualitative scale of the amount of reducing sugars released (g/L): −, 0.00 < 0.005; +, 0.05 < 0.200; ++, 0.200 < 0.300; +++, >0.300. Although the release of reducing sugars was undetected for four enzymes, with identification numbers (ID) 69, 73, 77 and 82, they were included in this selection because their predicted substrates (1,3-α-glucans; agar and neoagarooligosaccharides; 1,3-β-glucans and insoluble 1,3-β-glucans, respectively) are major constituents of *C. vulgaris* cell walls (Chen et al. 2013; Safi, Zebib, et al. 2014). Within this group of enzymes, CAZymes with ID 36, 47 and 60 exhibited the highest release of reducing sugars from the marine biomass, whereas the remaining enzymes displayed a low to moderate capacity to attack the complex polysaccharides.

Table 4.1. Screening of the selected individual CAZymes-sulfatases and Mix in *Chlorella vulgaris* cell wall disruption.

ID	Name	Category	E.C	Main Substrate	Reducing Sugars Released Scale
5	Cellulose 1,4-β-cellobiosidase	Cellobiohydrolases	3.2.1.91	Phosphoric acid-swollen cellulose, Avicel and others forID of insoluble cellulose	++
10	Laccase	Laccases	1.3.3.5	2,20-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	+
14	Laminarinase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans such as laminarin	+
16	Chitinase 1	Chitinases & Chitosanases	3.2.1.14	Chitin and chitosan	++
18	Oligoalginate lyase	Alginate lyases	4.2.2.	Low-viscosity alginate	+
25	β-1,3-1,4-glucanase P2	1,3-1,4-β-Glucanases	3.2.1.73	1,3-1,4-β-glucans	+
29	Algal laminarin-specific β-glucanase / laminarinase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans, such as laminarin, and display low activity on mixed linked glucans	++
32	Endo-β-1,3(4)-glucanase	1,3-1,4-β-Glucanases	3.2.1.6	1,3-1,4-β-glucans, such as lichenan and laminarin.	+
33	β-1,3-glucanase / laminarinase	1,3-β-Glucanases	3.2.1.39	Laminarin	++
36	Chitosanase	Chitinases & Chitosanases	3.2.1.132	Chitosan	+++
37	Endo-β-2,6-fructanase	Fructanases	3.2.1.65	Levans	+
38	Cellobiohydrolase	Cellobiohydrolases	3.2.1.91	Amorphous and crystalline cellulose	+
42	Trans-sialidase B	Sialidases	3.2.1.18	Sialic acids from complex carbohydrates and glycoprotein human alpha-1 (AGP)	++
47	Chitosanase	Chitinases & Chitosanases	3.2.1.132	Soluble and colloidal chitosan	+++
50	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	+
60	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	The 1,4-β-glycosidic bond of cellooligosaccharides, also hydrolysis nonreducing end of chitooligosaccharides (Glc-PNP)	+++
66	Alginate lyase	Alginate lyases	4.2.2.3	Polyguluronate and polymannuronate	+
69	α-1,3-Glucanase	α-Glucosidases	3.2.1.59	1,3-α-glucan	-
73	Exo-β-agarase D	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	-

77	Endo-β-1,3-glucanase	Laminarinases	3.2.1.39	1,3-β-glucans	-
78	Keratan sulfate hydrolase / keratanase II	Acetylglucosaminidases	3.2.1.103	Cartilage keratan sulfate and cornea keratan sulfate	+
81	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	Lactose, GlcNAc2, GlcNAc3, cellobiose, cellotriose, colloidal chitin, cellulose, lichenan, laminarin and xylan	+
82	β-1,3-Glucanase B	Laminarinases	3.2.1.39	Insoluble 1,3-β-glucan	-
85	β-Galactosidase	β-Galactosidases	3.2.1.23	β-galactosides	+
86	Lytic transglycosylase	Peptidoglycan lytic exotransglycosylases	4.2.2.n1	1,4-β-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine residues	+
92	Endo-rhamnogalacturonan lyase	Rhamnogalacturonan lyases	4.2.2.23	Rhamnogalacturonan	++
93	Peptidoglycan N-acetylmuramic acid deacetylase	Acetylglucosamine deacetylases	3.5.1.104	Peptidoglycan	+
95	Lysozyme	Lysozymes	3.2.1.17	Peptidoglycans	+
101	Lysozyme (CPE1314)	Lysozymes	3.2.1.17	Peptidoglycan containing muramic acid δ-lactam	+
Mix	**Exo-β-glucosaminidase, Alginate lyase, Peptidoglycan N-acetylmuramic acid deacetylase and Lysozyme (CPE1314) (ID 60, 66, 93 and 101, respectively)**				1.21 g/L

Each enzyme is presented with the identification number (ID), project identification, category, Enzyme Commission (E.C) number, main substrate and a qualitative scale of reducing sugars released. The enzymatic constitution of the Mix is also presented, as well as the value of the reducing sugars released in g/L. The qualitative scale is based on the amount of reducing sugars released (g/L): -, 0.00 < 0.005; +, 0.05 < 0.200; ++, 0.200 < 0.300; +++, > 0.300.

4.3.2. Composition of a four-enzyme constituted Mix based on reducing sugars released

With the purpose of finding synergistic actions between the individual enzymes identified, the 29 enzymes presented in Table 4.1 were combined and tested in a mixture for the capacity to release reducing sugars from the microalgae. A mixture (Mix) consisting of four-enzymes was found to be the most restricted combination in terms of enzyme numbers and displaying the highest level of released sugars. This Mix was composed of an exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme (CPE1314) and is presented in detail in Table 4.1. When this Mix was incubated with *C. vulgaris* suspension, a value of 1.21 g/L ($p < 0.001$) of reducing sugars released was observed, which represents an increase of 1.6-fold in relation to the highest value found in the individual enzyme screening. The rates for released sugars were found to be: for Mix *versus* Control = 333.7%; for Mix *versus* exo-β-glucosaminidase = 38.1%; for Mix *versus* alginate lyase = 198.4%; for Mix *versus* peptidoglycan N-acetylmuramic acid deacetylase = 248.1%; for Mix *versus* lysozyme (CPE1314) = 248.1%.

4.3.3. Thermostability and proteolysis assays

The four enzymes that constitute the Mix treatment were subjected individually to different temperatures to test their thermostability. Figure 4.1 illustrates the variation of protein concentration across the range of temperatures tested. At 37 and 40 °C, representing the internal temperature of mammals and poultry, respectively, all enzymes maintained their stability. However, the stability of ID 93 and ID 66 decayed abruptly from 37 and 40 °C, respectively. ID 66 even reached complete degradation at 55 °C, while ID 60 and ID 101 remained stable up to 80 °C. To investigate the capacity of the four enzymes to resist to the proteolytic attack, to which feed enzymes are subjected in the animal gastrointestinal tract, the same enzymes were treated with pancreatin at 37 °C. Table 4.2 shows the proteolytic resistance of these enzymes. ID 60 and ID 101 had partial resistance over the entire assay time; in turn, ID 66 and ID 93 showed complete degradation after 15 minutes.

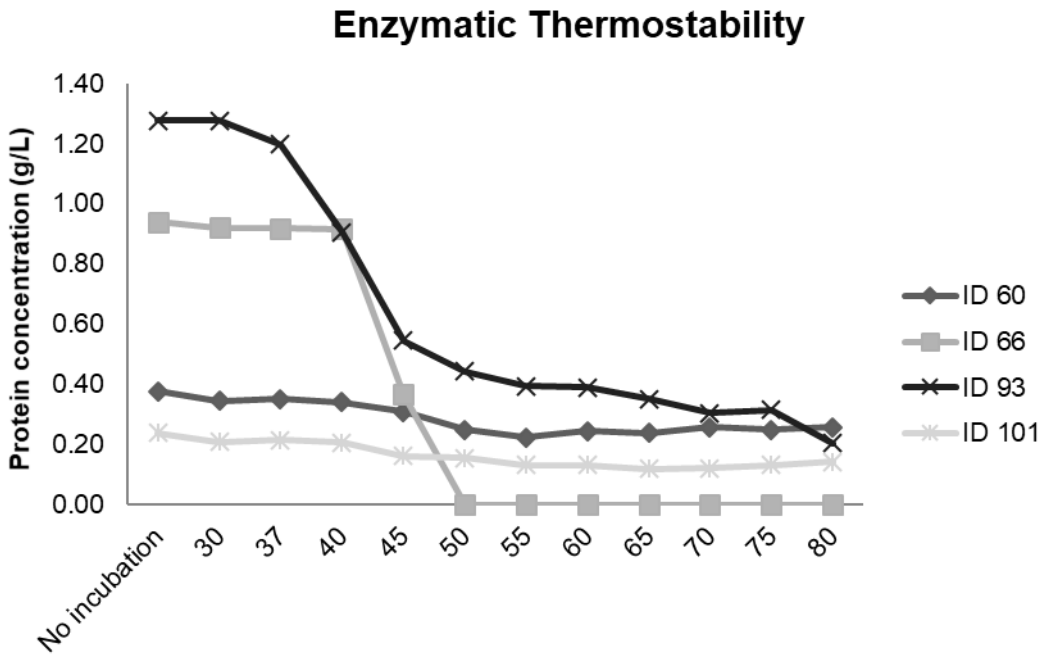

Figure 4.1. Thermostability analysis for the four enzymes constituting the Mix at different temperatures (30 to 80 °C) and for the control without incubation.

Table 4.2. Proteolysis resistance for each one of the four enzymes that constitute the Mix.

ID	Time (min)				
	15	**30**	**60**	**90**	**120**
60	+	+	+	+	+
66	-	-	-	-	-
93	-	-	-	-	-
101	+	+	+	+	+

Each enzyme, at a concentration of 1 g/L, was subjected to the proteolytic action of pancreatin, which was incubated at a final concentration of 2.5 g/L. The reactions were incubated at 37 ºC, at regular intervals of 15 min for 120 min. Results are presented at periods of 15, 30, 60, 90 and 120 min of incubation for each enzyme. The qualitative scale on proteolysis resistance is based on SDS-PAGE gels visualisation: -, no resistant (only fragmentation bands); +, partially resistant (protein and fragmentation bands).

4.3.4. Effect of Mix treatment on *Chlorella vulgaris* cell number and cell wall integrity

No significant differences were found on the number of cells observed between the control and the Mix ($p > 0.05$) (Figure 4.2A). The number of cells counted was around 20000 cells for both treatments (Figure 4.2B and C). The fluorescence intensity was reduced by 47% (Figure 4.2D; $p < 0.001$) when *C. vulgaris* was incubated with the Mix (Figure 4.2F), compared to the control (Fig. 4.2E).

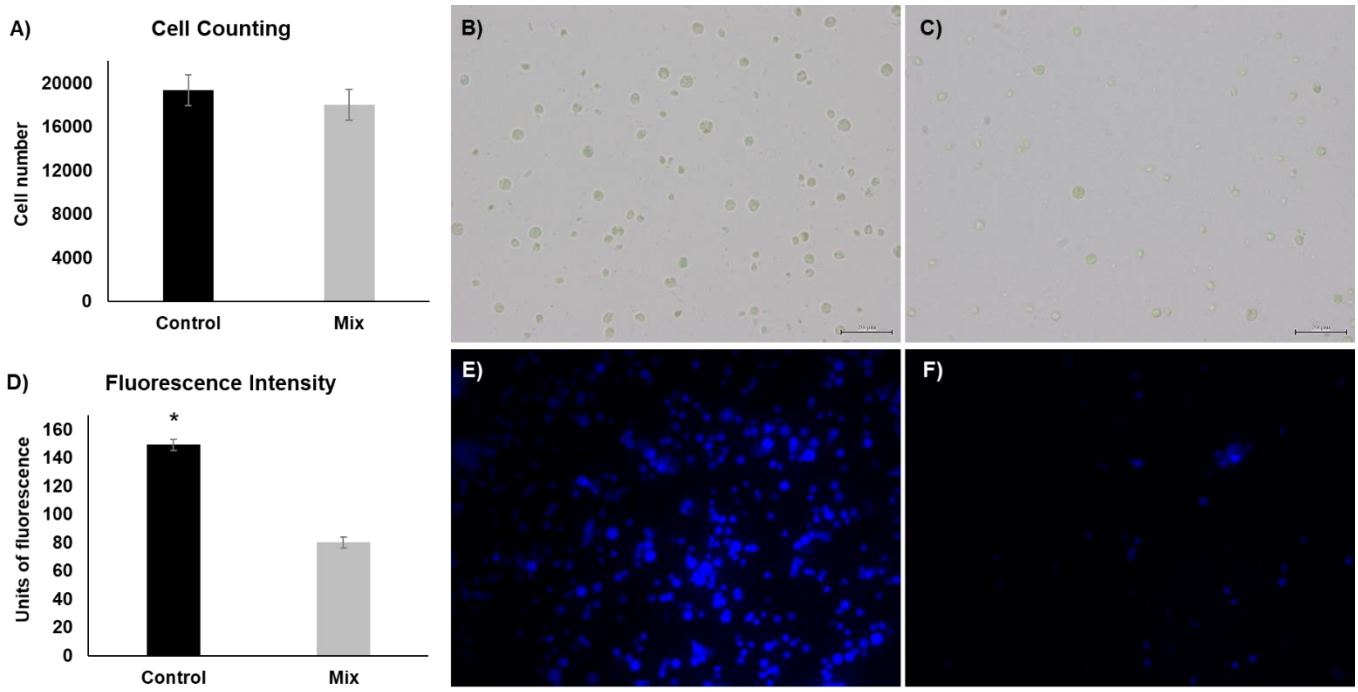

Figure 4.2. A. Cell counting using a Neubauer chamber for control and Mix treatments. B and C. Light microscopy images (×400) of *Chlorella vulgaris* suspension for control and Mix treatments, respectively (scale bar: 20 µm). D. Fluorescence intensity derived from Calcofluor White staining for control and Mix treatments. Asterisk denotes statistical difference at $p < 0.001$. E and F. Fluorescence images (×400) of *Chlorella vulgaris* suspension stained with Calcofluor White for control and Mix treatments, respectively.

4.3.5. Effect of Mix treatment on the release of oligosaccharides from *Chlorella vulgaris* cell wall

Figure 4.3 shows the chromatogram on the release of oligosaccharides from *C. vulgaris* cell wall after treatment with control enzyme mixtures and the Mix enzymes identified in this work. A large peak in the oligosaccharides region was observed in the Mix treatment chromatogram (Figure 4.3B) in relation to the control (Figure 4.3A), corresponding to an 8-fold increase of oligosaccharides amount ($p < 0.001$; Figure 4.3C).

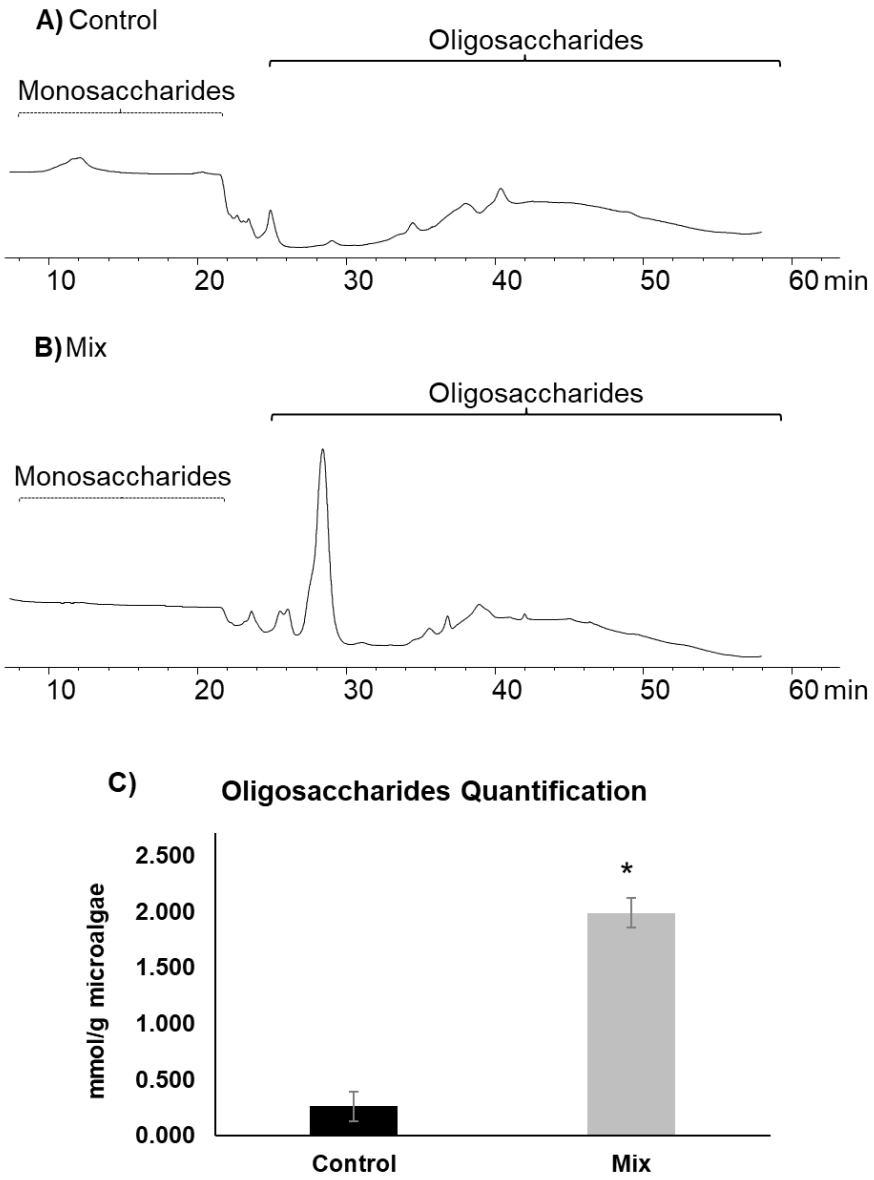

Figure 4.3. Illustrative chromatogram obtained by the HPLC analysis of supernatants for control (A) and Mix (B) treatments. Monosaccharides and oligosaccharides regions are shown. The quantification of oligosaccharides is graphically displayed in (C). Asterisk denotes statistical difference at $p < 0.001$.

4.3.6. Effect of Mix treatment on the release of proteins

In order to understand if the treatment with the Mix triggered the release of proteins from *C. vulgaris* cells to the external environment, the protein content in supernatant and residue fractions was determined (Table 4.3). In the supernatant, the Mix treatment caused a 23.4-fold increase in protein content relative to the control ($p < 0.001$), whereas in the residue, the Mix treatment led to a 1.7-fold decrease relative to the control ($p < 0.001$).

4.3.7. Effect of Mix treatment on the release of chlorophylls and carotenoids

Following the rationale of the previous point, the release of pigments from *C. vulgaris* cells to the external environment was determined in supernatant and residue fractions (Table 4.3). No significant variations were observed for chlorophylls ($p > 0.05$). Total carotenoids displayed significant differences with a 1.1-fold increase in the supernatant fraction of the Mix treatment relative to the control ($p = 0.032$).

4.3.8. Effect of Mix treatment on the release of fatty acids

The fatty acid content in residue and supernatant fractions, after incubation with the Mix treatment, was analysed to understand if the activity of Mix in the cell wall favoured the release of fatty acids from *C. vulgaris* cells to the external environment (Table 4.3). For the supernatant fraction, the predominant fatty acids were SFA > MUFA > PUFA > *n*-6 PUFA > *n*-3 PUFA, while for the residue higher MUFA percentages were found, in the following order: MUFA > SFA > PUFA > *n*-6 PUFA > *n*-3 PUFA. In the supernatant, the percentage of 18:0 was increased in the control relative to the Mix treatment ($p = 0.009$). Conversely, the percentages of 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:3*n*-3 and *n*-3 PUFA were found increased in the Mix treatment in comparison to the control ($p = 0.014$, $p = 0.028$; $p = 0.003$, $p = 0.015$ and $p = 0.015$, respectively). For the residue fraction, the Mix treatment presented higher percentages of 16:1*c*7, 16:1*c*9, 18:2*n*-6, 18:3*n*-3 and *n*-6 PUFA ($p = 0.043$, $p = 0.003$, $p = 0.041$, $p = 0.044$ and $p = 0.033$, respectively), and lower percentages of 16:0 ($p = 0.002$) and 22:2*n*-3 ($p = 0.033$) in relation to the control.

Table 4.3. Content of total proteins, chlorophylls, carotenoids and fatty acids of the supernatant and residue fractions derived from the incubation of *Chlorella vulgaris* with control and Mix treatments.

	Supernatant				**Residue**			
	Control	**Mix**	**SEM**	***p*-value**	**Control**	**Mix**	**SEM**	***p*-value**
Total proteins (mg/g microalgae)	14.6	341.2	17.01	< 0.001	776.4	453.8	17.03	< 0.001
Chlorophyll *a* (mg/g microalgae)	0.109[1]	0.116[1]	0.006	0.429	2.12[2]	2.86[2]	0.301	0.158
Chlorophyll *b* (mg/g microalgae)	0.154[1]	0.153[1]	0.010	0.948	1.27[2]	2.07[2]	0.388	0.217
Total chlorophylls (mg/g microalgae)	0.263[1]	0.269[1]	0.016	0.799	3.39[2]	4.93[2]	0.684	0.187

Total carotenoids (mg/g microalgae)	0.076[1]	0.083[1]	0.002	0.032	0.346[2]	0.268[2]	0.034	0.185
Total fatty acids (mg/g microalgae)	2.24	2.67	0.417	0.496	23.8	26.4	1.42	0.249
Fatty acid composition (% total fatty acids)								
14:0	1.81	1.21	0.216	0.097	1.37	1.30	0.039	0.223
16:0	43.7	44.4	1.59	0.773	23.7	21.8	0.26	0.002
16:1*c*7	0.170	0.512	0.070	0.014	4.17	4.59	0.116	0.043
16:1*c*9	2.17	2.84	0.165	0.028	9.52	11.01	0.226	0.003
17:0	1.72	1.24	0.142	0.053	0.527	0.394	0.044	0.075
17:1*c*9	0.902	3.02	0.307	0.003	6.34	6.53	0.237	0.589
18:0	32.6	27.1	1.04	0.009	7.27	5.17	1.221	0.269
18:1*c*9	6.64	7.56	1.235	0.617	14.3	14.4	0.08	0.291
18:1*c*11	1.79	1.61	0.378	0.741	10.0	9.93	0.277	0.844
18:2*n*-6	3.21	4.96	0.804	0.174	11.3	12.4	0.30	0.041
18:3*n*-6	0.850	0.999	0.220	0.648	0.103	0.127	0.035	0.634
18:3*n*-3	0.719	1.18	0.097	0.015	9.64	11.0	0.385	0.044
20:0	1.59	1.12	0.203	0.155	0.333	0.265	0.036	0.236
22:0	2.19	2.32	0.297	0.769	0.292	0.232	0.024	0.131
22:2*n*-3	nd	nd	-	-	0.430	0.164	0.068	0.033
Others	0.868	0.941	0.199	0.806	0.915	0.778	0.039	0.046
∑ SFA	83.5	77.3	1.94	0.063	33.5	29.2	1.52	0.093
∑ MUFA	11.7	15.6	1.48	0.114	44.3	46.5	0.83	0.116
∑ PUFA	4.78	7.14	0.741	0.065	21.4	23.7	0.67	0.055
∑ *n*-3 PUFA	0.719	1.18	0.097	0.015	10.1	11.2	0.38	0.085
∑ *n*-6 PUFA	4.06	5.96	0.767	0.130	11.4	12.5	0.293	0.033

Two mL of microalgae suspension was incubated with the four enzymes which constitute the Mix at a final concentration of 20 mg/L for each enzyme. The control treatment took the same amount of PBS. Incubations were done overnight at 37 °C and 140 rpm. After incubations, supernatant and residue fractions were separated by centrifugation. Only fatty acids whose percentage was >0.5% are presented; nd – not detected.

[1] Values measured in phosphate buffered saline (PBS)

[2] Values measured after extraction with acetone.

4.4. Discussion

To test the hypothesis that nutrients bioavailability of *C. vulgaris* could be largely improved after disruption of its recalcitrant cell wall, a large library of 178 CAZymes and 22 sulfatases, with well-defined and carefully thought-out enzymatic characteristics, was established by recombinant expression in *E. coli* cells. These 200 enzymes were selected taking into account the composition of the known matrix polysaccharides of microalgae cell walls, which comprises pectin, chitin agar, alginates or the aliphatic polymer algenan (Scholz

et al. 2014). The selected enzymes were produced in a HTP platform that involves gene synthesis, gene cloning, protein expression and protein purification. These enzymes were screened individually to degrade *C. vulgaris* cell wall, which was firstly assessed by measuring the release of reducing sugars. In the next stage, the 29 recombinant enzymes able to degrade *C. vulgaris* cell wall (see Table 4.1) were tested in combination to obtain the maximum disruption of *C. vulgaris* cell wall. As a result of these combinations, a four-enzyme mixture (Mix) was identified as the most active in the degradation of *C. vulgaris* cell wall and applied throughout.

The selected Mix was composed of four recombinant enzymes, an exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme. The exo-β-glucosaminidase, included in the category of glucosaminidases (Honda et al. 2011), has cellooligosaccharides and chitooligosaccharides as main substrates. The alginate lyase belongs to the family 5 of PL and has polyguluronate and polymannuronate as main substrates (Yoon et al. 2000; Yamasaki et al. 2005; Cantarel et al. 2009). The peptidoglycan N-acetylmuramic acid deacetylase, which is included in the category of acetylglucosamine, has peptidoglycan as main substrate (Blair and van Aalten 2004). Finally, lysozyme, also known as muramidase belongs to GH family 25 (Cantarel et al. 2009) and peptidoglycan containing muramic acid δ-lactam is the main substrate of this enzyme (Chen et al. 1997). The four enzymes constituting the Mix were biochemically characterised in terms of their thermostability and resistance to proteolysis. Both ID 60 and ID 101 were stable throughout the range of temperatures tested and resistant to the proteolytic action of pancreatin. The tertiary structure of protein, which confers thermotolerance to enzymes, could also confer inherent proteinase resistance, as demonstrated by Fontes et al. (1995). In contrast, enzymes ID 66 and ID 93 were shown to be sensitive to temperature rise and to proteolysis.

With the aim of evaluating the capacity of enzymes to digest the *C. vulgaris* cell wall for lipid extraction, Gerken et al. (2013) focused on the inhibition of *C. vulgaris* growth by a variety of enzymes. *C. vulgaris* is typically sensitive to chitinases and lysozymes, both enzymes degrading polymers containing N-acetylglucosamine. This observation corroborates our results with the introduction of a lysozyme, a glucosaminidase and an acetylglucosamine deacetylases in the Mix. Even if the composition of *C. vulgaris* cell wall is not entirely known, it is formed by a complex matrix constituted by glucosamine or galactose and mannose, and a broad range of pentose and hexose sugars (Lee et al. 2017). As discussed by Baudelet et al. (2017), certain viruses can infect *C. vulgaris*, digest the host cell wall, penetrate and let the newly synthesised virus to be released (Meints et al. 1984; Reisser and Kapaun 1991). Baudelet et al. (2017) referenced the identification of cell wall degrading alginate lyase coding

genes in the genome of *C. vulgaris* infecting virus. These insights remit to the importance of alginate lyase for *C. vulgaris* cell wall digestion.

The Mix was proven effective by the increase in reducing sugars released, indicating a likely synergistic effect of these enzymes, as described by Phong et al. (2018), and known when enzyme mixes degrade carbohydrate mixtures. Fu et al. (2010) with the aim to evaluate the capacity of an immobilised cellulase to hydrolyse the cell wall of *Chlorella* sp. under different conditions also used the measurement of reducing sugars. As *Chlorella vulgaris* has been described as having a residual content of polysaccharides inside the cell (Fu et al. 2010), the oligosaccharides found came from the disruption of cell wall instead of cell interior. Moreover, when applying the enzymatic mixture treatment to *Chlorella vulgaris* cells, it is expected that the first structure to be affected and partially or entirely disrupted would be the cell wall with the concomitant release of reducing sugars, easily measurable by the DNSA method.

No significant differences were observed in *C. vulgaris* cell number after treatment with the four-enzyme Mix. However, the fluorescence intensity was reduced by 47% with the Mix, indicating that these exogenous enzymes do not lead to the complete degradation of the cell wall, although cell wall integrity was affected to a major degree. Safi, Ursu, et al. (2014) and Safi et al. (2015) used the same fluorochrome on different species of microalgae, including *C. vulgaris*, before and after different cell wall disintegration methods (*e.g*. high-pressure and bead milling) were applied. This clear change in cell structure was reinforced in our study by an increase of oligosaccharides amount after the treatment with the Mix, as reported by Heo et al. (2017). Those authors observed a dramatic increase of glucose content in *C. vulgaris* after an osmotic shock treatment, which was related to an efficient cell wall disruption. Conversely, in our study, there was no complete degradation of carbohydrates from the cell wall, since a complex mixture of oligosaccharides rather than single sugars was obtained.

The Mix treatment effect on *C. vulgaris* cell wall was a massive release of (hydro-) soluble proteins found in the supernatant, which was counterbalanced by a considerable decrease of proteins in the residue. These results agree with Safi, Ursu, et al. (2014) and Safi et al. (2015), who observed an increase in soluble protein concentration after the application of different mechanical and chemical cell wall disruption methods in *C. vulgaris*. *C. vulgaris* has a high protein content, up to 68% (Liu and Chen 2014), with great nutritional quality since its amino acid composition meets the human dietary requirements proposed by WHO and FAO (Safi, Zebib, et al. 2014).

The Mix treatment also promoted the beneficial release of total carotenoids to the supernatant. Carotenoids, in particular β-carotene, astaxanthin, cantaxanthin and lutein, have various therapeutic properties, such as prevention of retina degeneration (Fernández-Sevilla et al. 2012) and regulation of blood cholesterol (Granado et al. 2003), which are associated

with their antioxidant activity (Gouveia et al. 2006) and account for 1% in *C. vulgaris* (Mendes et al. 1995; Safi et al. 2015). Chlorophyll is the most abundant pigment in *C. vulgaris*, reaching 1–2% of the microalga dry weight. Even though the Mix treatment promoted the release of total carotenoids to the supernatant, no variation was observed for chlorophylls *a* and *b*. Safi et al. (2015) showed that the disruption of cell wall through the application of several mechanical and chemical methods allowed to release chlorophylls and carotenoids to the aqueous phase (Safi et al. 2015), and at least for carotenoids, their results concur with ours. We speculate that the Mix treatment was unable of penetrating the phospholipid bilayer of the chloroplast in which pigments, such as chlorophylls and primary carotenoids, are embedded inside the thylakoids, therefore justifying the absence of differences for chlorophylls (Safi et al. 2015). Alternatively, the presence of chlorophylls and carotenoids in the supernatant indicates the formation of micellar structures (Safi et al. 2015), which are in line with their amphiphilic characteristics sharing different degrees of polarity (Háda et al. 2012).

The fatty acid content and composition described herein for *C. vulgaris* cells agree with previous reports (Zheng et al. 2011; Yeh and Chang 2012), regardless the enzymatic treatment. Several studies, including those of Heo et al. (2017), Zheng et al. (2011), Cho et al. (2013) and Liang et al. (2012), were performed to improve the yield of lipid extraction from microalgae. A substantial cell wall disruption was observed by Cho et al. (2013) using a mixture of cellulases and β-glucosidases, and by Zheng et al. (2011) using a mixture of snailase, lysozyme and cellulase. In both cases, the enzymatic treatment led to an increase in lipid extraction efficiency, highlighting in the case of Zheng et al. (2011), the good performance exhibited by lysozyme. In our study, the focus was not on whether the Mix led to an increase in lipid extraction yield but, instead, on the release of fatty acids from *C. vulgaris*, through the disruption of microalgae cell wall. The major differences were found at the level of some MUFA, with a higher release of 16:1*c*7, 16:1*c*9 and 17:1*c*9, when *C. vulgaris* was submitted to the Mix treatment, justifying a higher percentage in the corresponding supernatant. The same applies to ALA (18:3*n*-3), an essential *n*-3 LCPUFA, with important health properties, in particular for the prevention of cardiovascular diseases, cancer, autoimmune diseases and type 2 diabetes (Calder 2012; Abedi and Sahari 2014). Due to its benefits, the increase release of ALA when using this Mix deserves to be further exploited.

4.5. Conclusion

The results reported in this work indicate that this four-enzyme Mix has capacity to partially degrade *C. vulgaris* cell wall. These findings open new opportunities to develop a novel generation of biocatalysts to supplement diets for monogastric animals, in particular

those incorporating *C. vulgaris* microalga. Data indicate that exogenous enzymes may disrupt microalgae cell walls to a significant extent, allowing the release of trapped nutrients with important nutritional value. Consequently, exogenous enzymes may promote the use of microalgae in animal diets at higher incorporation levels (> 1%), leading to the release of highly beneficial bioactive compounds in an economically viable way. Further work is ongoing at our research laboratories to assess how effective these combined enzyme activities are for the supplementation of monogastric diets with *C. vulgaris* microalga as a feed ingredient. In addition to the animal feed industry, these results may increase the yield in obtaining valuable constituents of *C. vulgaris* for other biotechnological industries, in particular those related with biofuel, food and nutraceutical applications.

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Chapter 5 – A HIGH DIETARY INCORPORATION LEVEL OF *CHLORELLA VULGARIS* IMPROVES THE NUTRITIONAL VALUE OF PORK FAT WITHOUT IMPAIRING THE PERFORMANCE OF FINISHING PIGS

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Contribution of Diogo Coelho to this paper:

Diogo Coelho collaborated in animal experiment, tissues sampling and performed the production performance and meat quality parameters measurements. Moreover, proceeded to the laboratory analysis, data processing and statistical analysis. Finally, Diogo Coelho collaborated in the results interpretations and wrote the manuscript.

A HIGH DIETARY INCORPORATION LEVEL OF *CHLORELLA VULGARIS* IMPROVES THE NUTRITIONAL VALUE OF PORK FAT WITHOUT IMPAIRING THE PERFORMANCE OF FINISHING PIGS

Abstract

The influence of a high inclusion level of *Chlorella vulgaris*, individually and supplemented with two CAZymes mixtures, in finishing pig diets was assessed on zootechnical performance, carcass characteristics, pork quality traits and nutritional value of pork fat. Forty crossbred entire male pigs, sons of Large White x Landrace sows crossed with Pietrain boars, with an initial live weight of 59.1 ± 5.69 kg were used in this trial. Swines were randomly assigned to one of four dietary treatments (n = 10): cereal and soybean meal-based diet (control), control diet with 5% *C. vulgaris* (CH), CH diet supplemented with 0.005% Rovabio® Excel AP (CHR) and CH diet supplemented with 0.01% of a four-CAZyme mixture (CHM). Animals were slaughtered, after the finishing period, with a BW of 101 ± 1.9 kg. Growth performance, carcass characteristics and meat quality traits were not influenced (p > 0.05) by the incorporation of *C. vulgaris* in the diets. However, the inclusion of the microalga in finishing pig diets increased some lipid-soluble antioxidant pigments and *n*-3 PUFA, and decreased the *n*-6:*n*-3 ratio of fatty acids, thus ameliorating the nutritional value of pork fat. Moreover, the supplementation of diets with the CAZymes mixtures did not change (p > 0.05) neither animal performance nor meat quality traits, indicating their inefficacy in the increase of digestive utilization of *C. vulgaris* by pigs under these experimental conditions. It is concluded that the use of *C. vulgaris* in finishing pig diets, at this high incorporation level, improves the nutritional value of pork fat without compromising pig performance.

Key words: *Chlorella vulgaris*; CAZymes; finishing pigs; growth performance; pork quality; fat composition.

5.1. Introduction

Pork industry is currently facing the big challenges of feeding sustainability and the unhealthy image of fat. In fact, pork production is about 38% of the total amount of meat produced in the world, being is the most commonly consumed meat in different European, American, and Asian countries (Zhang et al. 2020). Moreover, the combination between rise of global population and the increase in income, will double the overall demand for animal-derived products by 2050, including pork (FAO 2007). The increased demand for these products will necessarily bring dramatic consequences in terms of sustainability, as cereal grains and soybean food crops are the two main conventional feedstuffs for animal feeding (FAO 2011). Therefore, alternative feed ingredients are needed to sustain animal agriculture and human food security (Ekmay et al. 2014; Taelman et al. 2015).

In addition, pork is frequently considered unhealthy due to the lower proportions of PUFA and lipid-soluble antioxidant vitamins, and higher percentages of SFA (Morgan et al. 1992). However, it is well established that pig diet provides an effective approach for altering the fat composition of pork, thereby modifying the impact of human dietary fat intake from pork (Wood and Enser 1997). Functionally, the most important *n*-3 fatty acids are EPA (20:5*n*-3) and DHA (22:6*n*-3), although the roles for DPA (22:5*n*-3) are now also emerging (Kaur et al. 2011). Lipid-soluble antioxidant vitamins comprise vitamin E homologues (tocopherols and tocotrienols) and vitamin A and its precursors (some carotenoids, including β-carotene). In general, the intakes of EPA and DHA are typically small and much lower than the recommended values (Calder 2017). This fact raised substantial interest in food enrichment with EPA and DHA, by using feed ingredients from marine origin in animal nutrition.

Microalgae, an important aquatic resource, could be a good sustainable alternative to conventional feedstuffs, since they have similar nutritional compositions (Liu and Chen 2014). *Chlorella vulgaris* is a freshwater eukaryotic green microalga. This microalga, one of the most cultivated microalgae worldwide, is known for its high biomass productivity, relative ease of cultivation and a balanced nutritional composition, making it an attractive alternative for monogastric diets (Kotrbáček et al. 2015). In particular, regarding fatty acid profile, *C. vulgaris* displays a high percentage of SFA, mainly myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0). In addition, *C. vulgaris* presents an interesting content in some *n*-6 PUFA (18:2*n*-6 and 18:3*n*-6) and ALA (18:3*n*-3), but much less quantity of EPA and DHA (Batista et al. 2013). However, *C. vulgaris* cell wall is composed by a diverse and complex matrix of cross-linked insoluble carbohydrates (Baudelet et al. 2017). Thus, the incorporation of *C. vulgaris* in monogastric diets could be a problem since the recalcitrant cell wall is largely indigestible, impairing the bioavailability of its valuable nutrients (Teuling et al. 2019).

Exogenous CAZymes are now completely accepted as feed supplements for monogastric livestock species to improve feed nutritive value and enhance animal performance and health (Ravindran and Son 2012). In addition to cereal cell walls, several *in vitro* studies demonstrated the ability of CAZymes to degrade microalgae cell walls (Sander and Murthy 2009; Zheng et al. 2011; Cho et al. 2013; Gerken et al. 2013). Recently, Coelho et al. (2019) described a four-CAZyme mixture, composed by an exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme, that was shown to disrupt microalgae *C. vulgaris* cell walls to a significant extent, in *in vitro* assays, enabling the release of trapped nutrients with important nutritional value.

Therefore, the supplementation with the four-CAZyme mixture mentioned above could enable the incorporation of *C. vulgaris* in monogastric diets, at high incorporation levels (>2% in diet), without impairing animal performance and health. In line with this, the aim of this study was to assess how the dietary incorporation of *C. vulgaris* at a 5% high level, supplemented or not with two exogenous CAZyme mixtures (the commercially available Rovabio® Excel (ADISSEO, Antony, France) AP and the four-CAZyme mixture described by Coelho et al. (2019), influences finishing pigs' performance, carcass characteristics, and pork quality traits.

5.2. Material and Methods

5.2.1. Production of recombinant four-CAZyme mixture

The genes encoding the four recombinant CAZymes that compose the mixture (exo-β-glucosaminidase, alginate lyase, peptidoglycan N-acetylmuramic acid deacetylase and lysozyme) were cloned according to Coelho et al. (2019). Briefly, BL21 *E. coli* cells were transformed with the generated recombinant plasmids and were grown on Luria-Bertani media, at 37 °C under agitation (190 rpm) to mid exponential phase (absorbance was measured at λ=595 nm as being 0.4 – 0.6). Isopropyl β-d-thiogalactoside was added to a final concentration of 1 mM in order to induce recombinant gene expression. Cells were incubated overnight at 19 °C with agitation (140 rpm). After induction, the culture media was centrifuged and the protein extracts were prepared by ultrasonication, followed by centrifugation and freeze dried. The four-CAZyme protein extracts were mixed in equal weight proportions at a final level of 0.01%.

5.2.2. Animal care, experimental design and experimental diets

The trial was conducted at the facilities of Unidade de Investigação em Produção Animal (Instituto Nacional de Investigação Agrária e Veterinária (UEISPA-INIAV, Santarém). The experimental procedures were reviewed by the Ethics Commission of the Centro de Investigação Interdisciplinar em Sanidade Animal/Faculdade de Medicina Veterinária (CIISA/FMV) and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Alimentação e Veterinária), following the appropriated European Union guidelines (2010/63/EU Directive). The staff members involved in animal trial hold license for conducting experiments on live animals from the Portuguese Veterinary Services.

Forty crossbred entire male pigs, sons of Large White × Landrace sows crossed with Pietrain boars, were obtained from a commercial farm. Before the beginning of the trial, pigs were submitted to an adaptation period of one week. Then, pigs with an initial weight of 59.1 ± 5.69 kg were randomly distributed into 10 pens with 4 animals in each pen (7.8 m2). Pens were equipped with one stainless steel nipple and four creep feeders separated by a system of gates, thus allowing individual feed intake control. The 4 experimental diets were randomly assigned to the four animals within each pen, with each animal in each pen receiving a different diet, thus being the pig the experimental unit. Pigs had access to feed and water ad libitum. The experimental diets were: cereal and soybean meal-based diet (Control), control diet with 5% of *C. vulgaris* supplied by Allmicroalgae (Natural Products, Portugal) (CH), control diet with 5% of *C. vulgaris* supplemented with 0.005% of Rovabio® Excel AP (Adisseo, Antony, France) (CHR), and control diet with 5% of *C. vulgaris* supplemented with 0.01% of four-CAZyme mixture (CHM).

The ingredient composition of the experimental diets is described in Table 5.1, and their chemical composition is presented in detail in Table 5.2. For further information on the feed analysis see details below.

Table 5.1. Ingredients and additives of the experimental diets (%, as fed basis).

Ingredients (%)	Experimental diets			
	Control	**CH**	**CHR**	**CHM**
Corn	56	56	56	56
Soybean meal	19.3	11.7	11.6	11.7
Barley	10	10	10	10
Sunflower meal	5.4	6.8	6.8	6.8
Wheat	5	5	5	5
Calcium carbonate	1.3	1.2	1.2	1.2

Soybean oil	0.5	0.2	0.2	0.2
Wheat bran	0.4	1.7	1.66	1.65
Salt	0.4	0.4	0.4	0.4
Vitamin–trace mineral premix[1]	0.4	0.4	0.4	0.4
Dicalcium phosphate	0.26	0.36	0.36	0.36
Sodium bicarbonate	0.1	0.1	0.1	0.1
Betaine-HCl	0.15	0.15	0.15	0.15
Mould inhibitor mixture[2]	0.075	0.075	0.075	0.075
Fatty acid mixture[3]	0.075	0.075	0.075	0.075
L-Lysine	0.41	0.57	0.57	0.57
L-Threonine	0.1180	0.2000	0.2000	0.2000
DL-Methionine	0.0712	0.1080	0.1080	0.1080
L-Tryptophan	0.0064	-	-	-
Chlorella vulgaris	-	5	5	5
Mix of 4 CAZymes	-	-	-	0.01
Rovabio® Excel AP	-	-	0.005	-

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

[1] VitaTec (Tecadi, Santarém, Portugal). Provided the following nutrients per kg of diet:

Premix provided per kg of complete diet: 6000 IU vitamin A; 1500 IU vitamin D_3; 15 mg vitamin E (acetate $_{DL}$-alpha-tocopherol); 0.3 mg vitamin B_2; 3.75 mg vitamin B_{12}; 0.1 mg biotin; 12 mg calcium pantothenate, 15 mg nicotinic acid; 0.75 mg folic acid; 200 mg choline chloride; 15 mg Cu (cupric sulphate pentahydrate); 100 mg Zn (zinc oxide); 35 mg Mn (manganese oxide); 0.7 mg I (potassium iodide); 0.05 mg Co (basic cobaltous carbonate mono hydrous); 0.2 mg Se (sodium selenite); 80 mg Fe(ferrous carbonate); and 0.2 mg butylated hydroxyl-toluene.

[2] Yeast extracts, high absorbent clay mineral, plant derivatives, calcium propionate and antioxidant premix (Escent® S, Innovad, Berchem, Belgium).

[3] Esterified butyric acid, medium chain fatty acid, plant extract and essential oil (Lumance®, Innovad, Berchem, Belgium).

Table 5.2. Chemical composition of *Chlorella vulgaris* and experimental diets.

	Microalga	**Experimental diets**			
Item	***C. vulgaris***	**Control**	**CH**	**CHR**	**CHM**
Metabolizable energy (kcal/kg DM[1])	3557	3576	3540	3644	3547
Proximate composition (%)					
Dry matter	93.1	90.0	89.7	89.5	90.0
Crude protein	42.8	14.0	15.9	15.2	15.2

Starch	1.86	45.5	45.3	44.7	47.4
Crude fat	8.73	2.60	3.00	3.10	3.10
Crude fibre	1.52	4.60	5.00	5.30	5.20
NDF	1.05	13.7	13.9	12.7	13.7
ADF	0.286	4.90	5.50	5.50	5.90
Ash	11.8	4.03	4.70	4.60	4.60
Amino acid composition (%)					
Alanine	2.77	0.682	0.848	0.806	0.776
Arginine	3.89	0.890	1.11	1.03	0.969
Asparagine	0.062	0.023	0.022	0.015	0.018
Aspartate	3.04	1.00	1.08	1.11	1.01
Cysteine	0.665	0.292	0.268	0.237	0.248
Glutamate	4.07	2.33	2.22	2.21	2.10
Glutamine	0.016	n.d.	n.d.	n.d.	n.d.
Glycine	1.72	0.544	0.687	0.614	0.584
Histidine	0.654	0.512	0.593	0.528	0.489
Hydroxyproline	0.741	0.880	1.33	1.19	1.16
Isoleucine	1.26	0.478	0.536	0.521	0.482
Leucine	2.45	0.942	1.05	1.03	0.984
Lysine	2.63	1.04	1.43	1.42	1.32
Methionine	0.451	0.116	0.124	0.144	0.088
Phenylalanine	1.49	0.578	0.634	0.621	0.587
Proline	1.87	1.06	1.04	1.04	1.01
Serine	1.56	0.689	0.771	0.727	0.679
Threonine	2.32	0.761	0.989	1.00	0.943
Tryptophan	0.471	0.156	0.172	0.147	0.133
Tyrosine	1.18	0.429	0.495	0.470	0.437
Valine	3.52	1.20	1.43	1.32	1.26
Fatty acid profile (% total fatty acids)					
14:0	1.13	0.150	0.218	0.190	0.190
16:0	17.2	16.3	16.6	16.3	16.5
16:1*c*9	3.90	0.228	1.14	0.989	0.972
17:0	0.234	0.189	0.182	0.153	0.154
17:1*c*9	0.610	0.038	0.704	0.739	0.732
18:0	3.00	2.89	3.29	3.11	3.08

18:1*c*9	11.7	27.4	27.4	27.6	27.5
18:1*c*11	nd	0.885	1.70	1.38	1.42
18:2*n*-6	11.2	48.1	44.1	45.1	44.9
18:3*n*-3	10.1	2.57	3.47	3.28	3.28
20:0	0.174	0.528	0.513	0.517	0.500
20:1*c*11	0.127	0.292	0.288	0.320	0.320
20:5*n*-3	nd	nd	nd	nd	nd
22:0	0.060	0.304	0.294	0.262	0.266
22:1*n*-9	nd	0.155	0.155	0.131	0.149
22:6*n*-3	nd	nd	nd	nd	nd
Diterpene profile (µg/g)					
α-Tocopherol	19.2	16.5	18.7	19.4	16.5
α-Tocotrienol	nd	4.84	3.70	3.88	4.36
β-Tocopherol	0.340	0.380	0.268	0.244	0.258
γ -Tocopherol	0.520	3.53	2.74	2.35	2.65
γ-Tocotrienol	0.560	7.23	5.93	7.30	6.02
δ-Tocopherol	0.360	0.340	0.331	0.312	0.314
δ-Tocotrienol	nd	0.287	0.230	0.246	0.247
Pigments (µg/g)					
β-Carotene	198	1.19	7.10	7.40	6.49
Chlorophyll *a*[2]	906	4.31	127	139	126
Chlorophyll *b*[3]	171	7.46	33.9	36.6	34.2
Total Chlorophylls[4]	1077	11.8	161	176	160
Total Carotenoids[5]	228	3.97	36.5	39.5	34.9
Total Chlorophylls + carotenoids[6]	1305	15.7	198	215	195

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

ME – metabolized energy; DM – dry matter; NDF – neutral detergent fibre; ADF – acid detergent fibre; nd – 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).

[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) = (1000 × A470 nm - 1.90 ×Ca - 63.14 × Cb) /214.

[6] Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

5.2.3. Animal performance, slaughter, and sampling

During the experiment, supplied feed and refusals were recorded daily, whereas pig were weighed weekly just before feeding, with the purpose of calculate the ADFI, the ADG, the FCR and G:F. Food was withdrawn from animal 17 to 19 h before slaughter. Animals were slaughtered at a BW of 101 ± 1.9 kg, after a trial period of 41 ± 7.8 days, at the Unidade de Investigação em Produção Animal experimental slaughterhouse (Santarém, Portugal), with electrical stunning followed by exsanguination. The HCW was measured in order to calculate carcass yield. Perirenal and mesenteric fat depot was removed and weighed. *Longissimus lumborum* muscle was collected from the right carcass side between the third and fifth lumbar vertebras, minced, immediately vacuum packed and stored at -20 °C, to assess meat quality, and at -80 °C, for meat oxidative stability determinations.

At 24 h *post mortem*, backfat thickness was measured in the left side of carcass at the last rib position (P2) (the most representative location), last lumbar vertebra (L6) and second sacral vertebra (S2), using a calibrated engineering calliper (150mm Electronic Digital Vernier Calliper CE ROHS) as described by (Frederick 1972). The loin was excised from the left side of carcass, between the last cervical and L6 lumbar vertebras, weighted and sliced into 2.5-cm-thick chops for sensory evaluation, shear force measurements, colour and drip loss determinations. Chops were vacuum packed, frozen and stored at -20 °C until sensory analysis and shear force measurements.

5.2.4. Microalga and experimental diets analyses

Experimental diets were collected 3 times during the entire trial. AOAC (2000) methods were used to determine the proximal composition of *C. vulgaris* microalga and experimental diets. Samples were dried at 103 °C until reach constant weight to determine DM. Crude protein of samples was calculated through the determination of the nitrogen content (N) by the Kjeldahl method using the factor 6.25 × N following the method 954.01 (AOAC 2000). Ash and starch contents of samples were determined according to the method 942.05 (AOAC 2000) and Clegg (1956) procedure, respectively. Crude fat of samples was determined after automatic Soxhlet extraction with petroleum ether (Gerhardt Analytical Systems, Königswinter, Germany). Crude fibre, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined by the method 989.03 (AOAC 2000). Metabolizable energy (ME) was calculated according to Noblet et al. (1989).

The amino acid composition of *C. vulgaris* and experimental diets was determined according to the method 994.12 (AOAC 2005) and quantified by HPLC (Agilent 1100, Agilent Technologies, Avondale, PA), as described by Henderson et al. (2000). The FAME profile of

C. vulgaris and experimental diets were analyzed by one-step extraction and acid transesterification, followed by gas chromatography using heneicosaenoic acid (21:0) methyl ester as the internal standard (Sukhija and Palmquist 1988).

The diterpene profile of *C. vulgaris* and experimental diets was analysed by direct saponification, using a single *n*-hexane extraction followed by HPLC analysis (Prates et al. 2006). The determination of pigments in *C. vulgaris* and experimental diets was performed according to Teimouri et al. (2013), with slight modifications as described in Pestana et al. (2020). The quantification of pigments in *C. vulgaris* and experimental diet samples were performed according to Hynstova et al. (2018).

5.2.5. Meat quality traits

The pH and temperature of *longissimus lumborum* muscle were measured in the right carcass side at 45 min and 24 h *post mortem* using a pH meter equipped with a penetrating electrode (HI8424, Hanna Instruments, Woonsocket, RI, USA). Meat colour was measured on the cut surface of *longissimus lumborum* section, 24 h *post mortem*, using a colorimeter (Minolta CR-400, Konica Minolta, Tokyo, Japan) with the illuminant D65, at an observer angle of 2° and 1 cm diameter of measurement area. Three measurements on different locations per sample were recorded following the CIE colour convention L* (lightness), a* (redness) and b* (yellowness) system after 1 h of blooming at 4 °C (Madeira et al. 2013).

Drip loss of fresh *longissimus lumborum* muscle was performed according to Hope-Jones et al. (2012). The amount of drip measured between 24 h and 144 h *post mortem* was expressed as a percentage of the initial mass of the sample, and calculated through the difference between the sample mass at the beginning and end of the storage period.

5.2.6. Cooking loss and shear force measurements

Meat cooking loss and shear force were determined according to the procedure adapted from Honikel (1998) and Oillic et al. (2011). Frozen meat samples were thawed at 2 ± 1 °C overnight, weighed and cooked in a water bath at 80 ± 0.5 °C until reaching the temperature of 75 ± 0.5 °C in the geometric centre, using an internal thermocouple (Thermometer Omega RDXL4SD, Manchester, USA). The samples were cooled for 20 h (2 ± 1 °C), weighed in order to calculate the cooking loss, and longitudinally cut in the fibre axis parallel to muscle fibre direction into 8 to 12 cores, with a 1-cm^2 cross-section area for shear force determinations. Cooking loss, expressed as percentage, was calculated by difference of the weights before and after cooking divided by the initial weight of the sample (AMSA 2016).

The Warner-Bratzler shear force (WBSF) was measured in a texture analyser (TA-XT Plus Texture Analyser; Stable Micro Systems, Surrey, UK) with a Warner-Bratzler shear device with a 30-kg compression load cell, trigger force was 25 g and crosshead speed during pre-test, test and post-test set were 5.0, 2.0 and 10.0 mm/s, respectively. Force and distance were recorded at 200 points/s and analysed with the Version 6.1.16 of Exponent software (Stable Micro Systems, Surrey, UK). The value of the peak shear force of cores from each sample was determined and averaged to obtain a single WBSF value per sample.

5.2.7. Trained sensory panel analysis

A trained sensory panel with five sessions was used to evaluate meat sensory characteristics. The eleven panellists were selected and trained according to Cross et al. (1979). For each session, meat samples were thawed at 2 ± 1 °C overnight and cooked at 170 ± 5 °C in a Ceramic Contact Grill 1.6 kW (UNOX Catering Equipment, Padova, Italy) with an internal thermocouple in each sample (Thermometer Omega RDXL4SD, Manchester, USA) until reached 71 °C in the geometric centre. After 10 min of stabilization at 40 °C, the sample was trimmed of the six external surfaces, included connective tissue, cut into 1×1×1 cm subsamples and maintained, on individual covered plates, in an incubator at 40 °C until tasting (no longer than 30 min) (Almeida et al. 2018). Samples were randomly distributed across sections and the attributes evaluated were juiciness, tenderness, flavour intensity, off-flavour, flavour acceptability and overall acceptability. These attributes were classified on a grading scale from 1 (extremely dry, tough, soft, weak or unacceptable) to 8 (extremely juicy, tender, strong, positive and positive), with the exception of off-flavour quantified from 0 (absence) or 1 (presence) (Belk et al. 2015).

5.2.8. Determination of total cholesterol and diterpene profile in meat

The simultaneous quantification of total cholesterol, β-carotene and vitamin E homologues (tocopherols and tocotrienols) in *longissimus lumborum* samples was performed, in duplicate, as previously described by Prates et al. (2006). Muscle samples were submitted to a saponification reaction in a water bath at 80 °C for 15 min under agitation. Afterwards, the diterpenes were extracted with *n*-hexane and analysed by HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA), using a normal-phase silica column (Zorbax RX-Sil, 250 mm × 4.6 mm i.d., 5 µm particle size, Agilent Technologies Inc.). The HPLC analysis was performed using UV-Visible photodiode array detector for cholesterol (λ=202 nm) and β-carotene (λ=450 nm) coupled to fluorescence detector for tocopherols and tocotrienols

(excitation λ=295 nm and emission λ=325 nm). Standard curves of peak area *versus* concentration was used to quantify total cholesterol, β-carotene and vitamin E homologues contents in meat samples.

5.2.9. Determination of pigments in meat

The contents of chlorophyll *a*, chlorophyll *b* and total carotenoids were measured according to the procedure of Teimouri et al. (2013) modified by Pestana et al. (2020). One g of each sample was incubated overnight with 10 mL of acetone (Merck KGaA, 249 Darmstadt, Germany) under agitation at room temperature in absence of light. Then, samples were centrifuged at 3345 × *g* for 5 min and the absorbance was measured in the supernatants using a UV-Visible spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, Little Chalfont, UK). The results were calculated according to Hynstova et al. (2018), as described above for *C. vulgaris* microalga and experimental diets.

5.2.10. Determination of total lipid content and fatty acid composition

Longissimus lumborum muscle samples were lyophilized (-60 °C and 2.0 hPa) using a lyophilizator Edwards Modulyo (Edwards High Vacuum International, Crawley, UK) for total lipids determination according to Folch et al. (1957). Total lipid content was determined gravimetrically, in duplicate, by weighing the fat residue obtained after solvent evaporation. Then, the fat residue was re-suspended in dry toluene and submitted to sequential alkaline and acid transesterification procedure at 50 °C for 30 and 10 min, respectively, to convert FA into FAME (Raes et al. 2001). FAME were separated through gas chromatography (HP7890A Hewlett-Packard, Avondale, PA) comprising a Supelcowax® 10 capillary column (30 m × 0.20 mm internal diameter, 0.20 µm film thickness; Supelco, Bellefonte, PA, USA) and a flame ionization detector as described by Madeira et al. (2014). For FAME identification, a reference standard (FAME mix 37 components, Supelco Inc.) was used and confirmed by gas chromatography with a mass spectrometry detector using a GC-MS QP2010-Plus (Shimadzu, Kyoto, Japan). FAME were quantified by the internal standard method using heneicosanoic acid (21:0) methyl ester as internal standard. The FA identified were expressed as percentage of total FA.

5.2.11. Determination of meat lipid oxidation

The extent of meat lipid oxidation was evaluated at day 0, 4 and 8 *post mortem* (storage at 4 °C), by quantifying thiobarbituric acid reactive substances (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 (Fluka, Neu Ulm, Germany), as a precursor of malonaldehyde, and the results were presented as mg of malonaldehyde per kg of meat (Madeira et al. 2014). In addition, lipid peroxidation levels in meat were also measured by the concentration of TBARS, after chemical oxidation through a ferrous-hydrogen peroxide system, as described by Mercier et al. (2004). The TBARS were quantified after 0, 30, 120 and 300 minutes of oxidation induction following the method described above.

5.2.12. Statistical analysis

All data were checked for normal distribution and variance homogeneity. Data were analysed by analysis of variance (ANOVA) using the PROC GLM of SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA) and measurements over time analysed with PROC MIXED of SAS. The statistical model considered the dietary treatment the fixed effect and the pig the experimental unit. Least square means for multiple comparisons were generated using the PDIFF option adjusted with the Tukey-Kramer method. The significance level was set at $p < 0.05$.

5.3. Results and Discussion

5.3.1. Feed intake, growth performance and carcass characteristics of pigs

Data on feed intake, growth performance and carcass traits of finishing pigs are shown in Table 5.3. Growth performance variables had no significant differences among animals fed with different experimental diets ($p > 0.05$). The average values of ADG, ADFI and FCR were 1.02 kg, 2.62 kg and 2.59, respectively. No significant differences in carcass characteristics were obtained among the experimental groups ($p > 0.05$), with the exception of perirenal fat ($p = 0.026$). The Control group displayed a higher value of perirenal fat than the group fed with the *C. vulgaris* diet (+34%).

We assessed, for the first time, the impact of a high dietary level (>2% in diet) of *C. vulgaris*, individually and combined with two exogenous CAZymes, on pig performance. In fact, some studies reported the use of *C. vulgaris* in pig diets but at much lower levels (1% in the diet or lower), compared with the 5% incorporated in the current trial (Baňoch et al. 2012; Yan et al. 2012; Furbeyre et al. 2017). Baňoch et al. (2012) investigated the effect of a very low level (0.0002%) of incorporation of *C. vulgaris* in female pigs, with an initial weight of 30 kg, and found no significant differences in ADG, HCW, lean muscle thickness and backfat thickness. Later, Furbeyre and colleagues (2017) showed no significant effects on ADG, ADFI and FCR, by using 1% of *C. vulgaris* in weaned piglet diets, with an initial weight of 9.1 kg, during 14 days. In another study, the same authors assessed the effect of oral supplementation with *C. vulgaris* (385 mg/kg BW) on growth and digestive health of weaning piglets and also found no significant changes in ADG, ADFI and G:F (Furbeyre et al. 2018). In addition, a study conducted in growing pigs, with an initial weight of 26.6 kg and *C. vulgaris* incorporation of 0.1% and 0.2% in the diet, described an increase of ADG with the lower dietary level without significant variations in ADFI and G:F (Yan et al. 2012). In the present study, no significant effects on zootechnical parameters and carcass characteristics were obtained, which indicates that dietary incorporation of 5% *C. vulgaris* does not compromise the productive parameters of finishing pigs. Moreover, the dietary supplementation with exogenous carbohydrases, aiming at improving *C. vulgaris* digestibility by finishing pigs, does not seem to be necessary at this high incorporation level.

Table 5.3. Effect of experimental diets on growth performance and carcass characteristics of pigs.

Item	Control	CH	CHR	CHM	SEM	*p*-value
Growth performance						
Initial weight (kg)	62.8	56.1	58.4	59.4	1.79	0.075
Final weight (kg)	101	101	101	101	0.643	0.927
ADFI (kg)	2.56	2.67	2.65	2.60	0.052	0.409
ADG (kg)	0.959	1.08	1.01	1.04	0.037	0.141
FCR	2.69	2.49	2.63	2.55	0.079	0.286
G:F (kg/kg)	0.374	0.404	0.382	0.398	0.011	0.244
Carcass characteristics						
HCW (kg)	80.1	79.5	79.3	78.9	0.735	0.703
Carcass yield (%)	77.4	77.1	76.9	76.8	0.430	0.749
Perirenal fat (kg)	0.897^{b}	0.666^{a}	0.806^{ab}	0.711^{ab}	0.055	0.026
Mesenteric fat (kg)	0.525	0.530	0.572	0.583	0.024	0.231
P_2 backfat thickness (mm)	6.38	5.54	7.17	6.40	0.633	0.359
L6 backfat thickness (mm)	9.33	10.1	10.8	9.64	0.758	0.535
S2 backfat thickness (mm)	4.98	5.22	5.42	5.77	0.737	0.891
Loin weight (kg)	2.14	2.11	2.10	2.18	0.066	0.850
Drip loss (%)[1]	5.82	5.63	7.27	6.51	0.460	0.065

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

SEM – standard error of the mean; ADFI – average daily feed intake; ADG – average daily weight gain; FCR – feed conversion ratio; G:F – gain-feed ratio; HCW – hot carcass weight; P_2 – at the last rib position; L6 – at the last lumbar vertebra; S2 – at the second sacral vertebra.

[1] Measured as the amount of drip between 24 h and 144 h *post mortem*.

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

5.3.2. Pork quality traits and sensory evaluation

Data concerning the effect of experimental diets on quality traits of *longissimus lumborum* muscle from finishing pigs are presented on Table 5.4. Experimental treatments had no significant effect on temperature 45 min *post mortem*, pH 45 min and 24 h *post mortem*, color parameters, WBSF and cooking loss ($p > 0.05$). Table 5.5 summarizes the trained panel scores obtained for pork. No significant differences were obtained among experimental diets for the several items evaluated by the trained sensory panel ($p > 0.05$).

Similar results for meat quality traits were reported by Baňoch et al. (2012), who found that a 0.0002% level of incorporation of *C. vulgaris* in pig diets had no significant effect on

color, pH, cooking loss and drip loss of pork. Here, the dietary incorporation of 5% *C. vulgaris* did not change pork quality traits and sensory parameters, which is very important for the consumer acceptance of this meat. By contrast, Oh et al. (2014) observed an increase of b*, pH and shear force in breast meat, and an increase of L* and b* in leg meat, of male Pekin ducks fed with 0.1-0.2% *C. vulgaris* during 42 days. Therefore, pork quality traits seem to be less sensitive to the dietary inclusion of *C. vulgaris* than poultry meat characteristics, although both are meats-derived from monogastric animals. Finally, it was also indicated here that the dietary use of CAZyme mixtures does not affect pork quality characteristics.

Table 5.4. Effect of experimental diets on meat quality traits of *longissimus lumborum* muscle from pigs.

Item	Control	CH	CHR	CHM	SEM	*p*-value
Temperature (°C)						
45 min	39.9	39.8	39.7	40.0	0.246	0.911
pH						
45 min	6.11	6.34	6.12	6.28	0.109	0.351
24 h	5.49	5.54	5.50	5.51	0.016	0.260
Color measurements						
Lightness (L*)	57.0	56.5	57.9	56.9	0.976	0.791
Redness (a*)	6.50	5.68	6.28	6.39	0.600	0.770
Yellowness (b*)	7.26	6.46	7.24	7.07	0.526	0.679
WBSF (kg)	6.92	7.17	6.44	6.95	0.373	0.574
Cooking loss (%)	30.8	30.7	31.0	30.1	0.605	0.740

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

SEM – standard error of the mean; WBSF – Warner-Bratzler shear force.

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

Table 5.5. Effect of experimental diets on sensory panel scores of *longissimus lumborum* muscle from pigs.

Item	Control	CH	CHR	CHM	SEM	*p*-value
Tenderness	4.45	4.61	4.57	4.54	0.117	0.788
Juiciness	3.72	3.85	3.74	3.84	0.111	0.760
Flavour	4.09	4.20	4.29	4.20	0.109	0.649
Off-flavour	0.061	0.111	0.171	0.131	0.029	0.064
Flavour acceptability	5.55	5.29	5.36	5.32	0.104	0.260
Overall acceptability	5.23	5.22	5.13	5.10	0.101	0.756

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. SEM – standard error of the mean.

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

5.3.3. Vitamin E profile and pigments of pork

The effect of experimental diets on vitamin E profile and pigments of *longissimus lumborum* muscle from finishing pigs is shown in Table 5.6. Experimental diets did not contribute to significant differences on the diterpene profile ($p > 0.05$). Regarding pigments analysis, β-carotene was not detected in any group fed with experimental diets, and there were no significant differences among experimental groups for chlorophyll *a*, chlorophyll *b* and total chlorophylls ($p > 0.05$). However, for total carotenoids, there were significant differences between animals fed Control diet and pigs fed *C. vulgaris* diets ($p = 0.042$), with approximately the double content of meat carotenoids in animals fed with the microalga. This could be explained by the much higher level of carotenoids in *C. vulgaris* diets that in the control diet (about nine times). In addition, there was also significant differences among groups fed with experimental diets for total chlorophylls and carotenoids ($p = 0.038$), being the sum two-fold higher in the group fed with CHR diet compared with the control group; pork from animals fed with CH and CHM diets had intermediate values of total pigments.

α-Tocopherol was the major diterpene in all groups fed with the experimental diets, while the other vitamin E homologues were present at lower concentrations. Concerning pigments, β-carotene (a pro-vitamin A) was not detected in pork, which could indicate that β-carotene in the diet is quickly metabolized into vitamin A (Nogareda et al. 2016), as animals cannot synthesize carotenoids by themselves (Gouveia et al. 1996). *C. vulgaris*, due to the photosynthetic pathway, is also rich in pigments, such as chlorophylls and carotenoids. Despite the fact that β-carotene was not detected, the inclusion of 5% *C. vulgaris* in pig diets, combined or not with the two exogenous CAZyme mixtures, improved the carotenoid content of pork,

thus providing further nutritional benefits for consumers. Total carotenoids were strongly in conformity with diet composition. Similar results were reported by Lemahieu et al. (2013), who studied the effect of dietary supplementation of laying hens with different *n*-3 rich autotrophic microalgae, including *Chlorella*, on meat carotenoids. These authors reported that the transference of carotenoids from the microalgae to the meat provides an additional value for microalgae supplementation.

Table 5.6. Effect of experimental diets on vitamin E profile and pigments of *longissimus lumborum* muscle from pigs.

Item	Control	CH	CHR	CHM	SEM	*p*-value
Diterpene profile (µg/100 g)						
α-Tocopherol	95.4	73.6	74.9	79.4	6.2	0.062
γ-Tocopherol	3.5	3.7	3.5	3.2	0.2	0.441
γ -Tocotrienol	10.2	9.0	10.4	8.2	1.9	0.821
Pigments (µg/100 g)						
β-Carotene	nd	nd	nd	nd	-	-
Chlorophyll *a*	14.7	23.9	31.3	28.0	4.75	0.094
Chlorophyll *b*	27.7	47.2	56.9	54.7	9.00	0.109
Total chlorophylls	42.4	71.2	88.1	82.8	13.7	0.103
Total carotenoids	7.18^{a}	16.4^{b}	16.4^{b}	15.1^{b}	2.55	0.042
Total chlorophylls and carotenoids	49.6^{a}	87.6^{ab}	104^{b}	97.9^{ab}	13.9	0.038

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. SEM – standard error of the mean.

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

5.3.4. Total lipids, cholesterol and fatty acid composition of pork

Table 5.7 shows the effect of dietary inclusion of *C. vulgaris*, alone or combined with exogenous CAZymes, on total lipids, cholesterol and fatty acid composition of *longissimus lumborum* muscle from pigs. Pork contents of total lipids and cholesterol were not affected by experimental diets ($p > 0.05$). In addition, experimental diets promoted only significant differences in the percentage of some minor fatty acids (< 1% of total fatty acids). Control group had a higher percentage of the saturated fatty acid 10:0 relative to CH and CHM groups ($p = 0.013$). In contrast, the percentages of the monounsaturated fatty acid 14:1*c*9 and *n*-3 fatty acids 18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3 were generally lower in the

Control group relative to the *C. vulgaris* groups. Among microalga experimental groups, the group fed with CHM usually had the highest percentage of these unsaturated fatty acids. In fact, both percentages of DPA and DHA increased 1.6-fold for CHM diet comparing with the Control diet. Contrarily, α-linolenic acid had higher percentages in all microalga-fed animals relative to the control group (+48%).

Regarding partial sums of fatty acids, total *n*-3 PUFA in pork had a significant increase of approximately 50% in microalga-fed groups comparing with the Control group ($p = 0.001$). This increase reflects the individual effects of the predominant *n*-3 PUFA (α-linolenic acid, DPA and DHA). The other partial sums of fatty acids, as well as the PUFA:SFA ratio, were not affected by the dietary treatment. However, the *n*-6:*n*-3 ratio decreased in all microalga-fed groups, in an extension of 24%, comparing with the Control group ($p < 0.001$). Feeding pigs with 5% of *C. vulgaris* increased the *n*-3 PUFA content in pork, which showed a correspondence between dietary and deposited *n*-3 PUFA in muscle. This finding reveals the ability of muscle to capture the precursor α-linolenic acid from *C. vulgaris* diets and its ability to convert it into *n*-3 PUFA derivatives. The *n*-3 long-chain PUFA (*n*-3 LC-PUFA), such as EPA and DHA, are of great interest for human diets due to their recognized positive effects, which includes anti-atherogenic, anti-thrombotic and anti-inflammatory properties (Mason 2019). In fact, a well-balanced fatty acids intake is crucial to reduce the risk of cardiovascular and related diseases (Shahidi and Ambigaipalan 2018). However, the intake of *n*-3 PUFA remains relatively low in human populations. In Europe, *n*-3 PUFA consumption is inferior to the recommendations of several international health organisations, which advise consuming one *n*-3 PUFA for five *n*-6 PUFA (ANSES 2011). Although the intake of 250 mg per day already affords protection against cardiovascular diseases (Kris-Etherton et al. 2009), the recommended daily intake of *n*-3 LC-PUFA ranges from 140 to 667 mg/day (Molendi-Coste et al. 2011). Herein, the dietary inclusion of 5% *C. vulgaris* in pig diets, supplemented or not with the two CAZyme mixtures, could be an interesting source to supply these beneficial fatty acids to animals and humans, since the usual *n*-3 PUFA content in pig muscle is very low (about 0.41 to 0.68 g/100 g of total fatty acids) (Domínguez et al. 2019). In opposition to our findings, El-Bahr et al. (2020) found higher levels of *n*-3 fatty acids, particularly of EPA and DHA, in breast muscle of broiler chickens fed *Spirulina platensis* and *Amphora coffeaformis* compared to those fed *C. vulgaris* and control birds. Interestingly, fatty acid profile in the microalgae supplemented contrasted with that of poultry meat, since *C. vulgaris* had higher *n*-3 fatty acids than *S. platensis* and *A. coffeaformis* (El-Bahr et al. 2020).

Concerning the ratio of *n*-6:*n*-3 PUFA, pork from microalga-fed groups had lower values than that from Control group (-21%). Although these lower values are more health-protecting to the consumers, they are considerable higher (approximately 12) than the recommended ratio of 4 to prevent cardiovascular diseases (HMSO 1994).

Table 5.7. Effect of experimental diets on total lipid content, total cholesterol and fatty acid (FA) composition of *longissimus lumborum* muscle from pigs.

Item	Control	CH	CHR	CHM	SEM	*p*-value
Total lipids (g/100 g)	1.18	1.03	1.05	0.933	0.073	0.141
Cholesterol (mg/g)	0.363	0.363	0.361	0.367	0.015	0.993
FA composition (g/100 g FA)						
10:0	0.053^{b}	0.023^{a}	0.042^{ab}	0.023^{a}	0.007	0.013
12:0	0.056	0.045	0.053	0.051	0.006	0.536
14:0	1.05	0.952	0.994	0.904	0.045	0.126
14:1*c*9	0.034^{a}	0.062^{ab}	0.064^{ab}	0.068^{b}	0.008	0.021
15:0	0.081	0.072	0.067	0.069	0.007	0.519
DMA 16:0	0.089	0.047	0.054	0.140	0.029	0.107
16:0	23.4	22.8	23.2	22.5	0.279	0.119
16:1*c*7	0.335	0.352	0.338	0.388	0.015	0.065
16:1*c*9	2.94	2.67	2.79	2.42	0.131	0.054
17:0	0.432	0.435	0.417	0.460	0.038	0.882
17:1*c*9	0.340	0.369	0.363	0.334	0.023	0.647
DMA 18:0	0.045	0.019	0.067	0.076	0.032	0.597
DMA 18:1	0.023	0.006	0.034	0.039	0.020	0.637
18:0	11.9	11.6	11.9	12.2	0.373	0.698
18:1*c*9	37.3	36.1	36.7	34.8	0.933	0.270
18:1*c*11	3.99	3.94	3.91	3.79	0.072	0.260
18:2*n*-6	11.8	13.4	12.4	13.9	0.846	0.291
18:2*t*9*t*12	0.039	0.034	0.026	0.032	0.006	0.494
18:3*n*-6	0.121	0.129	0.123	0.133	0.014	0.934
18:3*n*-3	0.279^{a}	0.408^{b}	0.377^{b}	0.381^{b}	0.020	>0.001
18:4*n*-3	0.027^{a}	0.050^{b}	0.041^{ab}	0.058^{b}	0.006	0.004
20:0	0.167	0.154	0.161	0.171	0.007	0.422
20:1*c*11	0.604	0.593	0.594	0.595	0.033	0.996
20:2*n*-6	0.341	0.358	0.326	0.336	0.018	0.675
20:3*n*-6	0.362	0.415	0.383	0.457	0.035	0.270
20:4*n*-6	2.30	2.72	2.40	2.93	0.280	0.368
20:3*n*-3	0.056^{a}	0.080^{ab}	0.089^{b}	0.092^{b}	0.008	0.008
20:5*n*-3	0.064^{a}	0.119^{b}	0.114^{b}	0.112^{b}	0.015	0.042
22:0	0.068	0.070	0.069	0.088	0.008	0.240
22:1*n*-9	0.047	0.049	0.055	0.043	0.008	0.740

22:5*n*-3	0.266[a]	0.385[ab]	0.356[ab]	0.428[b]	0.040	0.036
22:6*n*-3	0.241[a]	0.328[ab]	0.342[ab]	0.393[b]	0.038	0.035
23:0	0.162	0.189	0.170	0.211	0.021	0.366
Others	0.946	1.03	1.02	1.35	0.227	0.615
Partial sums of FA (g/100 g FA)						
SFA	37.4	36.4	37.1	36.7	0.534	0.564
MUFA	45.6	44.2	44.8	42.4	1.09	0.213
PUFA	15.9	18.4	17.0	19.3	1.25	0.243
n-6 PUFA	14.9	17.0	15.6	17.8	1.17	0.306
n-3 PUFA	0.932[a]	1.37[b]	1.32[b]	1.46[b]	0.093	0.001
Ratios of FA						
PUFA:SFA	0.427	0.508	0.461	0.530	0.038	0.232
n-6:*n*-3	16.1[b]	12.3[a]	11.9[a]	12.3[a]	0.395	<0.001

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

SEM – standard error of the mean; FA – fatty acids; DMA – dimethylacetal; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA - polyunsaturated fatty acids.

SFA = Sum of (10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 23:0).

MUFA = Sum of (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).

PUFA = Sum of (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, 20:3***n***-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3).

n-3 PUFA = Sum of (18:3*n*-3, 18:4*n*-3, 20:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3).

n-6 PUFA = 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 $p < 0.05$.

5.3.5. Oxidative Stability of Pork

Table 5.8 displays the effect of experimental diets on oxidative stability of pig *longissimus lumborum* muscle after 0, 4 and 8 days of storage at 4 °C. Data showed that for 0 days of storage TBARS are not detected in any group fed with the different experimental diets, as well as for the group fed with CHR diet with 4 days of storage. Although TBARS are detected for the other groups at day 4, and for all groups after 8 days of storage, experimental diets did not cause significant effects among them with regard to meat oxidative stability ($p > 0.05$). To complement these results, TBARS were also quantified after 0, 30, 120 and 300 min of chemical induction of lipid oxidation, through a ferrous/hydrogen peroxide system. No significant differences were observed among experimental diets for each time of lipid oxidation induction ($p > 0.05$), in spite of a significant increase of TBARS concentration between 0 and 30 min of lipid oxidation induction ($p = 0.0001$). Figure 5.1 presents the values of TBARS after

0, 30, 120 and 300 min of chemical induction of pork lipid oxidation for each experimental diet. The chemical lipid oxidation induced by the Fenton reaction corroborates the values of TBARS found for pork with the conventional TBARS method. In the current study, all the TBARS values during storage were below to the 0.9 mg malondialdehyde/kg of meat reported by Jayasingh and Cornforth (2004) for ground and cooked pork meat. TBARS values above 0.5 mg malondialdehyde/kg of fresh meat are considered critical because at this level of lipid oxidation rancid odour and taste can be already detected by consumers (Wood et al. 2008). Our values were all below this critical point, with exception of pork from CHR group with 8 days of storage, which was slightly higher (0.517).

The inclusion of microalgae rich in antioxidants as natural feed ingredients in animal diet can be a promising and sustainable alternative to enhance not only the nutritional value and health aspects of pork lipids, decreasing the ratio *n*-6:*n*-3 PUFA, but also delaying meat susceptibility to lipid oxidation (Wojtasik-Kalinowska et al. 2018). However, PUFA represent the best candidates for the propagation of oxidative reactions that could depreciate the sensory and nutritional properties of foods (Martini et al. 2020). Herein, the incorporation of 5% *C. vulgaris* in pig diets did not protect pork lipids from peroxidation, which is probably related to similar contents of PUFA, in spite of an important increase of carotenoids in microalga-fed groups in comparison to the Control group. Baňoch et al. (2012) and Vossen et al. (2017) also documented no changes on pork and dry cured ham oxidative stability with the incorporation of 0.0002% and 0.3-1.2% levels of *Chlorella* sp., respectively. Notwithstanding *C. vulgaris* is an excellent source of antioxidant compounds, such as α-tocopherol and carotenoids, as previously documented by Safi, Zebib, et al. (2014), the oxidative stability of pork did not reflect the antioxidant activity of *C. vulgaris*. In addition, Müller et al. (2011) showed a large variation on the reactivity of the different types of carotenoids toward antioxidant activity. Therefore, changes in antioxidant activity are not only associated to the quantity of carotenoids but also with the specific characteristics of carotenoids identity (Goiris et al. 2012). This aspect deserves further investigation.

Table 5.8. Effect of experimental diets on oxidative stability of pig *longissimus lumborum* muscle after 0, 4 and 8 days of storage at 4 °C.

TBARS (mg MDA/kg meat)	Control	CH	CHR	CHM	SEM	*p*-value
Day 0	nd	nd	nd	nd	-	-
Day 4	0.027	0.047	nd	0.031	0.017	0.604
Day 8	0.186	0.174	0.517	0.160	0.142	0.234

Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

TBARS – Thiobarbituric acid reactive substances; MDA – malonaldehyde; SEM – standard error of the mean; nd – not detected.

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

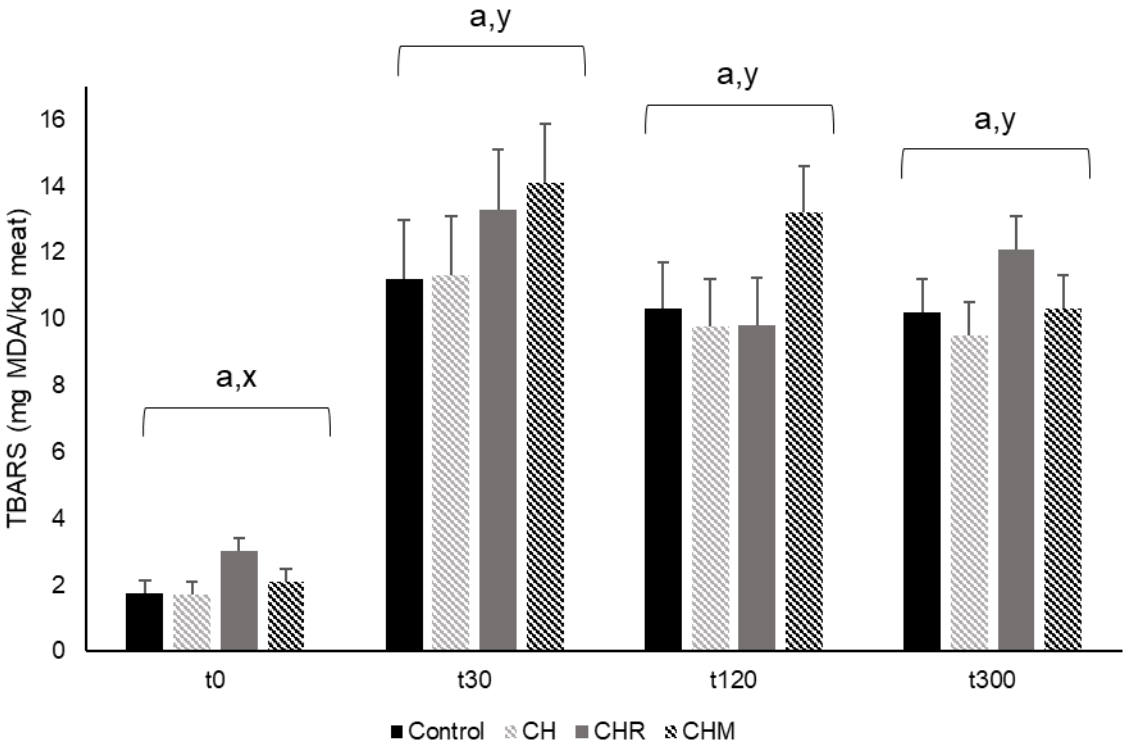

Figure 5.1. Determination of thiobarbituric acid reactive substances (TBARS) of pig *longissimus lumborum* muscle after 0, 30, 120 and 300 min of chemical induction of lipid oxidation. Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. Values with different letters within diet (a,b,…) and time (x,y,…) are significantly different ($p < 0.05$).

5.4. Conclusion

Dietary incorporation of 5% *C. vulgaris* did not negatively affect neither growth variables of finishing pigs nor carcass and meat quality traits (physicochemical and sensory analyses). In contrast, the inclusion of this microalga at this level in finishing pig diets improved the nutritional value of pork fat, through the increase of the beneficial lipid-soluble antioxidant pigments and *n*-3 PUFA, as well as the decrease of the *n*-6:*n*-3 ratio. In addition, the use of carbohydrases in the feed did not improve the digestive utilization of this microalga by pigs, at this incorporation level.

Overall, data indicate that *C. vulgaris* can be included in finishing pig diets up to 5%, with no need of feed enzymes supplementation, to increase pork fat nutritional value without impairing pig performance. As far as we know, this is the first study depicting the feasibility of the use of *C. vulgaris* as an alternative sustainable ingredient (incorporation at high levels) in swine feeding. In order to maximise both, the sustainability of swine diets and the pork nutritional quality, further research should be conducted with higher incorporation levels of *C. vulgaris*, combined or not with exogenous carbohydrases.

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Chapter 6 – IMPACT OF *CHLORELLA VULGARIS* AS FEED INGREDIENT AND CARBOHYDRASES ON THE HEALTH STATUS AND HEPATIC LIPID METABOLISM OF FINISHING PIGS

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Contribution of Diogo Coelho to this manuscript:

Diogo Coelho collaborated in animal experiment, tissues sampling and several laboratory analyses. Moreover, proceeded to data processing, statistical analysis, and interpretation of results. Finally, Diogo Coelho participated in the writing of manuscript.

IMPACT OF *CHLORELLA VULGARIS* AS FEED INGREDIENT AND CARBOHYDRASES ON THE HEALTH STATUS AND HEPATIC LIPID METABOLISM OF FINISHING PIGS

Abstract

Chlorella vulgaris is a promising green and sustainable feedstock alternative to the traditional feedstuffs for livestock industry. However, the implication of high dietary levels of this microalga on pigs' health and liver metabolism remains unknown**.** In this study, forty crossbred (Large White × Landrace sows crossed with Pietrain boars) entire male pigs were randomly allocated to the following experimental diets (*n*=10): cereal-soybean meal basal diet (control); 2) basal diet with 5% *C. vulgaris*; 3) basal diet with 5% *C. vulgaris* supplemented with 0.005% Rovabio® Excel AP; and 4) basal diet with 5% *C. vulgaris* supplemented with 0.01% of a preselected four-CAZyme mixture. The experimental trial lasted from 59.1 ± 5.69 kg of initial live weight to 101 ± 1.9 kg of slaughter weight. Data indicate that this high dietary level of *C. vulgaris* has an enormous impact on the profile of blood cells and plasma metabolites of finishing pigs. However, the most relevant health outcome observed under these experimental conditions was a strong immunosuppressive effect promoted by the microalga, which increases pigs' susceptibility to infection diseases. This decrease of pigs' immune response could be due to dose-specific immunoregulatory properties of *Chlorella* polysaccharides. In addition**,** the inclusion of *C. vulgaris* in the diet reduced the systemic antioxidant capacity of pigs. In turn, the dietary supplementation with the four-CAZyme mixture**,** but not with Rovabio® Excel AP, promoted a clear decrease on some blood parameters compared with the control group, including white blood cells, plasma lipids and immunoglobulins. Conversely, pigs fed the same diet had higher levels of glucose, insulin and insulin resistance. Regarding hepatic lipids, pigs fed diets incorporated with *C. vulgaris*, supplemented or not with the exogenous carbohydrases, had an increased hepatic content of *n*-3 PUFA, with a consequent health beneficial decrease on the *n*-6/*n*-3 ratio. In view of these findings, the use of *C. vulgaris* as feed ingredient appears to be safe under controlled experimental conditions but could be challenging in industrial production systems, with more stressful and less hygienic environments.

Key words: *Chlorella vulgaris*; Carbohydrate-Active Enzyme; finishing pigs; blood composition; hepatic lipid profile; immune response.

6.1. Introduction

Pork is one of the most consumed meats worldwide. Over the past few years, the livestock industry has developed feeding strategies to achieve higher production rates and improved pork quality to satisfy the current demand of consumers (FAO 2004; Madeira et al. 2015). Cereal grains and soybean food crops remain the two main conventional feedstuffs for animal feeding (FAO 2011). The significant increase in pork consumption leads to the intensification of these raw materials cultivation (Ekmay et al. 2014). As a result, the massive production of these feedstuffs and their direct competition with human consumption has high economic and environmental impacts (Manceron et al. 2014; Madeira et al. 2017).Therefore, sustainable and eco-friendly alternatives of feed ingredients are urgently needed for this new era of livestock industry (Ekmay et al. 2014; Taelman et al. 2015).

Pork is usually associated with a negative image of fat due to the high percentage of SFA and the low percentage of PUFA and lipid-soluble antioxidant vitamins (Morgan et al. 1992). It is important to reverse this pattern by increasing the content of omega-3 PUFA (*n*-3 PUFA) and lipid-soluble antioxidant vitamins in pork, because the consumption of these beneficial bioactive compounds by the general population is actually below the recommended limits (Calder 2017). A possible way to modify fat composition of pork is by implementing some feeding strategies (Wood and Enser 1997). In this regard, the inclusion of microalgae in animal feeds is a viable green approach, due to their well-balanced nutritional composition and richness in the beneficial antioxidants and *n*-3 PUFA (Christaki et al. 2011; Lum et al. 2013; Yaakob et al. 2014).

Chlorella vulgaris, a eukaryotic freshwater green microalga, is one of the most remarkable microalgae species and a promising sustainable alternative for monogastrics feeding (Liu and Chen 2014; Safi, Zebib, et al. 2014; Kotrbáček et al. 2015). Besides enhancing the nutritional value of diets, the incorporation of *C. vulgaris* has also been recognized as having hypocholesterolaemic, hepatoprotective, immunomodulatory and anti-inflammatory properties (Abdelnour et al. 2019; Abd El Latif et al. 2021). Until to date, studies exploiting the effect of dietary inclusion of *C. vulgaris* on circulatory and hepatic systems have been performed mainly using supplementation levels (<1% in diet) in poultry.

The major nutritional disadvantage of *C. vulgaris* is the recalcitrant cell wall, which is composed by a diverse and complex matrix of cross-linked insoluble carbohydrates (Baudelet et al. 2017). This recalcitrant cell wall is largely indigestible by monogastrics, thus impairing the bioavailability of the inner valuable nutritional compounds of *C. vulgaris* (Liu and Chen 2014; Teuling et al. 2019). In turn, the use of exogenous Carbohydrate-Active enZymes (CAZymes) as feed supplements for monogastric livestock species is well established for cereal-based diets (Jacela et al. 2009; Ravindran and Son 2012). In addition, several *in vitro*

studies have demonstrated the potential of CAZymes to disrupt microalga cell walls (Zheng et al. 2011; Cho et al. 2013; Gerken et al. 2013). In a recent study conducted by Coelho and colleagues (2019), a novel four-CAZyme mixture able to disrupt *C. vulgaris* recalcitrant cell wall, enabling the release of trapped value-added nutrients, was successfully described.

Our research group has recently shown that 5% of *C. vulgaris* incorporation in the diet improves the nutritional value of pork, without compromising the performance of finishing pigs (Coelho et al. 2020). However, the impact of *C. vulgaris* as feed ingredient (>1% in diet) on pigs' health and hepatic lipid metabolism remains to be established. In line with this, and using the same experimental design, we assessed herein the effect of 5% *C. vulgaris* incorporation in the diet, supplemented or not with the commercially available Rovabio® Excel AP and the four-CAZyme mixture described by Coelho et al. (2019) on blood cells, plasma metabolites and hepatic lipid metabolism of finishing pigs.

6.2. Material and Methods

6.2.1. Animal trial and experimental diets

The experimental trial was performed at Unidade de Investigação em Produção Animal do Instituto Nacional de Investigação Agrária e Veterinária (UEISPA-INIAV, Santarém, Portugal). All experimental procedures were reviewed by the Ethics Commission of Centro de Investigação Interdisciplinar em Sanidade Animal/Faculdade de Medicina Veterinária (CIISA/FMV) and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Alimentação e Veterinária, Portugal), in line with the European Union guidelines (2010/63/EU Directive). All the staff members involved in the animal experiment are licensed from the Portuguese Veterinary Services.

The animal trial was described in detail in a companion paper (Coelho et al. 2020). Briefly, forty crossbred entire male pigs, sons of Large White × Landrace sows crossed with Pietrain boars, were subjected to an adaptation period of 8 days to reduce stress and stable all metabolic conditions. Then, pigs with an initial body weight of 59.1 ± 5.69 kg were randomly allocated into 10 pens, each one with 4 pigs. Four experimental diets were randomly assigned to pigs within each pen, with each animal receiving a different diet, making the pig the experimental unit. The experimental diets were: Control, a cereal and soybean meal-based diet; CH, the control diet added 5% of *C. vulgaris* provided by Allmicroalgae (Natural Products, Portugal) (CH); CHR, the control diet added 5% of *C. vulgaris* supplemented with 0.005% of Rovabio® Excel AP (Adisseo, Antony, France); and CHM, the control diet added 5% of *C. vulgaris* supplemented with 0.01% of the preselected four-CAZyme mixture, composed by an

exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme (Coelho et al. 2019).

6.2.2. Analysis of *C. vulgaris* and experimental diets

During the entire trial, diet samples were collected three times. To assess the proximal chemical composition of *C. vulgaris* and experimental diets, AOAC (2000) methods were used. For DM determination, samples were dried at 103 °C until constant weight. For the calculation of crude protein of samples, the nitrogen content (N) was determined by the Kjeldahl method and the protein content was obtained using the factor 6.25 × N, following the method 954.01 (AOAC 2000). To determine the ash and starch content of samples, the method 942.05 (AOAC 2000) and Clegg (1956) procedure, respectively were applied. The determination of crude fat of samples was carried out after automatic Soxhlet extraction with petroleum ether (Gerhardt Analytical Systems, Königswinter, Germany). The analysis of crude fibre, ADF and NDF was performed by the method 989.03 (AOAC 2000). Feed ME was determined as described by Noblet et al. (1989).

Fatty acids in *C. vulgaris* and experimental diets were converted to FAME after extraction and acidic methylation. FAME were analysed by gas chromatography and heneicosaenoic acid (21:0) methyl ester was used as the internal standard (Sukhija and Palmquist 1988).

The diterpene composition of *C. vulgaris* and experimental diets was determined through HPLC analysis (Prates et al. 2006). The pigment composition was analysed according to Teimouri et al. (2013), with minor alterations implemented by Pestana et al. (2020). The pigment content of *C. vulgaris* and experimental diets was quantified as proposed by Hynstova et al. (2018).

The main ingredients and chemical composition of the experimental diets are shown in Table 6.1. The chemical composition of *C. vulgaris* and additional information on experimental diets is available elsewhere (Coelho et al. 2020).

Table 6.1. Main ingredients and chemical composition of experimental diets.

	Experimental diets[1]			
Item	**Control**	**CH**	**CHR**	**CHM**
Ingredients (% as fed basis)				
Corn	56	56	56	56
Soybean meal	19.3	11.7	11.6	11.7
Barley	10	10	10	10
Sunflower meal	5.4	6.8	6.8	6.8
Wheat	5	5	5	5
Soybean oil	0.5	0.2	0.2	0.2
Chlorella vulgaris	-	5	5	5
Mix of 4 CAZymes	-	-	-	0.01
Rovabio® Excel AP	-	-	0.005	-
Metabolizable energy (kcal/kg DM)[2]	3576	3540	3644	3547
Proximate composition (g/100 g)				
Dry matter	90.0	89.7	89.5	90.0
Crude protein	14.0	15.9	15.2	15.2
Starch	45.5	45.3	44.7	47.4
Crude fat	2.60	3.00	3.10	3.10
Crude fibre	4.60	5.00	5.30	5.20
Neutral detergent fibre	13.7	13.9	12.7	13.7
Acid detergent fibre	4.90	5.50	5.50	5.90
Ash	4.03	4.70	4.60	4.60
Fatty acid profile (% total fatty acids)				
C14:0	0.150	0.218	0.190	0.190
C16:0	16.3	16.6	16.3	16.5
C16:1*c*9	0.228	1.14	0.989	0.972
C17:0	0.189	0.182	0.153	0.154
C17:1*c*9	0.038	0.704	0.739	0.732
C18:0	2.89	3.29	3.11	3.08
C18:1*c*9	27.4	27.4	27.6	27.5
C18:1*c*11	0.885	1.70	1.38	1.42
C18:2*n*-6	48.1	44.1	45.1	44.9
C18:3*n*-3	2.57	3.47	3.28	3.28
C20:0	0.528	0.513	0.517	0.500
C20:1*c*11	0.292	0.288	0.320	0.320
C22:0	0.304	0.294	0.262	0.266
C22:1*n*-9	0.155	0.155	0.131	0.149
Diterpene profile (mg/100 g)				

α-Tocopherol	1.65	1.87	1.94	1.65
α-Tocotrienol	0.48	0.37	0.39	0.44
β-Tocopherol	0.038	0.026	0.024	0.026
γ -Tocopherol	0.35	0.27	0.23	0.26
γ-Tocotrienol	0.72	0.59	0.73	0.60
δ-Tocopherol	0.034	0.033	0.031	0.031
δ-Tocotrienol	0.029	0.023	0.025	0.025
Pigments (mg/100 g)				
β-Carotene	0.12	0.71	0.74	0.65
Chlorophyll *a*[3]	0.43	12.7	13.9	12.6
Chlorophyll *b*[4]	0.75	3.39	3.66	3.42
Total Chlorophylls[5]	1.18	16.1	17.6	16.0
Total Carotenoids[6]	0.40	3.65	3.95	3.49
Total Chlorophylls + Carotenoids[7]	1.57	19.8	21.5	19.5

[1.]Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR - basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. n.d. – not detected.

[2.]Metabolizable energy (kcal/kg dry matter) = 4412-11.06 × ash + 3.37 × crude fat - 5.18 × ADF.

[3.]Ca = 11.24 A662 - 2.04 A645.

[4.]Cb = 20.13 A645 - 4.19 A662.

[5.]Ca+b = 7.05 A662 + 18.09 A645.

[6.]Cx+c = (1000 A470 - 1.90 Ca - 63.14 Cb) / 214.

[7.](Ca+b) + (Cx+c).

6.2.3. Animals slaughter and sampling

Food was withdrawn from animal 17 to 19 h before slaughter. Pigs with a body weight of 101 ± 1.9 kg were slaughtered at the Unidade de Investigação em Produção Animal experimental slaughterhouse (Santarém, Portugal), using the same protocol reported by (Coelho et al. 2020). For haematology, blood samples were collected on anticoagulant ethylenediaminetetraacetic acid (EDTA) tubes. For the remained blood parameters, samples were centrifuged at 1500 x *g* for 15 min to obtain plasma and stored at -20 ºC. Liver samples were vacuum packed and kept at -20 ºC until analysis.

6.2.4. Blood parameters determination

Complete blood counts (red blood cells; white blood cells and thrombocytes) 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.

The haemoglobin concentration was measured by photometry with sodium lauryl sulphate as reagent. For white blood cells differential counting (%), the blood smears were stained with the May-Grünwald-Giemsa technique. At least 200 white blood cells were counted and classified.

The determination of total cholesterol, High-Density Lipoprotein (HDL) cholesterol, Low-Density Lipoprotein (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) was performed in a Modular Hitachi Analytical System (Roche Diagnostics, Mannheim, Germany), through diagnostic kits (Roche Diagnostics). For Very Low-Density Lipoprotein (VLDL) cholesterol and total lipids, Friedewald et al. (1972) and Covaci et al. (2006) formulas were applied, respectively. Insulin concentration was determined through 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 insulin resistance index (HOMA-IR): fasting serum glucose (mmol/L) multiplied by fasting serum insulin (mU/L) divided by 22.5 (Matthews et al. 1985). The immunoglobulin profile, including IgA, IgG and IgM was determined by immunoturbidimetry.

The total antioxidant capacity was assessed in plasma by the QuantiChrom Antioxidant Assay Kit (DTAC-100, Bioassay Systems, Hayward, CA, USA). The glutathione peroxidase (GPX) activity was measured in plasma through the EnzyChrom Glutathione Peroxidase Assay Kit (EGPX-100, Bioassay Systems). One unit of GPX is considered the amount of GPX that produces 1 µmol of glutathione disulphide (GS-SG) per min at pH = 7.6 and room temperature.

6.2.5. Hepatic total cholesterol and diterpene profile determination

Total cholesterol, pro-vitamin A (β-carotene) and vitamin E homologues (tocopherols and tocotrienols) were determined, in duplicate, in hepatic samples, according to Prates et al. (2006). After a saponification reaction, total cholesterol and diterpenes of liver samples were analysed by HPLC system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA, USA). Total cholesterol and β-carotene were detected using UV-Visible photodiode array detector (λ=202 nm and λ=450 nm, respectively), and tocopherols and tocotrienols using fluorescence detector (excitation λ=295 nm and emission λ=325 nm). The amount of total cholesterol, β-carotene and vitamin E homologues in hepatic samples was quantified using standard curves of peak area *versus* concentration.

6.2.6. Hepatic pigments determination

The quantification of chlorophyll *a*, chlorophyll *b* and total carotenoids was performed using the method described by Teimouri et al. (2013) with minor modifications implemented by Coelho et al. (2020). The results were calculated using the equations described by Hynstova et al. (2018), as applied above for *C. vulgaris* and experimental diets.

6.2.7. Hepatic total lipid content and fatty acid profile determination

For determination of total lipids and fatty acid composition, hepatic samples were lyophilised (-60 °C and 2.0 hPa, lyophilizator Edwards Modulyo, Crawley, UK). Total lipids, in duplicate, were gravimetrically quantified following Folch et al. (1957) protocol. Afterwards, the fat residue was re-suspended in dry toluene and submitted to successive alkaline and acid transesterification reaction to promote the conversion of FA into FAME (Sukhija and Palmquist 1988). The separation of FAME was performed through gas-liquid chromatography (GC-FID HP7890A Hewlett-Packard, Avondale, PA), as reported by Madeira et al. (2014). The reference standard (FAME mix 37 components, Supelco Inc.) was used for FAME identification, which was confirmed by gas chromatography combined with a mass spectrometry detector (GC-MS QP2010-Plus, Shimadzu, Kyoto, Japan). For FAME quantification, the internal standard method using heneicosanoic acid (21:0) methyl ester as internal standard was applied. FA were expressed as percentage of total FA.

6.2.8. Statistical analysis

The SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA) was used to analyse data by one-way analysis of variance (ANOVA) by the GLM procedure. All data were checked for normal distribution and variance homogeneity. The statistical model considered the feeding treatment the fixed effect and the pig the experimental unit. Least square means for multiple comparisons were generated using the PDIFF option adjusted with Tukey-Kramer in order to determine statistical differences among the experimental diets. The significance level was set at $p < 0.05$. In addition, blood parameters and hepatic lipid composition were subjected to principal component analysis (PCA) using the Statistica software (version 8.0; TIBCO, Palo Alto, CA, USA).

6.3. Results

6.3.1. Haematology, plasma metabolites and immunoglobulin profile

Blood cells, plasma metabolites and immunoglobulins of finishing pigs fed *C. vulgaris*, individually or combined with the mixtures of exogenous carbohydrases, are presented in Table 6.2. The number of white blood cells was lower in pigs fed the CHM diet comparing with the other experimental groups ($p < 0.001$). Pigs fed with CH and CHR diets had a higher proportion of granulocytes ($p < 0.001$) and, inversely, the percentage of lymphocytes was higher in pigs from the control group ($p < 0.001$). The number of red blood cells was lower in pigs fed with CH diet and higher in pigs fed with CHM and control ($p = 0.003$) diets. The concentration of haemoglobin was higher in the control group, lower in pigs fed with CH diet and intermediate in pigs fed with CHR and CHM ($p < 0.001$). The number of thrombocytes was significantly higher in pigs fed with CHM diet ($p = 0.001$).

For plasma lipids, total lipids ($p < 0.001$), TAG ($p < 0.001$), total cholesterol ($p < 0.001$), HDL-cholesterol ($p < 0.001$), LDL-cholesterol ($p < 0.001$) and VLDL-cholesterol ($p < 0.001$) decreased in pigs fed with the CHM diet. In addition, glucose levels were higher in pigs fed with CHM and CH diets ($p < 0.001$). The insulin levels were also increased in pigs fed with the CHM diet ($p < 0.001$), whereas for the other experimental groups the insulin level was below the methodological detection limit (< 0.4 mU/L). Consequently, the HOMA-IR for these experimental groups was in the range below 0.120 mmol/L × µU/mL, while pigs fed with the CHM diet had an increase of this parameter ($p = 0.001$). Pigs fed with the CHM diet also presented higher urea ($p = 0.004$) and creatinine values along with the control group ($p < 0.001$).

Regarding the hepatic function, pigs fed with control and CHM diets exhibited the lower values for ALT ($p < 0.001$) and pigs fed with CHR and CHM diets had the lower values for AST ($p = 0.001$). For ALP, pigs fed with diets incorporated with *C. vulgaris*, supplemented or not with CAZymes, presented lower values comparing to the control group ($p = 0.002$). For GGT, pigs fed with control and CHM diets had the lowest values ($p < 0.001$).

The immunoglobulin profile revealed that pigs fed with diets incorporating *C. vulgaris*, supplemented or not with carbohydrases, presented a decrease in the concentration of IgA and IgG ($p < 0.001$). The decrease in IgG was more pronounced in pigs fed with the CHM diet. The IgM levels decreased in pigs fed with the CH diet and, even more, with the CHM diet ($p < 0.001$).

Table 6.2. Effect of experimental diets on haematology, plasma metabolites and immunoglobulin profile of pigs.

	Experimental diets[1]					
Item	**Control**	**CH**	**CHR**	**CHM**	**SEM**	***p*-value**
Haematology						
White blood cells (×10^9/L)	21.6^{bc}	18.5^{ab}	22.4^{c}	16.8^{a}	0.975	<0.001
Leucogram (% white blood cells)						
Granulocytes	31.3^{a}	46.2^{c}	50.9^{c}	37.1^{b}	1.51	<0.001
Lymphocytes	66.7^{d}	51.4^{b}	42.0^{a}	60.1^{c}	3.03	<0.001
Monocytes	2.00	2.40	2.00	2.33	0.356	0.701
Red blood cells (×10^{12}/L)	8.10^{b}	6.94^{a}	7.60^{ab}	7.70^{b}	0.204	0.003
Haemoglobin (g/L)	141^{c}	118^{a}	131^{b}	130^{b}	2.87	<0.001
Thrombocytes (×10^9/L)	442^{ab}	389^{a}	414^{a}	510^{b}	23.6	0.001
Plasma metabolites						
Total lipids (g/L)[2]	3.84^{b}	4.15^{c}	4.02^{c}	2.62^{a}	0.038	<0.001
TAG (mg/L)	632^{b}	608^{b}	725^{c}	359^{a}	15.1	<0.001
Total cholesterol (mg/L)	854^{b}	1023^{c}	898^{b}	379^{a}	17.4	<0.001
HDL-cholesterol (mg/L)	391^{c}	358^{b}	393^{c}	110^{a}	7.76	<0.001
LDL-cholesterol (mg/L)	348^{b}	561^{c}	381^{b}	204^{a}	13.0	<0.001
VLDL-cholesterol (mg/L)[3]	126^{b}	122^{b}	145^{c}	71.8^{a}	3.03	<0.001
Glucose (mg/L)	808^{a}	966^{b}	725^{a}	1049^{b}	25.6	<0.001
Insulin (mU/L)	<0.4^{a}	<0.4^{a}	<0.4^{a}	0.727^{b}	0.058	<0.001
HOMA – IR (mmol/L × μU/mL)[4]	<0.12^{a}	<0.12^{a}	<0.12^{a}	0.19^{b}	0.013	0.001
Urea (mg/L)	242^{ab}	224^{a}	246^{ab}	259^{b}	6.22	0.004
Creatinine (mg/L)	18.0^{b}	10.7^{a}	13.3^{a}	17.1^{b}	0.85	<0.001
Total protein (g/L)	60.9	57.9	60.5	60.5	1.18	0.270
Hepatic markers						
ALT (U/L)	24.8^{ab}	35.0^{c}	31.4^{bc}	19.5^{a}	1.77	<0.001
AST (U/L)	46.2^{b}	41.5^{b}	38.6^{ab}	27.8^{a}	2.85	0.001
ALP (U/L)	221^{b}	158^{a}	161^{a}	158^{a}	12.4	0.002
GGT (U/L)	12.7^{a}	27.3^{b}	37.8^{c}	19.0^{a}	1.67	<0.001
Immunoglobulins						
IgA (mg/L)	88.3^{b}	47.4^{a}	55.3^{a}	54.2^{a}	5.03	<0.001
IgG (g/L)	4.50^{c}	2.94^{a}	3.46^{b}	2.91^{a}	0.130	<0.001
IgM (g/L)	1.65^{c}	1.32^{b}	1.36^{cb}	0.63^{a}	0.086	<0.001

[1.]Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR - basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. SEM – Standard error of the mean; TAG – triacylglycerols; HDL – high-density lipoproteins; LDL – low-density lipoproteins; VLDL –very low-density lipoproteins; ALT – alanine aminotransferase (EC 2.6.1.2); AST – aspartate

aminotransferase (E.C. 2.6.1.1); ALP – alkaline phosphatase (EC 3.1.3.1); GGT – gamma-glutamyltransferase (EC 2.3.2.13); IgA – immunoglobulin A; IgG – immunoglobulin G; IgM – immunoglobulin M.

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

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

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

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

6.3.2. Plasma antioxidant potential of pigs

Figure 6.1 displays the effect of experimental diets on plasma total antioxidant capacity (TAC) and glutathione peroxidase (GPX) activity. The CH group exhibited a lower level of TAC when compared with the control group ($p = 0.019$). TAC levels of CHR and CHM groups did not differ significantly from the levels of the control and CH groups ($p > 0.05$). The experimental diets promoted no changes in GPX activity ($p = 0.367$).

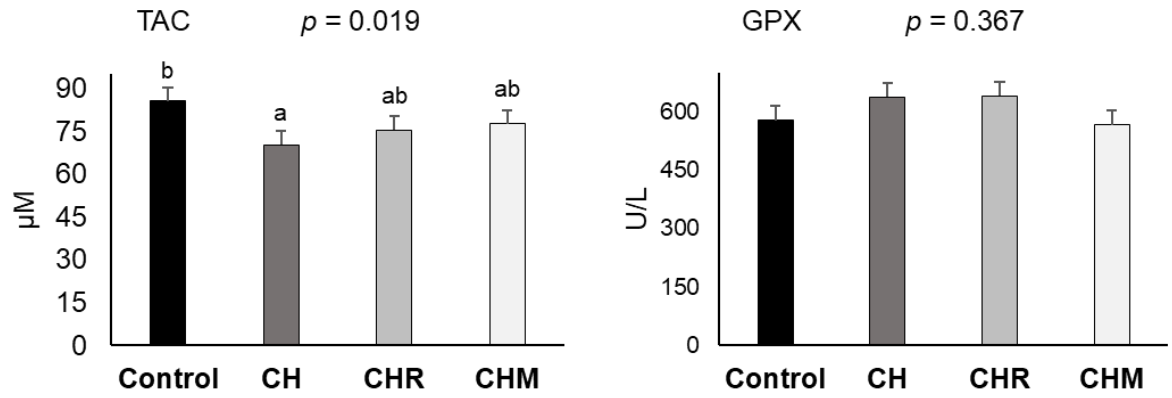

Figure 6.1. Effect of experimental diets on plasma total antioxidant capacity (TAC) and glutathione peroxidase (GPX) activity of pigs. One unit of GPX is the amount of GPX that produces 1 µmol of GS-SG per min at pH = 7.6 and room temperature. Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR - basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. [a, b] Values with different letters differ significantly at $p < 0.05$.

6.3.3. Lipid content, total cholesterol and fatty acid composition in the liver

The effect of experimental diets on hepatic lipid content, total cholesterol and fatty acid composition from finishing pigs is presented in Table 6.3. The experimental diets did not affect the content of total lipids ($p = 0.112$) and total cholesterol ($p = 0.061$). The predominant fatty acids found in the liver were: 18:0 (29.2-31.7%), 18:2*n*-6 (15.7-16.5%), 16:0 (15.4-16.3%), 18:1*c*9 (14.2-16.1%), and 20:4*n*-6 (11.0-13.1% of total fatty acids). However, only the percentage of some minor fatty acids (< 4% of total fatty acids) was significantly affected by the experimental diets, affecting 6 out of 32 fatty acids identified. The monounsaturated fatty

acids 18:1*c*11 and 22:1*n*-9, and the *n*-3 fatty acids 18:4*n*-3, 20:3*n*-3, 20:5*n*-3 andC22:6*n*-3, had a lower percentage in the control group relative to *C. vulgaris* groups ($p = 0.042$, $p = 0.011$, $p = 0.001$, $p = 0.003$, $p = 0.020$ and $p = 0.029$, respectively). Among *C. vulgaris* groups, pigs fed with the CHM diet had the highest percentage of these unsaturated fatty acids, except 20:5*n*-3 (eicosapentaenoic acid, EPA) and 22:6*n*-3 (docosahexaenoic acid, DHA). The highest percentages of EPA and DHA were found in pigs fed with the CH diet, being the amounts 1.5-fold and 1.3-fold higher, respectively, when compared with the control group.

As regards to the partial sums and ratios of fatty acids, the experimental diets promoted a significant increase of total *n*-3 PUFA in the liver, with an increase of 23% in pigs fed with the CH diet comparing to the control group ($p = 0.010$). This increase reflects the individual effects of *n*-3 PUFA, EPA and DHA. The experimental diets had no impact on the other partial sums of fatty acids or PUFA:SFA ratio. Conversely, all *C. vulgaris* groups had a significant decrease of 26% in the *n*-6:*n*-3 ratio, relative to the control ($p = 0.001$).

Table 6.3. Effect of experimental diets on hepatic total lipid content, total cholesterol and fatty acid composition of pigs.

	Experimental diets[1]					
Item	**Control**	**CH**	**CHR**	**CHM**	**SEM**	***p*-value**
Total lipids (g/100 g)	2.85	2.43	2.72	2.52	0.129	0.112
Cholesterol (g/100 g)	0.197	0.195	0.185	0.179	0.005	0.061
Fatty acid composition (g/100 g FA)						
12:0	0.009	0.008	0.010	0.011	0.003	0.887
14:0	0.215	0.184	0.238	0.213	0.025	0.497
14:1*c*9	0.017	0.034	0.018	0.016	0.008	0.333
15:0	0.095	0.097	0.100	0.092	0.006	0.824
DMA C16:0	0.010	0.014	0.020	0.044	0.010	0.095
16:0	15.4	15.4	15.7	16.3	0.444	0.432
16:1*c*7	0.349	0.345	0.379	0.356	0.031	0.868
16:1*c*9	0.671	0.613	0.694	0.662	0.047	0.666
17:0	0.883	0.927	0.908	0.818	0.080	0.788
17:1*c*9	0.254	0.235	0.271	0.260	0.021	0.670
DMA C18:0	0.017	0.035	0.025	0.053	0.013	0.252
DMA C18:1	0.000	0.004	0.003	0.007	0.003	0.435
18:0	29.2	31.7	29.9	29.3	0.912	0.215
18:1*c*9	16.1	14.2	15.8	15.9	0.606	0.139
18:1*c*11	1.95[a]	2.16[ab]	2.15[ab]	2.34[b]	0.091	0.042
18:2*t*9*t*12	0.017	0.023	0.021	0.030	0.005	0.350
C18:2*n*-6	15.7	15.9	16.5	16.2	0.540	0.779
18:3*n*-6	0.169	0.148	0.109	0.130	0.022	0.269

18:3*n*-3	0.212	0.279	0.308	0.277	0.034	0.259
18:4*n*-3	0.005[a]	0.056[b]	0.071[b]	0.087[b]	0.013	0.001
20:0	0.058	0.063	0.058	0.056	0.003	0.568
20:1*c*11	0.130	0.122	0.120	0.127	0.006	0.591
20:2*n*-6	0.278	0.299	0.285	0.280	0.019	0.862
20:3*n*-6	0.408	0.452	0.434	0.410	0.043	0.870
20:4*n*-6	13.1	12.0	11.0	11.5	0.684	0.175
20:3*n*-3	0.029[a]	0.066[b]	0.052[ab]	0.075[b]	0.008	0.003
20:5*n*-3	0.193[a]	0.291[b]	0.263[ab]	0.275[ab]	0.022	0.020
C22:0	0.048	0.058	0.056	0.062	0.004	0.114
22:1*n*-9	0.042[a]	0.073[ab]	0.063[ab]	0.099[b]	0.011	0.011
22:5*n*-3	1.01	1.19	1.25	1.14	0.088	0.284
22:6*n*-3	0.633[a]	0.829[b]	0.735[ab]	0.764[ab]	0.044	0.029
23:0	0.085	0.099	0.099	0.096	0.007	0.553
Others	2.75	2.06	2.47	2.02	0.222	0.080
Partial sums of fatty acids (g/100 g FA)						
SFA[2]	46.0	49.0	47.0	47.0	1.11	0.445
MUFA[3]	19.5	17.8	19.5	19.8	0.725	0.231
PUFA[4]	31.8	31.5	31.1	31.0	0.828	0.900
n-3 PUFA[5]	2.08[a]	2.71[b]	2.68[b]	2.61[ab]	0.141	0.010
n-6 PUFA[6]	29.7	28.8	28.3	28.5	0.733	0.553
Ratios of fatty acids						
PUFA:SFA	0.697	0.654	0.668	0.667	0.032	0.801
n-6:*n*-3	14.7[b]	10.8[a]	10.8[a]	11.3[a]	0.691	0.001

[1.] Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR - basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. SEM – standard error of the mean; FA – fatty acids; DMA – dimethylacetal; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

[2.] Sum (C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0 and C23:0).

[3.] Sum (C14:1*c*9, C16:1*c*7, C16:1*c*9, C17:1*c*9, C18:1*c*9, C18:1*c*11, C20:1*c*11 and C22:1*n*-9).

[4.] Sum (C18:2*n*-6, C18:3*n*-6, C18:2*t*9*t*12, C18:3*n*-3, C18:4*n*-3, C20:2*n*-6, C20:3*n*-6, C20:4*n*-6, C20:3*n*-3, C20:5*n*-3, C22:5*n*-3 and C22:6*n*-3).

[5.] Sum (C18:3*n*-3, C18:4*n*-3, C20:3*n*-3, C20:5*n*-3, C22:5*n*-3 and C22:6*n*-3).

[6.] Sum (C18:2*n*-6, C18:3*n*-6, C20:2*n*-6, C20:3*n*-6 and C20:4*n*-6).

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

6.3.4. Diterpene and pigment contents in the liver

The effect of experimental diets on hepatic content of diterpenes and pigments is presented in Table 6.4. α-Tocopherol was affected by experimental diets ($p = 0.001$), showing a decrease of approximately 36% in all *C. vulgaris* groups relative to the control. On the contrary, γ-tocopherol and pigments were not influenced by diets ($p > 0.05$). β-Carotene was undetected in any feeding treatment.

Table 6.4. Effect of experimental diets on hepatic α-tocopherol, γ-tocopherol and pigments of pigs.

	Experimental diets[1]					
Item	**Control**	**CH**	**CHR**	**CHM**	**SEM**	***p*-value**
Diterpene profile (µg/100 g)						
α-Tocopherol	314[b]	212[a]	199[a]	225[a]	19.7	0.001
γ-Tocopherol	5.2	7.6	6.5	5.4	0.76	0.115
Pigments (µg/100 g)						
Chlorophyll-*a*[2]	11.4	12.5	14.1	11.8	2.35	0.863
Chlorophyll-*b*[3]	25.8	16.8	23.4	15.7	4.03	0.235
Total chlorophylls[4]	37.2	29.3	37.4	27.6	5.93	0.523
Total carotenoids[5]	95.3	113	107	117	6.56	0.124
Total chlorophylls + Carotenoids[6]	132	142	144	144	8.65	0.739

[1.] Experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR -basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes. SEM – standard error of the mean; n.d. – not detected.

[2.] Ca = 11.24 A662 - 2.04 A645.

[3.] Cb = 20.13 A645 - 4.19 A662.

[4.] Ca+b = 7.05 A662 + 18.09 A645.

[5.] Cx+c = (1000 A470 - 1.90 Ca - 63.14 Cb) / 214.

[6.] (Ca+b) + (Cx+c).

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

6.3.5. Principal component analysis (PCA)

To assess the main source of variation for systemic and hepatic parameters of pigs fed with different diets, a discriminant analysis was applied. The PCA performed with hepatic lipid composition did not show a good resolution among experimental diets (data not shown), whereas the PCA score plots of blood parameters, including plasma metabolites, hepatic markers, immunoglobulins and antioxidant capacity indicators, explained 64% of the total variation, with 45% for factor 1 and 19% for factor 2 Figure 6.2. Figure 6.2A and Table 6.5

show loadings for the first two factors obtained for each variable. The variables with the highest discriminant power on factor 1 were total lipids, HDL-cholesterol, total cholesterol and VLDL-cholesterol. The variables with the highest discriminant power on factor 2 were IgG, IgA, creatinine and ALP. As shown in Figure 6.2B, the control and the CHM groups were clearly discriminated from the remaining groups. The same did not apply to CH and CHR groups, which were overlapped, thus suggesting that the blood profiles of these feeding groups were partially similar. The control group was clustered in the negative area along both factors (quadrant *c*) and, inversely, the CH group was clustered in the positive area along factor 2 (quadrant *a*). The CHR and CHM groups were more dispersed and allocated in quadrants *a-c* and quadrants *b-d*, respectively.

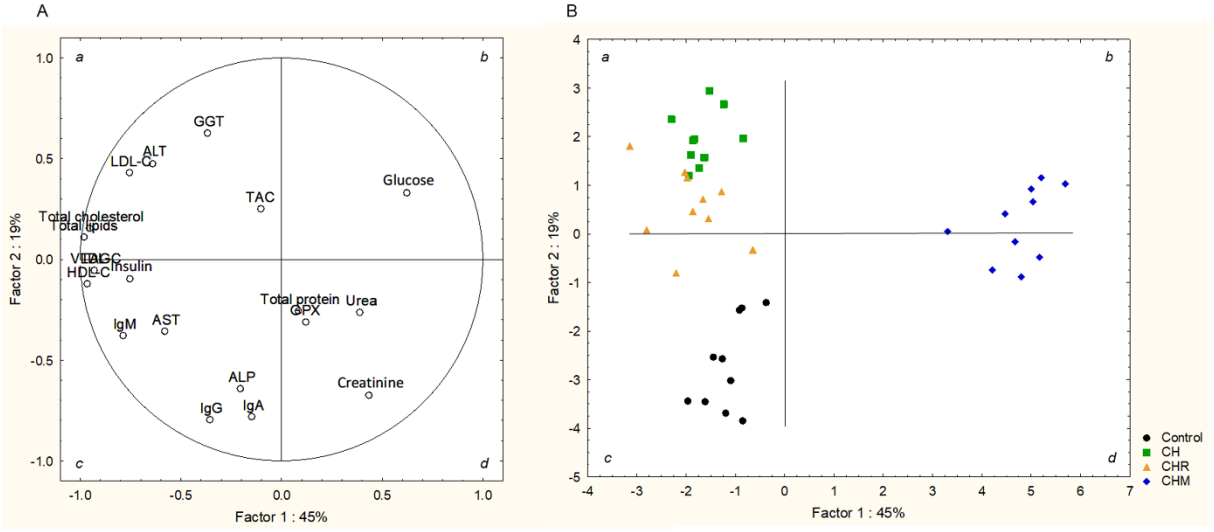

Figure 2

Figure 6.2. Loading plot of the first and second principal factors of the pooled data (A) and component score vectors (B) using plasma metabolites from pigs fed with experimental diets: Control - corn-soybean basal diet; CH - basal diet plus 5% *C. vulgaris*; CHR - basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM - basal diet plus 5% *C. vulgaris* + 0.01% mix of 4 CAZymes.

Table 6.5. Loadings for the first two principal factors.

Variables	Factor 1	Factor 2
Total lipids	-0.981	0.111
TAG	-0.931	-0.054
Total Cholesterol	-0.955	0.154
HDL-cholesterol	-0.967	-0.121
LDL-cholesterol	-0.756	0.431
VLDL-cholesterol	-0.931	-0.054
Glucose	0.622	0.331
Insulin	-0.755	-0.096
Urea	0.388	-0.263
Creatinine	0.433	-0.674
Total protein	0.083	-0.256
ALT	-0.642	0.475
AST	-0.581	-0.356
ALP	-0.205	-0.641
GGT	-0.369	0.627
IgA	-0.149	-0.780
IgG	-0.357	-0.796
IgM	-0.788	-0.379
TAC	-0.103	0.251
GPX	0.120	-0.311

TAG - triacylglycerols; HDL - high-density lipoproteins; LDL - low-density lipoproteins; VLDL - very low-density lipoproteins; ALT - alanine aminotransferase (EC 2.6.1.2); AST - aspartate aminotransferase (E.C. 2.6.1.1); ALP - alkaline phosphatase (EC 3.1.3.1); GGT - gamma-glutamyltransferase (EC 2.3.2.13); IgA - immunoglobulin A; IgG - immunoglobulin G; IgM - immunoglobulin M.

6.4. Discussion

In this study, it was approached for the first time the effect of a high level of *C. vulgaris* (5% incorporation in the diet) on blood cells, plasma biochemical markers and hepatic lipid metabolism in finishing pigs. In fact, the majority of studies available in the literature reported the influence of *C. vulgaris* as supplement (Baňoch et al. 2012; Yan et al. 2012; Furbeyre et al. 2018), and not as ingredient (> 1% in the diet), on pigs' growth performance, and not on health- or metabolic-related parameters. In addition, due to the recalcitrance of *C. vulgaris* cell wall, it was also assessed the effect of the supplementation with two mixtures of exogenous CAZymes on *C. vulgaris* diets: the Rovabio® Excel AP, a commercially available mixture; and a four-CAZyme mixture tested successfully *in vitro* by Coelho et al. (2019). The effect of these

experimental conditions on animal productive parameters and pork quality traits has been published already in a companion paper (Coelho et al. 2020). Briefly, 5% of *C. vulgaris* fed to finishing pigs improved the nutritional value of pork without impairing animal growth. Furthermore, at this level of *C. vulgaris* incorporation, the supplementation with Rovabio® or recombinant CAZyme mixture was unnecessary for pigs' productive performance maintenance.

Dietary supplementation of *Chlorella* in mammals, including humans, exhibited several beneficial activities, including antioxidant, antidiabetic, antihypertensive and antihyperlipidemic properties (Lee et al. 2008; Bito et al. 2020). Herein, the plasma lipid profile was largely affected by the experimental diets. In fact, a positive reducing effect on total lipids, total cholesterol, LDL-cholesterol, VLDL-cholesterol and TAG was consistently found in pigs fed with the four-CAZyme mixture. Total cholesterol exceeded the reference figures for pigs (Jackson and Cockcroft 2002a) across all experimental diets, except for *C. vulgaris* supplemented with the four-CAZyme mixture. Several reports have documented the cholesterol- and lipid-lowering ability of *Chlorella*, indicating that this microalga may either inhibit the intestinal absorption of cholesterol or promote the catabolism of cholesterol through the up-regulation of hepatic cholesterol 7α-hydroxylase expression (Shibata et al. 2007; Lee et al. 2008). In addition, Abdelnour et al. (2019) suggested that the decrease of serum lipemia could be explained by a decline in acetyl-CoA enzyme fusion, which is required for fatty acids biosynthesis. A recent meta-analysis on the effect of *Chlorella* supplementation on cardiovascular risk factors suggests that this microalga improves total cholesterol, LDL-cholesterol, and fasting blood glucose levels, but not TAG and HDL-cholesterol levels, which partially agree with our own data (Bito et al. 2020). These protective effects of *Chlorella* on blood lipemia have been attributed to the synergism between multiple nutrients and antioxidant compounds (Bito et al. 2020).

Glucose was found increased in *C. vulgaris* dietary groups, alone or supplemented with the four-CAZyme mixture. In turn, the increment observed on insulin levels accompanied the increment on glucose levels, promoting a higher insulin resistance of the four-CAZyme mixture. Although small variations were found for urea and creatinine, with higher levels in pigs fed with the four-CAZyme mixture, the levels are still within the normal range reported by Jackson and Cockcroft (2002b) and, therefore, lacking clinical relevance. The hepatic markers AST and ALP were consistently reduced in the four-CAZyme mixture group, when compared with the control group. Even so, the enzyme activities observed are in line with the published reference figures for pigs (31-58 for ALT, 32-84 for AST, 120-400 for ALP and 10-52 U/L for GGT, respectively) (Jackson and Cockcroft 2002a), indicating unaffected liver function. Contrarily to our data, Yan et al. (2012) reported no effects of fermented *Chlorella* supplementation on red blood cells, white blood cells and lymphocytes in growing pigs (Yan et al. 2012). In our case,

red blood cells, white blood cells and lymphocytes were higher in the control group when comparing to pigs fed *C. vulgaris* supplemented with the four-CAZyme mixture.

C. vulgaris is thought to enhance the immune function and acts as an anti-inflammatory agent because of its ability to reduce the secretion of cytokines with inflammatory activity (Furbeyre et al. 2018; Machmud et al. 2020). Our data indicate that *C. vulgaris*, regardless the presence of carbohydrases, decreased IgA, IgG and IgM concentrations, reinforcing this microalga ability for modulating the humoral immune response. *C. vulgaris* supplemented with the four-CAZyme mixture had 2.6-fold lower levels of IgM than the control group. Our results are not in line with previous studies that showed higher levels of plasma IgA and IgG (Kang et al. 2013), and of IgG and IgM (An et al. 2016) in broiler chickens. Kang et al. (2017) also documented that the administration of a *Chlorella* by-product increased the plasma concentration of IgA, IgG and IgM in broilers. Although the mechanism through which *Chlorella* is an immune system booster of animals remains unknown, some possible mechanisms have been proposed. Sugiharto and Lauridsen (2016) and Barkia et al. (2019) proposed that omega-3 fatty acids are among the biological active compounds that confer to *Chlorella* immune-enhancing properties in broiler chickens. Abdelnour et al. (2019) also suggested that antioxidants, β-carotene and vitamin B_{12} available in this microalga can modulate the immune function of broilers. The former authors also hypothesized that peptides and fibre of *C. vulgaris* lead to an increase of plasma IgA, IgM, and IgG levels in broiler chicks. Furthermore, immunoregulatory polysaccharides in *Chlorella*, such as β-glucan and immurella, may have the capacity to enhance the immune response of broilers (Mason 2001; Pugh et al. 2001). Thus, *C. vulgaris* composition can stimulate immunoglobulins, by producing B cells in the gut-associated lymphoid tissue and increasing IgA, IgG and IgM concentrations in the plasma of animals. However, in the current study, *C. vulgaris* was used in a much higher level (5% incorporation in the diet) and the results indicate a strong immunosuppressive effect, with the consequent increase of pigs' susceptibility to bacterial, viral and fungal infections. Therefore, it seems that microalga components affect immunoglobulins production of pigs in a dose-specific manner. The digestive action of the four-CAZyme mixture on *C. vulgaris* polysaccharides with immunoregulatory properties promoted an even lower production of immunoglobulins. Although microalga gave rise to a strong decrease of immunoglobulins (-53% for IgG, the main plasma Ig), the values found are higher than those described for weaning piglets (+45% for IgG), which are animals with an immature immune system (Hedegaard et al. 2016; Madeira et al. 2021).

In the pig, the cholesterol synthesis and fatty acid metabolism, including fatty acid oxidation and *de novo* lipogenesis, occur mainly in the liver, with *de novo* lipogenesis occurring also in the adipose tissue (Nafikov and Beitz 2007; Meadus et al. 2011; De Tonnac et al. 2016). The experimental diets had no effect on hepatic total lipids and cholesterol concentration.

However, the inclusion of 5% *C. vulgaris* in pig diets promoted an increase in the percentage of some fatty acids, mainly the beneficial EPA and DHA, with a consequent increase of 23% in *n*-3 PUFA sum and a decrease of 27% in *n*-6:*n*-3 ratio. Enser and colleagues (2000), aiming to increase the *n*-3 PUFA of pork through linseed feeding, observed a concomitant increase of *n*-3 PUFA, mostly EPA and DHA, and a decrease of the *n*-6:*n*-3 ratio. In resemblance to *C. vulgaris* microalga, the linseed presents a high percentage of α-linolenic acid (18:3*n*-3, ALA) (Batista et al. 2013; Lewinska et al. 2015). Similarly, Smink et al. (2012) with growing pigs fed with combined low and high levels of linoleic acid (18:2*n*-6, LA) and ALA in a 2 × 2 factorial design, documented also an increase of some individual *n*-3 fatty acids, and consequently, a rise of the *n*-3 PUFA sum, especially in pigs fed higher levels of ALA. De Tonnac and Mourot (2018) used *Schizochytrium* sp., a DHA-rich microalga, in addition to linseed, to enhance pigs' performance and technological, nutritional and sensorial traits of pork. These authors found that both dietary linseed and *Schizochytrium* sp. increased hepatic *n*-3 PUFA and decreased the *n*-6:*n*-3 ratio. Similar results were reported by Komprda and colleagues (2020), who described an increase of EPA, DHA and the partial sum of *n*-3 PUFA, as well as a decrease of *n*-6:*n*-3 ratio, in the liver of pigs fed with 8% of fish oil. Like microalgae and linseed, fish oil is a natural rich source of *n*-3 PUFA (Komprda et al. 2020). Herein, the increase of *n*-3 PUFA sum in the liver of pigs fed with *C. vulgaris* could be explained through *de novo* lipogenesis by the increased intake of ALA, the precursor in the biosynthetic pathway of *n*-3 long chain PUFA, such as EPA and DHA (Smink et al. 2012).

Vitamin E is the collective term for a group of tocopherols and tocotrienols. *α*-Tocopherol is the most prevalent compound, showing the highest antioxidant activity and, therefore, the highest preventing capacity of lipid peroxidation (Brigelius-Flohé and Traber 1999). Even so, there is an evident lack of studies exploiting the effect of incorporating microalgae in pig diets on hepatic vitamin E and pigments. Tao et al. (2018) revealed no significant differences on α-tocopherol content in the liver of broilers fed with 10% of defatted *Nannochloropsis oceanica*. Although *C. vulgaris* and *N. oceanica* have a similar content of vitamin E (Andrade et al. 2018; Zanella and Vianello 2020), herein the hepatic content of α-tocopherol was significantly lower in pigs fed with *C. vulgaris*. Carotenoids, like vitamin E, are one of the widespread and ubiquitous lipid-soluble pigments that play an important role as antioxidants (Nabi et al. 2020). These molecules display a high versatility of biological functions increasing the interest of its use as feed additives by livestock industry. Carotenoids cannot be naturally synthesized by the animal body, but they are absorbed and deposited through food intake (Nabi et al. 2020). Our results demonstrated that the experimental diets had no effect on the hepatic content of carotenoids. Contrarily, An et al. (2014) reported a consistent increase of lutein and total carotenoids in the liver of laying hens fed with 1% *C. vulgaris* or 1% lutein fortified *C. vulgaris* compared with the control group. Thus, 5% of dietary *C. vulgaris*

incorporation was not enough to promote changes in the hepatic content of carotenoids in finishing pigs, despite the high content of carotenoids in this microalga.

Concerning the assessment of systemic redox balance in pigs, experimental diets did not change the enzymatic activity of GPX. This enzyme integrates the first line of the antioxidant defence system and it is involved in the mechanisms of protection of haemoglobin, red blood cell enzyme activity and biological cell membranes against oxidative stress (Waggiallah and Alzohairy 2011). Contrarily, total antioxidant capacity values were lower in pigs fed with *C. vulgaris* alone when compared with the control group, although pigs fed with *C. vulgaris* supplemented with both exogenous CAZymes restored TAC to similar values to those found in the control group, pointing towards a positive effect of the carbohydrases used. TAC is used as an accurate assessment of redox status *in vivo*, being a classical marker of global antioxidant defence (McMichael 2007). The results herein obtained can be explained through two distinct mechanisms: one concerns the fact that the action of the exogenous CAZymes promoted the degradation of *C. vulgaris* cell wall, which led to a higher bioavailability of antioxidant carotenoids from *C. vulgaris*, justifying the increase of TAC in plasma; the other mechanism is related to the stimulant effect of TAC by the biological activity of alginate oligosaccharides in pigs, as reported in several studies (reviewed by Ming et al. 2021). *C. vulgaris* cell wall has alginate in its composition (Suda et al. 1999). Through the enzymatic activity of four-CAZyme mixture (composed by an alginate lyase among other enzymes), the alginate could be decomposed into alginate oligosaccharides that are absorbed at the gastrointestinal tract and have a stimulating effect on TAC. The two mechanisms proposed here are in line with the results previously obtained *in vitro* for the four-CAZyme mixture, particularly the capacity of this enzymatic cocktail to release carotenoids and oligosaccharides from the microalga (Coelho et al. 2019).

6.5. Conclusion

Our results demonstrate that *C. vulgaris* as ingredient in the diets of finishing pigs has a huge impact on blood cells profile and plasma metabolites. Although these variations do not compromise pigs' health, in general, the strong immunosuppressive effect promoted by the microalga at this incorporation level is a major finding. The increased susceptibility of pigs to infection diseases could be ascribed to dose-dependent immunoregulatory properties of *Chlorella* polysaccharides, as supported by the additional decrease of immunoglobulins promoted by the four-CAZyme mixture diet. Moreover, dietary *C. vulgaris* by itself seems to reduce systemic antioxidant capacity of pigs. The results of *Chlorella*-based diet supplemented with the preselected four-CAZyme mixture, in contrast to commercial Rovabio® Excel AP,

indicate an important role of this carbohydrase cocktail in the digestion of *C. vulgaris* cell wall, with the release of compounds with lipid- and cholesterol-lowering capacity, resulting on a clear discrimination of this feeding treatment. Concerning hepatic lipids, our data indicate that high dietary levels of *C. vulgaris*, regardless the presence of feed carbohydrases, increase the hepatic content of *n*-3 PUFA sum in pigs, leading to a health beneficial improvement of the *n*-6:*n*-3 ratio. Even though these results indicate that *C. vulgaris* is a safe feedstock for swine industry, the clinical significance of the higher susceptibility of pigs to infections found here is critical and should be further assessed in industrial production systems, with identical or higher levels of this microalga incorporation under challenging conditions of stress and hygiene.

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Chapter 7 – GENERAL DISCUSSION

Currently, pork is the most consumed meat worldwide, despite its consumption being practically equivalent with poultry products (Macho-González et al. 2020; Zira et al. 2020). However, the rise in global population combined with the increase in income will double the overall demand for animal-derived products by 2050, with particular impact on the most consumed meats worldwide. Consequently, the pig industry will face new challenges (Madeira et al. 2017; Chriki and Hocquette 2020). In addition, this increased demand for pork will be particularly dramatic for livestock agriculture, as corn and soybean food crops are the two main conventional feedstuffs for pig feeding. Apparently, the current allocations of corn and soybean are unsustainable and its intensive production is linked to land degradation, water deprivation (Madeira et al. 2017; Rauw et al. 2020). Moreover, increasingly informed consumers attribute a negative connotation to pork due to its unfavourable fatty acid profile, with a low proportion of *n*-3 PUFA (Verbeke et al. 2010; Verbeke et al. 2011; Dugan et al. 2015). Moreover, the WHO reported that a large part of the human population, including European, does not consume the recommended levels of *n*-3 PUFA (Tocher et al. 2019).

Arthrospira platensis and *Chlorella vulgaris*, are two taxonomically distinct microalgae. *A. platensis* is a prokaryotic cyanobacterium and *C. vulgaris* is a photoautotrophic eukaryotic green microalgae (Beheshtipour et al. 2012; Bernaerts et al. 2018). Consequently, these microalgae exhibit different chemical composition and cellular characteristics. In comparison, *A. platensis* presents a high protein content and low fat content, whereas *C. vulgaris* contains a higher fat content and lower protein content (Tokuşoglu and Ünal 2003). Moreover, the *C. vulgaris* cell wall is more recalcitrant then the *A. platensis* cell wall (Williams and Laurens 2010; Safi et al. 2013). In addition, these two microalgae are the two most studied microalgae with a major commercial importance (Garrido-Cardenas et al. 2018; Camacho et al. 2019; Matos et al. 2020). Therefore, these microalgae could be appropriate alternatives to conventional feedstuffs for pig production due to its well-balanced nutritional composition and a low environmental impact production technology (Madeira et al. 2017; Camacho et al. 2019).

Several studies have tested the incorporation of *C. vulgaris* into pig diets at supplemental levels (1% or lower in diet). However, its recalcitrant cell wall decreases its digestibility by pigs and, therefore, makes the scale up of the incorporation level unfeasible in *in vivo* assays (Madeira et al. 2017; Amorim et al. 2021). CAZymes have shown positive results in microalga cell wall disruption in *in vitro* assays (Cho et al. 2013) and its use as feed supplements in pig diets its well-established (Ravindran and Son 2012).

The two first studies reported in this thesis aimed to develop two CAZymes mixtures able to degrade *A. platensis* and *C. vulgaris* cell wall in *in vitro* trials. Then, the two other

studies, based on one *in vivo* experiment with finishing pigs, aimed to evaluate the effect of a high dietary incorporation level of *C. vulgaris* and the supplementation of the pre-developed and selected CAZyme mixture indicated for *C. vulgaris* cell wall disruption. The parameters analysed were the productive performance, meat quality traits and pork vitamin E, pigments, lipid, cholesterol and fatty acid profile, blood parameters, plasma antioxidant potential and hepatic content in diterpenes, pigments and fatty acid profile from finishing pigs.

7.1. Development of CAZyme Mixtures to Disrupt *A. platensis* and *C. vulgaris* Cell Wall

The first part of this thesis encompasses the development of CAZyme mixtures as cell wall disruption method of *A. platensis* and *C. vulgaris* to be applied in *in vivo* tests and enable a high dietary incorporation level of microalgae in finishing pigs feed.

The selection method of the most suitable enzymes for cell wall disruption process of the two microalgae under study began with the establishment of a large library, composed by 178 CAZymes and 22 sulfatases (Supplementary Material 1). The construction criteria of this enzymatic library took into account the composition of the know polysaccharide matrix of microalgae, which comprises pectin, chitin agar, alginates, glucosamine, peptidoglycan or the aliphatic polymer algenan and sulphate groups (Tomaselli 1997; Gerken et al. 2013; Scholz et al. 2014). Therefore, these enzymes have well-defined and carefully thought-out characteristics. The selected enzymes were produced in an HTP platform that involves gene synthesis, gene cloning, protein expression and protein purification. Then, an initial individual screening of enzymes was performed in order to identify which enzymes from the library were more suitable for *A. platensis* and *C. vulgaris* cell wall disruption based on release of reducing sugars from microalgae cell wall. A group of 26 and another of 29 individual enzymes with a potential capacity to disrupt these microalgae cell wall was obtained for *A. platensis* and *C. vulgaris*, respectively.

Several *in vitro* studies have reported the existence of synergistic effects between enzymes in microalga cell wall disruption. Multiple-enzyme complexes demonstrated to be more effective than the action of individual enzymes on microalgae cell wall and in microalga product recovery, due to the different compounds present on microalgae cell wall (Gerken et al. 2013; Huo et al. 2015; Zuorro et al. 2016). In addition, Ravindran and Son (2012) in its review indicate that the next generation of enzymes applied on animal feed will be those with multiple enzyme activities rather than individual enzymes. In line with this, the enzymes obtained in the initial screening were combined in mixtures according to natural subtracts of enzymes, organism and production yield. It was important that the enzymes had as substrates

compounds that were present in the cell wall of the microalgae under study, isolated from marine organisms and with good production yields. Moreover, the mixture must contain the lowest number of enzymes that guarantee the best possible result to allow its practical applicability. The efficiency of the mixtures in the degradation of cell walls was tested according to the release of reducing sugars. A two-CAZyme mixture composed by two GH, a lysozyme and an α-amylase, were obtained for cell wall disruption of *A. platensis*, while a four-CAZyme mixture constituted by an exo-β-glucosaminidase (GH), an alginate lyase (PL), a peptidoglycan N-acetylmuramic acid deacetylase (CE) and a lysozyme (GH), was selected for cell wall disruption of *C. vulgaris*.

Although both mixtures have lysozyme in their composition, it is isolated from different organisms, since each lysozyme displayed more effectiveness for the respective microalgae. The lysozyme in the two-CAZyme mixture was isolated from *Escherichia coli* and the lysozyme in the four-CAZyme mixture was isolated from *Clostridium perfringens*. With an alignment analysis with Clustal Omega tool from the European Bioinformatics Institute, using protein and DNA sequences of both lysozymes, was observed that these enzymes display considerable differences in molecular sequences, justifying the different efficiency in cell wall disruption of both microalgae.

The selected mixtures were fully characterized according to its ability to degrade the microalgae cell wall (release of reducing sugars and oligosaccharides, cell number and fluorescence intensity) and release of its internal compounds (proteins, pigments and FA) for extracellular medium.

For *A. platensis*, the two-CAZyme mixture promoted an increase of the amount of reducing sugars released up to 2.42 g/L in microalgae treated relative to no treatment (control), which represents a 1.24-fold increase in relation to the highest value observed in the individual screening, as well as a 7-fold increase of oligosaccharides amount from microalga cell wall. Was also observed a 36% reduction of fluorescence intensity. The mixture also promoted the release of 1.34-fold more in protein content relative to the control. Similarly, the mixture promotes the release of several FA, such as *n*-6 PUFA, in particular 18:2*n*-6, 18:3*n*-6, 22:2*n*-6 and MUFA in rates of 1.15, 1.12, 1.63 and 1.30-fold superior, respectively, comparing with control group. The release of chlorophyll a was also improved by the two-CAZyme mixture, increasing 1.14-fold relative to control. The treatment of *C. vulgaris* with four-CAZyme mixture induce the release of reducing sugars up to 1.21 g/L from microalga cell wall relative to control, representing a 1.6-fold increase in relation to the highest value observed in the individual screening. Moreover, the treatment with the mixture led to an 8-fold increase in oligosaccharides release from microalga cell wall and reduced the fluorescence intensity by 47%. The treatment with the mixture was successful in releasing proteins about 23.4-fold higher compared to control, and the beneficial 18:3*n*-3 fatty acid at a rate of 1.64-fold superior

when compared with control group. The release of total carotenoids was also increased in 1.09-fold superior comparing with control group.

The enormous diversity of chemical and structural composition between *A. platensis* and *C. vulgaris* cell walls corroborate the different complexity between CAZyme mixtures. Since *C. vulgaris* presents a more complex and rigid cell wall in comparison with *A. platensis* (Safi, Ursu, et al. 2014; Baudelet et al. 2017), the CAZyme mixture suitable for *C. vulgaris* cell wall disruption is composed by a high diversity of enzymatic activities compared to the CAZyme mixture for *A. platensis*.

The effectiveness of *A. platensis* cell wall disruption by the two-CAZyme mixture is in line with several studies that report the disruption of *A. platensis* cell wall through enzymatic method. The peptidoglycan containing muramic acid δ-lactam which composes the *A. platensis* cell wall, as well as other cyanobacteria, is the main subtract of lysozyme (Sotiroudis and Sotiroudis 2013; Babu et al. 2018). Mehta et al. (2015) described a complete cell wall lysis technique of different cyanobacteria strains which involves the incorporation of lysozyme. Also Aikawa et al. (2013) in its study concluded that the addition of lysozyme promoted the disruption of *A. platensis* cell wall allowing an efficient extraction of internal glycogen and its subsequent conversion to bioethanol, with the production yield reached 86% of the theoretically expected amount (Aikawa et al. 2013). Although the cell wall of *A. platensis* is mostly composed by peptidoglycan, the incorporation of α-amylase in the mixture increased its efficiency due to the presence of a highly branched α-1,4-polyglucan, denominated as cyanophycean starch (Pulz and Gross 2004), deeply located in the cyanobacterial cell wall with an irregular whitish spherical form (Lang 1968; Pyo et al. 2013).

Otherwise, the four-CAZyme mixture for disruption of *C. vulgaris* cell wall requires more and different enzyme activities. The composition of the four-CAZyme mixture is in alignment with different studies, in which enzymatic mixtures were applied to degrade the cell wall of *C. vulgaris*. Gerken and collegues in 2013 observed that *C. vulgaris* cell wall was sensitive to enzymes degrading polymers containing N-acetylglucosamine, justifying the inclusion of lysozyme, a glucosaminidase and an acetylglucosamine deacetylases, which are enzymes with capacity to degrade polymers containing N-acetylglucosamine (Gerken et al. 2013). Furthermore, they established that the *C. vulgaris* cell wall is composed by glucosamine (Lee et al. 2017). In addition, Baudelet et al. (2017) referenced the identification of cell wall degrading alginate lyase coding genes in the genome of *C. vulgaris* infecting virus, which confirm the importance of alginate lyase in the disruption of cell wall and, consequently, in the four-CAZyme mixture. In a recent work, Canelli et al. (2021) aimed to define an optimal combination of enzymes to increase the lipid and protein accessibilities of *C. vulgaris*. To assess the disruption efficacy of enzymatic treatment, the release of cellular material was evaluated by measuring the total carbon and total nitrogen released in the supernatant after

the treatment and compared to the control (no treatment). The cell integrity was evaluated by the determination of particle size and the lipid and protein accessibilities were measured. The authors reported that chitinase, rhamnohydrolase and galactanase caused the highest release of microalgae cellular material. Produced a slight increase of 9.5% in protein accessibility but no increase in lipid accessibility in comparison to the control (Canelli et al. 2021). On the contrary, the four-CAZyme mixture, in addition to proteins, was also effective on release of FA and total carotenoids.

The enzymes that compose both mixtures were biochemically characterized, namely their thermostability and proteolysis resistance, with the aim to evaluate *in vivo* applicability. In the two-CAZyme mixture, the α-amylase displays higher thermotolerance and proteolysis resistance when compared with lysozyme. In the four-CAZyme mixture, the exo-β-glucosaminidase and the lysozyme exhibit higher thermotolerance and proteolysis resistance when compared with alginate lyase and peptidoglycan N-acetylmuramic acid deacetylase. The enzymes with higher thermotolerance also display higher proteolysis resistance as the tertiary structure of protein, which confers thermotolerance to enzymes, could also confer inherent proteinase resistance, as demonstrated by Fontes et al. (1995).

Taken together, these results confirm the efficiency of this HTP approach to assess *A. platensis* and *C. vulgaris* cell wall disruption by enzymatic digestion and development of two CAZymes mixtures able to disrupt these microalgae cell wall whose efficiency is in alignment with other studies and the enzymes activity with the chemical composition of these microalgae cell wall. Therefore, these enzymatic mixtures are suitable to be applied not only in *in vivo* trials, but also for other industrial processes.

An overview of the main results obtained in the first part of this study is presented in Table 7.1.

Table 7.1. Overall findings of the development of CAZyme mixtures able to disrupt *A. platensis* and *C. vulgaris* cell walls. The results are compared with the respective control. The biochemical results are compared within enzymes.

Item	*A. platensis*	*C. vulgaris*
	Two-CAZyme mixture	**Four-CAZyme mixture**
Cell wall integrity	↑ Reducing sugars = Cell number ↓ Fluorescence intensity ↑ Oligosaccharides	↑ Reducing sugars = Cell number ↓ Fluorescence intensity ↑ Oligosaccharides
Release of compounds	↑ Proteins ↑ Chlorophyll *a* ↑ 18:2*n*−6 ↑ 18:3*n*−6 ↑ 22:2*n*−6 ↑ ∑ MUFA ↑ ∑ *n*−6 PUFA	↑ Proteins ↑ Carotenoids ↑ 16:1*c*7 ↑ 16:1*c*9 ↑ 17:1*c*9 ↑ 18:3*n*–3 ↑ ∑ *n*−3 PUFA
Thermotolerance	+ α-amylase − Lysozyme	+ Exo-β-glucosaminidase + Lysozyme − Alginate lyase − Peptidoglycan N-acetylmuramic acid deacetylase
Proteolysis	+ α-amylase − Lysozyme	+ Exo-β-glucosaminidase + Lysozyme − Alginate lyase − Peptidoglycan N-acetylmuramic acid deacetylase

CAZymes – carbohydrate–Active enzymes; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; ↑ – increase; ↓ – decrease; = – not changed; + – more resistant; −, – less resistant.

As demonstrated above, both mixtures developed in chapters 3 and 4 proved to be equally effective in the cell wall disruption of *A. platensis* and *C. vulgaris*, respectively. Therefore, the *C. vulgaris* microalga was selected for the *in vivo* trails based on its more well-balanced nutritional composition, such as proteins, pigments and lipids, namely in *n*-3 PUFA, when compared with the *A. platensis* (Batista et al. 2013). Thus, *C. vulgaris* is more suitable for enriching pork with *n*-3 PUFA and antioxidants, which is a key objective of the study.

7.2. Impact of a High Dietary Incorporation Level of *C. vulgaris* and Supplementation with the four-CAZyme mixture on Finishing Pigs

The second part of this study involved an *in vivo* trial, where crossbred entire male pigs, sons of Large White × Landrace sows crossed with Pietrain boars, with an initial live weight of 59.1 ± 5.69 kg were used. In this experiment, the effect of a high incorporation level of *C. vulgaris* alone or supplemented with the four-CAZyme mixture in several parameters was evaluated for the first time. The parameters analysed were: productive performance, carcass characteristics, meat quality traits, sensorial analysis, oxidative stability of pork, and chemical composition of *longissimus lumborum*, namely diterpene profile, pigments content, lipid content, total cholesterol and fatty acid profile. In addition to the productive and meat quality parameters, the impact of the high incorporation level of *C. vulgaris* alone or supplemented with the four-CAZyme mixture on health status and hepatic composition in diterpenes, pigments, lipid content, total cholesterol and fatty acid profile was also analysed since the effect of a high dietary incorporation level of *C. vulgaris* on these parameters of finishing pigs remains to be elucidated. The health status of finishing pigs was assessed through analysis of haematology, plasma metabolites, plasma hepatic markers, immunoglobulins profile, and plasma antioxidant potential. The four diets tested in this experiment were isoenergetic and isoproteic. The control diet was a corn-soybean basal diet with 19.3% (as fed basis) of soybean meal. To investigate the effect of *C. vulgaris*, 5% of *C. vulgaris* powder was added to the other three diets in a partial substitution of soybean meal by the microalga. One of the three *C. vulgaris* incorporated diets was supplemented with 0.005% of Rovabio® Excel AP, a commercially available mixture of CAZymes with xylanase and β-glucanase activities. The other *C. vulgaris* incorporated diet was supplemented with 0.01% of four-CAZyme mixture developed in the first part of this study to analyse the *in vivo* effect of the enzymatic mixture on *C. vulgaris* cell wall disruption to overcome the low digestibility of *C. vulgaris* by pigs (Madeira et al. 2017). The remaining *C. vulgaris* diet was only incorporated with the microalgae. The *C. vulgaris* incorporated diets presents 11.7% (as fed basis) of soybean meal, therefore, this experiment tested the partial substitution of this traditional feedstuff by *C. vulgaris*.

Several studies in literature indicate that the dietary incorporation of *C. vulgaris* has no effect on the productive parameters of pigs (Baňoch et al. 2012; Furbeyre et al. 2017; Furbeyre et al. 2018). With the exception, in the study conducted by Yan et al. (2012), the authors observed that a dietary incorporation of 0.1% dosage of *C. vulgaris* improved ADG of growing pigs with an initial weigh of 26.6 kg in a trial lasting 6 weeks. However, to date, studies in the literature only report the dietary incorporation of *C. vulgaris* in pigs at supplementation levels (1% or lower in diet) (Madeira et al. 2017). Abril et al. (2003), in a study performed with weaned castrated male pigs with 9.07 kg fed with 1.10-5.51% of *Schizochytrium* sp. for 27 days, and

0.39-1.94% during 13 days, reported that these higher levels of incorporation of this microalga did not cause significant differences in the production parameters. Only few studies investigated the cell wall composition of *Schizochytrium* sp., describing a thin non-cellulosic cell wall with galactose as the principal monosaccharide, less rigid than *C. vulgaris* (Bernaerts et al. 2018). In line with these studies, our expectations were that the high dietary incorporation level (5%) of *C. vulgaris* would impair the production performance of finishing pigs and the supplementation with four-CAZyme mixture to reverse this effect. However, in our experiment, the high dietary incorporation level of 5% of *C. vulgaris* supplemented or not with the CAZyme mixtures did not promote significant differences in production parameters of finishing pigs compared to the control, perhaps due to the fact that finishing pigs have digestive and metabolic machinery to digest *C. vulgaris* at this level of incorporation without the need for supplementation with feed enzymes. The limiting level of dietary incorporation of *C. vulgaris* without impairing the productive performance of pigs needs further investigation. Moreover, the dietary incorporation of *C. vulgaris* has no significant effect on carcass characteristics, which was also reported in the study of Baňoch et al. (2012). Nonetheless, at a dietary incorporation level of 5% of *C. vulgaris* the productive performance and carcass characteristic of finishing pigs is not impaired. This was a major outcome of this research with central importance for producers since at this level of incorporation, the cost-effective of pig production is not compromised.

In addition, the dietary incorporation of *C. vulgaris*, alone or supplemented with the CAZyme mixtures, had no significant influence on meat quality traits and sensorial attributes. In the study conducted by Baňoch et al. (2012), the authors observed similar results with female pigs with an initial weight of 30 kg fed with a very low level (0.0002%). The authors verified that this incorporation level had no significant effect on colour, pH, cooking loss and drip loss of pork (Baňoch et al. 2012). In our study, despite the incorporation of a high level of *C. vulgaris* and the supplementation with enzymes, these characteristics of pork remained unchanged, which is very important for the consumer acceptance of this meat. Interestingly, Oh and colleagues in 2014 observed an increase of b*, pH and shear force in breast meat, and an increase of L* and b* in leg meat, of male Pekin ducks fed with 0.1–0.2% C. vulgaris during 42 days. This demonstrates that pork quality traits seem to be less sensitive to the dietary inclusion of *C. vulgaris* than poultry meat characteristics, although both are meats-derived from monogastric animals.

The pork composition in vitamin E homologues (tocopherols and tocotrienols) was not significantly altered by microalgae incorporation and by enzyme supplementation, which agree with the diterpene content of the experimental diets. On the contrary, the chlorophylls content in *C. vulgaris* incorporated diets its superior to the control; however, there was no significant increase of chlorophylls in pork from animals fed with *C. vulgaris* incorporated diets comparing

to the control. On the other hand, the inclusion of 5% *C. vulgaris* in pig diets, combined or not with the two exogenous CAZyme mixtures, improved the carotenoid content of pork to about double, thus providing further nutritional benefits for consumers. The animals are incapable to synthesize carotenoids *in vivo* and these molecules exhibit a high antioxidant activity and play a versatile biological role that contributes to different therapeutic effects, including anticancer, immunomodulators, anti-inflammatory, antibacterial, antidiabetic and neuroprotective (Nabi et al. 2020). Total carotenoids were strongly in conformity with diet composition, with *C. vulgaris* being rich in carotenoids such as astaxanthin, cantaxanthin and lutein (Deenu et al. 2013; Safi, Zebib, et al. 2014). The difference between pork content of chlorophyll and carotenoids may be due to the difference in polarity of the molecules, with chlorophyll being more polar (Macías-Sánchez et al. 2007) and present less affinity for pork intramuscular fat. The enrichment of meat in carotenoids through dietary incorporation of *C. vulgaris* its well documented for poultry (Lemahieu et al. 2013; Alfaia et al. 2021). Despite the higher content of β-carotene, a pro-vitamin A, in *C. vulgaris* incorporated diets, this pigment was not detected in pork, which could indicate that β-carotene in the diet is quickly metabolized into vitamin A (Nogareda et al. 2016). Hence, pork enriched in carotenoids will have a positive impact on acceptability and a health benefit of consumers.

The fatty acid profile of pork was also improved trough the incorporation of *C. vulgaris* on finishing pigs' diets. The content of several *n*-3 PUFA, including ALA, EPA and DHA, was superior in *longissimus lumborum* of animals fed with *C. vulgaris* diets as well as the partial sum of *n*-3 PUFA and, consequently, was observed a decrease of *n*-6:*n*-3 ratio in this animals. These findings were reported for the first time by our study since only Sardi et al. (2006) and Vossen et al. (2017) reported an increase in EPA and DHA content and a decrease of *n*-6:*n*-3 ratio in meat of pigs fed with *Schizochytrium* sp. incorporated diets at a low incorporation level (0.25 – 1.2%). Moreover, these results are in line with the good proportions in *n*-3 PUFA of *C. vulgaris* and in *C. vulgaris* incorporated diets, mainly ALA, with *C. vulgaris* incorporated diets to present 30% more ALA then the control diet. Although EPA and DHA have not been detected in diets incorporated with *C. vulgaris*, the presence of this *n*-3 PUFA in pork indicates the capacity of muscle to capture the precursor ALA from *C. vulgaris* diets and its ability to convert it into *n*-3 PUFA derivatives EPA and DHA via its biosynthetic pathway (Smink et al. 2012; Dugan et al. 2015). The daily recommended values for *n*-3 PUFA are around 500 mg/day (Tocher et al. 2019), although the intake of 250 mg/day already affords protection against cardiovascular diseases (Kris-Etherton et al. 2009). The recommended *n*-6:*n*-3 ratio to prevent cardiovascular diseases is around 4-5 (Oonincx et al. 2020; Trbović et al. 2020). The meat of pigs fed with *C. vulgaris* presents 6.39 mg of *n*-3 PUFA per 100 g, a value 10% higher than the control which corresponds to 2.56% of the recommended intake to prevent cardiovascular diseases. In FA proportions, the animals fed with *C. vulgaris* displays a value of 1.57-fold

higher in *n*-3 PUFA than the control. Moreover, the animals fed with *C. vulgaris* diets presents a *n*-6:*n*-3 ratio of 12, which represents a decrease of 24% comparing with control, being 2.4-fold higher in comparison with the recommended value while the value of control is 3.22-fold higher than the recommended value. The *n*-3 PUFA, mainly EPA and DHA are of great interest for human diets due to their recognized positive effects, which includes anti-atherogenic, anti-thrombotic, and anti-inflammatory properties (Mason 2019). Thus, the incorporation of 5% of *C. vulgaris* on finishing pig diets demonstrated to be able to improve the *n*-3 PUFA content of pork facilitating the consumption of recommended values with health benefits for consumers. The supplementation with CAZyme mixtures had only significant effects on DHA content of pork in animals fed with diet supplemented with the four-CAZyme mixture.

PUFA are reported as more susceptible to peroxidation, with meat rich in *n*-3 PUFA being more susceptible to lipid oxidation during storage (Andrés et al. 2001). In line with these, our expectations were that the increase of *n*-3 PUFA in pork decreasing the oxidative stability of pork. However, with this experiment, we observed that the diets incorporated with *C. vulgaris* alone or supplemented with CAZymes had no significant influence on oxidative stability of pork. We hypothesize that this was due to the antioxidant activity of carotenoids present in meat from diets incorporated with *C. vulgaris*. Nonetheless, the chemical induction of lipid oxidation demonstrated that there was no lower oxidation in animals fed with *C. vulgaris* diets, being in agreement with Müller et al. (2011) that showed a large variation on the reactivity of the different types of carotenoids toward antioxidant activity. Therefore, changes in antioxidant activity are not only associated to the quantity of carotenoids but also with the specific characteristics of carotenoids identity (Goiris et al. 2012). Baňoch et al. (2012) also observed that the very low dietary incorporation level of *C. vulgaris* had no influence on the oxidative stability of pork. On the contrary, Dlouhá et al. (2008) observed that the supplementation with 0.00003% of a sodium enriched *Chlorella* promoted the decrease of the content of oxidation products in breast muscle, contributing to increase the oxidative stability of this meat. Thus, the oxidative stability response is dependent of species and microalga incorporation level. Although this aspect deserves further investigation, these findings suggests that this high incorporation level of *C. vulgaris* not have a negative impact on oxidative stability of pork. Moreover, the increase in *n*-3 PUFA did not cause sensory changes of pork, contrary to what is described by Pereira and Vicente (2013). This aspect contributes to a positive impact on consumer acceptability. Therefore, these data indicate that the dietary incorporation of *C. vulgaris* at a level of 5% is suitable to increase pork fat nutritional value without impairing pig performance.

To assess the effect of this high dietary incorporation level of *C. vulgaris* on health status of finishing pigs, the blood parameters were analysed. The haematological analysis reveal that, despite the experimental diets promoting significant changes on white blood cells

number, percentage of granulocytes and lymphocytes, red blood cells number, haemoglobin and thrombocytes, these values remain in the reference range (Jackson and Cockcroft 2002b). In the plasma metabolites, the antihyperlipidemic property of *Chlorella* previously described (Bito et al. 2020) was evident in pigs fed with *C. vulgaris* incorporated diet supplemented with the four-CAZyme mixture. Only animals fed this diet present the total cholesterol value in the reference range (Jackson and Cockcroft 2002a; Kaneko et al. 2008), while the animals fed with the other diets present a superior value for this parameter. Several mechanisms are proposed to explain how *Chlorella* can promote the decrease of plasma lipemia, including the ability of this microalga in inhibit the intestinal absorption of cholesterol, the capacity to promote the catabolism of cholesterol through the up-regulation of hepatic cholesterol 7α-hydroxylase expression, or the effect of *C. vulgaris* in decline the acetyl-CoA enzyme fusion, which is required for FA biosynthesis (Shibata et al. 2007; Lee et al. 2008; Abdelnour et al. 2019). These mechanisms rely on nutrient and antioxidant composition of *C. vulgaris* (Bito et al. 2020). Thus, the decrease of plasma lipemia of pigs fed with *C. vulgaris* incorporated diet supplemented with the four-CAZyme mixture comparing with other diets one of the major outcomes of this research. This finding suggests that the disruptive action of *C. vulgaris* cell wall performed by the CAZymes mixture is essential to release the antihyperlipidemic compounds presents in *C. vulgaris* (Bito et al. 2020). The experimental diets also promote significant differences on glucose, insulin, urea and creatinine. However, these levels are still within the normal range (Jackson and Cockcroft 2002a; Kaneko et al. 2008) and, therefore, lacking clinical relevance. Moreover, the hepatic markers AST and ALP were consistently reduced in the four-CAZyme mixture group, when compared with the control group. However, all hepatic markers values are in line with the published reference figures for pigs (Jackson and Cockcroft 2002a) indicating unaffected liver function. These data indicating that for plasma lipemia and hepatic markers, the supplementation with the four-CAZyme mixture was preponderant to release the *C. vulgaris* compounds involved in these mechanisms. Interestingly, our study demonstrated a consistent decrease of plasma immunoglobulins in animals fed with *C. vulgaris* incorporated diets, which is not in agreement with what is described for *C. vulgaris* since this microalga presents various immunostimulant activities (Kitada et al. 2009) Several studies have demonstrated an immunostimulant effect of *C. vulgaris* in poultry (Kang et al. 2013; An et al. 2016; Kang et al. 2017). The mechanisms proposed to explain how *Chlorella* is an immune system booster of animals rely on the composition of *C. vulgaris* in *n*-3 PUFA, antioxidants, β-carotene, vitamin B12, peptides, fibre, β-glucan and immurella, all immunoregulatory compounds with immunostimulant properties (Pugh et al. 2001; Abdelnour et al. 2019; Barkia et al. 2019). Therefore, it seems that microalga components affect immunoglobulins production of pigs in a dose-specific manner, since a high dietary incorporation level of *C. vulgaris* exhibit an effect contrary to that observed in trials with

poultry, where there was the incorporation of lower doses. In line with this, although this high dietary level of microalga incorporation can increase the susceptibility of the animals to bacterial, viral and fungal infections did not change the remaining parameters of the health status of the animals. Even so, this strong immunosuppressive effect may have negative consequences in a livestock production scenario, and therefore, its associated mechanisms need further investigation.

The plasma antioxidant potential was evaluated with the measurement of GPX activity and TAC. The GPX activity was not significantly altered by diets, whereas TAC was lower in animals fed with *C. vulgaris* incorporated diet alone compared with control. The animals fed with *C. vulgaris* incorporated diets supplemented with CAZymes mixtures exhibit TAC values significantly equal to animals fed the control diet and *C. vulgaris* diet. Two different mechanisms are proposed to explain these results. One mechanism relies on the ability of the CAZymes mixtures in disrupt the *C. vulgaris* cell wall and release the internal carotenoids with antioxidant capacity. The other mechanism involves the stimulant effect of TAC by the biological activity of alginate oligosaccharides in pigs, as reported in several studies (reviwed by Ming et al. 2021). As *C. vulgaris* cell wall presents in its composition alginate (Suda et al. 1999) and the four-CAZyme mixture have an alginate lyase, consequently, the enzymatic activity of four-CAZyme mixture could promote the decomposition of alginate into alginate oligosaccharides that are absorbed at the gastrointestinal tract and have a stimulating effect on TAC. These mechanisms, that can act synergistically, are in line with the results previously reported of development of the four-CAZyme mixture, particularly the capacity of this enzymatic cocktail to release carotenoids and oligosaccharides from the microalga. Thus, the action of the enzymatic mixtures promoted the slight increase of TAC values, equalling in significance the values of control.

The PCA score plots of blood parameters explained 64% of the total variation, with 45% for factor 1 and 19% for factor 2 and the animals fed with control diet and with *C. vulgaris* incorporated diet supplemented with the four-CAZyme mixture were clearly discriminated from the remaining animals, indicating the major influence of this diet on blood parameters.

Different studies demonstrated that feeding pigs with feedstuffs rich in *n*-3 PUFA, including ALA, promoted the increase of *n*-3 PUFA, such as EPA and DHA on the liver, accompanied with the decrease of *n*-6/*n*-3 ratio (Enser et al. 2000; Smink et al. 2012; De Tonnac and Mourot 2018; Komprda et al. 2020). In line with these studies, our expectations were that the high dietary incorporation level (5%) of *C. vulgaris* would increase the hepatic content of *n*-3 PUFA, which was proved by the *in vivo* trail. We observed an increase in 51% of EPA, 31% of DHA, 23% of sum of *n*-3 PUFA and a decrease of 27% in *n*-6/*n*-3 ratio, comparing with control. Therefore, as in pork, there was an accumulation of *n*-3 PUFA in liver. Herein, the increase of *n*-3 PUFA in the liver of pigs fed with *C. vulgaris* could be explained

through the *de novo* lipogenesis, that occur mainly in the liver (Meadus et al. 2011; De Tonnac et al. 2016), by the increased intake of ALA, the precursor in the biosynthetic pathway of *n*-3 long chain PUFA, such as EPA and DHA (Smink et al. 2012). The supplementation with CAZyme mixtures was not essential for the modification of FA profile of liver.

Contrary to what was observed in pork, the pigments of liver were not significantly affected by the incorporation of *C. vulgaris* alone or supplemented with the CAZymes mixtures. On the other hand, the hepatic α-tocopherol content was significantly lower in animals fed with *C. vulgaris* diets in comparison with control while in pork this parameter has not been changed. Although there is an evident lack of studies exploiting the effect of incorporating microalgae in pig diets on hepatic vitamin E and pigments content, Tao et al. (2018) revealed no significant differences on α-tocopherol content in the liver of broilers fed with 10% of defatted *Nannochloropsis oceanica*, a microalga with similar content in vitamin E compared to *C. vulgaris*. Moreover, An et al. (2014) reported a consistent increase of lutein and total carotenoids in the liver of laying hens fed with 1% *C. vulgaris* or 1% lutein fortified *C. vulgaris* compared with the control group. These data confirm the different response to dietary incorporation of *Chlorella* between poultry and pigs. In addition, seems that animals fed with *C. vulgaris* incorporated diets have more difficulty in fixing the dietary α-tocopherol on liver, and therefore this fact needs further elucidation.

The Table 7.2 features an overview of the major outcomes obtained in the second part of this study.

Table 7.2. Major outcomes of the *in vivo* trial with the dietary incorporation of *C. vulgaris* and supplementation with the two CAZyme mixtures in finishing pigs. The results are compared with the respective control.

Item	CH	CHR	CHM
Productive performance and carcass characteristics	= All parameters	= All parameters	= All parameters
Meat quality traits and sensory attributes	= All parameters	= All parameters	= All parameters
Meat diterpene profile and pigment content	= Diterpene profile ↑ Total carotenoids	= Diterpene profile ↑ Total carotenoids	= Diterpene profile ↑ Total carotenoids
Meat FA profile	↑ 18:3*n*-3 ↑ 20:5*n*-3 = 22:6*n*-3 ↑ *n*-3 PUFA ↓ *n*-6:*n*-3 ratio	↑ 18:3*n*-3 ↑ 20:5*n*-3 = 22:6*n*-3 ↑ *n*-3 PUFA ↓*n*-6:*n*-3 ratio	↑ 18:3*n*-3 ↑ 20:5*n*-3 ↑ 22:6*n*-3 ↑ *n*-3 PUFA ↓*n*-6:*n*-3 ratio
Meat oxidative stability	= TBARS	= TBARS	= TBARS
Blood parameters	↑ Total lipids ↑ Total cholesterol ↓ IgA ↓ IgG ↓ IgM	↑ Total lipids = Total cholesterol ↓ IgA ↓ IgG = IgM	↓ Total Lipids ↓ Total cholesterol ↓ IgA ↓ IgG ↓ IgM
Plasma antioxidant potential	↓ TAC	= TAC	= TAC
Hepatic diterpene profile and pigment content	↓ α-Tocopherol = Pigment content	↓ α-Tocopherol = Pigment content	↓ α-Tocopherol = Pigment content
Hepatic FA profile	= 18:3*n*-3 ↑ 20:5*n*-3 ↑ 22:6*n*-3 ↑ *n*-3 PUFA ↓ *n*-6:*n*-3 ratio	= 18:3*n*-3 = 20:5*n*-3 = 22:6*n*-3 ↑ *n*-3 PUFA ↓*n*-6:*n*-3 ratio	= 18:3*n*-3 = 20:5*n*-3 = 22:6*n*-3 = *n*-3 PUFA ↓*n*-6:*n*-3 ratio

CV – basal diet plus 5% *C. vulgaris*; CHR – basal diet plus 5% *C. vulgaris* + 0.005% Rovabio® Excel AP; CHM – basal diet plus 5% *C. vulgaris* + 0.01% four-CAZyme mixture; FA – fatty acids; PUFA – polyunsaturated fatty acids; TBARS – thiobarbituric acid reactive substances; IgA – immunoglobulin A; IgG – immunoglobulin G; IgM – Immunoglobulin M; TAC – total antioxidant capacity; ↑ – increase; ↓ – decrease; = – not changed.

Chapter 8 – CONCLUSIONS, IMPLICATIONS AND FUTURE PERSPECTIVES

8.1. Conclusions

The results obtained in the first part of this study indicate that the two- and four-CAZyme mixtures, developed through an HTP methodology of gene cloning and protein expression, are capable of efficiently degrade *A. platensis* and *C. vulgaris* cell walls, respectively. The composition of the two-CAZyme mixture is a lysozyme and an α-amylase; and the four-CAZyme mixture is constituted by an exo-β-glucosaminidase, an alginate lyase, a peptidoglycan N-acetylmuramic acid deacetylase and a lysozyme. We verified that the action of this two CAZyme mixtures on microalgae cell wall enhance the release of trapped bioactive compounds with important nutritional value from microalgae, such as proteins, pigments and fatty acids. These findings open new opportunities to develop a novel generation of biocatalysts to supplement diets for monogastric animals, in particular those incorporating these microalgae at a high dietary level. Thus, these enzymes may constitute a good approach to improve the bioavailability of these microalgae nutrients for monogastric diets, in particular, and to facilitate the cost-effective use of microalgae by the feed industry, in general.

In the second part of this study, was performed an *in vivo* trial with finishing pigs to test the effect of a high dietary incorporation level of the selected microalga on productive parameters, meat quality and composition, health status and on liver composition. As discussed above, the *C. vulgaris* was selected for the animal trial not only due to the efficiency of the four-CAZyme mixture in disrupt this microalga cell wall, but also due to its superior nutritional value compared with *A. platensis*. Thus, we performed an *in vivo* trial with finishing pigs to test a high dietary incorporation level of 5% of *C. vulgaris*, alone or supplemented with two CAZymes mixtures, the commercially available Rovabio® Excel AP and the four-CAZyme mixtures developed in the first part. The data from this *in vivo* trial allow us to conclude that this dietary incorporation level of *C. vulgaris* did not impair the growth performance, carcass characteristics, meat quality traits and oxidative stability of pork. In addition, the 5% incorporation of *C. vulgaris* in finishing pig diets improves the nutritional value of pork fat, through the increase of the beneficial lipid-soluble antioxidant pigments and *n*-3 PUFA accompanied with the decrease of the *n*-6:*n*-3 ratio. In addition, the use of CAZymes in the feed does not influence these parameters. In contrast, the supplementation with four-CAZyme mixture is fundamental to decrease blood lipemia to the reference value and promote a slight increase in plasma total antioxidant capacity due to its capacity to disrupt the *C. vulgaris* cell wall and release of compounds with lipid- and cholesterol-lowering capacity, resulting on a clear discrimination of this feeding treatment. In addition to blood lipemia, this incorporation

level of *C. vulgaris* has a huge impact on haematological profile, plasma metabolites and hepatic markers. However, these values remain in the reference range, not compromising pigs' health, in general. Conversely, we observed a strong immunosuppressive effect promoted by the microalga at this incorporation level. The increased susceptibility of pigs to infection diseases could be associated to dose-dependent immunoregulatory properties of *Chlorella* polysaccharides. Finally, we observed that the incorporation of *C. vulgaris* promotes an increase of the hepatic content of *n*-3 PUFA sum in pigs, leading to a health beneficial improvement of the *n*-6:*n*-3 ratio, regardless the presence of feed enzymes.

Overall, we concluded that the partial substitution of the traditional feedstuff soybean meal by 5% of *C. vulgaris* in finishing pigs does not impair the growth performance, carcass characteristics, meat quality traits and oxidative stability of pork, which is positive for both producers and consumers. In addition, it was showed that the incorporation of *C. vulgaris* improves the *n*-3 PUFA content and *n*-6/*n*-3 ratio in pork, with positive impact on consumers' health, and in liver. The dietary *C. vulgaris* supplemented with a four-CAZyme mixture has a positive impact on blood lipemia; however, the microalga incorporation promotes a strong immunosuppressive effect.

8.2. Implications and Future Perspectives

The development of two CAZyme mixtures with capacity to disrupt *A. platensis* and *C. vulgaris* in the first part of this study may contribute to improve the bioavailability of these microalgae compounds in monogastric diets, namely in high microalgae incorporation levels. Thus, these enzyme mixtures encompass the enzymatic method to facilitate the cost-effective use of microalgae by the feed industry. In addition to the animal feed industry, these results may increase the yield in obtaining valuable constituents of *A. platensis* and *C. vulgaris* for other biotechnological industries, namely those related with biofuel, food, cosmetic and pharmaceutical applications. However, it will be important to explore additional CAZyme mixtures with possible additional capacity to disrupt these microalgae cell wall. Moreover, the thermotolerance and proteolysis resistance of enzymes that composes both mixtures can be optimized through enzymatic engineering by site-specific alteration of the aminoacidic sequence.

Furthermore, the findings observed in the *in vivo* trial may trigger the development of pork products enriched in the beneficial *n*-3 PUFA and carotenoids, through the substitution of traditional feedstuffs by the microalga *C. vulgaris*, without compromising growth performance and normal meat characteristics, what is of major importance to producers and consumers. Nevertheless, with the aim to maximize both, the sustainability of pig diets and the pork

nutritional quality, further research should be conducted with higher incorporation levels of *C. vulgaris*. This higher incorporation level may contribute to improve the enrichment of pork in *n*-3 PUFA. It is also important to investigate what is the limiting level of incorporation of *C. vulgaris* from which the supplementation with the four-CAZyme mixture is essential for the maintenance of normal productive performance of finishing pigs. However, it is important that this increase of *C. vulgaris* incorporation level do not impair the meat quality traits and its sensory attributes. Further research is also needed to understand how this high dietary incorporation level of *C. vulgaris* influences the muscle and hepatic lipid metabolism, namely *de novo* lipogenesis involved in EPA and DHA biosynthesis. Moreover, due to the appreciable mineral composition of *C. vulgaris*, it is important to analyze whether a high level of incorporation of *C. vulgaris*, supplemented or not with the CAZymes mixture, promotes changes in the mineral profile of pork.

The dietary incorporation of 5% of *C. vulgaris* supplemented with the four-CAZyme mixture allowed to decrease the blood lipemia and did not have a negative impact on pig health, assesssed through theconventional plasma biochemical indicators. Even though these results indicate that *C. vulgaris* is a safe feedstock for pig industry, the clinical significance of the higher susceptibility of pigs to infections found here is critical and should be further assessed in industrial production systems, with identical or higher levels of this microalga incorporation under challenging conditions of stress and hygiene.

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SUPPLEMENTARY MATERIAL 1

Supplementary Material S1. CAZymes-sulfatases library used for the initial screening.

ID	Name	Category	E.C	Main Substrate	Organism
1	Licheninase	1,3-1,4-β-Glucanases	3.2.1.73	1,3-1,4-β-glucans	*Clostridium thermocellum*
2	Cellulose 1,4-β-cellobiosidase	Cellobiohydrolases	3.2.1.91	Phosphoric acid-swollen cellulose, Avicel and others forms of insoluble cellulose	*Clostridium cellulolyticum*
3	Glucan endo-1,3-β-D-glucosidase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans such as laminarin	*Clostridium thermocellum*
4	Chitinase	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Clostridium thermocellum*
5	Cellulose 1,4-β-cellobiosidase	Cellobiohydrolases	3.2.1.91	Phosphoric acid-swollen cellulose, Avicel and others forms of insoluble cellulose	*Clostridium thermocellum*
6	-	Mini-Cellulosome	3.2.1.73	Variety of cellulosic substrates	*Clostridium thermocellum*
7	Endo-β-N-acetylglucosaminidase	Acetylglucosaminidases	3.2.1.96	Mammalian high mannose N-glycans (HMNG), such as Man9GlcNAc2	*Bacteroides thetaiotaomicron* VPI-5482
8	Cellulase	Cellulases	3.2.1.4	1,3-1,4-β-glucans and soluble 1,4-β-glucans	*Bacillus. subtilis* LN
9	β-1,4-glucanase	Cellulases	3.2.1.4	Amorphous cellulose (PASC)	*Clostridium thermocellum F7*
10	Laccase	Laccases	1.3.3.5	2,20-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	*Bacillus subtilis subsp. subtilis* str. 168
11	Unsaturated rhamnogalacturonyl hydrolase	Rhamnogalacturonases	3.2.1.172	Unsaturated rhamnogalacturonan (RG)	*B. subtilis subsp. subtilis* str. 168
12	Laccase / Multicopper oxidase	Laccases	1.10.3.-	2,20-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)	*Escherichia coli* K-12 MG1655
13	Unsaturated rhamnogalacturonyl hydrolase	Rhamnogalacturonases	3.2.1.172	Rhamnogalacturonan oligosaccharides	*Bacteroides thetaiotaomicron* VPI-5482
14	Laminarinase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans such as laminarin	*Thermotoga maritima* MSB8

15	GlcNAc-α-1,4-Gal-releasing endo-β-galactosidase	Galactosidases	3.2.1.-	GlcNAc-α-1,4-Gal-β-1,3-GalNAc-α1-Ser/Thr	*Clostridium perfringens* ATCC 10543
16	Chitinase 1	Chitinases & Chitosanases	3.2.1.14	Chitin and chitosan	*Bacillus licheniformis* DSM 13 = ATCC 14580
17	Endo-β-1,3-glucanase	1,3-β-Glucanases	3.2.1.39	Lichenan and laminarin	*Pyrococcus furiosus* DSM 3638
18	Oligoalginate lyase	Alginate lyases	4.2.2.	Low-viscosity alginate	*Saccharophagus degradans* 2-40
19	Xylanase D / Lichenase	1,3-1,4-β-Glucanases	3.2.1.73 3.2.1.8	1,3-1,4-β-glucans, in particular lichenan	*Ruminococcus flavefaciens* 17
20	Endo-β-1,3-glucanase / Laminarinase	1,3-β-Glucanases	3.2.1.39	Laminarin	*Thermotoga petrophila* RKU-1
21	β-porphyranase B	Porphyranases	3.2.1.178	Porphyran	*Zobellia galactanivorans* DsiJT
22	Laminarinase A	1,3-β-Glucanases	3.2.1.39	Laminarin	*Thermotoga neapolitana*
23	α-1,3-L-(3,6-anhydro)-galactosidase / α-neoagarobiose hydrolase	Agarases & Carragenases	3.2.1.159	Neoagaro-hexaose, -tetraose and -biose	*Zobellia galactanivorans* DsiJT
24	Endo-guluronate lyase	Poly α-guluronate lyases	4.2.2.11	Sodium alginate	*Zobellia galactanivorans* DsiJT
25	β-1,3-1,4-glucanase P2	1,3-1,4-β-Glucanases	3.2.1.73	1,3-1,4-β-glucans	*Paenibacillus polymyxa* SC2 / WY100
26	Alginate lyase / Poly(β-mannuronate) lyase	Alginate lyases	4.2.2.3	Alginates	*Saccharophagus degradans* 2-40
27	Poly-MG alginate lyase	Alginate lyases	3.7.3.48	Alginate (preferably poly(MG) block structure)	*Pseudomonas aeruginosa* PAO1
28	Exochitinase	Chitinases & Chitosanases	3.2.1.14	Glycol chitin	*Bacillus cereus* ATCC 14579
29	Algal laminarin-specific β-glucanase / Laminarinase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans, such as laminarin, and display low activity on mixed linked glucans	*Zobellia galactanivorans* DsiJT
30	β-agarase D	Agarases & Carragenases	3.2.1.81	Agarose	*Zobellia galactanivorans* DsiJT

31	κ-carrageenase	Agarases & Carragenases	3.2.1.83	κ-carrageenan	*Zobellia galactanivorans* DsiJT
32	Endo-β-1,3(4)-glucanase	1,3-1,4-β-Glucanases	3.2.1.6	1,3-1,4-β-glucans, such as lichenan and laminarin.	*Bacillus halodurans* C-125
33	β-1,3-glucanase / Laminarinase	1,3-β-Glucanases	3.2.1.39	Laminarin	*Bacillus halodurans* C-125
34	Licheninase	1,3-1,4-β-Glucanases	3.2.1.73	1,3-1,4-β-glucans	*Ruminococcus flavefaciens*
35	Endo-levanase	Fructanases	3.2.1.65	Levans	*B. subtilis subsp. subtilis str.* 168
36	Chitosanase	Chitinases & Chitosanases	3.2.1.132	Chitosan	*B. subtilis subsp. subtilis str.* 168
37	Endo-β-2,6-fructanase	Fructanases	3.2.1.65	Levans	*B. thetaiotaomicron* VPI-5482
38	Cellobiohydrolase	Cellobiohydrolases	3.2.1.91	Amorphous and crystalline cellulose	*Clostridium thermocellum* ATCC 27405 / DSM 1237
39	Lytic transglycosylase A	Peptidoglycan lytic exotransglycosylases	4.2.2.n1	Murein glycan strands and insoluble, high-molecular weight murein sacculi	*Escherichia coli str. K-12 substr.* MG1655
40	Pullulanase	Glucosidases	3.2.1.41 I	Pullulan	*Thermotoga maritima* MSB8
41	Exo-α-sialidase	Sialidases	3.2.1.18	Sialic acids from complex carbohydrates; glycoprotein human α-1 (AGP)	*Clostridium perfringens* A99
42	Trans-sialidase B	Sialidases	3.2.1.18	Sialic acids from complex carbohydrates and glycoprotein human α-1 (AGP)	*Streptococcus pneumoniae* TIGR4
43	PaCel6A (GH6-2)	Cellobiohydrolases	3.2.1.91	Avicel and carboxymethyl cellulose	*Podospora anserina*
44	PaCel6C (GH6-4)	Cellobiohydrolases	3.2.1.91	Avicel, crystalline cellulose and carboxymethyl cellulose	*Podospora anserina*
45	-	Carbohydrate Binding Module	-	Linear 1,3-β-glucans with occasional decorations of 1,6-β-glucose side-chains	*Clostridium thermocellum*
46	-	Carbohydrate Binding Module	-	Undecorated linear 1,3-β-glucans or decorated with 1,6-β-glucose side-chains	*Ruminococcus flavefaciens*

47	Chitosanase	Chitinases & Chitosanases	3.2.1.132	Soluble and colloidal chitosan	*Bacillus cereus* ATCC 14579
48	Sialidase	Sialidases	3.2.1.18	Sialic acids from oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates	*Bacteroides fragilis* YCH46
49	α-galactosidase	α-galactosidases	3.2.1.22	α-galactose from decorated polysaccharides	*Bacteroides ovatus*
50	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	*Bacteroides ovatus*
51	Levanase	Levanases	3.2.1.80	Levans	*Bacillus subtilis*
52	Levanase	Levanases	3.2.1.80	Levans	*Bacillus subtilis*
53	α-1,2-L-fucosidase	Fucosidases	3.2.1.51	1,2-α linkages and 1,3-α linkages of fucose carbohydrates	*Bifidobacterium longum subsp. infantis* ATCC 15697 = JCM 1222
54	Chitin oxidative-cleaving enzyme	Lytic polysaccharide monooxygenases	1.-.-.-IIa.chitin	Soluble chitooligosaccharides and α- and β-chitin	*Enterococcus faecalis* V583
55	Glycosphingolipid β-N-acetylgalactosaminidase	Acetylgalactosaminidases	3.2.1.53	pNP-β-GalNAc	*Paenibacillus sp.* TS12
56	β-galactosidase	β-Galactosidases	3.2.1.23	p-nitrophenyl-β-D-galactopyranoside, but not p-nitrophenyl-α-D-galactopyranoside	*Victivallis vadensis ATCC BAA-548*
57	β-agarase	Agarases	3.2.1.81	Agarose	*Pseudoalteromonas sp.* CY24
58	N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase	Acetylglucosamine deacetylases	3.5.1.89	1-D-myo-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside	*Mycobacterium tuberculosis* H37Rv
59	Endoglycoceramidase I	Endoglycoceramidases	3.2.1.123	The glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids	*Rhodococcus sp.* C9
60	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	The 1,4-β-glycosidic bond of cellooligosaccharides, also hydrolysis nonreducing end of chitooligosaccharides (Glc-PNP)	*Photobacterium profundum* SS9
61	Xanthan lyase	Xanthan lyases	4.2.2.12	Xanthan	*Paenibacillus alginolyticus* XL-1

62	Alginate lyase	Alginate lyases	4.2.2.11	Alginates	*Agarivorans sp.* JAM-A1m
63	β-agarase	Agarases	3.2.1.81	Agar	*Agarivorans sp.* JA-1
64	α-1,3-3,6-anhydro-L-galactosidase	Agarases	3.2.1.-	Agarose oligosaccharides	*Bacteroides plebeius* DSM 17135
65	β-agarase	Agarases	3.2.1.81	Agarose	*Bacteroides plebeius* DSM 17135
66	Alginate lyase	Alginate lyases	4.2.2.3	Polyguluronate and polymannuronate	*Sphingomonas sp.* A1
67	α-N-acetylgalactosaminidase	Acetylgalactosaminidases	3.2.1.49	GalNAcα1-Ser	*Bifidobacterium bifidum* JCM 1254
68	α-1,6-mannanase	α-Mannanases	3.2.1.101	1,6 linkages in α-mannans	*Bacillus circulans* TN31
69	α-1,3-glucanase	α-Glucosidases	3.2.1.59	1,3-α-glucan	*Bacillus circulans* KA-304
70	β-N-acetylglucosaminidase 20B	Hexosaminidases	3.2.1.52	β-N-acetylglucosamine	*Saccharophagus degradans* 2-40
71	β-galactosidase	β-Galactosidases	3.2.1.23	β-D-galactosides	*Bacillus subtilis subsp. subtilis str.* 168
72	Pectate lyase	Pectate lyases	4.2.2.2 4.2.2.9	Polygalacturonic acid (PGA)	*Bacillus licheniformis* 14A
73	Exo-β-agarase D	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Saccharophagus degradans* 2-40
74	α-L-fucosidase	Fucosidases	3.2.1.51	Fucosyl-α-1,6-N-acetylglucosamine	*Lactobacillus casei* BL23
75	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	Chitobiose and nonreducing terminal glycosidic bond of chitooligosaccharides	*Thermococcus kodakarensis* KOD1
76	α-mannosidase	α-Mannosidases	3.2.1.113	1,2-α-mannobiose on Man9GlcNAc2	*Caulobacter sp.* K31
77	Endo-β-1,3-glucanase	Laminarinases	3.2.1.39	1,3-β-glucans	*Arthrobacter sp.* NHB-10
78	Keratan sulfate hydrolase / Keratanase II	Acetylglucosaminidases	3.2.1.-	Cartilage keratan sulfate and cornea keratan sulfate	*Bacillus circulans* KST202

79	Glucuronyl esterase 15A	Glucuronyl esterases	3.1.1.-	Glucuronyl crosslinks between hemicelluloses and lignin	*Zobellia galactanivorans*
80	Feruloyl esterase	Feruloyl esterases	3.1.1.73	Ferulate crosslinks between hemicelluloses and lignin	*Thermoanaerobacter mathranii subsp. mathraniistr.*A3
81	Exo-β-glucosaminidase	Glucosaminidases	3.2.1.165	Lactose, GlcNAc2, GlcNAc3, cellobiose and cellotriose, as well as colloidal chitin, cellulose, lichenan, laminarin and xylan	*Pyrococcus horikoshii* OT3
82	β-1,3-glucanase B	Laminarinases	3.2.1.39	Insoluble 1,3-β-glucan	*Lysobacter enzymogenes* N4-7
83	α-L-rhamnosidase	Rhamnogalacturonases	3.2.1.40	p-nitrophenyl α-L-rhamnopyranoside	*Sphingomonas paucimobilis* FP2001
84	N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy-α-glucopyranoside deacetylase	Acetylglucosamine deacetylases	3.5.1.89	1-D-myo-inosityl-2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins)	*Mycobacterium smegmatis str.* MC2 155
85	β-galactosidase	β-Galactosidases	3.2.1.23	β-galactosides	*Thermotoga maritima* MSB8
86	Lytic transglycosylase	Peptidoglycan lytic exotransglycosylases	4.2.2.n1	1,4-β-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine residues	*Escherichia coli* K-12
87	Exo-polygalacturonase	Polygalacturonases	3.2.1.67	Polygalacturonic acid (PGA)	*Thermotoga maritima* MSB8
88	β-N-acetylglucosaminidase	Hexosaminidases	3.2.1.52	N-acetyl-β-D-hexosaminides	*Clostridium perfringens str.* 13
89	Keratan-sulfate endo-β-galactosidase / Keratanase	β-Galactosidases	3.2.1.103	Internal endo-β-galactosyl linkages in keratan sulfate and glycoconjugates with N-acetyl-lactosamine repeating units	*Sphingobacterium multivorum*
90	Rhamnogalacturonate lyase	Rhamnogalacturonan lyases	4.2.2.-	Rhamnogalacturonan	*Dickeya dadantii* 3937
91	Bifunctional muramidase soluble-lytic transglycosylase	Peptidoglycan lytic exotransglycosylases	4.2.2.n1 3.2.1.17	N-acetylated glycan	*B. subtilis subsp. subtilis str.* 168
92	Endo-rhamnogalacturonan lyase	Rhamnogalacturonan lyases	4.2.2.23	Rhamnogalacturonan	*B. subtilis subsp. subtilis str.* 168

93	Peptidoglycan N-acetylmuramic acid deacetylase	Acetylglucosamine deacetylases	3.5.1.104	Peptidoglycan	*B. subtilis subsp. subtilis str.* 168
94	α-1 2-mannosidase / Mannosyl-oligosaccharide α-1,3-mannosidase	α-Mannosidases	3.2.1.-	Mannosyl-oligosaccharides	*Bacteroides thetaiotaomicron*
95	Lysozyme	Lysozymes	3.2.1.17	Peptidoglycans	*Escherichia coli*
96	Rhamnogalacturonan lyase	Rhamnogalacturonan lyases	4.2.2.-	Rhamnogalacturonan	*Bacteroides thetaiotaomicron*
97	α-galactosidase A	α-Galactosidases	3.2.1.22	Galactose containing carbohydrates	*Escherichia coli str.* K-12 *substr.* MG1655
98	β-N-acetylglucosaminidase	Hexosaminidases	3.2.1.52	Chitooligomers such as di-N-acetylchitobiose and tri-N-acetylchitotriose, and synthetic substrates	*Thermotoga maritima* MSB8
99	α-galactosidase	α-Galactosidases	3.2.1.22	Substrates with galactopyranoside as the glycone	*Thermotoga maritima* MSB8
100	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	*Thermotoga maritima* MSB8
101	Lysozyme (CPE 1314)	Lysozymes	3.2.1.17	Peptidoglycan containing muramic acid δ-lactam	*Clostridium perfringens str. 13*
102	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	*Bacteroides ovatus*
103	Hyaluronidase	Hyaluronidases	3.2.1.35	Hyaluronic acid	*Clostridium perfringens* CPN50
104	Lysozyme	Lysozymes	3.2.1.17	Peptiglycans	*Escherichia coli*
105	Pectinesterase (Cthe_2949)	Pectinesterases	3.1.1.11	This protein is involved in step 1 of the subpathway that synthesizes 2-dehydro-3-deoxy-D-gluconate from pectin	*Clostridium thermocellum* ATCC 27405
106	Polysaccharide lyase family 8	Polysaccharide lyases	4.2.2.5	Eliminative degradation of polysaccharides containing 1,4-β-D-hexosaminyl and 1,3-β-D-glucuronosyl linkages to disaccharides containing 4-deoxy-β-D-gluc-4-enuronosyl groups	*Pseudopedobacter saltans* DSM 12145
107	α-L-rhamnosidase	Rhamnogalacturonases	3.2.1.40	p-nitrophenyl α-L-rhamnopyranoside	*Bacteroides thetaiotaomicron*

108	D-4,5-unsaturated β-glucuronyl hydrolase (BT3687)	Glycoside Hydrolases	3.2.1.-	Hydrolase activity	*Bacteroides thetaiotaomicron*
(2)3	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	*Geobacillus stearothermophilus* T-1
(2)4	Endo-β-1,4-galactosidase	β-Galactosidases	3.2.1.-	β-galactosides	*Streptococcus pneumoniae* TIGR4
(2)6	Chitinase D	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Streptomyces coelicolor* A3(2)
(2)7	Chitinase G	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Streptomyces coelicolor* A3(2)
(2)9	β-galactosidase	β-Galactosidases	3.2.1.23	β-galactosides	*Vibrio* sp. (strain EJY3)
(2)10	β-agarase	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Bacteroides uniformis* NP1
(2)11	β-porphyranase	Porphyranases	3.2.1.178	Porphyran	*Bacteroides uniformis* NP1
(2)12	β-porphyranase B	Porphyranases	3.2.1.178	Porphyran	*Bacteroides plebeius* DSM 17135
(2)13	Agarase	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Microbulbifer thermotolerans*
(2)14	κ-carrageenase	Agarases & Carragenases	3.2.1.83	κ-carrageenan	*Pseudoalteromonas carrageenovora*
(2)15	β-porphyranase	Porphyranases	3.2.1.178	Porphyran	*Bacteroides plebeius* DSM 17135
(2)16	Agarase	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Bacteroides uniformis* NP1
(2)18	α-neoagaro-oligosaccharide hydrolase	Oligosaccharide hydrolases	3.2.1.159	Neoagarooligosaccharides	*Bacteroides uniformis* NP1
(2)20	Ulvan lyase	Ulvan lyases	4.2.2.-	Marine polysaccharide ulvan	*Pseudoalteromonas* sp
(2)21	Acetylglucosamine-6-sulfatase	Acetylglucosamine-sulfatases	3.6.1.14	Hydrolysis of the 6-sulfate groups of the N-acetyl-D-glucosamine 6-sulfate units of heparan sulfate and keratan sulfate	*Aquimarina agarilytica*

(2)22	Putative arylsulfatase 2	Arylsulfatases	3.1.6.1	Phenol sulfate	*Aquimarina spongiae*
(2)23	Arylsulfatase	Arylsulfatases	3.1.6.1	Phenol sulfate	*Bacteroides plebeius*, strain DSM 17135
(2)24	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Bacteroides uniformis* NP1
(2)25	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Bacteroides uniformis* NP1
(2)26	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Bacteroides uniformis* NP1
(2)27	Putative arylsulfatase 2	Arylsulfatases	3.1.6.1	Phenol sulfate	*Cellulophaga baltica*
(2)28	Putative arylsulfatase 2	Arylsulfatases	3.1.6.1	Phenol sulfate	*Echinicola pacifica*
(2)29	Putative arylsulfatase 2	Arylsulfatases	3.1.6.1	Phenol sulfate	*Formosa agariphila* KMM 3901
(2)30	Putative arylsulfatase 2	Arylsulfatases	3.1.6.1	Phenol sulfate	*Labilibacter marinus*
(2)31	Acetylglucosamine-6-sulfatase 3	Acetylglucosamine-sulfatases	3.6.1.14	Hydrolysis of the 6-sulfate groups of the N-acetyl-D-glucosamine 6-sulfate units of heparan sulfate and keratan sulfate	*Labilibacter marinus*
(2)32	Arylsulfatase A	Arylsulfatases	3.1.6.8	Hydrolyzes galactose-3-sulfate residues in a number of lipids and hydrolyzes ascorbate 2-sulfate and many phenol sulfates.	*Lewinella agarilytica*
(2)33	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Pseudoalteromonas atlantica* T6c
(2)34	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Pseudoalteromonas atlantica* T6c
(2)35	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Pseudoalteromonas atlantica* T6c
(2)36	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Pseudoalteromonas atlantica* T6c

(2)37	Putative arylsulfatase	Arylsulfatases	3.1.6.1	Phenol sulfate	*Bacteroides ovatus* ATCC 8483
(2)38	Putative β-agarase	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Bacteroides ovatus* ATCC 8483
(2)39	Putative secreted sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Bacteroides thetaiotaomicron* VPI-5482
(2)40	Putative Chitinase	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Bacteroides thetaiotaomicron* VPI-5482
(2)42	α-neoagaro-oligosaccharide hydrolase	Oligosaccharide hydrolases	3.2.1.159	Neoagarooligosaccharides	*Saccharophagus degradans* 2-40 DSM 17024
(2)43	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Thermotoga maritima* MSB8
(2)44	Putative α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	*Thermotoga maritima* MSB8
(2)45	Sulfatase	Sulfatases	3.1.6.-	It can remove sulfate from the C-6 position of glucosamine	*Zobellia galactanivorans* DsiJT
(2)46	β-porphyranase A	Porphyranases	3.2.1.178	Porphyran	*Zobellia galactanivorans* DsiJT
(2)47	Exo-chitosanase	Chitinases & Chitosanases	3.2.1.132	Soluble and colloidal chitosan	*Anabaena fertilissima* RPAN1
(2)48	Chitosanase II	Chitinases & Chitosanases	3.2.1.132	Soluble and colloidal chitosan	*Streptomyces griseus* HUT 6037
(2)49	Endoxylanase	1,3-1,4-β-Glucanases	3.2.1.8	Xylans	*Bacteroides eggerthii* DSM 20697
(2)50	β-agarase (AgaY) (extracellular)	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Janthinobacterium sp.* SY12
(2)51	Chitinase (ChiA)	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Rhodothermus marinus* PRI378
(2)52	Chitinase (Chi25)	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Streptomyces thermoviolaceus* OPC-520

(2)53	Mucin-desulfating sulfatase	Sulfatases	3.1.6	Sulfuric ester hydrolase activity	*Zobellia galactanivorans* DsiJT
(2)54	Mucin-desulfating sulfatase	Sulfatases	3.1.6	Sulfuric ester hydrolase activity	*Zobellia galactanivorans* DsiJT
(2)55	AgaraseB	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Zobellia galactanivorans* DsiJT
(2)56	AgaraseA	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Zobellia galactanivorans* DsiJT
(2)57	β-porphyranase A	Porphyranases	3.2.1.178	Porphyran	*Zobellia galactanivorans* DsiJT
(2)58	β-porphyranase C	Porphyranases	3.2.1.178	Porphyran	*Zobellia galactanivorans* DsiJT
(2)59	β-porphyranase D	Porphyranases	3.2.1.178	Porphyran	*Zobellia galactanivorans* DsiJT
(2)60	β-porphyranase E	Porphyranases	3.2.1.178	Porphyran	*Zobellia galactanivorans* DsiJT
(2)61	α-neoagaro-oligosaccharide hydrolase	Oligosaccharide hydrolases	3.2.1.-	Neoagarooligosaccharides	*Zobellia galactanivorans* DsiJT
(2)62	α-neoagaro-oligosaccharide hydrolase	Oligosaccharide hydrolases	3.2.1.-	Neoagarooligosaccharides	*Zobellia galactanivorans* DsiJT
(2)63	α-1,3-L-(3,6-anhydro)-galactosidase	Oligosaccharide hydrolases	3.2.1.-	Neoagarooligosaccharides	*Zobellia galactanivorans* DsiJT
(2)64	ι-carrageenase	Agarases & Carragenases	3.2.1.157	Hydrolyzes ι-carrageenans, sulfated 1,3-α-1,4-β galactans from red algal cell walls	*Zobellia galactanivorans* DsiJT
(2)65	ι-carrageenase	Agarases & Carragenases	3.2.1.157	Hydrolyzes ι-carrageenans, sulfated 1,3-α-1,4-β galactans from red algal cell walls	*Zobellia galactanivorans* DsiJT
(2)66	ι-carrageenase	Agarases & Carragenases	3.2.1.157	Hydrolyzes ι-carrageenans, sulfated 1,3-α-1,4-β galactans from red algal cell walls	*Zobellia galactanivorans* DsiJT
(2)67	κ-carrageenase	Agarases & Carragenases	3.2.1.83	κ-carrageenan	*Zobellia galactanivorans* DsiJT
(2)68	Endo-1,3-β-glucanase	1,3-β-Glucanases	3.2.1.39	Lichenan and laminarin	*Zobellia galactanivorans* DsiJT

(2)69	Alginate lyase	Alginate lyases	4.2.2.11	Alginates	*Zobellia galactanivorans* DsiJT
(2)70	Alginate lyase	Alginate lyases	4.2.2.11	Alginates	*Zobellia galactanivorans* DsiJT
(2)71	Endo-1,3-β-glucanase	1,3-β-Glucanases	3.2.1.39	Lichenan and laminarin	*Zobellia galactanivorans* DsiJT
(2)72	α-amylase	Amylases	3.2.1.1	Endohydrolysis of (1->4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1->4)-alpha-linked D-glucose units	*Thermotoga maritima* MSB8
(2)73	α-amylase	Amylases	3.2.1.1	Endohydrolysis of (1->4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1->4)-alpha-linked D-glucose units	*Thermotoga maritima* MSB8
(2)74	α-amylase	Amylases	3.2.1.1	Endohydrolysis of (1->4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1->4)-alpha-linked D-glucose units	*Thermotoga maritima* MSB8
(2)75	α-glucuronidase	Glucuronidases	3.2.1.139	Glucuronic acid from the xylan backbone	*Thermotoga maritima* MSB8
(2)76	α-L-fucosidase	Fucosidases	3.2.1.51	Fucosyl-α-1,6-N-acetylglucosamine	*Thermotoga maritima* MSB8
(2)77	β-glucuronidase	Glucuronidases	3.2.1.31	Hydrolase activity, hydrolyzing O-glycosyl compounds	*Thermotoga maritima* MSB8
(2)78	β-N-acetylglucosaminidase	Hexosaminidases	3.2.1.52	Chitooligomers such as di-N-acetylchitobiose and tri-N-acetylchitotriose, and synthetic substrates	*Thermotoga maritima* MSB8
(2)79	Exo-polygalacturonase	Oligogalacturonate hydrolases	3.2.1.67	Polygalacturonase activity	*Thermotoga maritima* MSB8
(2)80	Laminarinase	1,3-β-Glucanases	3.2.1.39	1,3-β-glucans such as laminarin	*Thermotoga maritima* MSB8
(2)81	Pectate lyase	Pectate lyases	4.2.2.22	Polygalacturonic acid (PGA)	*Thermotoga maritima* MSB8
(2)82	α-neoagaro-oligosaccharide hydrolase	Oligosaccharide hydrolases	3.2.1.-	Neoagarooligosaccharides	*Saccharophagus degradans* 2-40 DSM 17024

(2)83	β-agarase II	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Saccharophagus degradans* 2-40 DSM 17024
(2)84	Alginate lyase	Alginate lyases	4.2.2.11	Alginates	*Saccharophagus degradans* 2-40 DSM 17024
(2)85	Chitin-binding protein	-	-	The cellulose-binding function has been demonstrated in many cases. Several of these modules have been shown to also bind chitin or xylan.	*Saccharophagus degradans* 2-40 DSM 17024
(2)86	Chitinase A	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Saccharophagus degradans 2-40* DSM 17024
(2)87	Chitinase C	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Saccharophagus degradans 2-40* DSM 17024
(2)88	β-agarase	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Saccharophagus degradans 2-40* DSM 17024
(2)89	β-agarase	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Saccharophagus degradans 2-40* DSM 17024
(2)90	Chitinase B	Chitinases & Chitosanases	3.2.1.14	Chitin and pNP-chitotriose	*Saccharophagus degradans 2-40* DSM 17024
(2)91	Exo-β-agarase A	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Saccharophagus degradans 2-40* DSM 17024
(2)92	Exo-β-agarase D	Agarases	3.2.1.81	Agarose and neoagarooligosaccharides	*Saccharophagus degradans 2-40* DSM 17024
(2)93	Oligoalginate lyase	Alginate lyases	4.2.2.	Low-viscosity alginate	*Saccharophagus degradans 2-40* DSM 17024
1604	Galacto-N-biose / Lacto-N-biose phosphorylase	Glycoside Hydrolase	2.4.1.211	1,3-β-galactosyl-N-acetylhexosamine phosphorylase activity	*Bifidobacterium longum subsp. longum* JCM 1217

1453	Cellodextrin glucohydrolase	Glucosidases	3.2.1.21	Hydrolysis of terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose	*Clostridium cellulolyticum* H10 [B]
1113	Trehalose-6-phosphate hydrolase (TreC)	Glycoside Hydrolase	3.2.1.93	Hydrolyzes trehalose-6-phosphate to glucose and glucose 6-phosphate	*Escherichia coli* str. K-12 substr. MG1655
(2)2	β-1,3-glucanase / Laminarinase	1,3-β-Glucanases	3.2.1.39	Laminarin	*Thermobifida fusca* YX
(2)8	β-galactosidase	β-Galactosidases	3.2.1.23	β-galactosides	*Bacteroides uniformis* NP1
(2)17	α-agarase (AgaA)	Agarases	3.2.1.158	Endohydrolysis of 1,3- α -L-galactosidic linkages in agarose, yielding agarotetraose as the major product	*Alteromonas agarilytica*