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Rational use of dietary enzymes and lipids to improve broiler performance and meat quality

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À minha família

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RESUMO

Efeito da suplementação enzimática e lipídica de dietas para frangos no desempenho produtivo e na qualidade da carne

Uma melhor adequação da qualidade dos produtos animais, em concreto da carne de frango, às necessidades nutricionais dos consumidores, associada a uma maior eficiência de transformação dos alimentos para animais em produtos edíveis, são aspectos da maior importância prática na avicultura moderna e suscitam uma análise científica detalhada. Neste trabalho estudou-se a aplicação de um módulo de ligação ao β-glucano (CBM11), acoplado a três enzimas diferentes (GH26GH5 e GH16, ambas pertencentes ao *Clostridium thermocellum*, e a GH5, pertencente ao *Celvibrio mixtus*) na melhoria do valor nutritivo de dietas à base de cevada para frangos de carne. Foram também determinadas as propriedades bioquímicas e a estrutura cristalográfica do CBM da família 42 do *Clostridium thermocellum, Ct*CBM42A. Os resultados demonstraram que o CBM11 tem um efeito importante no direccionamento do módulo catalítico das enzimas ao substrato, que resulta num aumento da performance zootécnica dos frangos de carne. No entanto, esse efeito parece estar dependente da dose enzimática aplicada. Demonstrou-se também que a composição das cevadas, principalmente a actividade endo-β-glucanásica, influencia o efeito da suplementação enzimática. Em cevadas com actividade endo-β-glucanásica alta a suplementação enzimática tem um efeito redundante não se obtendo melhoria da performance dos frangos de carne. O estudo do CBM42 revelou que se trata dum CBM do tipo C, com três subdomínios (α, β e γ), com afinidade para o arabinoxilano (nas suas cadeias laterais de arabinose) e arabinano. O subdomínio γ parece ser o responsável pela afinidade ao arabinoxilano enquanto o subdomínio β juntamente com o γ parecem interagir pela afinidade ao arabinano, revelando-se como um módulo potencialmente interessante para uma futura utilização na suplementação enzimática de dietas à base de trigo para frangos. Foram efectuados ensaios com frangos de carne cujas dietas foram suplementadas com semente de linho extrudida e um subproduto de algas marinhas (DHA gold™) para estudar os seus efeitos no perfil dos ácidos gordos da carne e na qualidade da carne. Também se avaliou a extensão da bioconversão dos percursores ácidos linoleico (LA) e linolénico (LNA) nos seus homólogos de cadeia longa. Os resultados mostraram que a conversão dos ácidos gordos não é eficiente e por isso a suplementação directa com uma fonte de ácidos gordos de cadeia longa parece ser a melhor opção para melhorar o conteúdo de ácidos gordos ómega-3 de cadeia longa. No entanto, a qualidade da carne pode estar afectada negativamente em doses de incorporação elevadas de DHA gold™.

Palavras-chave: Frangos de carne, Parede celular, Módulos de ligação a hidratos de carbono, Polissacáridos não-amiláceos, Ácidos gordos n-3 de cadeia longa, Qualidade da carne.

ABSTRACT

Rational use of dietary enzymes and lipids to improve broiler performance and meat quality

The importance of carbohydrate-binding modules (CBMs) and the use of novel enzymes with specific catalytic activities to improve the nutritive value of barley based diets for broilers and the effectiveness of a lipidic supplementation to improve the levels of benefic fatty acids in broilers meat remain to be investigated. In this work we studied the importance of a β-glucan binding domain (CBM11) when appended to three different enzymes (GH26GH5 and GH16, belonging to *Clostridium thermocellum*, and GH5, belonging to *Celvibrio mixtus*) to improve the nutritional quality of barley-based diets for broilers. In addition, the crystal structure and biochemical properties of a family 42 carbohydrate binding module (CBM) from *Clostridium thermocellum*, termed *Ct*CBM42A, were investigated. Data presented here revealed that CBM11 has an important target effect in directing the appended catalytic modules to their target substrates, resulting in an improvement in broiler performance. However, this effect seems to be dependent on the level of supplementation. In addition, barley composition, namely its endogenous β-glucanase activity, influences the response to enzyme supplementation. Thus, exogenous enzymes were shown to be ineffective when used to supplement barleys expressing high endogenous β- glucanase activity. *Ct*CBM42A revealed to be a type C CBM with three subdomains (α, β and γ), with affinity for arabinoxylan (arabinose side chains) and arabinan. The γ subdomain seems to dominate ligand recognition for arabinoxylan while the β and γ subdomains cooperate in arabinan recognition. Thus, *Ct*CBM42A is potentially a good candidate for strategies aimed at improving the nutritive value of wheat-based diets for broilers. In order to improve the fatty acid profile of poultry meat, two different lipidic sources, extruded linseed and a subproduct of a marine alga (DHA gold™), were used to supplement broiler diets. This experiment allowed the evaluation of the metabolic rates of the biosynthetic pathway of long-chain ómega 3 polyunsaturated fatty acids (LC n-3 PUFA).The supplementation of broiler diets with DHA gold™ and extruded linseed showed that conversion of linolenic acid in LC n-3 PUFA is not effective and, consequently, direct supplementation with LC n-3 PUFA seems to be the best option to enrich and improve LC n-3 PUFA in broilers meat. However, higher incorporation dosages of DHA gold™ could affect meat quality.

Key-words: Broilers, Plant cell wall, Carbohydrate-binding modules, Non-starch polysaccharides, Long-chain n-3 fatty acids, Meat quality.

INTERNATIONAL PEER-REVIEWED PAPERS

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LIST OF ABBREVIATIONS AND SYMBOLS

Å	Angstrom
a*	Redness (colour dimension)
A_{600}	absorbance at 600 nanometers
AA	Arachidonic acid
ADF	Acid detergent fibre
ADL	Lignin acid detergent
AGE	Affinity gel electrophoresis
Ala	Alanine
Asp	Aspartate
b*	Yellowness (colour dimension)
BHT	Butylated Hydroxytoluene
BSA	Bovine serum albumin
C. mixtus	*Celvibrio mixtus*
C. thermocellum	*Clostridium thermocellum*
C18PUFA	Polyunsaturated fatty acids with 18 carbons
C20PUFA	Polyunsaturated fatty acids with 20 carbons
C22PUFA	Polyunsaturated fatty acids with 22 carbons
$CaCl_2$	Calcium chloride
CAZymes	Carbohydrate-active enzymes
CBD	Cellulose-binding domain
CBM	Carbohydrate-binding module
CBM11	Family 11 Carbohydrate-Binding Module
CBM13	Family 13 Carbohydrate-Binding Module
CBM3	Family 3 Carbohydrate-Binding Module
CBM42	Family 42 Carbohydrate-Binding Module
GE	Gross energy
Cel5A	Cellulase 5A
CelA	Cellulase A
CLA	Conjugated linoleic acid
Coh	Cohesin
CP	Crude protein
cP	centiPoise
CtGlc16A	*Clostridium thermocellum* Glucanase 16A
CtLic26A-Cel5E	*Clostridium thermocellum* Lichenase 26A-Cellulase 5E
CVD	Cardiovascular disease
d	day
Da	Dalton

DFD	Dry, firm and dark
DHA	Docosahexaenoic acid (22:6 n-3)
DM	Dry Matter
DNSA	3,5-dinitrosalicylic acid
Doc	Dockerin
dp	polymerization degree
DPA	Docosapentaenoic acid (22:5)
DTT	Dithiothreitol
EC	Enzyme Comission
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid (20:5 n-3)
EST	Expressed Sequence Tags
EU	European Union
EU27	European Union with 27 countries
FCR	Feed conversion ratio
g	gram
GH	Glycoside hydrolase
GH16	Family 16 Glycoside Hydrolase
GH2	Family 2 Glycoside Hydrolase
GH43	Family 43 Glycoside Hydrolase
GH54	Family 54 Glycoside Hydrolase
GH93	Family 93 Glycoside Hydrolase
GI	Gastro-intestinal
h	hour
HCl	Hydrochloric Acid
His	Histidine
His6	Six histidines in tandem
IMAC	Immobilized Metal Affinity Chromatography
kg	kilograms
L*	Lightness (colour dimension)
LA	Linoleic acid (18:2 n-6)
LB	Luria Bertani
LA:LNA	Linoleic acid/linolenic acid ratio
LC-PUFA	Long-chain polyunsaturated fatty acid
Leu	Leucine
LNA	Linolenic acid (18:3 n-3)
MDA	Malondialdehyde

ME	Metabolizable energy
mg	milligram
min	minute
mL	milliliter
mM	milliMolar
mm	millimeter
MUFA	Monounsaturated fatty acid
MUFA:SFA	Monounsaturated fatty acid/saturated fatty acid ratio
n-3	Omega 3
n-6	Omega 6
n-6:n-3	Total omega 3 fatty acids/total omega 6 fatty acids ratio
NaHCO3	Sodium bicarbonate
NDF	Neutral Detergent Fibre
nm	nanometer
NSP	Non-starch polysaccharides
ºC	degree Celsius
PC buffer	Phosphate/Citrate buffer
PCR	polymerase chain reaction
pdb	protein data bank
pH	negative decimal logarithm of the hydrogen ion activity in a solution
phe	Phenylalanine
pH_{ult}	ultimate potential of Hydrogen
PSE	Pale, Soft and Exudative
PUFA	Polyunsaturated Fatty Acids
R. flavefaciens	*Ruminococcus flavefaciens*
rmsd	root-mean-square deviation
rpm	rotation per minute
rcf	relative centrifugal force (g)
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
Ser	Serine
SFA	Saturated Fatty Acid
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Tyr	Tyrosine
U	Enzymatic unities

w/v	weight per volume
w/w	weight per weight

1. BIBLIOGRAPHIC REVIEW AND OBJECTIVES

1.1. INTRODUCTION

Poultry meat has an increasing global importance in human diets due to its healthier characteristics and due to its lower price for consumers, when compared with other meats. Monogastrics do not have the ability to digest plant cell wall polysaccharides. Soluble NSPs, such as arabinoxylan and β-glucans found in wheat and barley, respectively, express anti-nutritive properties that can be alliviated by the inclusion of cellulases and hemicellulases in broiler diets. Today, commercial enzymes used by the feed industry contain a broad range of substrate specificities to ensure a broad spectrum of action. However, in some particular circunstances this may be unnecessary since removal of the antinutritive soluble NSPs can result from the action of individual enzymes expressing the correct speificity. The advent of recombinant DNA technology chalenges the development of the appropriate enzymes for various biotechnological applications, including feed production. In nature, plant cell wall can be degrade either in aerobic or in anaerobic environments. Anaerobic organisms produce a large repertoire of enzymes which are organized in a highly elaborate nanomachine for the degradation of plant cell walls that was termed the cellulosome. Plant cell wall degrading enzymes are modular proteins containing both catalytic and non-catalytic domains conected by flexible linker sequences. Non-catalytic modules are predominantly involved in carbohydrate recognition and were termed Carbohydrate-Binding Modules (CBMs)
Here we have evaluated the importance of CBMs in the function of enzymes used to supplement broiler diets. In addition, we have studied different n-3-fatty acids sources to improve meat fatty acid profile in broilers.

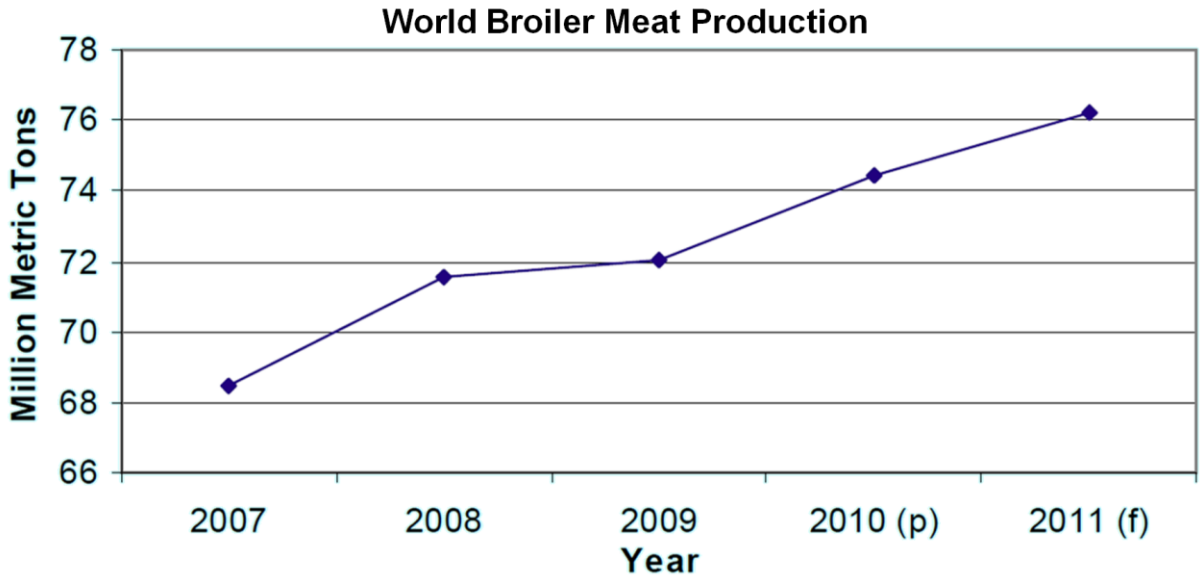
This thesis is divided in 5 chapters. The first chapter revises our current knowledge on the avian sector, globally and in the Portuguese market. Then a focus on the plant cell wall, their composition, different strategies and mechanisms of degradation and the importance of this knowledge in poultry production was provided. Finally, meat quality was reviewed with a special attention to meat lipid profile and its implications on meat quality. At the end of the bibliographic review, the objectives of this work are clearly defined. Chapters 2, 3 and 4 are organized in papers based on scientific manuscripts, already published or submitted to international peer reviewed journals. Each chapter is composed by an abstract, introduction, description of experimental procedures, results, discussion and conclusions. Finally, chapter 5 aims to discuss and conclude, in an integrated form, the results obtained in each of the 4 previous chapters. Finally, future perspectives for the advancement of this work wil be provided.

1.2. CHARACTERIZATION OF AVIAN SECTOR

1.2.1. Economic characterization

The growing demand for relatively low-cost, healthy and convenient meat products triggered a dramatic increase in the consumption of poultry meat (USDA, 2010). This increase is accompanied by a subtle decrease in the intake of red meat (the majority originated from cattle), which has been associated with cardiovascular diseases and some cancer types (WHO, 2004). The increasing demand of poultry meat is due to several factors beside the health issue, which include the low price, low-fat meat and nutritive value, the inexistence of religious or culture restrains and the relatively easiness to prepare. In addition, broiler producers ensure a low-cost production, rapid growth (low FCR), and relative easy management which makes this sector highly efficient. This trend is similar over the world. In United States of America broiler meat production increased 2% between 2009 and 2010 (USDA, 2010), and it is expected to increase the same proportion between 2010 and 2011 (Figure 1.1).

Figure 1.1|World broiler meat production (in million metric tons) during the last five years.



2011 information is forecasted; Production measured in weight of an animal after slaughter and removal of most internal organs, head, and skin. Source: adapted from USDA (2010) reports.

The 2008 data from the European Union (EU27) also reveal an increase in poultry consumption, where United Kingdom and Spain lead this tendency despite rising prices due to higher feeding costs (Eurostat, 2008; USDA, 2009). Overall, worldwide chicken meat is likely to keep replacing the more expensive red meat.

Table 1.1|Gross human *per capita* consumption of meat in the European Union (EU) during 2007 (kg).

	Cattle	Poultry	Pigs	Sheep and Goats	Fish and Seafood
EU [1]	8.8	21.8	41.3	3.0	23.6
BE	22.1	21.1	51.8	1.8	-
BG	-	-	-	-	2.8
CZ	-	2.3	-	-	10.4
DK	27.5	21.7	52.1	1.3	23.1
DE	12.7	16.6	53.9	1.0	12.3
EE	-	-	-	-	20.3
IE	17.7	31.0	38.5	5.4	17.7
EL	16.7	19.3	28.4	11.5	23.1
ES	15.5	32.1	60.9	5.2	45.5
FR	26.8	23.0	34.4	4.2	29.2
IT	25.0	15.3	39.0	1.5	24.8
CY	-	-	-	-	28.7
LV	-	-	-	-	9.6
LT	10.0	11.8	26.1	0.2	54.5
LU	32.5	11.5	44.1	1.7	-
HU	-	-	-	-	4.3
MT	26.4	22.9	33.0	2.5	39.1
NL	19.1	18.6	42.4	1.4	23.8
AT	18.2	18.7	57.0	1.2	11.8
PL	6.6	19.8	48.1	0.1	10.0
PT	18.4	29.8	44.2	3.1	55.3
RO	-	-	-	-	3.2
SI	-	-	-	-	7.5
SK	-	-	-	-	6.5
FI	18.8	16.2	33.7	0.4	30.6
SE	23.8	13.9	36.1	1.1	27.7
UK	21.1	29.8	21.6	6.4	21.2

In orange is highlighted the main consumer country in EU (Spain) and in red the Portugal consumption that closely approximates the largest consumer. [1)] Average of available countries using previous reference periods when no data were available for 2007. *Source:* adapted from Eurostat, Food: From farm to fork statistics (2008).

It is well established that *per capita* annual consumption of poultry meat varies substantially around the globe, ranging from 0.7 kg in India to 44 kg per year in the USA (Ravindran, 2010), and in Europe, from 2.3 kg in Czech Republic to 32.1 kg in Spain (Eurostat, 2008) (Table 1.1). In addition United States of America, China and Brazil became new centres of broiler production (Windhorst, 2007), and Brazil is the main exporter of poultry meat to the European Union. In 2008, chicken production in the EU increased, fuelled by the rising export demand (exports grew significantly (+16%) exceeding imports) and internal consumption. Russia, Saudi Arabia and Ukraine remain the major customers of EU chicken meat (USDA, 2009) and the main EU exporters are France and Benelux. The avian influenza decreased the demand of poultry meat during the first half of 2006 (Magdelaine *et al.*, 2008). As a result of a low commercial stock, prices raised during 2006 and 2007, primarily in

Benelux (USDA, 2009). However, data shows a rapid increase in confidence in poultry products and in authorities and health organizations (Magdelaine *et al.*, 2008).

The situation in Portugal is similar to the described European panorama. Meat is an important protein source in Portuguese diet and the consumption of poultry meat has been steadily rising in the past years. In addition, cattle meat decreased its importance in Portuguese feeding habits, despite the higher consume of pork meat. Portugal is one of the most important poultry meat consumers in Europe (29.8 kg per capita in 2007, (Eurostat, 2008) and is self-sufficient (INE, 2009). Following the European trend, broiler meat represents 96% of poultry meat slaughtered and approved for consumption in Portugal.

1.2.2. Production Systems

Broilers can be raised following two major production systems that will be refereed here, for simplification, as intensive or extensive (Figure 1.2). Intensive systems allow producing more kg of meat in less time (normally in 5-6 weeks). Intensive systems are cage-free, where the birds are on floor with dry litter, with slats and lower density. There are semi-intensive systems, which provide a yard for outdoor access. Extensive systems or alternative systems offer an ample space with outdoor access which promotes natural behaviours (soil scratching, pecking, running, and flying) and exercise (Appleby & Hughes, 1991). Birds are exposed to sunlight and fresh air, hence contributing to reduce stress. Extensive systems can be free-range or traditional free-range, as defined by the European Union Commission (EC, 2001). Traditional free range systems are more restricted than free range systems. For example, there is a minimum slaughter age, which is 56 days for free range systems and 81 days for traditional free range systems. Birds can be in fixed or portable houses, in pasture hens or in integrated systems. Traditional free range systems have less animal density and fewer houses per exploration than free range systems and need to use slow-growing genotype animals. Chicken meat from intensive systems is cheaper than that originated from alternative systems. However, extensive systems produce meat with add-value associated with sustainability, environment, animal welfare and health concerns. Beyond these concerns, the meat prices of alternative systems increase because the longer time of production of these animals.

Figure 1.2|Example of an intensive system and an extensive system (with a fixed house).

Source: Interaves S.A.

1.2.3. Breeds

Chicken breeds can be divided into three categories: egg laying stocks, meat-type birds and dual purpose breeds. Each type of bird is genetically developed to satisfy the best attributes for which they are intended. Currently, meat-type breeds are highly engineered and instead of breeds, they are hybrid varieties resulting from combinations of many different breeds. The combination of breeds is selected to produce a variety (strain) with meat characteristics that the producer desires most accordingly with consumers preference. Some breeds grow faster and larger while others emphasis traits like larger breast meat yield, more efficient feed conversion or more resistance to disease. The strains are named after the breeding companies that genetically develop them, like Arbor Acres, Ross, Peterson and Hubbard, to name a few. These strains are specialized in broiler producing and they have a fast growing genotype. The choice of the strain is also dependent on the production system. In intensive systems, fast-growing strains are preferably used instead of the slow-growing strains, which are more appropriated to alternative systems where the production period is longer. In extensive systems, breed selection is more related with animal's behaviour and welfare (Fanatico, 2006, 2010).

1.2.4. Feeding and management

Animal feeding is an important factor determining the efficiency of animal performance and meat quality. Through feeding, this sector has been improved since the last 50 years, decreasing the feed conversion ratio and the time needed to obtain a broiler with the market body weight. Feeding is also an important economic issue. In 2005, in Portugal, 28.5% of the total manufacture of feed for livestock was for broilers and chickens (INE, 2009). The aim in animal feeding is to maximize production, at a minimum cost, providing all the necessary nutrients to face maintenance, growth, development, reproduction and production needs. Birds have a short production cycle and currently have been slaughtered with bigger weights. Today, consumers are more interested in issues such as health, food safety and traceability.

Thus, considering all its implications it is clear that bird feeding is an important and complex matter. Birds are largely dependent on feeding contribution since they can not synthesize essential amino acids and vitamins and do to their reduced ability to digest cellulosic materials. In birds, feed intake is regulated by diet energy content. Increments in feed energy are also accompanied by a reduction in intake, so that total consumption of metabolizable energy remains almost the same. In addition, diet content on some amino acids influence intake, since birds compensate a lower content in amino acids such as methionine and cysteine, by improving feed consumption. Feed must be balanced between energy and protein. A high energy feed should contain a protein level also elevated to face the amino acids needs. Intake is also dependent on temperature and feed appearance. High temperature beyond the thermal neutrality leads to a linear reduction in feed intake. In contrast, low temperature causes an increase in feed intake and a higher feed conversion ratio. In addition, pelleted feed implies a high intake, less waste and a better feed conversion ratio. Feed formulation differs in different production stages and usually starter, grower and finisher feeds are used. For guidance, the main feedstocks for birds are cereals, which are usually incorporated at a rate of 40 to 60%. In Portugal, the most common cereal used in birds feed is maize due to its high content in starch, which is highly digestible by poultry and contains low levels of anti-nutritive factors. However, because the price of maize is frequently high this cereal may be substituted by wheat, sorghum (low-tannin varieties) or barley (Ravindran, 2010).

1.3. ENZYMATIC SUPPLEMENTATION OF DIETS FOR POULTRY

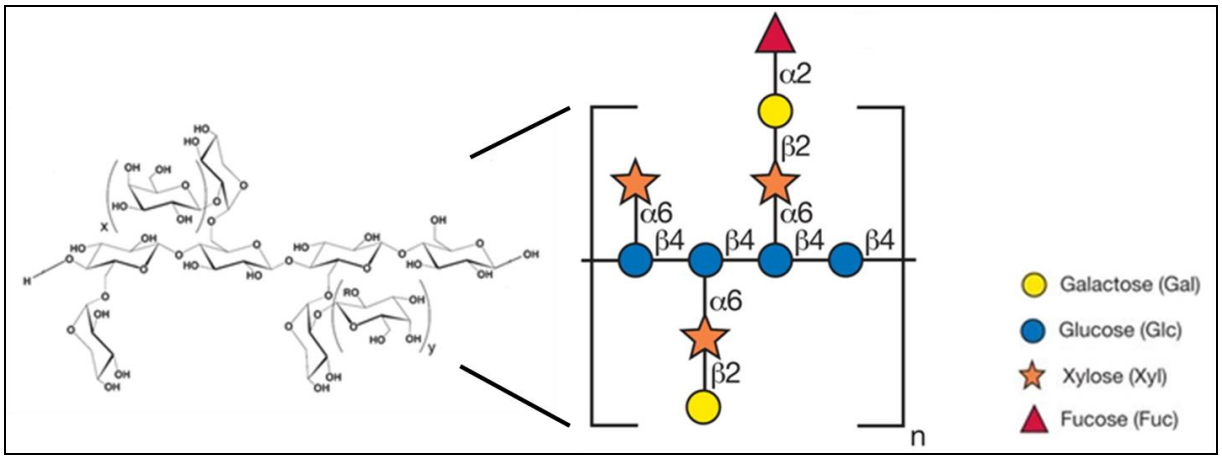
1.3.1. Plant cell wall components

The main energy sources in chicken diets are carbohydrates and lipids. The main carbohydrates in poultry feed are starch and structural polysaccharides found in plant cell walls. The plant cell wall is composed predominantly by carbohydrates (up to 90% of the dry weight) together with smaller amounts of structural glycoproteins (2-10%), phenolic esters (<2%), ionically and covalently bound minerals (1-5%) and enzymes (O'Neill & York, 2003). The main plant cell wall polysaccharides are cellulose, hemicellulosic polysaccharides and pectins. Primary cell walls are chemically defined as the insoluble material after extraction with buffers and organic solvents. Starch is removed by an α-amylase treatment and pectins and hemicelluloses are solubilised with aqueous buffers and strong alkali, respectively.

There are two main types of plant cell walls: type I, which are rich in xyloglucan and/or glucomannan with 20-35% of pectin, and type II, rich in arabinoxylan with less than 10% of pectins. Type I cell wall is more common in dicotyledonous, monocotyledonous non-gramineas and gymnosperms, while type II plant cell walls appear in poaceas, like rice and barley. Hemicelluloses include xyloglucan, xylan, mannan and arabinogalactan. They are

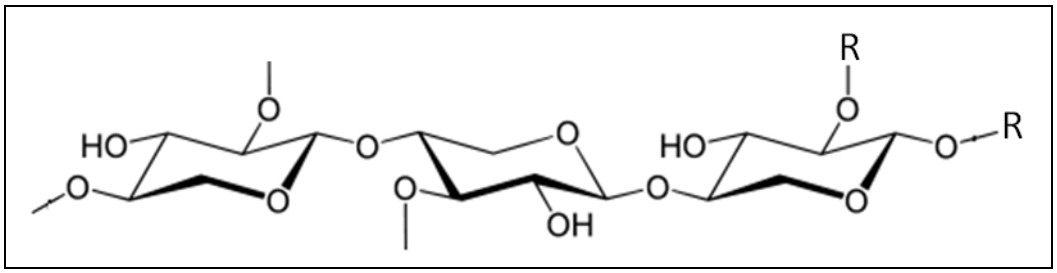
structurally homologous to cellulose. They have a backbone composed of 1, 4 linked β-D-pyranosil residues with O4 in equatorial orientation. Xyloglucan is the most abundant hemicellulosic polysaccharide in the plant cell wall of non-gramineas and has a backbone 1,4-linked β-D-Glc*p* residues, where more than 75% are branched bearing α-D-Xyl*p* residues at O6 (Figure 1.3). Xylans (Figure 1.4) are more common in gramineas and in secondary cell walls of woody plants. Branches and other modifications in their structure prevent hemicelluloses from forming microfibrils as it is observed in cellulose (Cosgrove, 2005).

Figure 1.3|Xyloglucan chemical structure



Xiloglucan general structure formed from β-1-4 glucopyranose residues with various branched residues. Adapted from Martinez-Fleites, *et al*., (2006).

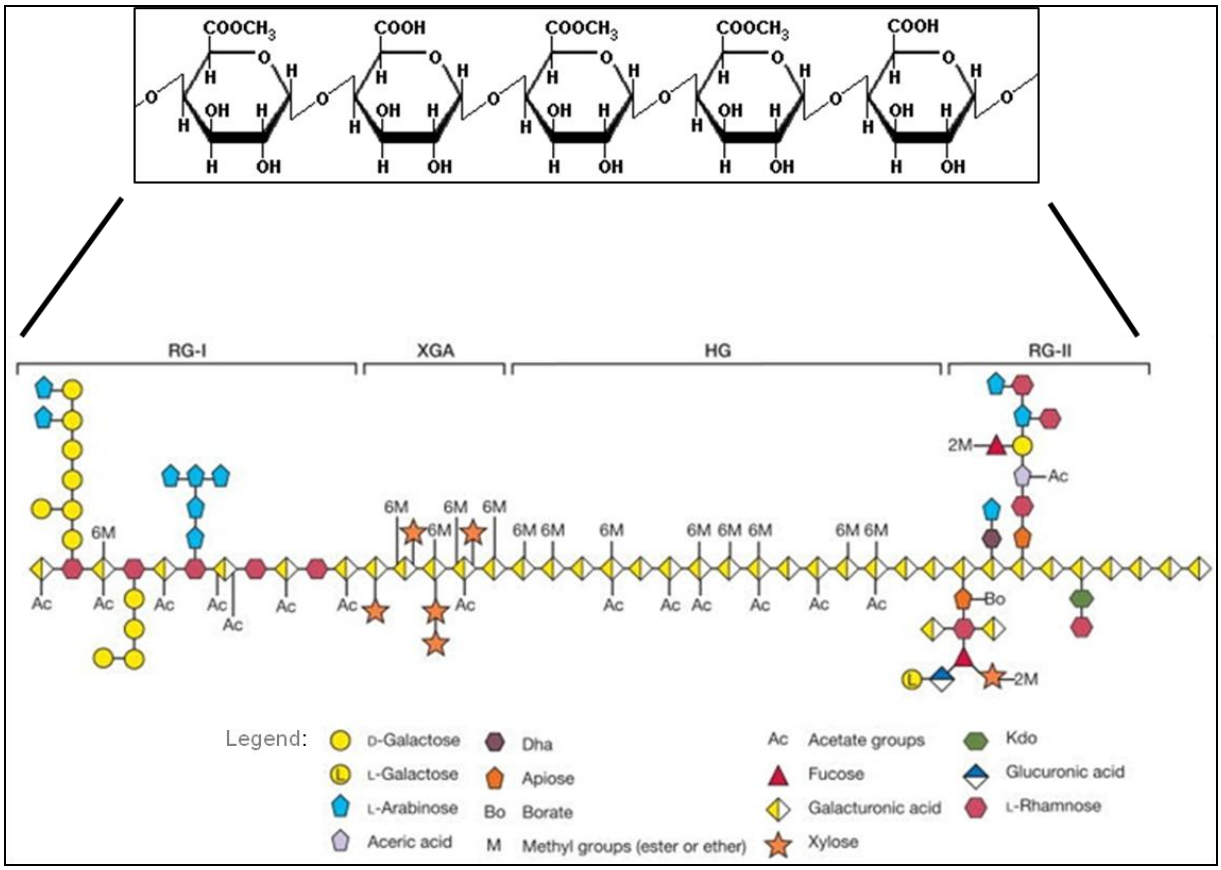
Figure 1.4|Xylan basic chemical structure



Basic structure of xylan formed from β-1-4 linked xylopyranose units where R can be various residues such as gluconic acids, arabinose, galactose, methyl, feruloyl, etc. Adapted from Evans & Hedger (2001).

There are three pectic polysaccharides types: homogalacturonan, substituted galacturonans and rhamnogalacturonan (Figure 1.5). Homogalacturonan constitutes more than 60% of pectins in dicotyledonous and non-gramineas (O'Neill & York, 2003). It has been proposed that they promote wall flexibility while binding to the surface of cellulose (Cosgrove, 2005).

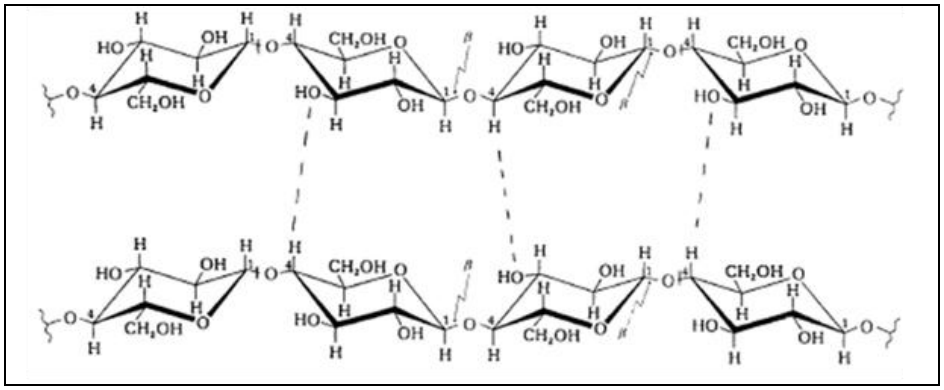
Figure 1.5|Chemical structure of pectins



Pectin is a polymer of α-Galacturonic acid with a variable number of methyl ester groups (schematic structure above). Schematic structure below of pectin showing the three main pectic polysaccharides: rhamnogalacturonan I (RG-I, *left*) and rhamnogalacturonan II (RG-II, *right*), at each side of a homogalacturonan (HG) chain. A region of substituted galacturonan, known as xylogalacturonan (XGA), is also shown between the HG and the RG-1. Dha, 3-Deoxy-D-lyxo-heptulosaric acid. Adapted from Etzler & Mohnen (2009).

Cellulose amounts to approximately 30% of the total polysaccharides in plant cell wall. Cellulose microfibrils are tough, inelastic fibres wrapped in layers (lamellae) within the plant cell wall. Cellulose is composed by 1,4-linked β-D-glucosyl residues (Figure 1.6). Many parallel glucans snap to form a crystalline microfibril that is mechanically strong and highly resistant to enzymatic attack. These long crystalline ribbons are aligned with each other giving a structural bias to the cell wall (Cosgrove, 2005).

Figure 1.6|Cellulose general chemical structure



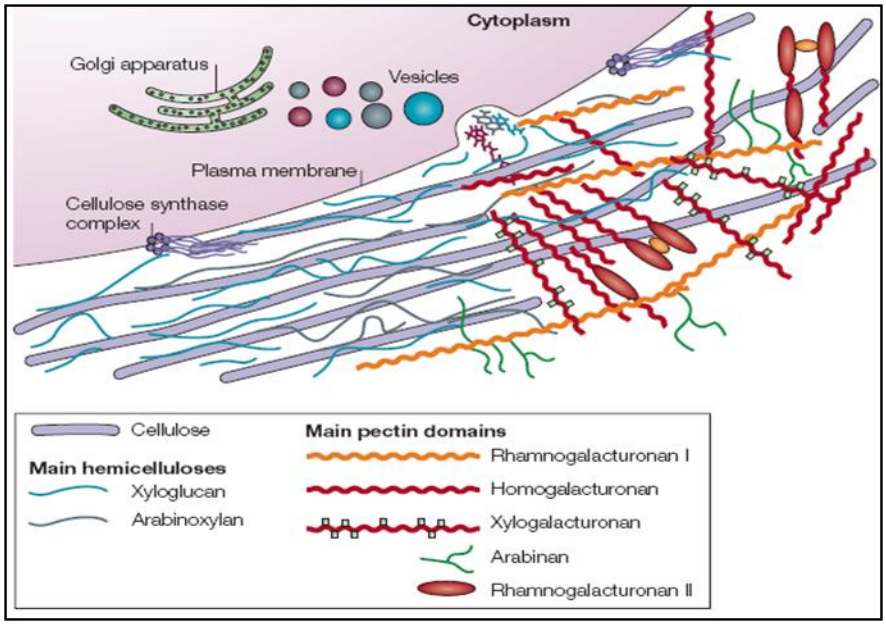
Cellulose is formed from β-1-4 linked cellobiose units, with hydrogen bonding between parallel chains.*Source:http://www.doitpoms.ac.uk/tlplib/wood/structure_wood_pt1.php*

There are other plant cell wall components, such as glycoproteins (may have a structural function), arabinogalactan proteins – AGPs (involved in the expansion and differentiation of cell wall function), enzymes (related with plant cell wall metabolism and breakage of hydrogen bonds between xyloglucan and cellulose thus regulating wall expansion – expansins) and minerals.

1.3.1.1. Plant cell wall models

The more recent models describing plant cell wall structure, proposed by Ha *et al*. (1997) and McCann *et al*. (1990) among other authors, suggest the absence of covalent linkage between the wall matrix polymers. These authors emphasize the importance of non-covalent interactions between wall polymers and propose two independent interacting networks: the pectic polysaccharides network and the xyloglucan and cellulose network. The pectic network seems to have a scaffolding function, preventing macromolecules, such as enzymes, to diffuse into inappropriate sites. It confers wall macromolecules orientation and may function as a sensor of mechanical stress or elastic strain in the cell wall. In addition, it seems to control wall porosity and wall thickness. Pectins, glue cells together in an adhesive layer called middle lamella. This network forms hydrated gels that push microfibrils apart, easing their sideways slippage during cell growth, while also locking them in place when growth ceases. The xyloglucan and cellulose network is believed to play a primary load-bearing function in the cell wall. This network although strong is also resilient. Xyloglucan connects and coat the cellulose microfibrils, limiting its aggregation and linkage, regulating the mechanical properties of the cell wall. Xyloglucan maintains the order spacing of the cellulose microfibrils by its rigidity.

Figure 1.7|Exemplified structure of a primary cell wall.



Cellulose microfibrils (purple rods) are synthesized by large hexameric complexes in the plasma membrane, whereas hemicelluloses and pectins are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles. In most plant species the main hemicellulose is xyloglucan (blue). The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan. Pectin domains are believed to be covalently linked together and to bind to xyloglucan by covalent and non-covalent bonds. Neutral pectin polysaccharides (green) are also able to bind to cellulose surfaces. *Source*: adapted from Cosgrove, 2005.

1.3.2. Plant cell wall degradation

Polysaccharide degradation consists in the cleavage of glycoside bonds, leading to a decrease in the polymerisation degree of those carbohydrates. The chemical and physical complexity of plant cell walls restrict their accessibility to enzyme attack. Only a restricted number of microorganisms have acquired the ability to deconstruct these structural carbohydrates (Fontes & Gilbert, 2010). The plant cell wall-degrading apparatus produced by aerobic and anaerobic microorganisms differ considerably in their macromolecular organization and their process efficiency (Warren, 1996). Aerobic microorganisms produce free enzymes. Most of these enzymes are modular, with one or more catalytic modules, linked to other non-catalytic domains mainly carbohydrate-binding modules (CBMs) that bind their appended domains to their target substrates (Warren, 1996). Anaerobic microorganisms produce organized systems of plant cell wall degrading enzymes in which the proteins are located in multi-enzymatic complexes. Anaerobic environments impose numerous energetic constrains, leading to an effective selective pressure that led to the formation of highly efficient plant cell wall degrading nanomachines (Fontes & Gilbert, 2010). These structures are known as cellulosomes. The naturally occurring carbohydrate substrates are often insoluble. Thus, microorganisms use extracellular enzymes, free or associated in cellulosomes, to convert the polysaccharides into soluble products that are transportable into the cells (Wilson, 2008).

1.3.2.1. Substrates

Cellulose has β-1, 4-glycosidic linkages in a “zigzag” arrangement between oxygen bridges. Their parallel arrays are held together predominantly by strong interactions: hydrogen bonds and hydrophobic stacking between the sugar rings. There are, however, two types of cellulose: crystalline and amorphous. Cellulose is crystalline when molecules are tightly packed and amorphous when they are loosely packed. The crystalline areas are more insoluble and inaccessible to enzymatic attack than the amorphous areas, making the hydrolysis more complex and difficult (Warren, 1996). The actual substrate of most cellulases is not pure cellulose but rather plant cell wall cellulose, which are extremely diverse macromolecules both among different plants and in different cell types in a single plant species. Other cell wall components are bound to cellulose protecting the polysaccharide from hydrolysis. Mannans and xylans are less crystalline and shorter than cellulose molecules. These characteristics facilitate enzymatic attack. Mannans are β-1, 4 linked polymers of mannose and they are relatively flexible. Xylans are β-1, 4 linked xylopyranose polymers that form twisted ribbons. Different xylans are variously substituted with acetyl, arabinofuranoside and glucuronosyl residues (Warren, 1996).

1.3.2.2. CAZymes

Carbohydrate-active enzymes, usually termed as CAZymes for simplification, are usually modular proteins containing one or various catalytic domains connected with CBMs through flexible linker sequences. The most common type of catalytic domains are glycoside hydrolase (GH) and attack β-glycosidic or α-glycosidic bonds. They can be retaining or inverting enzymes. Retaining enzymes catalyze either transglycosylation or hydrolysis reactions with retention of configuration at the anomeric center. In contrast, inverting enzymes do not catalyze transglycosylation reactions, only hydrolysis with the inversion of configuration at the anomeric center (McCarter & Withers, 1994). Glycoside hydrolases nomenclature has evolved in the past 20 years. It has been proposed by the International Union of Biochemistry and Molecular Biology (IUB, 1984) that nomenclature could be based on the type of reaction that enzymes catalyse and on their substrate specificity. Henrissat *et al*., (1991) also proposed a classification based on primary sequences similarities leading the organization of GHs by families. Glycoside hydrolases are classified in 123 families (CAZy database, February, 2011). All members of a family are predicted to share common properties: all are either inverting or retaining enzymes (Warren, 1996). Crystal structures revealed relationships between families. Three-dimensional structures are more conserved than the amino acid sequences hence there are enzymes with similar functions that belong to different families but with similar folds (Davies & Henrissat, 1995). The basic function of the enzymes is determined by the active site topology, with the inversion or retention of the

configuration and specificity determined by the amino acids lateral chains decorating the active site (Warren, 1996). Glycoside hydrolases display different specificities and attack substrates through different mechanisms reflecting the number of substrate binding sites within the active site of the enzymes. Active site in exo-acting enzymes is located in a tunnel and they cleave sequentially molecules of cellulose from one of the ends of the polysaccharide chain releasing cellobiose molecules. This hydrolysis is more efficient because there are two types of exo-acting enzymes: one that acts in the non-reducing end and other that acts in the reducing ends (Shen *et al.*, 1991). Endo-acting enzymes hydrolyse random bonds within the carbohydrate chains, thereby producing more ends for the exo-enzymes to act on. They have an open cleft that binds to the internal linkages in the carbohydrate molecule. Finally, endo-processive enzymes hydrolyse bonds within the chains but unlike the endo-acting enzymes, after the first cleavage they act as exo-acting enzymes. Exo-enzymes and endo-enzymes act in synergy, increasing the efficiency of the polysaccharide hydrolyse. The distinction of these two types of enzymes is not absolute, since some exo-enzymes have some, although low, endo-acting activity (Ståhlberg *et al.*, 1993).

Enzyme terminology was proposed by Henrissat *et al.*, (1998) based on the GH target substrate, where family designation will also been known, using the first three letters of the substrate, followed by the family number and by a uppercase letter that correspond to the order in which the catalytic domain were first reported. For example a family 5 GH will be named Cel5 or Man5, depending on its substrate that could be cellulose or mannose respectively and by Cel5A or Cel5B if there were two catalytic domains with the same specificity but reported at different times (Henrissat, 1998).

1.3.2.3. Non-catalytic modules

As described above, plant cell wall degrading enzymes contain, in addition to the catalytic domains, accessory non-catalytic modules, such as carbohydrate-binding modules (CBMs). Their function is independent of the catalytic modules and they are usually smaller in size (Warren, 1996). The main function of these modules is to maintain a high concentration of the enzymes at the surface of their target substrates (Warren, 1996). CBMs were shown to display a variety of ligand specificities as they can bind cellulose, mannan, xylan, xyloglucan, arabinan, among others. Other non- catalytic modules have been identified particularly those that are involved in protein-protein interactions, such as dockerins, or other modules with unkown function such as fibronectin-like or imunoglobuline-like sequences. Linker sequences separating the different modules in plant cell wall degrading enzymes are responsible for enzyme flexibility. Usually linker regions are rich in serine and threonine (Coutinho & Reilly, 1994) and may be glycosilated, which confer protection from proteolysis (Tomme *et al.*, 1995).

1.3.2.4. Carbohydrate-binding modules

Several carbohydrate-active proteins have acquired non-catalytic modules that interact very specifically with mono-, oligo- and polysaccharides. These modules are known as carbohydrate-binding modules (CBMs). CBMs are auxiliary domains with autonomous folding and specific capacity to recognize heterogeneous and complex carbohydrates. These modules may be found in proteins that recognize polysaccharides such as cellulose, chitin, β-glucans, among many other different sugars, such as lipo-polysaccharides (Guillén *et al.*, 2009). CBMs can be located at the N- or C- terminal end of CAZymes (Guillen et al., 2009). Their main function is to recognize and bind specifically to carbohydrates, enhancing the hydrolysis of insoluble substrate by bringing the catalytic domain into close proximity to the substrate facilitating polysaccharide disruption (Boraston *et al.*, 2004b; Guillén *et al.*, 2009; Hashimoto, 2006). Removal of CBMs leads to a reduction or total abolition in the capacity to bind carbohydrates resulting in a partial or complete loss in the catalytic activity. CBMs may also have a disruption function (albeit a controversial role). Binding of CBMs to a crystalline substrate leads to polysaccharide disorganization and improvement of substrate availability. Gao *et al.* (2001) suggest that the binding of the cellulose-binding domains (CBD) to cotton fibers leads to structural changes and release of short fibers. Wang *et al.* (2008) also found that the attachment of a CBM to cotton fibers promotes severe weakening of the cellulose-interchain hydrogen bonds. This disruptive function does not seem to be shared by all CBM's (Guillén *et al.*, 2009). For efficient polysaccharide hydrolysis, there is a need of a dynamic interaction between CBMs and their substrates, where the catalytic domain is first positioned in proximity to substrate through the CBM. Then, the catalytic domain is able to hydrolyze the polysaccharide chains inserted in the active site. CBMs can also be relocated to new regions on the ligand allowing a continuous hydrolysis of the substrate (Guillén *et al.*, 2009). CBMs are classified in families based on primary sequence similarities (grouped into 61 families, CAZy, February, 2011) and display substantial variation in ligand specificity (Boraston *et al.*, 2004b). Common folds are observed in proteins with different specificities (Guillén *et al.*, 2009). This diversity reflects the diverse ligand specificity of these modules. The most common fold in CBMs is the β-sandwich followed by the β-trefoil. An example of a β-sandwich conformation is the family 11 CBM, while family 42 CBM is an example of the β-trefoil fold. For example, *Clostridium thermocellum* CBM11 has a β-sandwich classical conformation with a distorted β-jelly roll fold consisting of two six-stranded anti-parallel β-sheets, which form a convex and concave side. The concave side forms a cleft with four tyrosines displaying a key-role in carbohydrate recognition (Carvalho *et al.*, 2004b). *Aspergillus kawachii* CBM42 has a β-trefoil conformation, with three segments (α, β and γ) of about 50 amino acids each. Each subdomain consists of four-stranded β-hairpin turns and one additional α-helix and has the capacity to recognize a single ligand (Miyanaga *et al.*,

2004; Miyanaga *et al.*, 2006). Thus, in contrast to CBM11, CBM42 display three ligand binding sites.

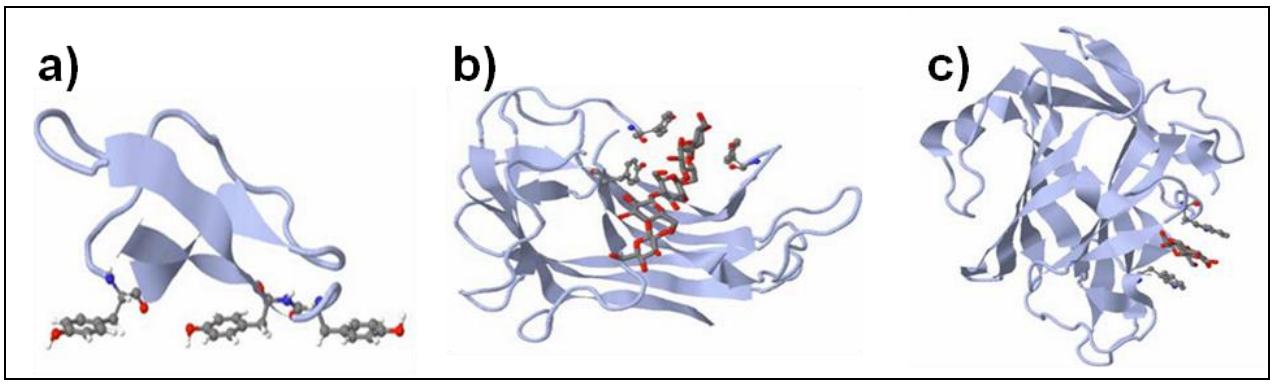
CBMs were also classified based on the topology of the carbohydrate binding site in types A, B or C. Type A CBMs have a flat or platform-like hydrophobic surface (Figure 1.8) composed of aromatic residues. They interact with the flat surfaces of crystalline polysaccharides (cellulose or chitin). Thus, type A CBMs show little or no affinity for soluble carbohydrates (Hashimoto, 2006) providing a distinctive property when compared with the other CBM types (Boraston *et al.*, 2004). In type A CBMs hydrogen bonds have little effect in ligand recognition which is dominated by stacking interactions. Type B CBMs bind amorphous cellulose or xylan and displays a cleft (Figure 1.8) in which aromatic residues interacts with free single polysaccharide chains. Aromatic side chains are oriented in such a way that forms twisted or sandwich platforms (Guillén *et al.*, 2009). The orientation of these amino acids is a key determinant of specificity of these CBM's (Hashimoto, 2006). Type B CBMs comprise several sub-sites that are able to accommodate the individual sugar units of the polymeric ligand. Binding proficiency of this class of CBMs is determined by the degree of polymerization of the carbohydrate ligand. Higher affinities are associated with up to hexasaccharides and lower or negligible affinities with oligosaccharides with a degree of polymerization of 3 or less (Boraston *et al.*, 2004). Contrary to type A CBMs, direct hydrogen bonds play a key role in defining the affinity and ligand specificity in type B CBM's (Boraston *et al.*, 2004). Type C CBM's (Figure 1.8) (or lectin-like CBM's) only bind to mono-, di- or trisaccharides moieties in polysaccharides due to steric restriction in the binding site (Guillén *et al.*, 2009). Hydrogen-bonding network between protein and ligand is also important in this CBM type. CBMs often reflect the specificity of the contiguous catalytic domain, at least for types B and C.

The importance of aromatic amino acids for binding, especially tryptophan but also highly conserved tyrosine residues, is well known. They form stacking interactions with the sugar rings resulting in strong Van der Waals interactions that stabilize the structure of protein-carbohydrate complexes (Guillén *et al.*, 2009). The side chains of polar residues may form hydrogen bonds with the sugar ligand, also stabilizing the interaction. On the other hand, the orientation of aromatic residues is responsible for the different ligand specificities of the families of CBMs, since it defines their topology. Subtle changes in the topology of the binding sites dictate ligand specificity and explain why CBMs with apparent similar structure recognize different ligands (Guillén *et al.*, 2009). Also the hydrogen bonds and calcium-mediated co-ordination play a key role in CBM ligand recognition (Boraston *et al.*, 2004). It has been extensively shown that calcium maintains the binding site in the correct conformation (Boraston *et al.*, 2004).

CBMs can be divided into two groups according to binding affinity: group I, where are the CBMs that bind tightly ($Ka>10^6\ M^{-1}$) and group II, where are the CBM's with weak interaction

(Ka<$10^6 M^{-1}$). These weak relations are often compensated by multiple clustered carbohydrate-binding sites (Boraston *et al.*, 2004) that can result from a single protein with multiple binding sites or association of two or more modules (random or in tandem). Interestingly, the appearance of this multiple CBMs seems to occur more often in thermo- or hyperthermophilic enzymes. This may allow overcoming the loss of binding affinity that accompanies most molecular interactions at elevated temperatures (Boraston *et al.*, 2003a).

Figure 1.8|Structures of the three different CBM types based on topology of carbohydrate binding site.



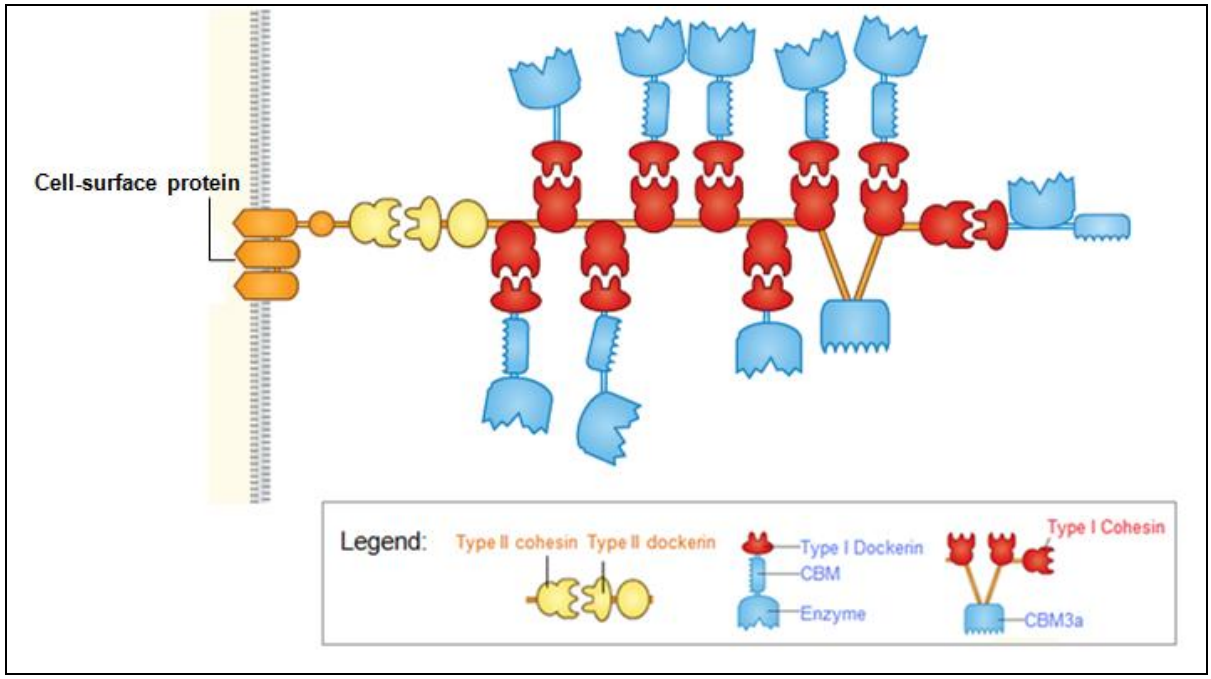
a) Type A CBM (CBM1) from *Trichoderma reesei* cellobiohydrolase I (PDB code 1CBH); b) Type B CBM (CBM4) from *Cellulomonas fimi* endo-1,4-glucanase C (PDB code 1GU3) and c) Type C CBM (CBM9) from *Thermotoga maritime* xylanase 10A (PDB code 1I82). *Source*: adapted from Guillén *et al*., 2009.

1.3.2.5. Cellulosomes

Cellulases and hemicellulases synthesized by anaerobic microorganisms, particularly from the genus *Clostridia* and rumen microorganisms frequently assemble into a large multienzyme complexes, with a molecular weight superior to 3 MDa, that were termed cellulosomes (Bayer *et al.*, 2004). The cellulosome is a cellulose-binding multienzyme complex (Figure 1.9) that mediates the degradation of cellulosic substrates. This complex contains cellulases, hemicellulases, pectinases and other enzyme activities that include polysaccharide lyases, carbohydrate esterases and protease inhibitors (Tamaru & Doi, 2001). This diversity of enzymes and their multimodular organization reflect the chemical and structural intricacy of the plant cell wall. It is well established that this multienzymatic complex is more efficient than the free enzymes produced by aerobic microorganisms when acting on recalcitrant substrates (Fontes & Gilbert, 2010). This efficiency is justified by the particular assembly of cellulosome and by the complexity of the multimodular cellulosomal enzymes. Cellulosome assembly is determined by protein-protein interactions. Cellulosomal catalytic components contain non-catalytic modules, called dockerins, which bind to cohesins located in a large non-catalytic protein that act as a scaffoldin (dockerins and cohesins from type I) (Fontes & Gilbert, 2010). The cohesin-dockerin interaction allows the integration of the hydrolytic enzymes into the complex and cohesins found in membrane associated proteins bind dockerins located in scaffoldings (cohesin and dockerins from type II). This interaction

allows binding of the complex to the surface of the bacterium (Figure 1.10) (Salamitou *et al.*, 1994; Tokatlidis *et al.*, 1991). A family 3 CBM located in the scaffoldin anchors the entire complex onto crystalline cellulose. This CBM potentiate the proximity between the cellulosomal catalytic units and the plant cell wall (Poole *et al.*, 1992).

Figure 1.9|Mechanism of cellulosome assembly.



Modular cellulases and hemicellulases produced by anaerobic microbes contain a type I dockerin appended to catalytic (enzyme) and noncatalytic carbohydrate-binding module (CBM). Type I dockerins bind the type I cohesins of a noncatalytic scaffoldin, providing a mechanism for cellulosome assembly. In general, scaffoldings also contain a cellulose-specific family 3 CBM (CBM3a) and a C-terminal divergent dockerin (type II) that target the cellulosome to the plant cell wall and the bacterial cell envelope, respectively. The linkers joining the modules in the scaffoldin and catalytic subunits are shown as orange and blue lines, respectively. *Source*: adapted from Fontes & Gilbert, 2010.

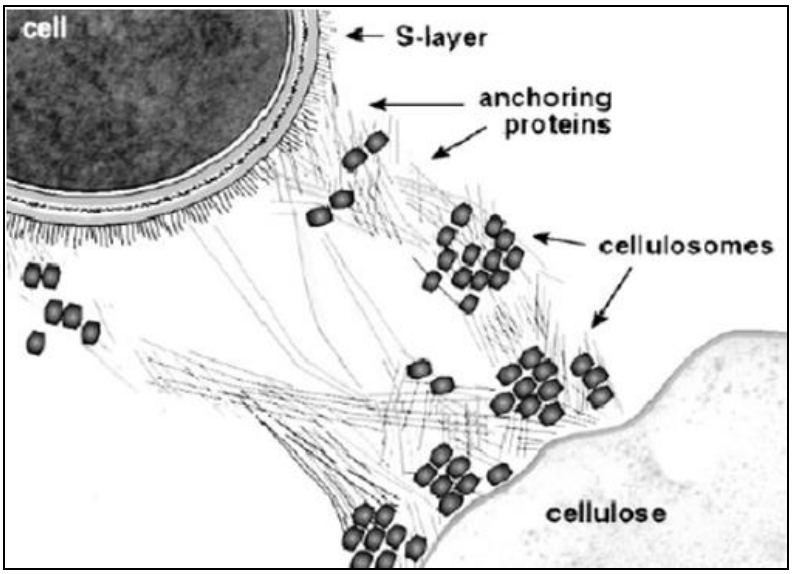
Dockerins are 70 aminoacid residues in size, containing two duplicated segments, each one with 22 residues containing a calcium ion that is required for stability and function (Choi & Ljungdahl, 1996; Salamitou *et al.*, 1994). Cohesins are 150-amino-acid-residue modules that are usually present as tandem repeats in scaffoldings (Fontes & Gilbert, 2010). Both dockerins and cohesins are highly homologous within the same species and the residues involved in protein:protein interaction are highly conserved. Type I and type II cohesins and dockerins do not interact, ensuring a clear distinction between the mechanism for cellulosome assembly and cell surface attachment (Fontes & Gilbert, 2010). Ligand specificities in type I cohesins-dockerins interactions were shown to vary between different species. This is in clear contrast with type II interactions, which demonstrated relatively extensive cross-species plasticity. The biological relevance of this promiscuity remains unknown (Fontes & Gilbert, 2010). Although the function of cohesins and dockerins in the cellulosome is well defined, these non-catalytic modules are also present in microorganisms that do not produce cellulosomes. Most of them are components of enzymes that are not

involved in carbohydrate metabolism and they are quite widespread in *Archaea*, *Bacteria* and *Eukarya* (Peer *et al.*, 2009). Cellulosomal catalytic modules are also associated with a large variety of CBMs. Besides the CBM3 that anchors the scaffoldin to the plant cell wall polysaccharide, there are several CBMs located in the cellulosomal enzymes that also potentiate the contact between the catalytic modules and the ligand (Poole *et al.*, 1992). Bacterial cellulosomes can be classified in two types: one that present multiple types of scaffoldins (for example *C. thermocellum*) and the other which contains a single scaffoldin (for example most of the mesophilic microorganisms) (Fontes & Gilbert, 2010). Single scaffoldin cellulosomes only contain type I cohesins and lack the type II dockerins and have six to nine catalytic components. The anchorage to the bacterial cell wall, is mediated through a yet still unknown mechanism (Fontes & Gilbert, 2010).

The cellulosome of *C. thermocellum* is one of the best characterized and one with the highest rates of cellulose hydrolysis. This cellulosome display a specific activity against crystalline cellulose that is 50 folder higher than the corresponding *Trichoderma* system (Demain *et al.*, 2005) and it produces 72 cellulosomal enzymes (Fontes & Gilbert, 2010). The primary scaffoldin from *C. thermocellum*, CipA, contains nine type I cohesins and a C terminal type II dockerin. The proteins that bind to the type II dockerin of CipA do not interact with the cellulosome catalytic components directly and are termed anchoring scaffoldins (Fontes & Gilbert, 2010). In *C. thermocellum* there are four anchoring scaffoldins containing one, two or seven type II cohesins (Fontes & Gilbert, 2010).

Some bacteria do not contain CBMs in their scaffoldin cellulosome, such as *R. flavefaciens*. This bacterium seems to have developed a different but still unknown mechanism for crystalline cellulose recognition (Rincon *et al.*, 2005). Fungal cellulosomes play a key role in fiber digestion in the rumen together with bacteria and other microorganisms. However, these cellulosomes are less characterized and they have some important differences to the bacteria cellulosomes (Fontes & Gilbert, 2010). Amino acid sequences of fungal dockerins are completely unrelated to the bacterium dockerins and usually they are present in two copies on fungal enzymes (Ljungdahl, 2008). Some studies suggest that cohesin modules might not be present in fungal cellulosomes (Fontes & Gilbert, 2010).

Figure 1.10|Ultrastructure of the *C. thermocellum* cell surface.



Schematic interpretation of the cellulose-bound cell surface shown in a protracted, antibody-labeled polycellulosomal protuberance. The cellulosome-specific label is mainly associated with the cellulose surface and connected to the cell via extended fibrous material. *Source*: Bayer *et al*., 1998.

1.3.3. Non-starch polysaccharides in poultry nutrition

Non-starch polysaccharides (NSPs) are a large variety of polysaccharides molecules, comprising some of the most representative compounds of the cell wall (Williams *et al.*, 1997). They can be insoluble or soluble in water. Insoluble NSPs are indigestible and have the ability to absorb large amounts of water and to maintain the normal motility of the gut. Soluble NSPs are more susceptible to biological hydrolysis especially in the last compartments of the birds' GI tract, such as the caecum. Soluble NSPs display an anti-nutritive effect for poultry due to the resulting increase in digesta viscosity. An increase in digesta viscosity causes a reduction in digesta passage rate and a modification in gut physiology that result in an enlargement of the GI tract (Choct, 1997). The lower passage rate also results in the proliferation of a fermentative anaerobic microflora in the upper compartments of the GI tract (Vahouny, 1982). This microflora can bind some proteins and form complexes that limit protein hydrolysis (Vahouny *et al.*, 1981). The normal and healthy microflora is composed by facultative anaerobic microorganisms in jejunum and strict anaerobic microorganisms in caecum. An increase in digesta viscosity influences performance because feed intake decreases as well as feed digestibility. The decrease in digestibility is due to an increase in size and stability of digesta layers without motility in mucosa surface (Chesson, 2001) leading to a minor contact between feed and enzymes, which leads to a decrease in nutrient availability (Bedford & Morgan, 1995). These consequences are more frequent when the carbohydrate degree of polymerization is higher. NSPs digestion depends on the animal (presence of microflora able to digest NSPs

increases with animal age), NSPs solubility, chemical structure of polysaccharide (linkage between sugars determines the fermentation extension of the different carbohydrates) and the amount of NSPs in the diet (anti-nutritive effects of NSPs are related with NSPs concentration in the diet) (Choct *et al.*, 1996).

As described above, there is a negative correlation between the diet content in NSPs and its nutritive value. Addition of cellulases and hemicellulases into monogastric animal diets reduces the degree of polymerization of NSP which then have a lower capacity to affect digest viscosity. Commercially available enzymatic mixtures contain a variety of enzyme specificities which cleave different polysaccharides acting in synergy (Zyla *et al.*, 1999). Enzymes need only to cleave the carbohydrate at a few places in the polysaccharide chain to greatly reduce the viscosity of solutions and thus enhance nutritive value (Williams *et al.*, 1997). This supplementation improve nutrients digestibility and feed intake, increasing animal's performance. The polysaccharide disruption release and provide nutrients increasing the feed metabolizable energy. To summarize, enzymatic supplementation can improve the nutritive value of cereals containing high levels of soluble NSP (Williams *et al.*, 1997).

Most cereals, such as wheat or barley, are rich in NSPs. Barley and oats are rich in β-glucans which are responsible for the low nutritive value of the diets based on these raw materials (Chesson, 2001). Feed supplementation with β-glucanases that cleave β1,3-1,4 linkages decrease the polymerization degree of β-glucans allowing a better use of the nutrients released, leading to an increase in intake and a decrease in the feed conversion ratio. Arabinoxylans are the major contributors to soluble NSP released from wheat, rye and triticale (Chesson, 2001). However, arabinoxylans can be structurally different, with different branching degrees and with different substituted residues. In the same way, also xylanases can show remarkably different patterns of activity depending on the structure of the substrate (Chesson, 2001). Choct and Annison (1990) classified different cereal grains based on their total NSP content from low to high as follows: rice, sorghum, maize, wheat, triticale, rye and barley. Much of the soluble NSP from wheat and barley grain derives from the thin walled endosperm cells and reflects the composition of the endosperm wall (Chesson, 2001). Feed enzymes have proved most efficacious against soluble substrates for which there is no endogenous competition (Chesson, 2001). Low rates of endogenous activity detectable in cereal flour (Cleemput *et al.*, 1997) appear to be constantly present during grain storage and may slowly degrade soluble NSPs. However, thermal processes of feed destroy endogenous activities and on the other hand promote the release of soluble NSPs (Chesson, 2001).

1.4. LIPID SUPPLEMENTATION OF DIETS FOR POULTRY

1.4.1. Meat physical parameters and meat quality

1.4.1.1. Carcass Yield

The growth rate of commercial broilers has changed enormously over the past 45 years (Havenstein *et al.*, 2003). Sherwood (1977) and Havenstein *et al.* (1994a) have shown that the majority of that change (85 to 90%) has been brought by the genetic selection practiced by commercial breeding industries. During the past decades, because consumer demand for white meat dominates the global market, poultry nutrition has been focused on increasing breast muscle yield, especially in the heavy-sized broilers. Also modern diets produce consistently better growth rates as well as higher abdominal fat levels (Havenstein *et al.*, 1994b). Dietary nutrition is the most critical factor in commercial broiler production, not only for its significant effect on growth performance, carcass quality and broiler health but also for its economic importance in broiler production (Brickett *et al.*, 2007; Scott, 2002). Increases in feed prices over the past years have encouraged producers to consider lowering the dietary nutrient level, especially that of crude protein (CP) and metabolizable energy (ME) (Zhao *et al.*, 2009). It has been suggested that slow-growing genotypes require less CP than do fast-growing genotypes (Morris & Njuru, 1990). Fast-growing genotypes allow a heavier carcass, breast, thigh, liver (associated with higher fat levels) and abdominal fat yields but lower heart relative weight (that indicate a more sensitive genotype to metabolic disorder such as Olkowski *et al.* (2005) suggested. On the other hand slower-growing genotypes are more adapted to low levels of CP and ME than fast-growing animals. Slow-growing genotypes are often more resistant to adverse conditions, metabolic disorders and leg problems and have lower mortality during the finishing period and less downgrading at slaughter than do fast-growing birds (Fanatico *et al.*, 2008; Julian, 1998).

Carcass yield is the proportion of the weight of the commercial carcass (without giblets) recovered from the bird live weight and is expressed in percentage. This value varies, usually between 64 to 68% for broilers (Pollock, 1997). Besides genetic and dietary nutrition, there are other factors that may affect the carcass yield, such as ante-mortem management, environment and holding conditions.

1.4.1.2. pH

Post-mortem pH is a very important parameter in meat quality. This pH is the ultimate pH (pH_{ult}) value that results when all stored glycogen is consumed during the passage from muscle to meat. This value is intimately correlated with the initial levels of glycogen reserves in the muscle (Bendall, 1973). Poultry are rich in white fibers and these ones have, in majority, an anaerobic metabolism which means more glycogen reserves and a final lower

pH than in beef, where red fibers predominate. When pH_{ult} is too low and is installed rapidly, there is a risk to obtain a pale, soft and exudative meat (PSE) that leads to a high meat reflectance (Swatland, 2008), reduced water-holding capacity and protein extractability (Offer, 1991). This will obviously affect meat acceptability (Woelfel *et al.*, 2002). On the other hand dry, firm and dark (DFD) meat is associated with a higher pH and a higher water-holding capacity. This pH is obtained in a shorter period as well. This type of meat is related with a poor storage quality (Allen *et al.*, 1997), since it is a more favourable environment for bacteria (Aberle *et al.*, 2001). pH_{ult} is dependent on several factors, such as breed, age, muscle, ante-mortem stress and carcass temperature. Along the birds' age, there is a conversion of some white fibers into red fibers, leading to a more cadenced lowering of pH. Despite of slow-growing birds have more red fibers, they are also more susceptible to stress than fast-growing birds conducting to a lower pH than the fast-growing animals. Thighs are richer in red fibers than breast muscle. Stress ante-mortem leads to a carcass acidification due to depletion of glycogen in the birds' muscles (Fletcher, 1995; Holm & Fletcher, 1997). Higher temperatures leads to an increase in enzymatic activity, which in turns leads to an increase in lactic acid production and to a pH decline at a faster rate (when carcasses are cooled to slowly) (Fernandez *et al.*, 1994).

1.4.1.3. Colour

Poultry meat colour is a critical food quality attribute. Colour is important for both consumers' initial selection and for the consumer final evaluation and ultimate acceptance of the cooked product upon consumption (Fletcher, 1999b). Broiler skin and broiler meat colours are influenced by numerous production, handling and processing factors (Fletcher, 1989; Froning, 1995). Colour is dependent on myoglobin content. Myoglobin can be oxidized (metamyoglobin, associated with brown colour), reduced (desoximyoglobin, associated with purple colour) or oxygenated (oximyoglobin, associated with red ideal colour). These different myoglobin states are dependent on the oxygen contact. Purple colour is characteristic of meat without oxygen contact. When that meat is exposable to oxygen, pigments reacted with it and oximyoglobin increases. Brown colour is associated with the total consume of oxygen and is related with a commercial depreciation.

Colour is also dependent on slaughter conditions (especially pH and temperature) and pigment content which in turns is dependent on breed, birds age (myoglobin increase with age), gender (females have higher pigments content) and physical activity (more physical activity is related with higher pigments concentrations). Colour can be measured by the CIELAB recommendations (C.I.E., 1978) and by the three principle colour dimensions – L*(lightness), a*(redness) and b*(yellowness). Berri, *et al*, (2001) found that genetic selection of broilers for increased meat yields also affected colour by increasing breast meat lightness (L*) and decreasing redness (a*). Because heme pigments increase with age, slow-growing

birds have redder meat than fast-growing animals, since the slow-growing birds are normally slaughtered older (Baeza *et al.*, 2002; Gordon & Charles, 2002). Nevertheless, other authors found an increase in yellowness (b*) and a decrease in redness in slow-growing birds (Fanatico *et al.*, 2007b; Quentin *et al.*, 2003; Santos *et al.*, 2005).

Barbut (1993) showed a significant negative correlation between muscle pH and lightness of turkey meat, suggesting that colour measurement could be used to detect cases of PSE-like condition in meat (Barbut, 1996). Several other authors suggested the possibility of using colour measurements to predict functional properties of poultry meat (Bianchi & Fletcher, 2002). The main predictable parameter to use is lightness value (L*) and it can be applied to evaluate the incidence of pale, soft and exudative (PSE) meat in poultry (Owens *et al.*, 2000). Boullianne and King (1995) observed that broiler meat with higher pH was darker, redder and was less yellow and brighter than meat with lower pH. High pH contributes also to higher water-binding capacity, hence making it appear darker (Cornforth, 1994). Stress, is a well-known contributor to PSE muscle condition in pigs and several studies also confirm this observation in turkeys (Ngoka & Froning, 1982; Owens & Sams, 2000). However, studies in broilers were not conclusive.

Relationships were also found between colour and shelf-life (Allen *et al.*, 1997) and between colour and composition of broiler breast meat (Qiao *et al.*, 2002). McKee and Sams (1997) and Woelfel (2002) established a relation between lightness and drip loss and that a cut-off of L*=53 and L*=54 respectively could discriminate breast meat with PSE properties. Meat colour also changes during processing and storage time. Skin and meat colour change dramatically, especially during the first 4 hours after slaughter. After 4 hours, the colours continue to change but at a slower rate (Petracci & Fletcher, 2002). On the other hand temperature during processing influences colour of poultry meat. Higher temperatures during ante-mortem (McKee & Sams, 1997) and product holding prior to steps such as deboning and storage, as well as delays in post-mortem chilling are associated with lighter meat colours (McKee, 2007). Skin colour plays an important role in consumer acceptance, mainly when carcasses are commercialized as a whole (Castaneda *et al.*, 2005).

Skin colour is also dependent on the genetic ability of the bird to produce melanin pigments in the dermis and epidermis, as well as to absorb and deposit carotenoid pigments in the epidermis (Fletcher, 1999). Carotenoids and xanthophylls are compounds responsible for skin colour in broilers and the most important source of pigmentation in poultry feed (Goodwin, 1954). A yellower skin is usually preferred since it is associated with a more sustainable and healthier production system. Fanatico *et al.* (2007b) showed that, in indoor systems slow-growing birds had significantly higher b* values than fast-growing birds, indicating more yellow skin and, when slow birds have access to outdoor, their skin became even more yellow than when indoors. This result was attributed to the fact that the slow-growing genotypes spent more time in the outdoor, were more active and foraged more than

fast-growing birds. However, standard diets do not supply sufficient pigments to obtain a yellower skin. Castaneda *et al*. (2005) showed that natural pigments are more effective than synthetic ones in increasing skin yellowness. The principal ingredient sources of carotenoids used during the past decades include yellow corn, corn gluten meal and dehydrated alfalfa meal. Ponte *et al.* (2004b) also showed the importance of forage consumption in skin pigmentation. Many new ingredients are being developed such as algae meals (Marusich & Bauernfeind, 1981) to improve yellower skin. Scalding temperature and time, at slaughter, may also affect negatively broiler skin colour (Fanatico *et al.*, 2007b; Graf & Stewart, 1953). Antioxidants can also affect carcass colour. Vitamin E was associated with an increase in meat colour scores (less light) (Sheldon *et al.*, 1997).

1.4.1.4. Sensory attributes

Texture is the most important attribute of poultry meat for consumer final satisfaction (Fletcher, 2002). Texture is a multidimensional attribute (Meilgaard *et al.*, 1999) and represents a complex set of characteristics that includes thickness, cohesiveness, chewiness and fracturability. The movement of jaws and tongue, the cutting, grinding and tearing action of the teeth during chewing all play a role in texture evaluation of meat by humans (McKee, 2007).

One of the most important textural properties in meat is tenderness, which is defined as the ease which a piece of meat can be cut and chewed (McKee, 2007). Tenderness is influenced by the size of the muscle fiber; the diameter of the muscle fiber is positively related to the tenderness of the meat (Pingel & Knust, 1993). This attribute is also influenced by the size of the conjunctive tissue and its collagen content, by the intramuscular fat, which are both dependent on genetics. Finally tenderness is also influenced by the production system and animal age, since tenderness tends to decrease as the animal ages because cross linking of collagen reduces collagen solubility (Fletcher, 2002). Muscle fiber size is associated with genotype and tenderness. Muscle fibers in fast-growing animals are more numerous and have a wider diameter than muscle fibers in slow-growing birds (Rémignon *et al.*, 1994). Smaller muscle fibers result in tender breast meat (Farmer *et al.*, 1997). Age and genotype can also affect fiber size. As slow-growing animals are used in alternative systems with later slaughter ages than fast-growing bird of intensive systems that are slaughtered at earlier ages, this may explain the differences in tenderness in these two genotypes and production systems. However, studies developed by Fanatico *et al*. (2006; 2007a) were unable to found differences in tenderness in different genotypes and production systems. Muscle shortening can cause toughening of poultry meat and is influenced by temperature and pH. Accumulation of lactic acid causes a decrease in pH and formation of actomyosin resulting in stiffening of the muscle proteins (McKee, 2007). Cohesiveness refers to the degree to which the sample deforms rather than rupturing when chewing (Meilgaard *et al.*, 1999). Exercise

may cause a strengthening of connective tissue fiber structure (Aberle *et al.*, 2001) suggesting that outdoor access results in meat that is more firm than meat produced indoor (Santos *et al.*, 2005). Juiciness is a property related to the fat content and water-holding capacity (McKee, 2007). Fillets of breast meat from slow-growing birds' are smaller and thinner in dimension promoting more surface area in relation to muscle mass exposed to the air, which can cause a higher drip loss leading to a dryer meat than in fast-growing lines (Fanatico *et al.*, 2005). On the other hand, the lower juiciness may also be related with the lower content of intramuscular fat in slow-growing genotypes (Fanatico *et al.*, 2007a).

Flavour is a combination of smell and taste. Meat flavour increases with age (Aberle *et al.*, 2001) but also genotype, sex, weight and production system have a considerable effect in this factor (Farmer, 1999). Fast-growing birds' deposit more intramuscular and intermuscular fat, which leads to a tendency of breast meat of this genotype to be more salty than meat of the slow-growing animals (Fanatico *et al.*, 2007a). Dark meat (thighs) also showed a more intense flavour due to its higher fat content and fat type (Chartrin *et al.*, 2006). Flavour increases after growth inflection occurs (age of which gain is maximum) when flavour precursors are deposited in muscle (Gordon & Charles, 2002). The conventional birds' are generally young, very tender and juicy but have less intense flavour (Le Bihan-Duval, 2003). When different genotypes (slow-growing animals versus fast-growing birds') were compared at same ages there were no differences in meat flavour (Touraille *et al.*, 1981). Forage may be associated with sweet flavour in slow-growing animals and pasture may have the potential to contribute to flavour (Fanatico *et al.*, 2007a). Diet manipulation could contribute to differences in flavour intensity, and on the other hand, to off-flavours appearance. These off-flavours can be associated with lipid oxidative stability and rancidity (when diet manipulation influence lipid profile of meat). Overall sensory is the sum of perceptions of tenderness, juiciness and flavour. Consumers are sensitive to meat appearance and quality. Tenderness seems to be the most important sensory parameter that commits the consumer decision and meat overall appreciation.

1.4.2. Lipids in poultry meat

1.4.2.1. Fatty acid composition

Fatty acids are distributed between neutral lipids and phospholipids in animal muscles (Gandemer, 2002). Phospholipids are an essential component of cell membranes and its amount remain fairly constant, or increases slightly, as the animal increases in fatness (Wood *et al.*, 2008). Neutral lipids (triacilglycerols), mainly rich in saturated and monounsaturated fatty acids, are found in the adipocytes located in the perymisium (Sanosaka *et al.*, 2008). Breast meat contains more lipids as phospholipids, whereas the thigh meat contains more triacilglycerols (Gonzalez-Esquerra & Leeson, 2000). Enrichment

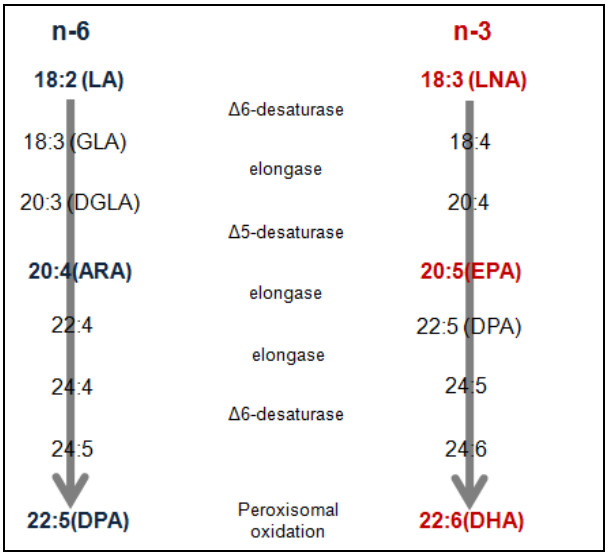
of breast meat in lipids may be difficult to achieve since the potential for depot is smaller (Betti *et al.*, 2009a). Enriched n-3 fatty acid broiler meat may be obtained through the incorporation of ingredients rich in these fatty acids in poultry diets. Diets containing flaxseed (Rebole *et al.*, 2006) or incorporating pasture (O'Sullivan *et al.*, 2004; Ponte *et al.*, 2008) lead to an improvement in the levels of α-linolenic acid (LNA) in broiler meat. On the other hand, diets incorporating fish oils or algae showed to increase eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) levels on meat (Komprda *et al.*, 2005; Lopez-Ferrer *et al.*, 2001b; Mooney *et al.*, 1998; Poureslami *et al.*, 2010b; Rymer *et al.*, 2010a). Hence, it is well established that diet composition influences directly the fatty acid profile of meat and its quality.

1.4.2.2. LC-PUFA conversion

Cardiovascular diseases (CVD) are one of the leading causes of death in men and women in developed countries. Consumption of saturated dietary fats and n-6 polyunsaturated fatty acids (PUFA) increases the risk of CVD, whereas consumption of n-3 PUFA may reduce CVD incidence (Simopoulos, 1997). Typical dietary ratios of n-6/n-3 in developed countries are now greater than 10:1 (Azain, 2004) which is greater than the recommended that should be close to 1:1 proportion (Simopoulos, 2002). Some studies suggest that n-6/n-3 ratio has no biological relevance in itself but rather the proportion of certain fatty acids, such as linoleic acid (LA, n-6) and α-linolenic acid (Griffin, 2008). LA: LNA ratio must be used instead of n-6/n-3 ratio (Griffin, 2008). Major sources of n-6 PUFA are vegetable oils, whereas n-3 PUFA sources include fish and some plants (Schmitz & Ecker, 2008).

Omega-3 fatty acids make up a family of essential fats that humans are unable to synthesize *de novo* (Arterburn *et al.*, 2006). Among PUFA, fatty acids that are essential for humans are α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) (Bezard *et al.*, 1994; Schmitz & Ecker, 2008). Since humans cannot synthesize these molecules, they must be provided in the diet. In mammalian cells, LA and LNA are metabolized to long-chain PUFA (LC-PUFA). The n-6 LA is converted to araquidonic acid (ARA-20:4n-6) and then converted to docosapentaenoic acid (DPA-22:5n-6) or eicosanoids (Schmitz & Ecker, 2008). The n-3 LNA is converted to stearidonic acid and eicosatetraenoic acid (20:4n-3) to form eicosapentaenoic acid (20:5n-3). EPA is further metabolized to DHA (22:6n-3) or eicosanoids (Bezard *et al.*, 1994; Schmitz & Ecker, 2008) (Figure 1.11).

Figure 1.11|Biochemical pathways for the interconversion of n-6 and n-3 fatty acids.



LNA, α-linolenic acid; LA,linoleic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid. *Source*: adapted from Arterburn, et al. (2006).

Eicosanoids from the n-6 pathway and ARA are metabolically more potent in promoting inflammation, platelet aggregation and immune and vascular reactivity than those eicosanoids from LC n-3 PUFAs and have been associated with CVD, inflammatory disorder and cancer (Emken *et al.*, 1994; Siddiqui *et al.*, 2005). To substantially depress the production of eicosanoids from ARA there is a need to consume approximately 1% LNA of the total energy requirement. LNA competes very effectively with LA for common desaturases and elongases that convert C18 PUFA to their C20 and C22 homologues (Emken *et al.*, 1994). For that reason, background diets influence the conversion of these fatty acids (Arterburn *et al.*, 2006) and there is a need to maintain an adequate ratio LA:LNA to guarantee the LNA oxidation. The conversion of LNA to EPA and DHA occurs primarily in the liver in the endoplasmatic reticulum and involves a series of elongation enzymes that sequentially add two carbon units to the fatty acid backbone and desaturation enzymes that insert double bonds into the molecule (Arterburn *et al.*, 2006). In vivo conversion of LNA into EPA and DHA is a relatively inefficient process. Some studies in humans show that males convert LNA to EPA on a rate between 0.3 to 8% and LNA to DHA on a rate less than 4%, while women convert up to 21% of LNA into EPA and up to 9% of LNA into DHA (Arterburn *et al.*, 2006).

LNA is present in various vegetable oils such as linseed, canola and soy oils (Arterburn *et al.*, 2006). On the other hand, EPA and DHA are found primarily in algae and fish. Algae are the most prolific producers of EPA and DHA in the ecosystem and several refined algal oils are particularly rich in DHA (Arterburn *et al.*, 2006). Omega-3 fatty acids are present in cell membranes and are incorporated primarily into phospholipids. These fatty acids, mainly the most unsaturated, can influence the biophysical properties of membranes (higher fluidity and

flexibility). Overall membrane fatty acid composition can have a large impact on cell and organ function as well as a wide variety of biological processes (Arterburn *et al.*, 2006). DHA is the most abundant n-3 fatty acid in membranes and is present in all organs, but is particularly abundant in neural tissue such as brain and retina. In adipose tissue, where fatty acids are stored as triacylglycerol, LA is the most abundant PUFA and LNA is the predominant n-3 fatty acid. Only very small amounts of EPA and DHA are present in adipose tissue, which suggests a limited storage capacity of these LC-PUFAs and implies the need for a continuous supply of these molecules through the diet (Arterburn *et al.*, 2006). Plasma phospholipid content varies in response to the dietary fatty acids intake. Arterburn *et al*. (2006), in studies with humans, showed that diets rich in LNA increases LNA plasma content, a small increase in EPA while DHA levels are undetectable. Supplementation with EPA originates an increase in EPA plasma content but there is no increase in DHA concentrations. Supplementation with DHA results in an increase in DHA until a limit from which saturation is reached, and a small increase in EPA due to retroconversion process is observed. These results suggest a limited conversion to LC-n-3PUFAs dependent on the LA:LNA ratio (Nitsan *et al.*, 1999) and that DHA and EPA can be effectively elevated only by dietary supplementation. Increase of LNA together with EPA and DHA, and reduction of vegetable oils with higher LA content are necessary to achieve a healthier diet in western industrialized countries (Simopoulos, 2000).

1.4.2.3. Cholesterol content

Cholesterol is present in animal cells and is an important regulator of membrane fluidity. This sterol is also the precursor of other important regulator sterols, such as bile acids. As cholesterol is insoluble, its prolonged presence in the blood promotes deposition in the blood vessels wall, which leads to atherosclerotic plaque formation (McDonald *et al.*, 1995). There is an urgent need to reduce cholesterol content in modern diets in industrialized countries and consumers are more concerned with the selection of low-cholesterol foods. Several studies have focused on the beneficial effects of a monounsaturated (MUFA) rich diet on cardiovascular health, by decreasing low-density lipoprotein cholesterol content in blood plasma, among others positive effects (Kris-Etherton *et al.*, 1999; Roche, 2001). Poultry has recognizable low levels of intramuscular fat and cholesterol in breast meat (Chizzolini *et al.*, 1999; Givens, 2005). In the past years, several supplements to decrease cholesterol content in poultry meat have been tested, such as garlic, copper (Konjufca *et al.*, 1997), chia seed (Ayerza *et al.*, 2002), alfalfa (Ponte *et al.*, 2004b), inulin (Velasco *et al.*, 2010), among others. Approximately 75% of the chicken's total cholesterol is in the HDL form (Gould & Siegel, 1985; Yu *et al.*, 1976). Peebles *et al.*(1997) suggested that age and sex plays a role in LDL metabolism, with a tendency to be higher in females and in older birds. Cholesterol content is influenced not only by lipid content but also by lipid quality in diets, since cholesterol can be

also synthesized *in vivo* (stearic acid is considered much less hypercholestereolemic compared to palmitic fatty acid, for example) (Grundy, 1997; Katan *et al.*, 1995). For this reason, there is a need to decrease the levels of saturated fatty acids (SFA) and increase the content of unsaturated fatty acids in the diet.

1.4.3. Oxidative stability and antioxidants

1.4.3.1. Antioxidants

Antioxidants prevent oxidation of unsaturated fatty acids. Antioxidant properties are common to a wide variety of compounds such as phenols, quinines, tocopherols, among others. To maximize the oxidative stability of meat, antioxidants are added to feed (Gray *et al.*, 1996; Jensen *et al.*, 1998) which is an efficient method to guarantee oxidative stability and shelf-life of meat (Maraschiello *et al.*, 1999). According to origin, antioxidants can be classified as synthetic or natural. Synthetic antioxidants have been widely used as food preservatives, because of their effectiveness and relatively low cost (Fellenberg & Speisky, 2006). The most used antioxidants are those derived from phenolic structures, like butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT), tertbutylhydroxiquinone (TBHQ) and dodecyl, propyl and octyl gallate (Fellenberg & Speisky, 2006). Ethoxyquin (ETOX) is another synthetic antioxidant with a non-phenolic structure. In contrast to the others, its consumption by humans is not allowed, but it is only used in animal diets such as in the preservation of aviary foods (Bailey *et al.*, 1996). On the other hand, natural antioxidants are generally molecules present in plants. Among the most important natural antioxidants are the tocopherols (or vitamin E- liposoluble) and ascorbic acid (or vitamin C- hidrosoluble). Other natural antioxidants are carotenes (β-carotene, lycopene, luthein, asta-, zea- and cantha-xantin), flavonoids (catechins, quercetin, rutin among others) and non-flavonic phenols (rosmanol, boldine among others) (Fellenberg & Speisky, 2006). Some studies indicated BHA and BHT as tumour promoters in contrast with vitamin E (α-tocopherol) that shows to be not carcinogenic (Chen *et al.*, 1992; Kahl & Kappus, 1993). Some studies reported the antioxidant properties of plant extracts, such as green tea, rosemary, grape seed, tomato extracts (Schwarz *et al.*, 2001; Smet *et al.*, 2008), oreganos (Luna *et al.*, 2010) among others. Pastures and cereals are also a good source of tocopherols and tocotrienols (Kerry *et al.*, 2000), and of β-carotene. α-tocotrienols were also shown to help lowering plasma cholesterol levels (Qureshi *et al.*, 1997) and to be powerful free-radical interceptors in cell membranes (Cabrini *et al.*, 2001). The type, quantity and quality of fat in the diet have a marked impact on antioxidants requirement (Applegate & Sell, 1996) that increase with dietary PUFA content (Sheehy *et al.*, 1994).

In live organisms, reduced glutathione (GSH; hydrosoluble tripeptide synthesised by poultry), along with vitamin C and E are responsible for lowering the oxidative stress (Fellenberg &

Speisky, 2006). GSH helps to stabilize free radicals and acts as a co-factor of glutathione peroxidase (GSHpx), an enzyme that is responsible for transforming lipohydroperoxides (LOOH- product of lipoperoxidation) into easily eliminated lipoalcohols. This enzyme contains selenium as a prosthetic group, which makes it highly dependent of the availability of this metal (Fellenberg & Speisky, 2006). Selenium is an important metal to include in diet management for avoiding oxidative stress in poultry and to ensure the efficient utilization of vitamin E (Peric *et al.*, 2009). This metal was also shown to influence meat quality by decreasing drip-loss in broilers (Peric *et al.*, 2009).

Apparently antioxidants that are more soluble in fat (vitamin E, BHA, BHT) would be absorbed more rapidly at intestinal level, and a certain quantity can be found deposited in tissues which allows efficient oxidative control of these tissues and of meat (Lin *et al.*, 1989). In contrast, in case of antioxidants with minor liposolubility (carotenoids, polyphenols) the absorption might be slower at intestinal level or its deposition in the fatty tissues less efficient (Fellenberg & Speisky, 2006).

1.4.3.2. Oxidative stability

Unsaturated fatty acids have a high propensity to oxidization, promoting the formation of hidroperoxids (LOOH), which are decomposed in other products with short chains, including free radicals. These free radicals attack more easily other fatty acids, forming new free radicals, thus increasing the oxidation rate. Formation of free radicals is catalysed by radiation and oxygen. The products of oxidation are shorter fatty acids, ketones, aldehydes, epoxides among others with some of these being potentially cytotoxic to live systems. Oxidation of fatty acids is responsible for unpleasant odours and off-flavours in meat, reducing meat quality (McDonald *et al.*, 1995).

Chicken meat containing relatively high amounts of PUFA presents a challenge for the food industry to maintain lipid oxidation stability during a prolonged storage time, particularly in aerobic conditions (Narciso-Gaytan *et al.*, 2011). Dahle *et al.,* (1962) showed that as the amount of double bonds increased in the carbon chain of fatty acids, so did the production of malondialdhyde (MDA) and the peroxide values. An increase in PUFA content influences lipid oxidation and can affect colour, flavour and oxidative stability during sub-optimal storage (Basmacioglu *et al.*, 2004; Bou *et al.*, 2001; Morrisey *et al.*, 1998). In addition, oxidative deterioration seems to be higher in dark meat (thigh) than in white meat (breast) (Rebole *et al.*, 2006). Oxidation increases as a result of oxidized lipids, oxidation of sensitive PUFA or a low intake of nutrients involved in the antioxidant defence system (Morrisey *et al.*, 1998). In muscle and fat tissue, oxidation continues post-mortem and affects the shelf-life of meat and meat products (Smet *et al.*, 2008). Lipid oxidation is one of the primary mechanisms of quality deterioration in meat products (Kanner, 1994).

1.4.4. Dietary lipid supplementation and meat quality

Monogastric animals do not have microorganisms either in quantity or in quality to modify extensively feed nutrients. Monosaccharides and fatty acids (FAs) are absorbed directly from the small intestine. Therefore, feed composition of monogastric diets is revealed almost directly in the lipid profile of meat (Dodson *et al.*, 2010). The content and composition of fat in animal products has become increasingly important to consumer´s perception of the meat healthfulness (Drackley, 2000). Following this idea, there is a need to develop efficient strategies to improve the composition and enhance health-related effects of animal products. For instance, these products can become better in quality terms by modifying their lipid content (Pisulewski, 2005). On the other hand, to ensure the efficiency of these strategies, there is a need to understand the background mechanisms involved with the lipid metabolism, specially the conversion cycle of the LC-PUFA, particularly the n-3 LC-PUFA that was shown to reduce the risk of several chronic diseases, including cardiovascular diseases (Lopez-Garcia *et al.*, 2004). White meat, such as chicken, is commonly preferred to red meat supposedly as a more healthy choice, due to its lower SFA content and consequently healthier unsaturated:saturated ratio (DeSmet & Raes, 2004). A number of nutritional strategies have been used to obtain PUFA-enriched meat. These methods focused on the alteration of fatty acid carcass composition and the improvement of poultry meat oxidative stability. In poultry, dietary fatty acids are absorbed unaltered from the small intestine and directly incorporated into tissue lipids. SFA and MUFA are, in part, synthesized endogenously and their concentration in carcass lipids is less influenced by dietary fat. Poureslami *et al*. (2010a) suggested that the primary strategy to achieve a healthier MUFA:SFA ratio is to increase MUFA instead of decrease dietary SFA. On the other hand, PUFAs cannot be synthesized in the body and their carcass concentration responds rapidly to dietary alterations (Pisulewski, 2005). Fatty acid profiles of carcass fat closely reflect those of the dietary fat. Poultry products enriched in n-3 fatty acids have been developed in an attempt to meet the growing consumer demand for functional food products which are those that promote health benefits beyond their nutritional value (Narciso-Gaytan *et al.*, 2011). Omega-3 fatty acids, particularly EPA and DHA, have shown functional properties that promote health benefits in humans including a reduction in the risk of cardiovascular problems, rheumatoid problems, depression, inflammation and some cancer types (Simopoulos, 2002; Tamura *et al.*, 1986). In addition, conjugated linoleic acid (CLA) was shown to benefit human and animal health by reducing obesity and some types of cancer (Krauss *et al.*, 2000; Wang & Jones, 2004).

1.5. OBJECTIVES

This work aims to elucidate several unresolved questions concerning the use of enzymes and lipids as dietary supplements to improve broiler performance and meat quality. The specific aims of this study were:

- To test the capacity of a family 11 CBM, which binds specifically to β-glucan, to improve the function of the modular cellulase *Ct*Lic26A-Cel5E to improve the nutritive value of barley-based diets (Chapter 2);

- To evaluate the efficacy of *Clostridium thermocellum* a glycoside hydrolase with a high catalytic activity for β-glucans – GH16 – (CtGlc16A) and its derivative construct *Ct*Glc16A-CBM11 to improve the performance of broilers fed barley-based diets (Chapter 2);

- To investigate endogenous factors in barley, specifically β-glucanase activity, viscosity and β-glucans content, that might affect the efficacy of exogenous enzymes used in broiler diets (Chapter 2);

- To characterize novel family 42 CBMs from *Clostridium thermocellum* cellulosome, which are present in three modular proteins *Cthe* 0015, *Cthe* 2138 and *Cthe* 2139, and evaluate their particular applicability to improve the efficacy of feed enzymes (Chapter 3);

- To study the effect of two different dietary lipid sources in the quality and sensory attributes of broiler meat (Chapter 4);

- To test the effect of two different lipid sources in the fatty acid profiles, cholesterol and lipid-soluble antioxidant vitamins of broiler meat (Chapter 4).

2. EFFECT OF ENZYMATIC SUPPLEMENTATION OF BARLEY-BASED DIETS IN BROILER PERFORMANCE

2.1. A FAMILY 11 CARBOHYDRATE-BINDING MODULE (CBM) IMPROVES THE EFFICACY OF A RECOMBINANT CELLULASE USED TO SUPPLEMENT BARLEY-BASED DIETS FOR BROILERS AT A LOWER DOSAGE RATES

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Abstract

Exogenous microbial β-1,3-1,4- glucanases and hemicellulases contribute to improving the nutritive value of cereals rich in soluble non-starch polysaccharides for poultry. In general, plant cell wall hydrolases display a modular structure comprising a catalytic module linked to one or more non-catalytic carbohydrate-binding modules (CBMs). Based on primary structure similarity, CBMs have been classified in 60 different families. CBMs anchor cellulases and hemicellulases into their target substrates, therefore eliciting efficient hydrolysis of recalcitrant polysaccharides. A study was undertaken to investigate the effects of a family 11 β-glucan-binding domain in the function of recombinant derivatives of cellulase *Ct*Lic26A-Cel5E of *Clostridium thermocellum* that were used to supplement a barley-based diet at lower dosage rates. The results showed that birds fed on diets supplemented with the recombinant *Ct*Lic26A-Cel5E modular derivative containing the family 11 CBM or the commercial mixture Rovabio™ Excel AP tended to display improved performance when compared to birds fed diets not supplemented with exogenous enzymes. It is suggested that at lower than previously reported enzyme dosage (10 U/kg vs 30 U/kg of basal diet), the β-glucan binding domain also elicits the function of the recombinant *Ct*Lic26A-Cel5E derivatives. Finally, the data suggest that exogenous enzymes added to barley-based diets act primarly in the proximal section of the gastrointestinal tract.

2.1.1. Introduction

In poultry, dietary soluble non-starch polysaccharides (NSP), such as arabinoxylans and β-glucans, are known to increase digesta viscosity leading to a lower efficiency of nutrient digestion and absorption (Smith & Annison, 1996) while contributing to reduce feed passage rates (van der Klis *et al.*, 1993). In addition, higher levels of recalcitrant polysaccharides in poultry digesta may cause anaerobic microbial proliferation in the small intestine leading to the production of toxins and deconjugation of bile salts, which are essential for the digestion of fat (Langhout, 1998; Misir & Marquardt, 1978). It is well established that inclusion of microbial cellulases and hemicellulases in wheat, barley and rye-based diets for simple-stomach animals improves the efficiency of feed utilization, enhances growth and contributes for a better use of low cost feed ingredients (Bedford, 2000; Chesson, 1993). It is usually agreed that plant cell wall hydrolases improve the nutritive value of cereal based diets rich in NSPs through a variety of mechanisms. Therefore, by efficiently contributing to decrease digesta viscosity that is associated with the intake of soluble NSPs, exogenous polysaccharidases have a positive effect in the rate of diffusion of substrates, digestive enzymes and nutrients (Bedford *et al.*, 1991; Bedford & Classen, 1992; Fengler & Marquardt, 1988; White *et al.*, 1981), while increasing feed intakes. In addition, cellulases and xylanases may promote the proliferation of beneficial microflora in the final compartments of simple-stomach animals' gastrointestinal (GI) tract, by increasing the quantity and/or the quality of the substrates available for fermentation (Apajalahti & Bedford, 1999; Bedford & Morgan, 1996). Finally, plant cell wall hydrolases may mediate their effects by releasing endosperm plant cell wall trapped nutrients that were otherwise unavailable for digestion (Hesselman & Aman, 1986). It is believed that the action of one or a conjunction of the above-mentioned effects may depend on the type of animal, diet and exogenous enzyme used.

Cellulases and hemicellulases are generally modular enzymes, containing discrete non-catalytic carbohydrate-binding modules (CBMs), which anchor the biocatalysts to the plant cell wall, linked to the enzyme catalytic domains via flexible linker sequences. By mediating a close and prolonged interaction between enzymes and plant carbohydrates, CBMs allow the appended catalytic domains to intimately contact its target substrates, therefore potentiating catalysis (Boraston *et al.*, 2004b). This proximity and targeting role of CBMs is of extreme importance for cellulase and hemicellulase function, as the complex interactions established between polysaccharides within the plant cell wall restrict their accessibility to enzymatic attack (Guerreiro *et al.*, 2008). CBMs are currently grouped into 60 sequence-based families (Coutinho & Henrissat, 2003) and were shown to display a large range of different ligand specificities. Therefore, CBMs that recognize cellulose, β-1,3-1,4-glucans, xylans, mannans, galactans, xyloglucans, arabinans and laminarins have been identified in a variety of cellulases and hemicellulases and the molecular determinates of binding specificity have been mostly elucidated. There is a paucity of information concerning the importance of non-

catalytic CBMs in the function of exogenous cellulases used to supplement cereal-based diets for simple stomach animals. Nevertheless, it has been shown that a family 6 xylan-binding domain is able to improve the efficacy of a microbial recombinant xilanase used to supplement wheat and rye-based diets for poultry (Fontes *et al.*, 2004). Animals supplemented with a bi-modular xylanase containing catalytic and xylan-binding domains grew significantly faster than animals fed on diets containing exclusively the xylanase catalytic domain. More recently, we showed that the family 11 β-glucan-binding domain located in the bi-functional cellulase *Ct*Lic26A-Cel5E from *Clostridium thermocellum* is unable to improve the efficacy of the appended catalytic domains (Guerreiro *et al.*, 2008). However, it was suggested that the high enzyme dosage rates used in the above mentioned study may have contributed to attenuate the effect of the non-catalytic β-glucan-binding domain.

The objective of this work was to compare the capacity of two truncated derivatives of *Clostridium thermocellum* bi-functional cellulase *Ct*Lic26A-Cel5E, consisting on the enzyme with two catalytic modules expressed as an individual entity or fused to its endogenous family 11 CBM, to enhance the performance of poultry fed on a barley based diet at lower enzyme incorporation levels. Together, the data presented here suggest that when microbial enzymes are supplemented at lower levels into barley based diets for broiler chicks the efficacy of the exogenous cellulases is improved by the presence of the family 11 β-glucan-binding domain. The contribution of CBMs to decrease the incorporation rates of feed enzymes in poultry diets is discussed.

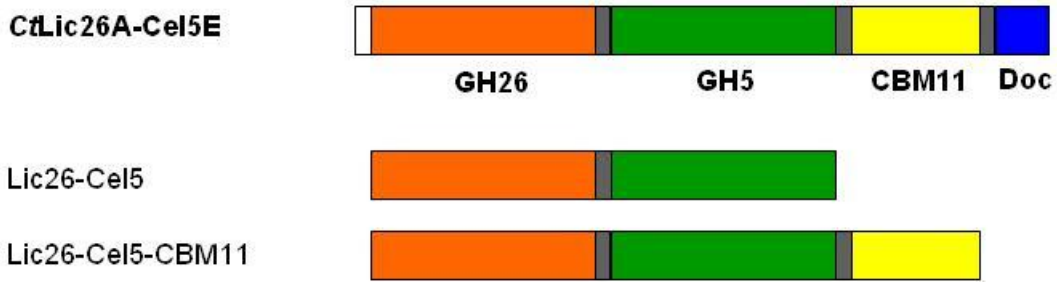
2.1.2. Materials and Methods

2.1.2.1. Enzyme Preparation

The molecular architecture of *Ct*Lic26A-Cel5E and its truncated recombinant derivatives used in this study are presented in Figure 2.1. The enzyme contains a N-terminal β-1,3-1,4-glucanase catalytic domain (GH26), followed by a β-1,4-cellulase second catalytic module (GH5), a family 11 carbohydrate-binding module (CBM11) and a C-terminal dockerin characteristic of other *C. thermocellum* cellulosomal enzymes (Taylor *et al*., 2005). The *Ct*Lic26A-Cel5E truncated derivatives Lic26-Cel5E-CBM11 and Lic26-Cel5E were hyperexpressed in *Escherichia coli* following the protocols described by Taylor *et al*. (2005). The recombinant plasmids, containing the Clostridial genes under the control of T7 promoters in the prokaryotic expression vector pET21a (Novagen, Darmstadt, Germany), were used to transform BL21 *Escherichia coli* cells. Recombinant *E. coli* strains were grown on Luria Bertani media to mid-exponential phase (A_{600nm} of 0.5) and recombinant gene expression was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1

mM. Cells were collected after 5 h induction at 37 ºC and protein extracts prepared by ultrasonication followed by centrifugation. The recombinant proteins were purified by metal-affinity chromatography as described by Fontes, *et al*., (2004). Both recombinant proteins, Lic26-Cel5E-CBM11 and Lic26-Cel5E, retain considerable catalytic activity at 40 ºC and are resistant to proteolytic degradation (Taylor *et al.*, 2005).

Figure 2.1|Domain organization of *Ct*Lic26A-Cel5A and its truncated derivatives Lic26-Cel5 and Lic26-Cel5-CBM11.



The β-glucanase (GH26), cellulase (GH5), β-glucan binding domain (CBM11) and the dockerin (Doc) are indicated. The gray and the white boxes represent the linker sequences and the signal peptide, respectively.

2.1.2.2. Animals, Diets and Management

The barley-based diet used in this study was formulated to contain adequate nutrient levels as defined by the NRC (1994) and its composition is presented in Table 2.1. The basal diet was supplemented with no enzyme (C0) or with 10 U/kg of Lic26-Cel5E or Lic26-Cel5E-CBM11. In addition, a fourth treatment corresponded to the supplementation of the basal diet with 15 U/kg of the commercial enzyme cocktail Rovabio^{TM} Excel AP (Adisseo, France; treatment termed Rov for simplification), which corresponds to an incorporation ratio of 50 g of enzyme per ton of feed. One hundred and sixty day-old male chicks (Ross 308) were divided into 40 battery brooders, with a capacity of 4 animals per pen, exposed to constant light for the duration of the trial. Water and a barley-based feed were available *ad libitum* throughout the experiment and were provided with automatic drinking nipples and a hanging feeder, respectively. The brooders were located in an environmentally-controlled room, which was daily adjusted to the recommended temperatures, according to standard brooding practice. Birds were individually weighed at the commencement of the experiment and were randomly assigned into one of the four treatments, with 10 replicates of four birds per treatment. Weekly, feed consumption and individual body weights were recorded. Feed conversion ratios were calculated by dividing the weight of feed consumed by the weight gain per pen, including the weight gain of any dead birds. Bird mortality was recorded daily. At the end of the experiment, at day 28, one bird per pen was slaughtered by an intravenous injection of an aqueous isotonic solution of 125 mg Tiopental Braun (Braun, Barcelona, Spain). The size of the various GI compartments was measured and digesta samples were

collected and stored at –20ºC for posterior analysis. Levels of cellulase and hemicellulase activity in the GI tract were measured as described below.

Table 2.1|Ingredient composition and calculated analysis of the cereal-based feed.

Ingredients	**%**
Barley	55.00
Soybean meal 47%	30.61
Soybean oil	5.73
Maize	5.45
Salt	0.25
Calcium carbonate	0.81
Dicalcium phosphate [1]	1.79
DL-Methionine	0.16
Mineral and vitamin premix [1]	0.20
Estimated nutrient content	
Energy (kcal ME/kg DM)	2900
Crude Protein (%)	20.80
Ether extract (%)	7.33
Crude cellulose (%)	4.87

[1] Contained 20% Ca and 18% P. [2] Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 2,100 IU; vitamin E, 20 mg; nicotinic acid, 30 mg; vitamin B_{12}, 0.12 mg; calcium pantothenate, 10 mg; vitamin K_3, 2 mg; thiamin, 1 mg; riboflavin, 4.2 mg; vitamin B_6, 1.7 mg; folic acid, 0.5 mg; biotin, 0.5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 ppm.

2.1.2.3. Analytical Procedures

Enzyme catalytic activity was determined at 40 ºC by measuring release of reducing sugars, following the method described by Taylor *et al*.,(2005), using barley β-glucan (Megazyme®, Ireland) as the substrate. One unit of catalytic activity is defined as the amount of enzyme required to release one µmole of product per min. The extract containing Rovabio™ Excel AP enzymes was prepared by ressuspending 75 mg of the enzyme mixture in 10 ml of 50 mM NaHepes buffer, pH 7.5, which was followed by an incubation o/n at 4 ºC with gentle agitation and a centrifugation at 13,000 rcf for 5 min. Previously to detection of β-glucanase activity, digesta samples were centrifuged and the supernatant recovered for analysis. Initially, qualitative analysis of cellulase activity in the digesta samples recovered from the various GI compartments was assessed in agar plates, using barley β-glucan (Megazyme®, Ireland) at 0.1% (w/v) final concentration, in 10 mM Tris-HCl pH 7.0. Catalytic activity was detected after 16 hours incubation at 37 ºC through the Congo Red assay plate, as described in Ponte *et al*., (2004a) and Mourão *et al.,* (2006). Zymogram analysis was performed as described by Fontes *et al*., (2004). Briefly, digesta proteins were separated through SDS-

PAGE in 10 % acrylamide gels containing 0.1% of barley β-glucan (Megazyme®, Ireland), according to Laemmli (1970). After electrophoresis, polypeptides were renatured by subjecting the gel to four 30 min washes in 100 mM sodium succinate, pH 6.3, containing 10 mM $CaCl_2$ and 1 mM DTT. The gel was incubated for 36 hours at 37 ºC, in the same buffer and proteins were stained in a solution comprising 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.4% (w/v) Coomassie Brilliant Blue R. After destaining, β-glucanase activity was detected using a 0.1% (w/v) Congo Red solution, for 15 min and washing with 1 M NaCl until excess dye was removed. Areas of catalytic activity appeared as colourless zones in a dark blue background after a quick wash in a 0.5% (v/v) solution of acetic acid. For measuring the viscosity of small intestine contents, samples were centrifuged for 10 min at 9000 rpm and the viscosity of the supernatant was measured using a Brookfield viscometer (Model LVDVCP-II, Brookfield Engineering Laboratories, Middleboro, MA) whose cup was maintained at 24 ºC.

2.1.2.4. Statistical Analysis

Statistical analysis of data related to birds performance was conducted by analysis of variance, using the General Linear Models procedure of SAS (SAS Inst. Inc., Cary, NC). Means with a significant F ratio were separated by the least significant difference test. The experimental unit was a cage of 4 birds. Unless otherwise stated, differences were considered significant when $P<0.1$.

2.1.3. Results and Discussion

The importance of *Ct*Lic26A-Cel5E family 11 β-glucan-binding domain in the function of the recombinant cellulase used to supplement a barley-based diet for broiler chicks was evaluated. The basal diet was supplemented with the required enzymes and used to feed broiler chicks *ad libitum* from days 1-28. During the experiment, the mortality rate was low (3.8 %) and was not related to treatments (anatomopathological results not shown).

2.1.3.1. Bird performance

Values concerning bird's body weight, weight gain, feed intake and feed conversion ratio are summarized in Table 2.2.

Table 2.2|Growth performance of broilers fed on a barley-based feed supplemented with recombinant β-glucanases displaying different molecular architectures

	C0	ROV	GH26-GH5	GH26-GH5-CBM11	SEM	p(*F*)
Body Weight (g)						
0d	45.6	45.4	45.9	45.4	0.19	0.180
7d	156.6	157.6	152.6	160.4	3.26	0.367
14d	382.7	400.0	379.8	405.5	8.85	0.096
21d	693.6	725.4	707.8	737.2	13.37	0.107
28d	1147[a]	1206[b]	1192[ab]	1238[b]	24.5	0.075
Weight Gain (g)						
0-7d	111.0	112.3	106.8	115.5	3.23	0.264
7-14d	226.1	242.4	227.3	245.1	6.61	0.082
14-21d	310.9	325.5	328.0	331.8	7.45	0.218
21-28d	452.9	480.3	483.8	500.4	15.42	0.184
0-28d	1101[a]	1160[b]	1146[ab]	1193[b]	24.5	0.073
Feed Intake (g)						
0-7d	139.1	144.1	136.8	137.5	7.27	0.876
7-14d	340.4	359.2	341.4	352.8	15.62	0.778
14-21d	543.8	572.7	578.3	568.5	17.48	0.511
21-28d	909.3	895.6	923.5	925.6	25.48	0.802
0-28d	1932	1972	1980	1984	58.2	0.915
Feed Conversion Ratio						
0-7d	1.27	1.23	1.30	1.19	0.038	0.168
7-14d	1.51	1.53	1.54	1.44	0.076	0.749
14-21d	1.75	1.76	1.78	1.72	0.043	0.760
21-28d	2.02	1.92	1.93	1.86	0.055	0.229
0-28d	1.76	1.72	1.75	1.67	0.037	0.240

During the entire experimental period, feed intake and feed conversion ratio were not significantly different between birds fed the different diets. In addition, weekly body weight and weight gain were not different between birds during the first 3 experimental weeks. However, during week four, birds receiving the commercial enzyme mixture and CtLic26A-Cel5E-CBM11 revealed better performance than birds of the negative control treatment. Therefore, even though the enzymes of the commercial mixture included a range of glucanases, cellulases and xilanases with different substrate specificities that were added at higher levels than the recombinant enzymes, body weight and weight gain were similar between these treatments. Since the individual bi-functional cellulase *Ct*Lic26A-Cel5E-CBM11 work as effectively as the commercial enzyme cocktail it is suggested that accessory non-glucanase activities, such as xylanase or mannanse, become obsolete in barley based

diets such as the one used in the present study. In addition, these results indicate that incorporating the CBM11 containing protein at lower doses (three fold decrease) when compared to the ones reported in a previous study (Guerreiro *et al.*, 2008) are adequate to improve the nutritive value of a barley-based diet for broiler chicken. It has been previously shown that a modular enzyme containing a family 6 CBM yields better broiler performance (Fontes *et al.*, 2004). In the present study, although birds fed on the modular enzymes with or without the β-glucan specific CBM and the commercial enzyme mixture did not reveal significant differences in final body weight, there is a clear numerical difference in the weight of birds receiving the diets supplemented with the recombinant enzyme, which indicates that *Ct*Lic26A-Cel5E-CBM11 is more effective in reducing the detrimental effects associated with the ingestion of β-glucan than *Ct*Lic26A-Cel5E. As reported previously by Guerreiro *et al*. (2008), our results highlight the capacity of single recombinant cellulases to improve the nutritive value of barley-based diets for poultry, questioning the need for using enzyme mixtures containing a large array of different enzyme specificities for targeting the anti-nutritive factors present in those diets. This is not completely unexpected, since data previously reported by Philip *et al*., (1995b) demonstrated that a recombinant single-domain cellulase, which originates also from the anaerobic bacterium *C. thermocellum*, was as efficient as a complex mixture of cellulases in improving the nutritive value of a barley-based diet for broilers. One of the major actions of feed cellulases is to decrease the degree of polymerization of soluble β-glucans, through the randomly cleavage of glycosydic bonds in the polysaccharide backbone. The reduction in carbohydrate chain length contributes to decrease the levels of digesta viscosity (Bedford & Morgan, 1996; Fengler & Marquardt, 1988).

Dietary fibre can influence the development and the size of digestive organs. It is well known that diets with high levels of soluble NSP induced considerable enlargements of some portions of the gastrointestinal tract (Brenes *et al.*, 1993; Mourão *et al.*, 2006; Petersen *et al.*, 1993) and pancreas and stimulated an increase in protein turnover rates (Danicke *et al.*, 2000). Since enzyme addition decreases digesta viscosity and therefore improves the feed passage rate and nutrient absorption, then, the relative weight of the digestive tract decreases leading to an increase on the carcass yield (Pettersson & Aman, 1989). Fuente *et al*., (1998) have found an equation relating the empty weight of the digestive tract to digesta viscosity. Therefore, the effects of the different dietary treatments in the relative length or weight of different organs and GI tract compartments of broiler chicken were evaluated and the respective data is presented in Table 2.3.

Table 2.3|Relative weight and length of GI tract and viscosity of digesta samples of broilers fed on a barley-based feed supplemented with recombinant β-glucanases displaying different molecular architectures

	C0	ROV	GH26-GH5	GH26-GH5-CBM11	SEM	p(*F*)
Relative Weight (g/100g BW)						
Crop	0.37	0.34	0.39	0.38	0.022	0.261
Gizzard	1.40	1.37	1.29	1.35	0.059	0.598
Liver	3.18	3.12	3.18	3.06	0.183	0.955
Relative Length (cm/100g BW)						
Duodenum	1.86	1.74	1.91	1.82	0.061	0.228
Jejunum	5.40	5.00	5.10	5.20	0.139	0.208
Ileum	5.48	5.15	5.46	5.51	0.171	0.381
Caecum	1.29	1.16	1.22	1.23	0.039	0.102
Content Viscosity (cP)						
Duodenum+ Jejunum	7.45[a]	4.96[b]	6.96[a]	6.15[ab]	0.650	0.053
Ileum	12.1	9.49	9.08	10.5	1.45	0.467

In the current study, the relative weights of crop, gizzard and liver and the relative length of the duodenum, jejunum, ileum and caecum of birds were not different between birds of the 4 dietary treatments. These data is in contrast with results reported by several other authors who have shown that enzyme addition decreases the digestive tract weight and/or length when expressed as a percentage of live weight (Brenes *et al.*, 1993; Petersen *et al.*, 1993; Viveros *et al.*, 1994). Since the percentages of soluble glucans in barley vary widely, it is possible that the levels of the soluble NSP present in the cereal used in this experiment were too small to have an impact in the size of the birds GI tract.

Digesta viscosity in the hindgut and foregut of birds fed the different dietary treatments was measured and the data is presented in Table 2.3. Ileum viscosity was shown to be identical in birds of the different treatments. However, digesta viscosity at the level of the duodenum and jejunum was smaller in birds supplemented with the commercial enzyme when compared with birds receiving *Ct*Lic26A-Cel5E. Although digesta viscosity in birds supplemented with the modular enzymes was not significantly different from the negative control, there is a numerical tendency for *Ct*Lic26A-Cel5E-CBM11 to contribute to decrease this digesta physical parameter. Therefore, it is suggested that presence of the non-catalytic

domain CBM improves the capacity of the appended catalytic domain to decrease the degree of polymerization of the soluble glucans, therefore leading to a lower viscosity. In fact, the viscosity of the duodenum and jejunum contents of birds fed the commercial mixture was similar to the viscosity in birds fed diets containing *Ct*Lic26A-Cel5E-CBM11, which agrees with the results considering the bird's body weight and weight gain.

Taken together, our results suggest that single purified recombinant cellulases or enzyme mixtures containing cellulases, can equally improve bird performance. Moreover, the addition of lower doses (10 U/kg vs 30 U/kg) of the modular recombinant enzymes than the ones used in a previous study (Guerreiro *et al.*, 2008) appears to be equally effective in improving performance of birds fed barley-based diets only if the recombinant enzyme contain a non-catalytic CBM. It is well established that CBMs contribute to enhance the activity of adjacent catalytic modules by increasing enzyme concentration on the substrate surface (Fernandes *et al.*, 1999; Gilbert *et al.*, 2002). This action is particularly important in plant cell wall hydrolases that need to be targeted to their specific substrates which are usually less accessible in the complex organization of the plant cell wall. In fact, Fontes *et al*., (2004) have found that a modular xylanase containing a family 6 CBM yields better animal performance than the enzyme's catalytic module alone. The family 11 CBM of *Ct*Lic26A-Cel5E binds both β-1,4- and β-1,3-1,4-mixed linked glucans (Carvalho *et al.*, 2004c). It is also well known that CBMs are particularly important for the hydrolysis of insoluble substrates (Gilbert *et al.*, 2002). We have previously shown (Guerreiro *et al.*, 2008) that, although the molar activity of the recombinant cellulases Lic26-Cel5E-CBM11 and Lic26-Cel5E against barley β-glucan is similar, the presence of the family 11 CBM potentiates the action of the modular cellulase against insoluble cellulose forms, such as Avicel. Since barley-based diets are relatively poor in insoluble polysaccharides, we envisaged that the major contribution of the family 11 CBM of *Ct*Lic26A-Cel5E to increase the efficiency of the recombinant enzyme would be related with the targeting of the associated catalytic domains to the anti-nutritive soluble β-glucans. Our results revealed that when *Ct*Lic26-Cel5E-CBM11 was supplemented to the diets, the viscosity of the duodenum and jejunum contents was not different from the viscosity of the contents of birds fed the commercial enzyme. In addition, birds fed diets with *Ct*Lic26-Cel5E showed no differences in body weight and weight gain in comparison to the negative control, while birds on *Ct*Lic26-Cel5E-CBM11 achieved higher body weights and weight gains than the negative control but similar to the commercial enzyme treatment. This, again, may indicate that the family 11 CBM positively affects the efficiency of the GH5 and GH26 catalytic domains *in vivo* at the present doses.

2.1.3.2. Recombinant β-glucanase stability *in vivo*

To evaluate the stability of the exogenous glycoside hydrolases during passage through the GI tract, β-glucanase activity was qualitatively determined in digesta samples collected in the various digestive compartments of 10 animals per treatment. The data, presented in Table 2.4, demonstrated that while caeca samples collected from birds of the group not receiving exogenous enzymes were positive for cellulase activity, no β-glucan degrading properties were detected in the contents of the other GI compartments.

Table 2.4|Qualitative detection of beta-glucanase activity in digesta collected from the gastrointestinal compartments of 40 broilers fed on a barley-based feed, supplemented with recombinant β-glucanases displaying different molecular architectures.

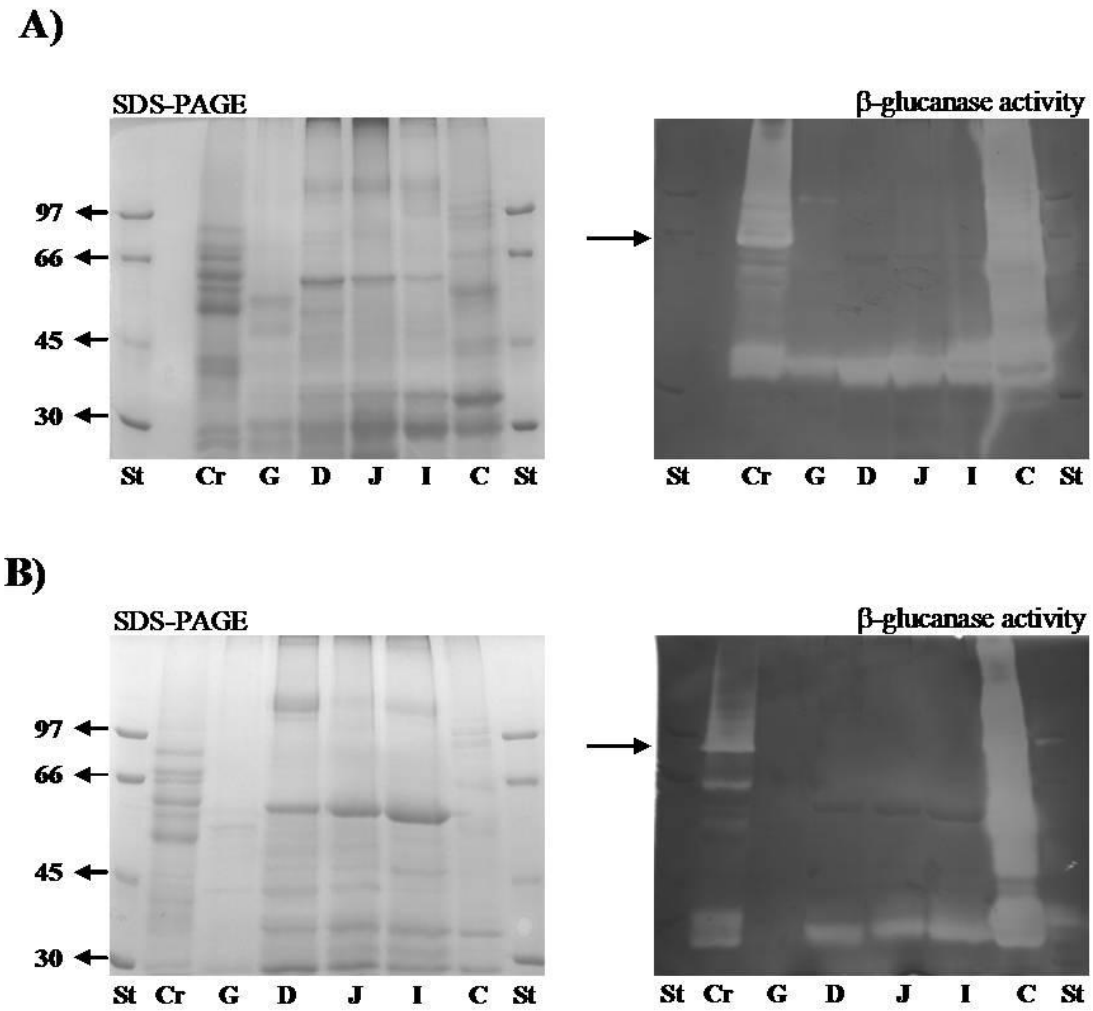
	C0	ROV	GH26-GH5	GH26-GH5-CBM11
Crop	-/+/-/+/-/-/-/-/-/+	+/+/+/+/+/-/-/-/-/+	+/+/+/-/+/+/+/+/+/+	+/+/+/+/+/+/+/-/+/+
Gizzard	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/+/+/-	+/-/+/+/-/-/-/+/-/-
Duodenum	-/-/-/-/-/-/-/-/-/-	+/-/-/+/-/-/+/+/-/-	+/-/-/+/-/+/+/-/-/-	-/+/-/-/+/-/-/+/-/-
Jejunum	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-	-/-/+/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-
Ileum	-/-/-/-/-/-/-/-/-/-	+/+/-/-/-/-/+/+/-/-	+/-/+/+/-/-/-/+/-/+	+/-/+/+/+/+/+/-/+/-/-
Caecum	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+

[1] Symbols refer to none (-) or detectable (+) β-glucanase activity.

However, β-glucanase activity could be detected along the entire digestive tract of most animals fed on diets supplemented with the plant cell wall hydrolases. Interestingly, β-glucanase activity was not detected in the gizzard of birds fed diets supplemented with the commercial enzyme, possibly because of the acidic conditions in this organ. Nevertheless, broilers fed *Ct*Lic26-Cel5E and, to a greater extent, *Ct*Lic26-Cel5E-CBM11 showed detectable beta-glucanase activity in the gizzard, suggesting that the modular enzymes used in the present study have a higher resistance to the acidic conditions that are prevalent in this portion of the digestive tract when compared with the commercial enzyme mixture.

To analyse potential changes in the molecular architecture of the recombinant cellulases during passage through the GI tract, digestive samples of birds of treatments receiving *Ct*Lic26-Cel5E-CBM11 and *Ct*Lic26-Cel5E were subjected to zymogram analysis. The data, displayed in Figure 2.2, suggest that both *Ct*Lic26-Cel5E-CBM11 and *Ct*Lic26-Cel5 are prone to proteolytic cleavage in the birds' GI tract, which occurs initially but moderately in the crop and then completely in the gizzard and in the following GI compartments.

Figure 2.2|Zymogram analysis of digesta samples collected from various regions of the GI tract of birds supplemented with the recombinant β-glucanases Lic26-Cel5 (Panel A) and Lic26-Cel5-CBM11 (Panel B).



Proteins were fractionated through SDS-PAGE and stained for β-glucanase activity after enzyme renaturation. The arrows depict the location of the non-truncated versions of the two recombinant enzymes in the stained gels. Abbreviations: St, low molecular weight protein standards; Cr, crop; G, gizzard; D, duodenum; J, jejunum; I, ileum; C, caecum

Therefore, in agreement with the data reported by Guerreiro *et al*., (2008), it is suggested that both recombinant enzymes are proteolytically cleaved in the linker regions connecting the Lic26, the Cel5 and the CBM11 modules, which contributes to release the two 32-35 kDa catalytic domains that still retain significant catalytic activity in the digestive tract. Experiments performed *in vitro* revealed that the proteolytic cleavage of Lic26-Cel5 does not affect the biological capability of the resulting catalytic domains to degrade soluble β-glucans (Guerreiro, unpublished data), as it was previously demonstrated by Taylor *et al*., (2005). Since we have suggested here that *Ct*Lic26-Cel5E-CBM11 looked more promising in yielding better bird performance than *Ct*Lic26-Cel5E, and that there is proteolityc cleavage of the

modular enzymes at the gizzard level, we believe that the CBM may exert its effect primarily at the crop level.

2.1.4. Conclusions

The results suggest that individual recombinant cellulases with a non-catalytic CBM added at lower doses than a commercial mixture are equally effective in improving the nutritive value of barley-based diets for poultry. At the present recombinant enzyme doses the modular enzyme containing a family 11 CBM, which is β-glucan specific, was more effective in reducing the anti-nutritive properties of β-glucans when compared with its truncated counterpart lacking the CBM. In addition, both recombinant enzymes were prone to proteolysis in the birds' gizzard and subsequent GI compartments leading to the conversion of the enzyme molecular architecture into two single-domain enzymes that are identical in birds of the two treatments. Therefore, considering the animal performance and the *in vivo* enzyme analysis reported here, it is suggested that *Ct*Lic26-Cel5E derivatives, and in general cellulases added to barley-based diets exert primarily their function at the initial portions of the GI tract.

2.2. THE THERMOSTABLE BETA-1,3-1,4-GLUCANASE FROM *CLOSTRIDIUM THERMOCELLUM* IMPROVES THE NUTRITIVE VALUE OF AN HIGHLY VISCOUS BARLEY-BASED DIET FOR BROILERS

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Abstract

Microbial β-1,3-1,4-glucanases improve the nutritive value of barley-based diets for poultry by effectively decreasing the degree of polymerization of the anti-nutritive soluble β-glucans. Glycoside hydrolases (GHs) acting on recalcitrant polysaccharides display a modular architecture comprising a catalytic domain linked to one or more non-catalytic carbohydrate-binding modules (CBM). GHs and CBMs have been classified in different families based on primary structure similarity (see CAZy webpage at www.cazy.org). The role of CBMs is to anchor the appended GHs into their target substrates, therefore eliciting the efficient hydrolysis of structural carbohydrates. Here we describe the biochemical properties of the family 16 GH from *Clostridium thermocellum*, termed *Ct*Glc16A. The enzyme belongs to the extracellular multi-enzyme complex of cellulases and hemicellulases, termed the cellulosome, secreted by the bacterium. Consistent with its origin, *Ct*Glc16A is a thermostable enzyme that specifically acts on β-1,3-1,4-glucans with a remarkable catalytic activity (38000 U/mg protein). *Ct*Glc16A individually or fused to the family 11 β-glucan-binding domain of celulase *Ct*Lic26A-Cel5E of *C. thermocellum* was used to supplement a highly viscous barley-based diet for broilers. The data showed that birds fed diets supplemented with the recombinant enzymes displayed an improved performance when compared to birds fed diets not supplemented with exogenous enzymes. However inclusion of the non-catalytic CBM had no influence in the capacity of *Ct*Glc16A to reduce the anti-nutritive effects of soluble β-1,3-1,4-glucan. It is suggested that CBMs might be effective exclusively when the exogenous enzymes are supplemented at lower dosage rates.

2.2.1. Introduction

Dietary soluble non-starch polysaccharides (NSP), such as arabinoxylans and β-glucans, are known to increase digesta viscosity leading, in poultry, to a lower efficiency of nutrient digestion and absorption (Smith & Annison, 1996) while contributing to reduce feed passage rates (van der Klis *et al.*, 1993). This later effect promotes a proliferation of anaerobic microbes in the small intestine leading to the production of toxins and deconjugation of bile salts, which are essential for the digestion of fat (Langhout, 1998; Misir & Marquardt, 1978). It is well established that inclusion of microbial plant cell wall hydrolases in wheat, barley or rye-based diets for simple-stomach animals improves the efficiency of feed utilization, enhances growth and contributes for a better use of low cost feed ingredients (Bedford, 2000; Chesson, 1993). Xylanases and β-1,3-1-4-glucanases act by reducing the degree of polymerization (dp) of soluble polysaccharides resulting in oligosaccharide products that do not affect digesta viscosity. Thus, the action of exogenous feed enzymes leads to a reduction of digesta viscosity having a consequent positive effect in the rate of diffusion of substrates, digestive enzymes and nutrients (Bedford *et al.*, 1991; Bedford & Classen, 1992; Fengler & Marquardt, 1988; White *et al.*, 1981). In addition, exogenous enzyme products promote the proliferation of a beneficial microflora in the final compartments of simple-stomach animals' gastrointestinal (GI) tract, as a result of the change in the quantity and/or the quality of substrates available for fermentation (Apajalahti & Bedford, 1999; Bedford & Morgan, 1996). Moreover, plant cell wall hydrolases contribute to release endosperm plant cell wall trapped nutrients that were otherwise unavailable for digestion (Hesselman & Aman, 1986).

Enzyme discovery is pivotal to potentiate a variety of biotechnological applications using plant cell wall hydrolases. The cellulase and hemicellulase complex expressed by the anaerobic thermophilic bacterium *Clostridium thermocellum*, referred to as the "cellulosome", is one of the most powerful and intricate plant cell wall degrading systems described to date (Fontes & Gilbert, 2010). These enzymes contain a non-catalytic dockerin domain that binds one of the nine cohesin domains found in a large non-catalytic protein scaffold defined as CipA. *Clostridium thermocellum* cellulosomal Glycoside Hydrolases (GH) are remarkably stable and active enzymes and although targeting primarily cellulosic substrates there are also cellulosomal examples of xylanases, arabinofuranosidases, xyloglucanases, mannanases, galactanases, β-glucanases and a variety of carbohydrate esterases and pectate lyases. The catalytic cellulosomal subunits also contain non-catalytic Carbohydrate Binding Modules (CBMs) that target β-glucans, xylans, uronic acids, chitin and pustulan (Carvalho *et al.*, 2004b; Charnock *et al.*, 2000b; Czjzek *et al.*, 2001; Dvortsov *et al.*, 2009; Montanier *et al.*, 2009a; Najmudin *et al.*, 2006) and thus direct the complex towards the target substrates of these enzymes. GHs and CBMs have been grouped into sequence-based families (Coutinho & Henrissat, 2003) and each GH or CBM family sometimes display a large range of different substrate/ligand specificities. Not only there is a need to discover

highly effective and stable cellulases and hemicellulases but also there is a paucity of information concerning the importance of non-catalytic CBMs in the function of exogenous cellulases used to supplement cereal-based diets for simple stomach animals. However, a family 6 CBM that binds xylan was shown to improve the efficacy of a microbial recombinant xylanase used to supplement wheat and rye-based diets for poultry (Fontes *et al.*, 2004). More recently, a family 11 CBM that is highly specific for β-1,3-1,4-glucans improved the efficacy of the associated catalytic domain used to supplement a barley-based diet (Guerreiro *et al.*, 2008; Ribeiro *et al.*, 2008). The higher efficacy of the modular enzyme allowed for a significant reduction in enzyme dosage.

The genome sequence of *C. thermocellum* revealed the presence of 72 cellulosomal polypeptides (defined as those containing type I dockerin domains that bind to the type I cohesin domains in CipA). Several of these enzymes remain to be characterized. The aim of this work was to characterize the GH16 cellulosomal enzyme from *C. thermocellum* in view of its potential to supplement barley-based diets for poultry. GH16 is an enzyme family containing predominantly β-1,4-1,3-glucanases. The capacity of a recombinant derivative of the enzyme, individually or fused to a β-glucan specific CBM, to improve the nutritive value of barley based diets for broilers was evaluated.

2.2.2. Material and Methods

2.2.2.1. Bacterial strains, plasmids and culture conditions

Escherichia coli strains XL10-Blue (Stratagene) and BL21 (Novagen) were cultured at 37ºC in Luria Bertani broth (LB) unless otherwise stated. Media were supplemented with 100 mg/L ampicillin to select for *E. coli* transformants. The plasmids employed in this work were pET21a (Novagen) and pNZY28 (NZYTech Ltd.).

2.2.2.2. Expression and purification of *Ct*Glc16A and *Ct*Glc16A-CBM11

DNA encoding *Ct*Glc16A catalytic domain (residues 31 - 251) was amplified through PCR from genomic DNA using the thermostable polymerase NZYDNA Change (NZYTech Ltd.) with primers CTCGCTAGCACTGTGGTAAATACGCC (forward) and CACCTCGAGATTATCTTGCGGAACAC (reverse) and cloned into *Nhe*I and *Xho*I restricted pET21a to generate pTR2. The clostridial gene was sequenced to ensure the integrity of the nucleic acid. The recombinant protein encoded by this plasmid contains a *C*-terminal His_6 tag. To fuse *Ct*Glc16A with a β-glucan specific CBM, the gene encoding the family 11 CBM of *Clostridium thermocellum* CtLic26A-Cel5E was amplified through PCR as described above and the following primers: Forward, 5´-CTCGTCGACCCAACTCCAAGACCGACC; Reverse, 5´-CACCTCGAGAGCACCAATCAGCTTGAT. The PCR product was cloned into pNZY28, sequenced to ensure that no mutations accumulated during PCR, and subsequently sub-

cloned into the *XhoI* site of pTR2, generating pTR3. Plasmid pTR2 encodes the single-domain β-glucanase *Ct*Glc16A while pTR3 encodes the modular enzyme fused to the Clostridial CBM11, which was termed *Ct*Glc16A-CBM11 (Figure 2.3). To express the Clostridial proteins, *E. coli* BL21 cells harbouring the appropriate recombinant plasmid were cultured in LB containing ampicillin at 37ºC to mid-exponential phase (A_{550} 0.6), at which point isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and the cultures were incubated for a further 5 h. The His_6-tagged recombinant proteins were purified from cell-free extracts by immobilized metal ion affinity chromatography (IMAC) as described previously (Fontes *et al.*, 2004).

Figure 2.3|Domain organization of *Ct*Glc16A and derivative containing a C-terminal family 11 CBM, termed *Ct*Glc16A-CBM11.

The β-glucanase (GH16), β-glucan binding domain (CBM11) and the dockerin (Doc) are indicated. The gray and the black boxes represent the signal peptide and linker sequences, respectively.

2.2.2.3. Source of carbohydrates

Polysaccharides were purchased from Megazyme International (Bray County Wicklow, Ireland), except oat spelt xylan and hydroxyethylcellulose, which were obtained from Sigma.

2.2.2.4. Enzyme assays

The activity of *Ct*Glc16A and *Ct*Glc16A-CBM11 against various polysaccharides was determined as described previously (Fontes *et al.*, 2000) by detecting the release of reducing sugars using the DNSA reagent. One unit of catalytic activity is defined as the amount of enzyme required to release one µmole of product per min. Unless otherwise stated, assays with *Ct*Glc16A were carried out in 50 mM sodium phosphate buffer, pH 6.5 at 60 ºC. To explore the pH profile of *Ct*Glc16A, 50 mM MES (2-(*N*-morpholino)ethanesulfonic acid) (pH 4-7), 50 mM Tris-HCL (pH 7-9.5), 50 mM $NaHCO_3$ (pH 8-10) buffers were used in enzyme assays that employed 0.2 % barley β-glucan as the substrate. For thermostability experiments, *Ct*Glc16A was incubated at temperatures ranging from 30 to 90ºC. After 20 min

at the required temperature, samples were withdrawn and residual activity was determined at 50ºC by measuring the amount of reducing sugar released from barley β-glucan as described above. Determination of temperature of maximal enzyme activity was performed by incubating the enzyme at temperatures ranging from 30 to 90ºC and measuring reducing sugar release as previously described. Resistance to proteolysis was tested essentially as described previously (Fontes *et al.*, 1995) by incubating the proteins with porcine pancreatine (Sigma P-1500) at 37ºC, and measuring residual enzyme activity as described for thermostability.

2.2.2.5. Animals, Diets and Management

The composition of the barley-based diet used in this study, which was formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Table 2.5. The basal diet was supplemented with no enzyme (treatment C0) or with 1000 U/kg of *Ct*Glc16A (treatment GH16) or with 1000 U/kg *Ct*Glc16A-CBM11 (treatment GH16-CBM11). The amount of recombinant enzymes used corresponds to the level of supplementation of the positive control enzyme (see below). Thus, a fourth treatment corresponded to the supplementation of the basal diet with a calculated 1000 U/kg of the commercial enzyme cocktail Rovabio^{TM} Excel AP (Adisseo, France; treatment termed C+ for simplification), which corresponds to an incorporation ratio of 50 g of enzyme per ton of feed as recommended by the manufacturer. The four diets were provided in the pelleted form and enzyme preparations were mixed with the feed just before administration to the animals. Animal experiments were conducted in accordance with Ethics Committee of CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Veterinária, Portugal), following the appropriate European Union guidelines (EEC, 1986).

Table 2.5|Ingredient composition and calculated analysis of the cereal-based feed.

Ingredients	%
Barley	61.5
Soybean meal 47%	29.40
Soybean oil	5.90
Sodium chloride	0.25
Calcium carbonate	0.84
Dicalcium phosphate 18%	1.74
DL-Methionine	0.17
Mineral and vitamin premix [1]	0.2
Calculated nutrient content	
Energy (MJ ME/kg DM)	12.1
Crude Protein (%)	20.8
Ether extract (%)	7.5
Crude cellulose (%)	5.1
Ash (%)	5.7
Methionine (%)	0.47

[1] Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 2,100 IU; vitamin E, 20 mg; nicotinic acid, 30 mg; vitamin B_{12}, 0.12 mg; calcium pantothenate, 10 mg; vitamin K_3, 2 mg; thiamin, 1 mg; riboflavin, 4.2 mg; vitamin B_6, 1.7 mg; folic acid, 0.5 mg; biotin, 0.5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 ppm.

For the animal experiment, 160 1-day-old male chicks (Ross 308) were divided into 40 battery brooders, with a capacity of 4 animals per pen, exposed to constant light for the duration of the trial. Water and diets were available *ad libitum* throughout the experiment and were provided from automatic drinking nipples and a hanging feeder, respectively. The brooders were located in an environmentally-controlled room, which was adjusted daily to the recommended temperatures according to standard brooding practice. Birds were individually weighed at the beginning of the experiment and were randomly assigned into one of the four treatments, with 10 replicates per treatment. Feed consumption and individual body weights were recorded. Feed conversion ratios were calculated by dividing the total feed consumed by the weight gain per pen, per week and at the end of the experiment, including the weight gain of any dead birds. Bird mortality was recorded daily. At the end of the experiment, at day 28, one bird per pen was slaughtered by an intravenous injection of an aqueous isotonic solution of 125 mg Tiopental Braun (Braun, Barcelona, Spain). The size of the various GI compartments was measured or weighed and digesta samples were collected and stored at –20 ºC for later analysis. The weight of the GI compartments was determined empty. Levels of β-glucanase activity in the GI tract were measured as described below.

2.2.2.6. Analytical Procedures

To standardize the number of enzyme units used to supplement the basal diets, the catalytic activity of the various exogenous enzymes, including the commercial mixture, was determined under identical experimental conditions as described above. The extract containing Rovabio^{TM} Excel AP enzymes was prepared by ressuspending 75 mg of the enzyme mixture in 10 ml of 50 mM NaHepes buffer, pH 7.5, followed by an incubation overnight at 4 ºC with gentle agitation and a centrifugation at 13,000 rcf for 5 min. Previously to detection of β-glucanase activity, digesta samples were centrifuged and the supernatant recovered for analysis. Initially, qualitative analysis of β-glucanase activity in the digesta samples recovered from the various GI compartments was assessed in agar plates, using barley β-glucan (Megazyme, Ireland) at 0.1% (w/v) final concentration, in 10 mM Tris-HCl pH 7.0. Catalytic activity was detected after 24 hours of incubation at 37 ºC through the Congo Red assay plate, as described in Ponte, *et al*., (2004a) and Mourão, *et al*., (2006). Zymogram analysis was performed as described by Fontes *et al*., (2004). Briefly, digesta proteins were separated through SDS-PAGE in 10% acrylamide gels containing 0.1% of barley β-glucan (Megazyme®, Ireland), according to Laemmli (1970). After electrophoresis, polypeptides were renatured by subjecting the gel to four 30 min washes in 100 mM sodium succinate, pH 6.3, containing 10 mM $CaCl_2$ and 1 mM DTT. The gel was incubated overnight at 37 ºC, in the same buffer and proteins were stained in a solution comprising 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.4% (w/v) Coomassie Brilliant Blue R. After destaining, β-glucanase activity was detected using a 0.1% (w/v) Congo Red solution, for 15 min and washing with 1 M NaCl until excess dye was removed. Areas of catalytic activity appeared as colourless zones in a dark blue background after a quick wash in a 0.5% (v/v) solution of acetic acid. For measuring the viscosity of small intestine contents, samples were centrifuged for 10 min at 7,500 rcf and the viscosity of the supernatant was measured using a Brookfield viscometer (Model LVDVCP-II, Brookfield Engineering Laboratories, Middleboro, MA) with a cup maintained at 24 ºC.

2.2.2.7. Statistical Analysis

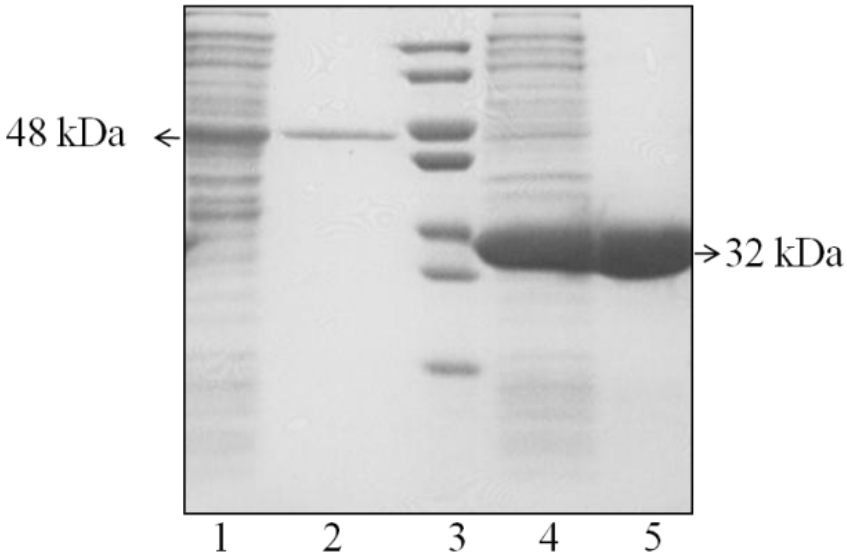
Statistical analysis of data related to birds performance was conducted by analysis of variance, using the General Linear Models procedure of SAS (SAS Inst. Inc., Cary, NC). Means with a significant F ratio were separated by the least significant difference test. The experimental unit was a cage of four birds. Unless otherwise stated, differences were considered significant when $P<0.05$.

2.2.3. Results and Discussion

2.2.3.1. Biochemical properties of *Ct*Glc16A

The gene encoding *Ct*Glc16A was amplified through PCR and cloned into the prokaryotic expression vector pET21a. The encoded recombinant protein, which was expressed at high levels in *E. coli*, was purified by IMAC to electrophoretic homogeneity (Figure 2.4).

Figure 2.4|Expression and purification of *C. thermocellum* β-1,3-1,4-glucanases *Ct*Glc16A and *Ct*Glc16A-CBM11.



The recombinant proteins were expressed in *Escherichia coli* and purified through Immobilized Metal Affinity Chromatography (IMAC) as described in Material and Methods. E. coli extracts of *Ct*Glc16A-CBM11 (lane 1) and *Ct*Glc16A (lane 4) were subjected to IMAC. Purified *Ct*Glc16A-CBM11 (lane 2) and *Ct*Glc16A (lane 5) were found to be >90% pure as judged through SDS-PAGE analysis. Protein markers (NZYTech Ltd) were loaded in lane 3.

Interrogation of the biochemical properties of *Ct*Glc16A shows that the enzyme does not exhibit any activity against soluble cellulosic substrates, Avicel, xylans, galactomannans, glucomannans or laminarin, but appears to be specific for β-1,3-1,4-glucans, such as lichenan and barley β-glucan (Table 2.6).

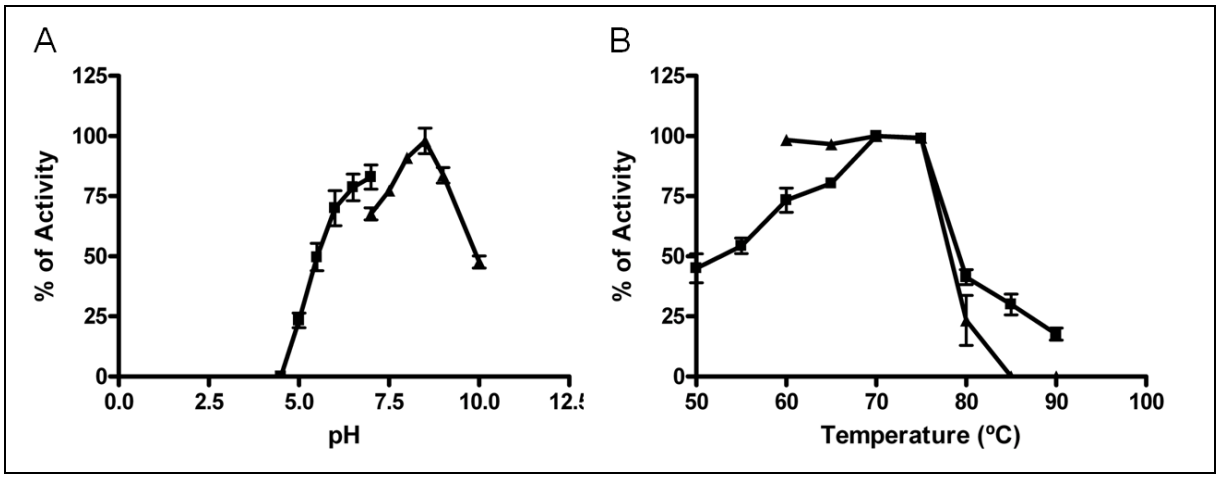
Table 2.6|Substrate specificity of *Ct*Glc16A.

Substrate (0.25%)	Qualitative activity
Hydroxyethylcellulose	-
Liquenan	+++
β-glucan	+++
Laminarin	++
Curdlan	+
Carboxymethylcellulose	-
Soluble xylan	-
Arabinoxylan	-
Glucuronoxylan	-
Birchwood xylan	-
Xyloglucan	-
Mannan	-
Galactomannan – locust bean	-
Galactomannan – 38:62	-
Galactomannan – carob	-
Arabinogalactan	-
Galactan – lupin	-
Arabinan	-
Glucomannan	-
Rhamnogalacturan – soybean*	-
Rhamnogalacturan – potato*	-
Pectic galactan - lupin	-
Pectic galactan - potato	-
Pectic – apples	-
Pectic - citrus	-
Polygalacturonic acid	-
Pustulan	-
Pullulan	-

The enzyme was incubated with a a variety of carbohydrates and enzyme activity evaluated through the capacity to release reducing sugars (labelled with a + in the table). Incapacity to degrade the tested polysaccharide is marked in the table with a -. ***** extracted without rhamnogalacturan enzymes

The optimal temperature of *Ct*Glc16A is ~70 °C, and the enzyme displays significant activity at a considerable wide pH range (pH 6-9) with maximal activity at pH 8.0 (Figure 2.5). In addition, the enzyme was found to be completely resistant to proteolytic inactivation in a 180 minute experiment (data not shown). Thus, the data confirm that *Ct*Glc16A is a typical β-1,3-1,4-glucanase. The enzyme is a remarkably active glucanase with a specific activity of 38000 U/mg of protein when assayed on barley β-1,3-1,4-glucan at the optimal temperature (70 °C). Its broad pH and temperature optimum and resistance to proteolytic inactivation suggest that the enzyme is particularly suited to resist the harsh conditions observed during feed processing and poultry digestion.

Figure 2.5|pH (panel A) and temperature (panel B) profiles of *Ct*Glc16A.



In Panel A, the enzyme was incubate at standard conditions in MES (■) or Tris (▲) buffers expressing different pHs and β-glucanase activity determined. In Panel B, *Ct*Glc16A activity was determined with barley β-glucan at different temperatures (■). For thermostability, the enzyme was incubated for 20 min at different temperatures and residual activity determined at 50ºC (▲).

2.2.3.2. *Ct*Glc16A improves the nutritive value of barley-based diets for broilers

A highly viscous barley (> 20 cP) presenting lower endo-β-glucanase activity (< 200 U/Kg) was selected for these studies. The barley-based diet was supplemented with the commercial and recombinant enzymes and used to feed broiler chicks *ad libitum* from days 1-28. During the experiment, the mortality rate was 5.6% and was not related to treatments. Values concerning bird's body weight, weight gain, feed intake and feed conversion ratio are summarized in Table 2.7. The data revealed that final body weight of birds fed with the basal diet supplemented with the three exogenous enzyme mixtures was significantly higher than birds of control group that were not subjected to a β-glucanase supplementation. Differences in body weight were visible as soon as day 7 and remained significant throughout the duration of the experiment. However, differences in weight gain were particularly acute at the first two weeks (P<0,0001) revealing that the exogenous enzymes are particularly effective in earlier stages of growth. In addition, animals receiving the exogenous enzymes expressed higher feed intakes suggesting that the highly viscous diet had a considerable impact in feed

passage rates that resulted in lower consumptions in non-supplemented birds. The lower feed intake expressed by animals fed on non-supplemented diets resulted in a smaller FCR in the 0-28d period for animals of this group. However, FCR were significantly smaller in supplemented animals in the first two weeks of the experiment, the period where feed enzymes revealed to be more effective. Together these data suggest that an improvement in body weight in animals receiving feed enzymes resulted not only from a higher feed intake but also from a better use of the feed ingredients. In addition the results demonstrate the capacity of both recombinant enzymes to improve the nutritive value of the barley-based diet. However, inclusion of the non-catalytic family 11 CBM in the Clostridial β-glucanase had no influence in the efficacy of the enzyme *in vivo*, at least when enzymes are employed at this rate of incorporation.

Table 2.7|Performance of broilers fed on a barley-based diet supplemented with different β-glucanase preparations.

	C-	C+	GH16	GH16-CBM11	SEM	p(*F*)
Body Weight (g)						
0d	42.8	42.7	42.5	43.0	0.26	0.667
7d	114.6^{a}	139.6^{b}	141.5^{b}	139.8^{b}	2.23	<0.0001
14d	249.7^{a}	353.9^{b}	363.1^{b}	351.1^{b}	6.78	<0.0001
21d	539.5^{a}	687.5^{b}	694.8^{b}	662.8^{b}	15.32	<0.0001
28d	940.6^{a}	1084^{b}	1074^{b}	1096^{b}	23.9	<0.0001
Weight Gain (g)						
0-7d	71.8^{a}	96.9^{b}	99.0^{b}	96.8^{b}	2.15	<0.0001
7-14d	135.1^{a}	217.4^{b}	221.7^{b}	211.4^{b}	5.74	<0.0001
14-21d	287.0^{a}	335.2^{b}	332.0^{b}	311.7^{ab}	10.07	0.006
21-28d	387.5^{a}	395.4^{b}	374.8^{b}	430.8^{ab}	14.74	0.064
0-28d	897.7^{a}	1045^{b}	1033^{b}	1053^{b}	24.1	0.0001
Feed Intake (g)						
0-7d	98.1^{a}	120.8^{b}	124.8^{b}	119.2^{b}	3.33	<0.0001
7-14d	225.1^{a}	305.0^{b}	324.3^{b}	318.7^{b}	7.23	<0.0001
14-21d	404.4^{a}	495.1^{b}	516.3^{b}	479.6^{b}	10.94	<0.0001
21-28d	607.3^{a}	649.3^{ab}	662.3^{b}	701.3^{b}	18.61	0.010
0-28d	1286^{a}	1570^{b}	1610^{b}	1580^{b}	30.7	<0.0001
Feed Conversion						
0-7d	1.37^{a}	1.25^{b}	1.26^{b}	1.23^{b}	0.030	0.013
7-14d	1.69^{a}	1.41^{b}	1.46^{b}	1.51^{b}	0.048	0.0001
14-21d	1.43^{a}	1.48^{ab}	1.57^{b}	1.55^{ab}	0.047	0.179
21-28d	1.58^{a}	1.66^{ab}	1.78^{b}	1.63^{a}	0.445	0.017
0-28d	1.44^{a}	1.51^{ab}	1.56^{b}	1.50^{ab}	0.027	0.027

C-, birds fed the basal diet not supplemented with an exogenous enzyme; C+, birds supplemented with a commercial enzyme mixture; GH16, birds supplemented with *C. thermocellum* recombinant cellulosomal enzyme *Ct*Glc16A; GH16-CBM11, birds supplemented with protein *Ct*Glc16A fused to a β-glucan binding domain, termed *Ct*Glc16A-CBM11.

Notwithstanding the fact that the commercial enzyme mixture included a range of different enzymes, including not only β-glucanases, but also cellulases and xylanases for example, performance of birds receiving enzymes from fermentative (commercial) or recombinant sources was similar. Since the recombinant cellulosomal enzymes function as effectively as the commercial enzyme cocktail, it is suggested that accessory non-glucanase activities, such as cellulase, xylanase or mannanase, are not required to improve the nutritive value of

barley based diets. As reported previously by Guerreiro, *et al*., (2008) and Ribeiro, *et al*., (2008), the results presented here reveal that there is no need to use enzyme mixtures containing a large array of different enzyme specificities for targeting the anti-nutritive factors present in barley-based diets. It is possible that the carbohydrate chains of soluble NSPs that are anti-nutritive for poultry are readily exposed for enzymatic attack and thus the action of the accessory enzymes, which could be involved in exposing the soluble glucans and arabinoxylans to the exogenous enzymes, is not required. Thus, acting individually the recombinant enzymes randomly cleave the glycosydic bonds in the polysaccharide backbone of their target substrates decreasing the degree of polymerization of soluble β-glucans. The reduction in carbohydrate chain length contributes to decrease the levels of digesta viscosity (Bedford & Morgan, 1996; Fengler & Marquardt, 1988) improving feed intake and the efficiency of nutrient utilization. Previously it was shown that when used at lower dosage rates the β-glucan specific family 11 CBM could contribute to improve the efficacy of the associated catalytic domain (Ribeiro *et al.*, 2008). In addition, it has been previously shown that a modular xylanase containing a family 6 CBM that is specific for xylans yields better broiler performance when animals are fed wheat or rye based diets (Fontes *et al.*, 2004). In the present study, birds fed with the modular enzyme containing the β-glucan specific CBM (*Ct*Glc16A-CBM11) did not reveal significant differences in performance when compared with animals of the group receiving the single domain enzyme (*Ct*Glc16A). It is possible that at the dosage rate used in this experiment the amount of enzyme is so high that would immediately saturated the anti-nutritive β-glucan with both the single and the two domain enzymes. In addition, the data presented here suggest that barley β-glucans are highly exposed carbohydrates making the need for a CBM, which target enzymes within complex macromolecular structures, highly redundant. CBMs contribute to enhance the activity of adjacent catalytic modules by increasing enzyme concentration on the substrate surface (Fernandes, *et al*., 1999; Gilbert, *et al*., 2002). This action is mostly important in plant cell wall hydrolases that need to be targeted to usually less accessible substrates which are hindered by the complex organization of the plant cell wall. We envisaged that the major contribution of the family 11 CBM would be related with the targeting of the associated catalytic domain (*Ct*Glc16A) to the anti-nutritive soluble β-glucans that are abundant in barley-based diets. The data suggest that the polysaccharide is quite accessible to enzyme attack being the presence of a CBM less important to enzyme function. However, this importance might raise if the enzyme is supplemented at lower dosage rates as it was recently demonstrated by Guerreiro and co-workers (2008).

Digesta viscosity in the hindgut and foregut of birds fed the four different diets was measured and the data is presented in Table 2.8.

Birds not supplemented with the exogenous enzymes presented high levels of digesta viscosity and the highest values were observed in the Ileum. Addition of exogenous enzymes had a dramatic effect in the physical properties of GI contents which displayed a reduced viscosity when measured in the various portions of the GI tract. However, both the recombinant enzymatic variants and the commercial enzymes had the same capacity to reduce digesta viscosity. It is well known that viscous digesta induce considerable enlargements of various portions of the gastrointestinal tract and pancreas (Brenes *et al.*, 1993; Petersen *et al.*, 1993) and stimulated an increase in protein turnover rates (Danicke *et al.*, 2000). Since enzyme addition decreases digesta viscosity and therefore improves feed passage rate and nutrient absorption, then, the relative weight of the digestive tract decreases leading to an increase on the carcass yield (Pettersson & Aman, 1989). Fuente, *et al*. (1998) have found an equation relating the empty weight of the digestive tract to digesta viscosity. The effects of the different dietary treatments in the length or weight of different organs and GI tract compartments of broiler chicken were evaluated and the respective data is presented in Table 2.8. As expected, relative weights and lengths of duodenum, jejunum and ileum of birds not receiving the exogenous β-glucanases were significantly larger when compared with animals of groups supplemented with the microbial glycoside hydrolases. These data are in agreement with results reported by several other authors who have shown that enzyme addition decreases the digestive tract weight and/or length when expressed as a percentage of live weight (Brenes *et al.*, 1993; Pettersson & Aman, 1993; Viveros *et al.*, 1994). The size or length of crop, gizzard, liver and caecum do not differ among the various groups. The data confirm that highly viscous diets have a dramatic effect in the size of GI tract which can be significantly reduced as a consequence of supplementation with exogenous enzymes. Taken together, our results suggest that a single purified recombinant β-1,3-1,4-glucanase, individually or fused with a non-catalytic β-glucan specific CBM module, or enzyme mixtures containing cellulases and other accessory enzyme activities, can equally improve bird performance when birds are fed highly viscous barley-based diets.

Table 2.8|Relative weight and length of the GI tract and viscosity of digesta samples of broilers fed on a barley-based feed supplemented with different exogenous β-glucanases.

	C-	C+	GH16	GH16-CBM11	SEM	p(*F*)
Relative Weight (g/100 g BW)						
Crop	0.38^{ab}	0.34^{b}	0.37^{ab}	0.45^{a}	0.032	0.135
Gizzard	1.16	1.19	1.26	1.19	0.062	0.733
Liver	2.93	2.75	2.59	2.72	0.122	0.288
Relative Weight (g/100 g BW)						
Duodenum	1.13^{a}	0.87^{b}	0.96^{b}	0.94^{b}	0.061	0.030
Jejunum	2.01^{a}	1.64^{b}	1.56^{b}	1.64^{b}	0.085	0.003
Ileum	1.84^{a}	1.31^{b}	1.42^{b}	1.46^{b}	0.075	<0.0001
Relative Length (cm/100 g BW)						
Duodenum	2.47^{a}	2.12^{b}	2.18^{b}	2.20^{b}	0.085	0.031
Jejunum	6.67^{a}	5.37^{b}	5.63^{b}	5.67^{b}	0.199	0.0003
Ileum	7.23^{a}	5.84^{b}	6.05^{b}	6.02^{b}	0.173	<0.0001
Caecum	1.42	1.31	1.35	1.34	0.046	0.386
Content Viscosity (cP)						
Duodenum+ Jejunum	28.5^{a}	5.43^{b}	6.69^{b}	4.49^{b}	2.000	<0.0001
Ileum	37.3^{a}	9.07^{b}	9.87^{b}	5.99^{b}	2.621	<0.0001

C-, birds fed the basal diet not supplemented with an exogenous enzyme; C+, birds supplemented with a commercial enzyme mixture; GH16, birds supplemented with *C. thermocellum* recombinant cellulosomal enzyme *Ct*Glc16A; GH16-CBM11, birds supplemented with protein *Ct*Glc16A fused to a β-glucan binding domain, termed *Ct*Glc16A-CBM11.

2.2.3.3. Recombinant β-glucanase stability *in vivo*

To evaluate the stability of the exogenous glycoside hydrolases during passage through the GI tract, β-glucanase activity was qualitatively determined in digesta samples collected in the various digestive compartments of ten animals per treatment. The data, presented in Table 2.9, demonstrated that while caecal samples collected from birds of the group not receiving exogenous enzymes were positive for cellulase activity, no β-glucan degrading properties were detected in the contents of the other GI compartments. However, β-glucanase activity could be detected along the entire digestive tract of most animals fed on diets supplemented with the plant cell wall hydrolases. The frequency of β-glucanase activity was especially higher in the crop, gizzard and duodenum of birds supplemented with modular enzymes. The results on the chi-square analysis show a significant difference at the 0.05 level of

confidence in the crop, gizzard, duodenum and ileum, indicating that in these compartments the type of enzyme added to the diet influenced the β-glucanase activity.

Table 2.9|Qualitative detection of cellulase activity in digesta collected from the gastrointestinal compartments of 40 broilers fed on a barley-based feed, supplemented with two different recombinant β-glucanases.

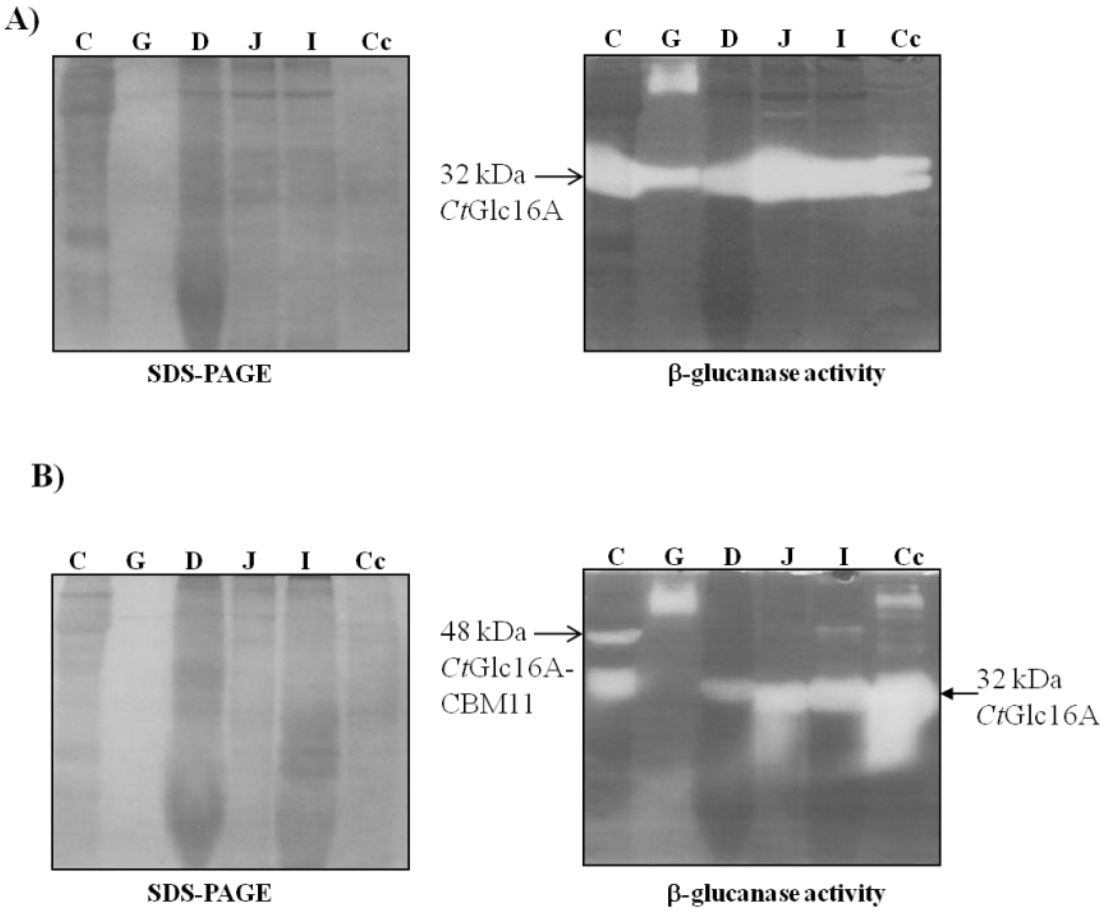
	C-	C+	GH16	GH16CBM11
Crop	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/-/+/+
Gizzard	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-	+/+/-/-/-/-/+/+/-/-	+/+/+/+/+/+/+/+/-/-
Duodenum	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-	+/+/-/-/+/+/-/-/-/-	+/+/+/+/+/+/+/+/-/-
Jejunum	-/+/-/-/+/+/-/-/-/-	-/-/-/-/-/-/-/-/-/-	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+
Ileum	+/+/+/-/-/-/+/-/-/-	-/-/-/-/-/-/-/-/-/-	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/-/+/+/+
Caecum	+/+/+/-/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+

Symbols refer to none (-) or detectable (+) β-glucanase activity.

C-, birds fed the basal diet not supplemented with an exogenous enzyme; C+, birds supplemented with a commercial enzyme mixture; GH16, birds supplemented with *C. thermocellum* recombinant cellulosomal enzyme *Ct*Glc16A; GH16-CBM11, birds supplemented with protein *Ct*Glc16A fused to a β-glucan binding domain, termed *Ct*Glc16A-CBM11.

To analyse potential changes in the molecular architecture of the recombinant cellulases during passage through the GI tract, digestive samples of birds of treatments receiving *Ct*Glc16A and *Ct*Glc16A–CBM11 were subjected to zymogram analysis. The data, displayed in Figure 2.6, suggest that *Ct*Glc16A–CBM11 is prone to proteolytic cleavage in the birds' GI tract, which occurs initially but moderately in the crop and then completely in the gizzard and in the following GI compartments.

Figure 2.6|Zymogram analysis of digesta samples collected from various regions of the GI tract of birds supplemented with the recombinant β-glucanases *Ct*Glc16A (panel A) and *Ct*Glc16A-CBM11 (panel B).



Proteins were fractionated through SDS-PAGE and stained for β-glucanase activity after enzyme renaturation. The arrows depict the location of the truncated and non-truncated versions of the two recombinant enzymes in the stained gels. Abbreviations: C, crop; G, gizzard; D, duodenum; J, jejunum; I, ileum; Cc, caecum

Therefore, in agreement with the data reported by Guerreiro, *et al*., (2008) and Ribeiro, *et al*., (2008), it is suggested that the modular enzyme is proteolytically cleaved in the linker region connecting the GH16 and the CBM11 modules, which contributes to release the CBM module from the catalytic region. Thus the data suggest that being the linker sequence susceptible to proteolytic cleavage it renders the modular enzyme into a single domain β-glucanase. The observed similar capacity of both recombinant enzymes to affect the nutritive value of the barley based diet may therefore result from the proteolysis suffered by the modular enzyme in the GI tract of broilers chicks which effectively result in two identical enzymes. Future research should be directed to the identification of linker sequences of different/reduced sizes and eventually different composition that may render the modular enzyme more resistant to proteolytic separation.

2.2.4. Conclusions

Data presented here suggests that individual recombinant β-glucanases or commercial enzyme mixtures are equally effective to improve the nutritive value of barley-based diet for poultry. At the present recombinant enzymes doses the modular enzyme containing a family 11 CBM, which is β-glucan specific, was not more effective in reducing the anti-nutritive properties of β-glucans when compared with its truncated counterpart lacking the CBM. Lack of effectiveness of the non-catalytic CBM may result from the high dosage rate of enzyme supplementation used in this study. In addition, it was revealed that the linker sequence separating the catalytic domain and the CBM in *Ct*Glc16A-CBM11 was prone to proteolysis in the birds GI tract, resulting in two enzymes with an identical molecular architectures. Thus, lack of resistance to proteolysis from the enzyme linker sequence affected the integrity of the modular enzyme and, since CBMs do not act in trans, the capacity of the non-catalytic module to improve the catalytic efficacy of the appended catalytic domain. Nevertheless, the data presented here describes a highly active and thermostable enzyme that was shown to be highly effective in rescuing the nutritive value of a viscous barley-based diet for broilers.

2.3. LEVELS OF ENDOGENOUS BETA-GLUCANASE ACTIVITY IN BARLEY AFFECT THE EFFICACY OF EXOGENOUS ENZYMES USED TO SUPPLEMENT BARLEY-BASED DIETS FOR POULTRY

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Abstract

To improve the nutritive value of barley-based diet for broilers, 2 experiments using 2 different barley lots were performed to evaluate the capacity of a mesophilic cellulase when fused to a β-glucan specific family 11 carbohydrate-binding module. The data revealed that the recombinant β-glucanase derivatives were not appropriate for feed supplementation because of a lack of stability at acidic pH levels. However, under the same experimental conditions, a commercial enzyme mixture improved the nutritive value of 1 of the cereal lots used. Analysis of the nutritive value of the 2 barleys revealed intrinsic differences in the levels of endogenous β-glucanase activity. These differences were extensively evident when the studies were expanded to a range of 64 barley lots. Thus, to clarify the effect of endogenous cellulases on the efficacy of exogenous β-glucanases used to supplement barley-based diets for poultry, 2 barley lots presenting low and high levels of endogenous plant cell wall-degrading enzymes were selected. These lots were used to prepare 2 barley-based diets, which were supplemented with or without a commercial enzyme product and fed to broiler chicks. The data revealed that the exogenous enzymes were effective when the basal diet presented low levels of endogenous β-glucanases but were unable to improve the nutritive value of the barley lot displaying higher β-glucanase activity. Thus, these studies suggest that levels of endogenous β-glucanases may affect the efficacy of exogenous enzymes used to improve the nutritive value of barley-based diets for broilers. The development of a quick β-glucanase assay that could be applied for cereal-based feeds may help identify those barley-based diets that are more responsive to the action of feed enzymes.

2.3.1. Introduction

Most cereals contain a significant proportion of soluble non-starch polysaccharides (NSP), which are known to display a variety of anti-nutritive properties for monogastric animals, particularly for poultry (Hesselman & Aman, 1986). Incorporation of exogenous β-1,3-1,4-glucanases, in barley, and β-1,4-xylanases, in wheat or rye-based diets improves the efficiency of feed utilization, enhances growth and contributes for a better use of low cost feed ingredients (Bedford, 2000; Chesson, 1993). Plant cell wall degrading enzymes contribute to reduce digesta viscosity that is associated with the intake of soluble indigestible carbohydrates, therefore improving the rate of diffusion of substrates, endogenous digestive enzymes and nutrients (Bedford *et al.*, 1991; Bedford & Classen, 1992; Fengler & Marquardt, 1988; White *et al.*, 1981). A reduction in digesta viscosity also increases the velocity of feed passage thus decreasing the proliferation of fermenting microbes in the upper regions of the gastrointestinal (GI) tract (van der Klis *et al.*, 1993) while improving feed intake. However, it is well known that in certain cases the inclusion of exogenous enzymes in diets containing a high percentage of wheat, barley or rye fails to have any effect in animal performance (Bedford, 2000). Numerous hypotheses have been advanced to explain this observation. There is strong evidence that the availability of energy from cereal grains in poultry is inversely related with the content of soluble NSPs (Villamide *et al.*, 1997). Thus, levels of NSPs may vary between different cereal lots resulting in cereals expressing different nutritive values. It is well established that a multitude of factors may affect cereal content in NSPs, which include cereal genotype and growing conditions, length of the cereal storage period, grain cultivar, growing season or soil type, among others (Villamide *et al.*, 1997). Although other factors may explain the unpredictable response to enzyme supplementation, such as the levels of endogenous cellulase and hemicellulase activities within the grain, these remain relatively uncharacterized.

Glycoside hydrolases (EC 3.2.1) that participate in the hydrolysis of plant cell wall carbohydrates display remarkably elaborate molecular architectures comprising both catalytic and non-catalytic CBMs. CBMs contribute to establish a close interaction between enzymes and plant polysaccharides allowing the appended catalytic domain to intimately contact its target substrates, thus potentiating catalysis (Boraston *et al.*, 2004). CBMs have been classified into sequence-based families in the CAZy database (Boraston *et al.*, 2004). The role of CBMs in the function of feed enzymes has been investigated. A family 6 xylan-binding domain was shown to improve the efficacy of a microbial recombinant xylanase, *in vivo*, when the enzyme was used to supplement wheat or rye-based diets for poultry (Fontes *et al.*, 2004). Thus, animals supplemented with a bi-modular xylanase containing both catalytic and xylan-binding domains grew significantly faster than animals fed on diets containing exclusively the xylanase catalytic domain. More recently, a family 11 CBM that is highly specific for β-1,3-1,4-glucans was shown to improve the efficacy of the associated

catalytic domain used to supplement a barley-based diet (Ribeiro *et al.*, 2008). The higher efficacy of the modular enzyme allowed for a significant reduction in enzyme dosage.

The primary objective of this work was to evaluate, in two replicated experiments, the capacity of a family 11 CBM that binds specifically to β-1,3-1,4-mixed linked glucans to improve the efficacy of a single domain cellulase from *Cellvibrio mixtus*. A different response to enzyme supplementation in these two initial experiments motivated the measurement of the levels of β-glucan, β-glucanase activity and viscosity in the two barley lots used. The experiments were extended to a range of more than 60 barleys. Since levels of endogenous plant β-glucanases were found to vary widely in barley, a third experiment was conducted where the capacity of exogenous enzymes to improve the nutritive value of barley-based diets containing different levels of plant β-glucanases was evaluated.

2.3.2. Materials and Methods

2.3.2.1. Enzyme Preparation

The gene encoding the family 11 CBM of *Clostridium thermocellum Ct*Lic26A-Cel5E was amplified through PCR from genomic DNA using the thermostable polymerase NZYPremium (NZYTech Ltd.) and the following primers: Forward, 5´-CTCGTCGACCCAACTCCAAGACCGACC; Reverse, 5´-CACCTCGAGAGCACCAATCAGCTTGAT. The PCR product was cloned into pNZY28, sequenced to ensure that no mutations accumulated during PCR, and subsequently sub-cloned into the XhoI site of pCF1 (Fontes *et al.*, 1997) generating pTR1. Plasmid pCF1 encodes the single-domain β-glucanase Cel5A while pTR1 encodes the uni-modular cellulase fused to the Clostridial CBM11, which was termed Cel5A-CBM11 (Figure 2.7). The two plasmids were used to transform BL21 *Escherichia coli* cells. Recombinant *E. coli* strains were grown on Luria Bertani media to mid-exponential phase (A_{600}nm of 0.5) and recombinant gene expression was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 1 mM. Cells were collected after 5 h induction at 37 ºC and protein extracts prepared by ultrasonication followed by centrifugation. The recombinant proteins were purified by metal-affinity chromatography as described by Fontes *et al*., (2004). Both recombinant proteins retain considerable catalytic activity at 40 ºC and are resistant to proteolytic degradation (Fontes *et al.*, 1997).

Figure 2.7|Domain organization of *Cellvibrio mixtus Cm*CelA and its recombinant derivative used in this study.

The cellulase (GH5) and β-glucan binding domain (CBM11) are indicated. The grey and the black boxes represent the signal peptide and linker sequences, respectively.

2.3.2.2. Animals, Diets and Management

The composition of the barley-based diets used in this study, which were formulated to contain adequate nutrient levels as defined by the NRC (1994), is presented in Table 2.10.

Table 2.10|Ingredient composition and calculated analysis of the cereal-based feed.

Ingredients	**%**
Barley	61.48
Soybean meal 47%	29.40
Soybean oil	5.90
Sodium chloride	0.25
Calcium carbonate	0.80
Dicalcium phosphate 18%	1.80
DL-Methionine	0.17
Mineral and vitamin premix [1]	0.20
Calculated nutrient content	
Energy (MJ ME/kg DM)	12.1
Crude Protein (%)	20.8
Ether extract (%)	7.5
Crude cellulose (%)	5.1
Ash (%)	5.7
Methionine (%)	0.47

[1] Mineral-vitamin premix provided the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 2,100 IU; vitamin E, 20 mg; nicotinic acid, 30 mg; vitamin B_{12}, 0.12 mg; calcium pantothenate, 10 mg; vitamin K_3, 2 mg; thiamin, 1 mg; riboflavin, 4.2 mg; vitamin B_6, 1.7 mg; folic acid, 0.5 mg; biotin, 0.5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 ppm.

In the first studies (experiments 1 and 2), two different barley lots were used to produce basal diets that were supplemented with no enzyme (treatment C0) or with 15 U/kg of Cel5A (treatment CelA) or Cel5A-CBM11 (treatment CelA-CBM11). This level of supplementation corresponds to the calculated level of supplementation of the positive control enzyme (see below). In addition, a fourth treatment included a basal diet supplemented with a calculated 15 U/kg of the commercial enzyme cocktail Rovabio™ Excel AP (Adisseo, France; treatment

termed C+ for simplification), which corresponds to an incorporation ratio of 50 g of enzyme per tonne of feed as recommended by the manufacturer. In the third experiment, two basal diets were produced using barley batches that contain high or low levels of endogenous β-glucanases. The diets were supplemented with or without the commercial enzyme product Rovabio™ Excel AP as described above. Thus, the effect of β-glucanase supplementation was tested in barley lots presenting high and low levels of endogenous β-glucanase. Basal diets were provided in the pelleted form and enzyme preparations were mixed with the feed just before administration to the animals. Animal experiments were conducted in accordance with the principles and specific guidelines presented in Guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

For each experiment, 160 1-day-old male chicks (Ross 308) were divided into 40 battery brooders, with a capacity of 4 animals per pen, exposed to constant light for the duration of the trial. Water and diets were available *ad libitum* throughout the experiment and were provided from automatic drinking nipples and a hanging feeder, respectively. The brooders were located in an environmentally-controlled room, which was adjusted daily to the recommended temperatures according to standard brooding practice. Birds were individually weighed at the beginning of the experiment and were randomly assigned into one of the four treatments, with 10 replicates per treatment. Weekly, feed consumption and individual body weights were recorded. Feed conversion ratios were calculated by dividing the total feed consumed by weight gain per pen, per week and at the end of the experiment, including the weight gain of any dead birds during the respective period. Bird mortality was recorded daily. At the end of the experiment, at day 28, one bird per pen was slaughtered by an intravenous injection of an aqueous isotonic solution of 125 mg Tiopental Braun (Braun, Barcelona, Spain). The size of the various GI compartments was measured or weighted and digesta samples were collected and stored at –20 ºC for later analysis. In experiments 1 and 2 the weight of the GI compartments was determined full. In contrast, in experiment 3 the various portions of the GI tract were emptied before weighting. Levels of β-glucanase activity in the GI tract were measured as described below.

2.3.2.3. Analytical Procedures

To standardize the number of enzyme units used to supplement the basal diets, the catalytic activity of the various exogenous enzymes, including the commercial mixture, was determined under identical experimental conditions. Catalytic activity was determined at 40 ºC by measuring reducing sugar released, following the method described by Fontes *et al*. (2000), using barley β-glucan (Megazyme, Ireland) as the substrate. One unit of catalytic activity is defined as the amount of enzyme required to release one µmole of product per min. The extract containing Rovabio™ Excel AP enzymes was prepared by ressuspending 75 mg of the enzyme mixture in 10 ml of 50 mM NaHepes buffer, pH 7.5, followed by an

incubation overnight at 4 ºC with gentle agitation and a centrifugation at 13,000 rcf for 5 min. Previously to detection of β-glucanase activity, digesta samples were centrifuged and the supernatant recovered for analysis. Initially, qualitative analysis of β-glucanase activity in the digesta samples recovered from the various GI compartments was assessed in agar plates, using barley β-glucan (Megazyme, Ireland) at 0.1% (w/v) final concentration, in 10 mM Tris-HCl pH 7.0. Catalytic activity was detected after 16 hours of incubation at 37 ºC through the Congo Red assay plate, as described in Ponte *et al*. (2004) and Mourão *et al*. (2006). For measuring the viscosity of small intestine contents, samples were centrifuged for 10 min at 7,500 rcf and the viscosity of the supernatant was measured using a Brookfield viscometer (Model LVDVCP-II, Brookfield Engineering Laboratories, Middleboro, MA) with a cup maintained at 24 ºC. Analyses for dry matter (DM; method 934.01), crude fat (920.39), crude protein (954.01), NDF (2002.04) and ADF/ADL (973.18) were performed according to the methods specified by Association of Official Analytical Chemists (1980).

A large range of different barley lots from different varieties was selected in September/October 2009 and levels of viscosity, and contents in β-glucan and β-glucanase were measured. All barleys were harvested in 2009 and thus could be considered as young barleys, since the assays were performed in the autumn of 2009. Barley content in β-glucan and levels of cereal β-glucanase activities were determined with the β-Glucan assay kit and the β-glucanase assay kits from Megazyme (Ireland), respectively. The method for determining β-glucanase activity followed the manufacturer protocol with a modification on the incubation period that was extended from 10 minutes to 3 hours. The method used for measuring the levels of viscosity followed the steps described above, although the barley samples were milled at 0.5 mm and mixed with 15 ml of PC buffer pH 6.5 at a vigorous shaking during 5 minutes before the start of the experiment.

2.3.2.4. Statistical Analysis

Statistical analysis of data related to birds performance was conducted by analysis of variance, using the General Linear Models procedure of SAS (SAS Inst. Inc., Cary, NC). Means with a significant F ratio were separated by the least significant difference test. The experimental unit was a cage of four birds. Unless otherwise stated, differences were considered significant when $P<0.05$. Regression analyses were conducted to test for linearity between level of β-glucans and viscosity, between level of β-glucans and β-glucanase activity, and between viscosity and β-glucanase activity in barley lots.

2.3.3. Results and Discussion

2.3.3.1. The recombinant derivatives of Cel5A from *C. mixtus* are unable to improve the nutritive value of barley-based diets for poultry

In contrast to the majority of plant cell wall degrading enzymes, Cel5A from *C. mixtus* is a unimodular enzyme which, despite being produced by a mesophilic bacterium, presents resistance to proteolytic inactivation and significant thermostability (Fontes *et al.*, 1997). Previously, it was shown that a family 11 CBM has the capacity to increasing the catalytic activity of the associated β-glucanase and cellulase catalytic domains *in vivo*, by improving the efficacy of the catalytic modules to enhance the nutritive value of barley-based diets for poultry (Guerreiro *et al.*, 2008; Ribeiro *et al.*, 2008). Here, we have investigated the capacity of a recombinant form of Cel5A from *C. mixtus* to decrease the levels of β-glucans found in barley based diets for poultry. In addition, the capacity of the family 11 CBM to enhance the efficacy of Cel5A was evaluated by engineering a recombinant fusion enzyme combining the two modules (Figure 2.7).

In experiment 1, body weight of birds fed with barley-based diets supplemented with or without the exogenous polysaccharidases was not significantly different at days 7, 14, 21 or 28 (final body weight) (Table 2.11) (bird mortality was 3.1%). There were no differences in weight gain among the different groups during the entire trial duration. In addition, feed intake and feed conversion ratios did not differ among the groups. Taken together, the results suggest that the exogenous enzymes of microbial (CelA and CelA-CBM11) or fungal origin (Rovabio^{TM} Excel AP, treatment C+) were unable to improve the nutritive value of the barley-based diet used in Experiment 1, which contained more than 60% (w/w) of barley.

Table 2.11|Performance of broilers fed on a barley-based diet supplemented with different β-glucanase preparations studied in Experiment 1.

	C-	C+	CelA	CelA CBM11	SEM	p(*F*)
Body Weight (g)						
0d	42.9	43	42.9	42.9	0.15	0.945
7d	135.9	137.3	130.4	131.4	2.57	0.182
14d	310.5	331.4	310.2	307.4	7.33	0.095
21d	594.5	627.8	591.3	589.1	11.59	0.077
28d	1029	1069	1015	1040	22.4	0.391
Weight Gain (g)						
0-7d	93.0	94.3	87.5	88.5	2.57	0.186
7-14d	174.6	194.1	179.8	176.1	5.56	0.070
14-21d	284.1	296.5	281.1	281.7	6.16	0.268
21-28d	434.2	440.7	423.9	451.3	15.59	0.655
0-28d	985.8	1026	972.2	997.4	22.32	0.391
Feed Intake (g)						
0-7d	122.0	127.1	123.2	120.6	3.66	0.636
7-14d	261.4	288.6	274.3	272.7	6.64	0.052
14-21d	438.6	455.8	444.4	432.3	14.65	0.709
21-28d	695.0	698.7	716.7	714.4	16.58	0.729
0-28d	1517	1570	1559	1540	32.8	0.683
Feed Conversion						
0-7d	1.32	1.36	1.41	1.36	0.044	0.545
7-14d	1.50	1.49	1.54	1.58	0.044	0.520
14-21d	1.55	1.54	1.59	1.56	0.033	0.708
21-28d	1.65	1.59	1.70	1.63	0.042	0.381
0-28d	1.57	1.54	1.60	1.63	0.036	0.257

C+, birds supplemented with an enzymatic commercial mixture; CelA, birds supplemented with *C. mixtus*, CelA; CelA-CBM11, birds supplemented with the protein CelA-CBM11; C-, birds not supplemented with an exogenous enzyme.

Exactly the same experiment was repeated (Experiment 2) using a basal diet including the same proportion of ingredients (as described in Table 2.10) but that was prepared using different barley, soybean meal and soybean oil; all other remaining components of the diet had the same origin as in experiment 1. Bird body weight, weight gain, feed intake and feed conversion ratio of experiment 2 are summarized in Table 2.12 (bird mortality was 1.9%).

Table 2.12|Performance of broilers fed on a barley-based diet supplemented with different β-glucanase preparations studied in Experiment 2.

	C-	C+	CelA	CelA CBM11	SEM	p(*F*)
Body Weight (g)						
0d	45.3	44.7	45.2	45.3	0.45	0.759
7d	141.2^{b}	157.3^{a}	132.3^{b}	133.6^{b}	4.73	0.002
14d	365.0^{b}	397.3^{a}	344.9^{b}	351.5^{b}	9.02	0.001
21d	699.2^{b}	750.8^{a}	664.3^{b}	677.1^{b}	16.10	0.003
28d	1172^{b}	1230^{a}	1113^{b}	1154^{b}	23.2	0.009
Weight Gain (g)						
0-7d	96.0^{b}	112.6^{a}	87.1^{b}	88.3^{b}	4.67	0.002
7-14d	223.8	240.1	212.6	218.0	8.60	0.146
14-21d	334.3^{ac}	353.5^{a}	319.4^{bc}	325.6^{bc}	8.10	0.029
21-28d	472.6	479.8	458.4	477.0	11.06	0.538
0-28d	1127^{ac}	1186^{a}	1078^{bc}	1109^{bc}	23.9	0.022
Feed Intake (g)						
0-7d	140.8	148.4	136.0	142.0	6.07	0.551
7-14d	327.1	338.5	322.7	335.2	9.65	0.640
14-21d	591.9	595.6	587.5	612.4	16.17	0.716
21-28d	917.7	877.5	910.1	917.6	21.69	0.513
0-28d	1978	1960	1956	2008	39.0	0.783
Feed Conversion						
0-7d	1.46	1.37	1.56	1.63	0.079	0.117
7-14d	1.48	1.44	1.52	1.55	0.058	0.535
14-21d	1.78	1.69	1.84	1.89	0.058	0.091
21-28d	1.94	1.84	2.00	1.93	0.049	0.158
0-28d	1.76^{ab}	1.66^{b}	1.82^{a}	1.82^{a}	0.038	0.014

C+, birds supplemented with an enzymatic commercial mixture; CelA, birds supplemented with *C. mixtus*, CelA; CelA-CBM11, birds supplemented with the protein CelA-CBM11; C-, birds not supplemented with an exogenous enzyme.

The data revealed that final body weight of birds fed with the basal diet supplemented with the commercial enzyme cocktail (Rovabio™ Excel AP, treatment C+) was significantly higher than birds of the other groups. Differences in body weight were visible as soon as day 7 and remained significant throughout the duration of the experiment. However, differences in weight gain were particularly acute at day 7 revealing that the exogenous enzymes are particularly effective in earlier stages of growth. In addition, there were no differences in feed intake between treatments although feed conversion ratio in animals receiving the fungal enzymes was smaller when compared with the other groups (0-28d). Thus, these data suggest that an improvement in body weight in animals receiving the commercial feed enzymes resulted from a better use of the feed ingredients rather from an increase in feed

intake. Taken together the results revealed that, in contrast to the commercial enzyme mixture, the recombinant enzymes were unable to improve the nutritive value of the barley based diet of experiment 2. In addition, broilers fed with the basal diets of experiments 1 and 2 responded differently to the addition of the fungal exogenous enzymes that were only effective in experiment 2.

It is well known that diets presenting high levels of soluble NSP induce considerable enlargements of some portions of the gastrointestinal tract (Brenes *et al.*, 1993) and stimulated an increase in protein turnover rates (Danicke *et al.*, 2000). Enzyme supplementation decreases digesta viscosity and therefore improves the feed passage rate and nutrient absorption. In addition, the relative weight of the digestive tract decreases leading to an increase in the carcass yield (Fuente *et al.*, 1998; Pettersson & Aman, 1989). Fuente *et al*. (1998) have found an equation relating the empty weight of the digestive tract to digesta viscosity. Therefore, the effects of dietary treatments in the relative length/weight of GI tract compartments of broiler chickens of Experiments 1 and 2 were evaluated. Enzyme supplementation had no effect on crop, gizzard and liver relative weights, on digesta viscosity or on the duodenum, jejunum and caecum relative lengths in experiment 1 (data not shown). In contrast, in experiment 2 jejunum and ileum relative lengths were significantly reduced ($P<0.05$) in birds receiving the commercial enzyme mixture when compared with animals of other treatments (Table 2.13).

Table 2.13|Relative weight and length of the GI tract and viscosity of digesta samples of broilers fed on a barley-based feed supplemented with different exogenous β-glucanases (data from Experiment 2).

	C-	C+	CelA	CelA CBM11	SEM	p(*F*)
Relative Weight (g/100 g BW)						
Crop	0.38[a]	0.29[b]	0.31[b]	0.35[ab]	0.021	0.051
Gizzard	1.37	1.26	1.34	1.33	0.089	0.840
Relative Length (cm/100 g BW)						
Duodenum	2.21	2.18	2.34	2.15	0.069	0.261
Jejunum	6.03[b]	5.34[a]	6.38[b]	6.01[b]	0.181	0.002
Ileum	6.40[ab]	5.94[a]	6.87[b]	6.14[ab]	0.220	0.030
Caecum	1.47	1.33	1.51	1.43	0.054	0.140
Content Viscosity (cP)						
Duodenum+ Jejunum	6.75	5.98	6.63	6.94	0.483	0.919
Ileum	12.5	9.55	12.5	12.1	0.97	0.670

C+, birds supplemented with an enzymatic commercial mixture; CelA, birds supplemented with *C. mixtus*, CelA; CelA-CBM11, birds supplemented with the protein CelA-CBM11; C-, birds not supplemented with an exogenous enzyme.

Thus, exogenous enzymes in treatment C+ contributed to reduce the levels of digestive anti-nutritive β-glucans which through a decrease in viscosity contribute to decrease the size of the digestive compartments. The commercial β-glucanase (treatment C+) although significantly contributing to improve broiler performance, had no impact in the viscosity of the intestinal contents (Table 2.13). Digesta viscosity was determined exclusively at the end of the experimental period, at day 28. At later stages of animal growth, the endogenous microflora might contribute to considerably reduce the chain length of soluble glucans, thus reducing digesta viscosity.

In contrast with previous reports (Guerreiro *et al.*, 2008; Philip *et al.*, 1995a; Ribeiro *et al.*, 2008) that confirmed the capacity of recombinant cellulases Cel5E (Philip *et al.*, 1995a) and *Ct*Lic26A-Cel5E (Guerreiro *et al.*, 2008; Ribeiro *et al.*, 2008) from *C. thermocellum* to improve the nutritive value of barley-based diets for poultry, data presented here suggest that Cel5A from *C. mixtus* is unable to reduce the anti-nutritive effects of soluble glucans present in barley-based diets. One of the major actions of feed β-glucanses is to decrease the degree of polymerization (dp) of β-1,3-1,4-glucans present in barley, through the randomly cleavage of glycosydic bonds of the polysaccharide backbone. The reduction in carbohydrate

chain length contributes to decrease the levels of digesta viscosity (Apajalahti & Bedford, 1999; Bedford & Morgan, 1996; Fengler & Marquardt, 1988). Data presented here suggest that the exogenous recombinant Cel5A derivatives were not catalytically active. Thus, to evaluate the stability of exogenous glycoside hydrolases during passage through the GI tract, β-glucanase activity was qualitatively determined in digesta samples collected in the various digestive compartments of ten animals per treatment. The data, presented in Table 2.14, revealed that the majority of digesta samples collected from birds receiving the commercial enzyme mixture expressed β-glucanase activity. However, the levels of enzyme activity present in the GI tract of birds supplemented with the recombinant enzymes were very small or below the assay detection limit, particularly after the crop. The lower activity of the recombinant enzymes in the crop when compared with animals supplemented with the fungal β-glucanases is difficult to explain considering that the crop presents a milder environment in terms of pH and proteases. It is possible that some degree of enzyme inhibition may have occurred as a result of the presence of specific Cel5A inhibitors in the diet, although this possibility was unexplored under the current work. As it were anticipated, birds not receiving exogenous enzymes only expressed significant levels of β-glucanase activity in the caecum. Thus, the data suggest that although a similar level of exogenous enzymes was added to the basal diets of treatments C+, Cel5A and Cel5A-CBM11, the activity of the recombinant enzymes is significantly reduced after the passage through the proventriculus. This suggests that Cel5A and its recombinant derivatives are particularly sensitive to denaturation under acidic conditions. Assays performed *in vitro* confirmed this hypothesis; the two recombinant derivatives were shown to retain only approximately 10% of their initial activity after incubation for 10 minutes at pH 3.

Taken together the data presented here revealed that the nutritive value of the barley lots used in the 2 experiments differ, since the diets reacted differently to the supplementation with exogenous cellulases. In addition, the recombinant enzymes derived from *C. mixtus* Cel5A are not appropriate for feed supplementation due to their limited stability at lower pHs.

Table 2.14|Qualitative detection of cellulase activity in digesta collected from the gastrointestinal compartments of 40 broilers fed on a barley-based feed, supplemented with recombinant cellulases displaying different molecular architectures (data from Experiment 2).

	C-	C+	CelA	CelACBM11
Crop	+/-/-/+/-/-/-/-/-/-	+/+/+/+/+/+/+/+/+/+	+/++/-/-/+/+/-/-/-	-/-/+/-+/-/+/-/+/+
Gizzard	-/-/-/-/+/-/-/-/-/+	+/+/+/+/-/+/+/+/-/+	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/+/-
Duodenum	-/-/-/+/-/-/-/-/-/-	+/+/-/+/-/-/+/+/+/-	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/-/-
Jejunum	-/-/-/-/-/-/-/-/-/-	+/+/-/-/-/-/+/-/+/+	-/-/-/-/-/-/-/-/-/-	-/-/-/-/-/-/-/-/+/-
Ileum	-/-/-/-/+/+/-/-/-/-	+/-/-/-/+/-/-/-/++	-/-/-/-/-/-/-/-/+/-	-/-/+/--/-/-/-/-/-
Caecum	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+	+/+/+/+/+/+/+/+/+/+

Symbols refer to none (-) or detectable (+) β-glucanase activity. C+, birds supplemented with an enzymatic commercial mixture; CelA, birds supplemented with *C. mixtus*, Cel5A; CelA-CBM11, birds supplemented with the protein Cel5A-CBM11; C-, birds not supplemented with an exogenous enzyme.

2.3.3.2. Different barley lots express different levels of endogenous β-glucanase activity

Data displayed above revealed that the exogenous enzymes of Rovabio™ Excel AP were unable to improve the nutritive value of the basal diet of experiment 1. Since diets of Experiments 1 and 2 contained over 60% of barley but present the same formulation it is suggested that the observed differential response to enzyme supplementation resulted from the different composition of the two barley lots used in experiments 1 and 2. To test this possibility, viscosity, β-glucan and β-glucanase contents of the two barley lots used in the above described experiments were determined. The data revealed that levels of β-glucan and viscosity are higher in the barley lot used in Experiment 2 (Barley of experiment 2 contained 3,44 % of β-glucan and 1,36 cP viscosity, while the barley lot of experiment 1 presented levels of β-glucan and viscosity of 2,26 % and 1,25 cP, respectively). In contrast, the barley lot used in experiment 1 presented almost five times more β-glucanase activity than the barley lot of the second experiment (589 U/Kg and 134 U/kg of β-glucanase activity in barleys of experiments 1 and 2, respectively). Thus, the data suggest that there is a variation in the levels of β-glucan and digesta viscosity but particularly of endogenous β-glucanase activity in the barleys used in experiments 1 and 2. These observed variations may explain the differential response to enzyme supplementation as it will be explored below.

Levels of viscosity, β-glucan and β-glucanase activity were determined in a large range of different barley lots to assess the variation in barley composition in relation to those factors (see Material and Methods section). The data, depicted in Table 2.15, revealed that there is a considerable variation in the levels of β-glucanase activity expressed by the different barley

lots, which ranged from more than 1300 U/kg to less than 60 U/kg (in Table 2.15, barleys B9 and B11 correspond to the cereals used in Experiments 1 and 2, respectively). A regression analysis to test the existence of linear effects between the level of β-glucan and viscosity, between the level of β-glucan and β-glucanase activity, and between viscosity and β-glucanase activity was performed. However, the coefficients of determinations were 0.019, 0.097, and 0.331, respectively, which indicates a low correlation between the variables. However, β-glucanase activity varies much wider than the levels of viscosity and β-glucans. Taken together, these observations suggest that endogenous levels of β-glucanase activity and not exclusively the content in β-glucans may affect the nutritive value of barley for poultry.

Table 2.15|Viscosity (cP) and levels of β-Glucan (%) and β-Glucanase activity (U/kg) in different barley lots.

Barley ID	Variety	β-Glucans	Viscosity	β-Glucanase activity
B1	Scarlett	2.96	1.97	554
B2	Braeman	2.83	1.23	930
B3	Braeman	3.52	1.86	569
B4	Braeman	3.14	1.71	1209
B5	Unk	3.43	1.41	1010
B6	Unk	2.62	2.12	541
B7	Unk	3.01	1.97	221
B8	Unk	2.89	1.53	339
B9	Scarlett	2.26	1.25	589
B10	Unk	2.35	1.23	672
B11	Unk	3.44	1.36	134
B12	Unk	2.50	1.23	1381
B13	Unk	2.80	1.46	1047
B14	Unk	3.02	1.41	1125
B15	Unk	2.50	1.46	1304
B16	Unk	3.24	2.05	174
B17	Unk	2.40	1.20	1202
B18	Unk	3.40	1.61	254
B19	Unk	3.20	1.43	1024
B20	Unk	3.24	1.46	843
B21	Unk	3.08	1.20	1064
B22	Scarlett	2.44	1.43	113
B23	Unk	2.58	1.49	287
B24	Braeman	2.68	1.23	407
W1	Esterel	1.65	2.02	108
W2	Regina	2.75	1.69	97
W3	Wintmalt	3.94	1.46	315
W4	Cartel	3.47	1.69	126
W5	Malice	3.41	1.77	95
W6	Metaxa	4.12	1.71	128
W7	Malicorne	3.57	1.84	123
W8	Manureva	3.87	1.89	113
W9	Melodica	3.62	1.64	60
W10	Violetta	3.91	2.00	66
W11	Vanessa	2.95	2.12	111
CS1	Scarlett	3.73	2.04	61
CS2	Prestige	2.99	2.10	78
CS3	Vivaldi	3.11	2.71	234
CS4	Maltasia	3.27	2.25	150
CS5	Kangoo	3.79	2.27	241
CS6	Jolika	3.06	2.12	1038
CS7	Belgravia	3.34	2.28	157
CS8	Thorgal	3.22	1.99	98
CS9	Cropton	3.07	1.99	296
CS10	Jennifer	2.84	1.82	436
CS11	Signora	2.98	2.22	177
CS12	Calcule	4.38	1.56	188
CS13	Concerto	3.08	2.07	173
CS14	Azalea	3.10	1.64	84
CS15	Primadonna	2.93	1.64	93
CS16	Pewter	3.40	2.04	100
CS17	Margret	3.97	2.33	57
IB1	Quench	3.65	1.84	52
IB2	Clairion	3.60	1.74	267
IB3	Concerto	3.78	2.15	77
IB4	Erlina	3.62	2.27	67
IB5	NFC106-119	3.28	1.87	58
IB6	180/02 5B	3.78	1.77	115
IB7	302/02-G	3.75	2.05	203

IB8	IN0616	3.86	2.68	67
IB9	SYN407-143	1.12	2.50	117
IB10	Charmay	2.39	2.25	133
IB11	Chogun	3.26	1.66	151
IB12	Parigus	3.67	1.38	111

Table 2.15 |Viscosity and levels of β-Glucan and β-Glucanase activity in different barley lots (continuation). Barley B9 was used in Experiment 1; Barley B11 was used in Experiment 2; Barleys B13 and B23 were used in Experiment 3. The Unk varieties are mixtures of several barley varieties. Barley ID with "B" refers to Portuguese barleys; barley ID with a "W" refers to European winter barleys; barley ID with a "CS" refers to European spring barleys and barley ID with a "IB" refers to Iberian spring barleys.

2.3.3.3. Levels of endogenous β-glucanase activity affect the nutritive value of barley based diets for poultry

Data presented above suggest that levels of endogenous plant β-glucanases in barley may affect the efficacy of exogenous cellulases used to supplement barley-based diets for poultry. To test this possibility two different barley lots were selected for a comparative study aiming at evaluating the capacity of an exogenous cellulase mixture to improve the nutritive value of barley-based diets with different levels of endogenous plant enzymes for broilers. The two barley lots selected were B13 (presenting High β-glucanase Activity; HA) and B23 (presenting Low β-glucanase Activity; LA) with barley B13 displaying approximately 4 times more β-glucanase activity than barley B23. In contrast, the viscosity and levels of β-glucan were similar in barleys B13 and B23 (Table 2.15). The chemical composition of the two selected barley lots was similar although levels of NDF were higher in barley HA (Table 2.16).

Table 2.16|Chemical composition of the two barleys used in experiment 3 (% DM).

	Barley	
Chemical composition	**Barley HA (B13)**	**Barley LA (B23)**
DM (%)	11.65	13.66
GE (MJ/kg DM)	16.30	16.05
CP (%)	9.15	9.00
Ash (%)	2.32	1.90
NDF (%)	25.73	20.35
ADF (%)	4.99	5.70
ADL (%)	1.56	1.57

Thus, the formula of Table 2.10 was used to produce two different barley-based diets using either barley B13 (diet HA) or B23 (diet LA). The two basal diets, *per se* or supplemented with the commercial enzyme mixture Rovabio™ Excel AP, were used to feed broiler chicks till day 28 as described in Material and Methods section (Experiment 3). The results of

Experiment 3, expressed as final body weight, weight gain, feed intake and feed conversion ratios are summarized in Table 2.17 (bird mortality was 3.6%). Final body weight of birds fed with the HA basal diet were significantly higher than that of animals fed with the LA diet. In contrast with what was observed in the two preceding experiments, final body weight of birds fed HA diets was much similar to what is expected in standard commercial conditions, confirming that barley B13 displayed a higher nutritive value when compared with the other barleys used in this study. The addition of exogenous β-glucanases had no impact in the weight gain of animals receiving the HA diet. In contrast, addition of exogenous enzymes to diet LA significantly improved bird final body weight, although birds did not reach the final weight of birds receiving the HA diets. There were no variations in feed intake among the four groups suggesting that barley source and exogenous enzymes lead to different efficiencies of nutrient utilization rather to an increase/decrease of feed intake. This was confirmed by analyzing the data on feed conversion ratios, which were substantially smaller for the groups receiving the HA diet and the group receiving the LA diet supplemented with the exogenous enzymes, when compared with the animals receiving the LA basal diet not supplemented with microbial enzymes. Taken together the data suggest that levels of endogenous β-glucanase activity may have a major impact on the nutritive value of barley-based diets. Hence, addition of exogenous β-glucanases may be only effective in the case of barley-based diets containing lower levels of endogenous plant cell wall degrading activity, such as barley B23.

Table 2.17|Performance of broilers fed on two different barley-based diets supplemented or not with a commercial β-glucanase preparation (data from Experiment 3).

	HA-	HA+	LA-	LA+	SEM	p(*F*)
Body Weight (g)						
0d	48.1	47.7	47.7	48.2	0.25	0.423
7d	154.5^{ba}	159.7^{a}	131.2^{c}	144.2^{b}	4.93	0.001
14d	404.2^{a}	421.3^{a}	293.9^{c}	361.3^{b}	11.46	<0.0001
21d	784.7^{a}	811.5^{a}	576.7^{c}	693.3^{b}	21.92	<0.0001
28d	1323^{a}	1305^{a}	992.7^{c}	1164^{b}	30.9	<0.0001
Weight Gain (g)						
0-7d	106.4^{ab}	111.9^{b}	84.0^{c}	96.1^{ac}	5.01	0.002
7-14d	249.7^{a}	261.2^{a}	162.3^{b}	217.3^{c}	7.74	<0.0001
14-21d	378.9^{c}	390.2^{c}	282.9^{a}	332.1^{b}	12.83	<0.0001
21-28d	533.6^{c}	493.1^{c}	415.9^{a}	470.6^{b}	14.13	<0.0001
0-28d	1275^{c}	1257^{c}	945.0^{a}	1116^{b}	30.92	<0.0001
Feed Intake (g)						
0-7d	141.7	144.3	153.0	132.9	10.20	0.585
7-14d	412.1	394.4	348.5	373.3	24.52	0.305
14-21d	721.1^{b}	681.8^{ab}	596.4^{a}	662.4^{ab}	37.42	0.141
21-28d	986.5	925.8	854.7	867.9	55.62	0.332
0-28d	1958	1888	1953	1875	79.3	0.831
Feed Conversion						
0-7d	1.27^{a}	1.25^{a}	1.83^{b}	1.40^{a}	0.063	<0.0001
7-14d	1.50^{a}	1.47^{a}	2.23^{b}	1.72^{a}	0.095	<0.0001
14-21d	1.75^{a}	1.70^{a}	2.15^{b}	2.01^{b}	0.086	0.002
21-28d	1.69^{a}	1.82^{a}	2.08^{b}	1.86^{ab}	0.090	0.033
0-28d	1.67^{a}	1.66^{a}	2.09^{b}	1.84^{a}	0.068	0.0002

HA+, barley-based diet displaying high endogenous β-glucanase activity supplemented with a enzymatic commercial mixture of cellulases, HA-, barley-based diet displaying high endogenous β-glucanase activity not supplemented with a enzymatic commercial mixture of cellulases. LA+, barley based diet displaying low endogenous β-glucanase activity supplemented with a enzymatic commercial mixture of cellulases; LA-, barley based diet displaying low endogenous β-glucanase activity not supplemented with a enzymatic commercial mixture of cellulases.

Notwithstanding the suggested implications of endogenous plant enzymes in the effectiveness of exogenous enzymes used to supplement barley diets, it is clear that diets used in experiment 3 may have presented unaccounted variations that may have affected the nutritive value of the two barley lots. A clear prove for the concept would be possible by comparing the nutritive value of a barley batch similar to B13 previously and after specifically inactivating the endogenous enzymes, for example through heating. However, although it can be anticipated that endogenous plant β-glucanases are thermolabile, it is clear that a relative thermostability exists since diets used in this study were subjected to pelleting where

temperatures can reach temperatures of 80 ºC. However, it is also expected that a considerable degree of enzyme-substrate protection exists which contributes to enhance the stability of the endogenous enzyme (Fontes *et al.*, 1995).

The effects of the different dietary treatments in the relative length or weight of different organs and GI tract compartments of broiler chickens of Experiment 3 were evaluated and the respective data are presented in Table 2.18. The data revealed lower relative lengths and weights for the duodenum, jejunum and ileum of birds receiving the HA diet when compared with birds of the LA treatment. In addition, supplementation of LA diet with β-glucanase activity had the ability to reduce organ sizes. As discussed above, these data are in agreement with results reported by several other authors who have shown that exogenous enzymes decrease digestive viscosity affecting the digestive tract weight and/or length when expressed as a percentage of live weight (Brenes *et al.*, 1993; Petersen *et al.*, 1993; Viveros *et al.*, 1994). Digesta viscosity in the hindgut and foregut of birds fed the different dietary treatments was measured and the data are presented in Table 2.18. Duodenum and jejunum viscosities were higher in digesta samples from birds receiving the LA diet not supplemented with exogenous enzymes when compared with the three other groups. Therefore, the data suggest that endogenous and exogenous enzymes have a similar capacity to reduce the dp of soluble glucans thus contributing for a considerable reduction in digesta viscosity. Since only one animal per pen was slaughtered for sample collection, as a result of logistic limitations, these values and those reported for experiments 1 and 2 should be viewed with some caution considering that variation among animals for the measured variables can be significantly high.

Table 2.18|Relative weight and length of the GI tract and viscosity of digesta samples of broilers fed on two barley-based diets supplemented or not with exogenous β-glucanases (data from Experiment 3).

	HA-	HA+	LA-	LA+	SEM	p(*F*)
Relative Weight (g/100 g BW)						
Crop	0.35	0.31	0.39	0.35	0.023	0.106
Gizzard	1.60^{ab}	1.71^{ac}	1.47^{bcd}	1.30^{d}	0.087	0.014
Relative Length (cm/100 g BW)						
Duodenum	2.09^{ab}	1.99^{a}	2.72^{c}	2.21^{b}	0.075	<0.0001
Jejunum	5.50^{a}	5.45^{a}	6.88^{b}	5.62^{a}	0.230	0.0002
Ileum	5.53^{a}	5.46^{a}	7.53^{b}	5.77^{a}	0.227	<0.0001
Caecum	1.27^{a}	1.26^{a}	1.60^{b}	1.39^{a}	0.053	0.0002
Content Viscosity (cP)						
Duodenum+Jejunum	3.46^{b}	2.81^{b}	8.61^{a}	3.60^{b}	0.437	<0.0001
Ileum	6.51^{b}	7.84^{b}	4.99^{b}	12.7^{a}	0.853	0.002

HA+, barley-based diet displaying high endogenous β-glucanase activity supplemented with a enzymatic commercial mixture of cellulases, HA-, barley-based diet displaying high endogenous β-glucanase activity not supplemented with a enzymatic commercial mixture of cellulases. LA+, barley based diet displaying low endogenous β-glucanase activity supplemented with a enzymatic commercial mixture of cellulases; LA-, barley based diet displaying low endogenous β-glucanase activity not supplemented with a enzymatic commercial mixture of cellulases.

Various authors have suggested that a range of factors such as animal age, microbial challenge, cereal genotype, cereal growing conditions or cereal storage length (Fuente *et al.*, 1998; Svihus & Gullord, 2002), may contribute to the reduction of effectiveness of exogenous β-1,3-1,4-glucanases used to supplement poultry diets. However, the molecular mechanisms underlying this lack of response, in a variety of circumstances, remain unknown. It is clear that if levels of β-glucans are smaller the resulting digesta viscosity will be lower and consequently enzymes have no margin to function (Campbell *et al.*, 1989; Choct, 2006). In addition, it is known that cereal genotype, growing conditions and storage length affect the levels of barley β-glucan. Data presented here revealed that barley may also express significant but variable levels of endogenous β-glucanases. The plant enzymes seem to be active under the conditions of the GI tract and thus contribute to a significant depolymerisation of the anti-nutritive β-glucans. For example, plant β-glucanases of barley B13 were sufficient to decrease duodenal viscosity of non-supplemented birds to the levels observed in animals supplemented with the exogenous enzyme. Thus, endogenous

enzymes may contribute to affect microbial enzyme effectiveness by reducing substrate availability to the exogenous enzymes. The role of endogenous β-glucanases in plant metabolism remains relatively unexplored. During germination of barley grains, the cell walls of the starchy endosperm are degraded by β-1,3-1,4-glucanases. This allows the amylases and proteases secreted from the surrounding aleurone or scutellar tissues to reach their starch and storage protein substrates inside the endosperm cells (Fincher *et al.*, 1986). The activation of β-glucanase activity most possibly contributes to improve the nutritive value of soaked or germinated barley (Svihus *et al.*, 1997). In these examples, the activity of the endogenous enzymes reduces the dp of the anti-nutritive beta-glucans resulting in an improvement of the diet nutritive value. However, data presented here suggest that the basal levels of expression of barley cellulases and β-1,3-1,4-glucanases before the germination process starts may vary and can reach significant values in a range of barley lots.

The molecular structure of barley cellulases remains to be identified. In addition, the barley genome sequence, which could reveal the complete set of plant cell wall hydrolase genes encoded by this cereal, is presently unknown. However, work developed in the last 20 years lead to the cloning of two β-1,3-1,4-glucanases encoding genes (Fincher *et al.*, 1986). In addition, 12 Expressed Sequence Tags (EST), which contain plant cell wall hydrolase genes, were identified in various plant tissues (data obtained from NCBI, GenBank). Cellulase and β-1,3-1,4-glucanase activities are of outmost importance during the germination of cereal grains to allow the degradation of cell walls of the nonliving starchy endosperm (Fincher *et al.*, 1986). However, levels of expression of genes encoding plant cell wall hydrolases in barley grains are presently unknown, since most studies have been performed in germinating seeds. Therefore, more work to characterize the expression profiles of genes encoding plant cell wall degrading enzymes in grains, particularly of β-1,3-1,4-glucanases and cellulases, is required. Differences in the expression pattern of the different genes should be evaluated with cereal lots from different origins. The collected data may contribute to the development of novel targets for barley breed improvement. Clearly the development of barley varieties expressing higher levels of endogenous cellulase activity would contribute to improve the nutritive value of the corresponding barley-based diets for monogastric animals. The development of novel barley varieties with increased β-1,3-1,4-glucanase activity could eventually contribute to preclude the requirement for feed supplementation of barley-based diets in monogastric nutrition.

2.3.4. Conclusions

Data presented here revealed that endogenous β-glucanases may be present in moderate levels in barley seeds and may remain active throughout the GI tract, thus contributing to reduce digesta viscosity and consequently the effectiveness of exogenous cellulases used to supplement poultry feed. In addition, the results also revealed that levels of endogenous β-glucanases vary largely between different barley lots, a variation that is more pronounced than the levels of β-glucans. Therefore, a variable response to enzyme supplementation may result, among other factors, from the variable levels of β-glucanase activity present in barley grains. The implementation of a quick assay to detect the levels of both barley β-glucan and β-glucanase may help rationalizing the supplementation of poultry feed with exogenous enzymes.

3. STRUCTURE OF A TYPE C CBM

3.1. FAMILY 42 CARBOHYDRATE-BINDING MODULES DISPLAY MULTIPLE ARABINOXYLAN-BINDING INTERFACES PRESENTING DIFFERENT LIGAND AFFINITIES

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Abstract

Interactions between carbohydrates and proteins play a key role in numerous biological processes. In general, enzymes that degrade plant cell wall polysaccharides display a modular architecture comprehending a catalytic domain bound to one or more non-catalytic carbohydrate-binding modules (CBMs). CBMs display considerable variation in primary structure and are grouped into 60 sequence-based families organized in the Carbohydrate-Active enZYme (CAZy) database. Here we report the crystal structure of CBM42A together with the biochemical characterization of two other members of family 42 CBMs from *Clostridium thermocellum*. CBM42A, CBM42B and CBM42C bind specifically to the arabinose side-chains of arabinoxylans and arabinan. The structure of CBM42A displays a beta-trefoil fold, which comprises 3 sub-domains designated as α, β and γ. Each one of the three sub-domains presents a putative carbohydrate-binding pocket where an aspartate residue located in a central position dominates ligand recognition. Intriguingly, only the γ sub-domains of CBM42A, CBM42B and CBM42C are required for binding arabinoxylan. In contrast, the recognition of arabinan by CBM42B and CBM42C seems to depend on the cooperative action of the β and γ sub-domains. The binding mechanism revealed by CBM42 is in contrast with that of the homologous CBM13, in which the recognition of complex polysaccharides is the result of the cooperative action of the three protein sub-domains.

3.1.1. Introduction

Plant cell wall polysaccharides represent the most abundant reservoir of organic carbon within the biosphere. Recycling of photosynthetically fixed carbon through the action of microbial plant cell wall hydrolases is, therefore, a fundamental biological process that has recently acquired considerable industrial importance (Himmel & Bayer, 2009). Development of second generation bio-fuels derived from lignocellulosic biomass highlights the need to dissect and understand the biological processes that result in the production of soluble sugars from plant cell wall structural polysaccharides. It is well established that the complex and intricate nature of plant cell walls restrict the access of enzymes to their target substrates, primarily cellulose and hemicellulose. To overcome their limited accessibility to plant carbohydrates, microbial cellulases and hemicellulases have acquired complex molecular architectures generally comprising catalytic domains and non-catalytic carbohydrate-binding modules (CBMs). The primary role of CBMs is to target the appended catalytic module to the proximity of its substrate, thereby potentiating catalysis and reducing the accessibility constrains (Boraston *et al.*, 2004). Carbohydrate modifying enzymes and their associate modules, which include CBMs, have been classified into sequence-based families in the CAZy database (Cantarel *et al.*, 2009). Currently there are 58 families of CBMs (October 2009) which recognize a variety of microbial, plant and mammalian glycans. Based on the topology of the carbohydrate-binding site, which complements the conformation of the target ligand, CBMs have been classified into three types (Boraston *et al.*, 2004). Thus, in type A modules, which interact with the flat surfaces of crystalline polysaccharides, the binding site comprises a planar hydrophobic platform that contains three exposed aromatic amino acids (Raghothama *et al.*, 2000). These CBMs show no significant affinity for soluble polysaccharides and the ligand specificity of CBM families that contain type A modules is, usually, invariant. In contrast, type B and type C CBMs recognize single carbohydrate chains either internally or at the termini, respectively, and present a ligand specificity that reflects the substrate specificity of the appended catalytic domain (Boraston *et al.*, 2003b; Charnock *et al.*, 2000a; Szabo *et al.*, 2001). Structural studies revealed that type B and C CBMs accommodate their target ligands in clefts or pockets, respectively (Boraston *et al.*, 2004; Montanier *et al.*, 2009b; Najmudin *et al.*, 2006a).

The three-dimensional structure of most CBMs conform to a β-sandwich fold in which a single ligand-binding site lies in a cleft located on the concave surface of the protein (Boraston *et al.*, 2004). Ligand plasticity in CBMs built on a β-sandwich platform usually results from subtle variations at the binding interface that confer capacity to accommodate heterogeneity in the composition and linkage of the sugar backbone *per se* or in the branches that may decorate the carbohydrate polymers (Henshaw *et al.*, 2004; Pires *et al.*, 2004). In contrast, a variety of CBMs have evolved the capacity to recognize their target

ligands at multiple binding sites, as exemplified by members of families CBM13 and CBM42, which assume a β-trefoil fold (Miyanaga *et al.*, 2006; Notenboom *et al.*, 2002). These modules, which are typical type C CBMs, show a sequential 3-fold internal repeat of ~45 amino acid residues comprising three sub-domains, denoted as α, β and γ, each one containing a discrete ligand binding site. Although CBM13 and CBM42 are built on a similar scaffold, the ligand-binding sites of the two structurally related families assume different topologies and locations within the protein. The three type C binding interfaces of CBM13 and CBM42 have a pocket-like topology, which is particularly suited to recognize small sugars (Fujimoto *et al.*, 2002; Miyanaga *et al.*, 2004). Surprisingly, members of CBM13 found in xylanases were shown to display a higher degree of affinity and specificity for insoluble xylan (Notenboom *et al.*, 2002; Scharpf *et al.*, 2002); subtle variations in the ligand-binding sites allow the α, β and γ sub-domains to bind cooperatively three different xylan strands of the insoluble macromolecule. In addition, members of family CBM42, were shown to bind specifically to the arabinose side-chain of arabinoxylans, while not interacting directly with the xylan backbone individually (Ichinose *et al.*, 2008; Miyanaga *et al.*, 2006). Both CBM13s found in xylanases and CBM42s located in arabinofuranosidases were shown to promote the activity of the appended catalytic domains against insoluble xylans (Ichinose *et al.*, 2008; Miyanaga *et al.*, 2006).

Clostridium thermocellum produces a remarkably complex functional nanomachine, termed the cellulosome, which efficiently degrades plant cell wall polysaccharides (Bayer *et al.*, 2004; Carvalho *et al.*, 2003). Cellulosome assembly results from the interaction of type I dockerin domains, present in cellulosomal cellulases and hemicellulases, and the cohesin domains of a large non-catalytic integrating protein, termed CipA, which acts as a molecular scaffold (Carvalho *et al.*, 2007; Pinheiro *et al.*, 2008). CipA contains a family 3 CBM that binds crystalline cellulose, thus anchoring the enzyme complex onto the plant cell wall (Tormo *et al.*, 1996). In addition, most cellulosomal enzymes also contain CBMs that bind a variety of carbohydrates allowing the individual catalytic units to interact with their specific target substrates (Bayer *et al.*, 2004). Inspection of *C. thermocellum* proteome revealed the presence of three CBM42 modules in cellulosomal enzymes. Cellulosomal CBM42s are specifically associated with enzymes containing GH43 catalytic domains. Here we report the structural and biochemical characterization of *C. thermocellum* cellulosomal CBM42s. The structure of one of these proteins was solved and was used to inform a mutagenesis study on the ligand specificity of the various CBM42. The data suggest that cellulosomal CBM42 display a restricted specificity for arabinose side-chains located on complex polysaccharides. In addition, the various CBM42 sub-domains recognize the target ligand with distinct affinities.

3.1.2. Material e Methods

3.1.2.1. Cloning, protein expression and purification

Genes encoding the family 42 CBM modules of *C. thermocellum* Cthe_0015 (CBM42A, residues 26 to 170), Cthe_2138 (CBM42B, residues 19 to 166) and Cthe_2139 (CBM42B, residues 477 to 614) were amplified through PCR from *C. thermocellum* YS genomic DNA, using the primers listed in Table 3.1. The PCR employed the thermostable DNA polymerase NZYPremium (NZYTech Ltd) and the forward and reverse primers contained engineered *Nhe*I and *Xho*I or *Nhe*I and *Sal*I restriction sites, respectively (Table 3.1). Amplified DNA was directly cloned into pNZY28 (NZYTech Ltd) and sequenced to ensure that no mutations were accumulated during the amplification. The genes were subsequently sub-cloned into *Nhe*I and *Xho*I restricted pET21a (Novagen) generating pCBM42A, pCBM42B, and pCBM42C, respectively. All recombinant derivatives contained a C-terminal His_6-tag. *Escherichia coli* BL21 DE3 cells harbouring all the recombinant expression vectors were cultured in Luria-Bertani broth at 37 $^{\circ}$C to mid-exponential phase (A_{600nm} 0.6) and recombinant protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside and incubation for a further sixteen hours at 25 $^{\circ}$C. The His_6-tagged recombinant proteins and their generated mutant derivatives (see below), were purified from cell-free extracts by immobilized metal ion affinity chromatography (IMAC) as described previously (Carvalho *et al.*, 2004; Pinheiro *et al.*, 2009). Purified proteins were buffer exchanged into 20 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 5 mM $CaCl_2$. SDS/PAGE showed that all the recombinant proteins were more than 95 % pure. For crystallization, CBM42A was further purified by size exclusion chromatography. Following IMAC, the protein was buffer exchanged into 50 mM Hepes-HCl buffer, pH 7.5, containing 200 mM NaCl and 2 mM $CaCl_2$ and then subjected to gel filtration using a HiLoad 16/60 Superdex 75 column (GE Healthcare) at a flowrate of 1 ml/min. Purified CBM42A was concentrated using an Amicon-10 kDa molecular weight centrifugal concentrator and washed three times into 2 mM $CaCl_2$.

Table 3.1|Primers used to obtain the genes encoding CBM42A, CBM42B, CBM42C and its respective mutant derivatives.

Engineered restriction sites and mutation points are depicted in bold.

Protein	Primer Sequence (5'→3')	Direction
CBM42A	CTC**GCTAGC**TCAACTAACCCGATAAC	FOR
	CAC**CTCGAG**TTGAGTATCCTCGCTG	REV
CBM42A_D41A	GAGAATGTCACGCCGGAAATG**GCG**TCCCAGTGGGAGTTGGTTCCG	FOR
	CGGAACCAACTCCCACTGGGA**CGC**CATTTCCGGCGTGACATTCTC	REV
CBM42A_S91A	GGAACTTCACTTTTTGCGGAA**GCG**GCAACATTTAAAATAGTACCC	FOR
	GGGTACTATTTTAAATGTTGC**CGC**TTCCGCAAAAAGTGAAGTTCC	REV
CBM42A_Y121A	CCCACAAGATATATAAGGCAT**GCG**AATTACTTATTAAGATTG	FOR
	CAATCTTAATAAGTAATT**CGC**ATGCCTTATATATCTTGTGGG	REV
CBM42A_D138A	GTGACAGAGCTTGACAGACAG**GCG**GCAACCTTTAAAATAATCAGC	FOR
	GCTGATTATTTTAAAGGTTGC**CGC**CTGTCTGTCAAGCTCTGTCAC	REV
CBM42AA26Y	CTAATATGTACATAAGACAT**TAC**AATTTTGATGCAAGGATAGAC	FOR
	GTCTATCCTTGCATCAAAATT**GTA**ATGTCTTATGTACATATTAG	REV
CBM42A_F28Y	GTACATAAGACATGCAAAT**TAC**GATGCAAGGATAGACGAG	FOR
	CTCGTCTATCCTTGCATC**GTA**ATTTGCATGTCTTATGTAC	REV
CBM42A_A26YF28Y	CCTAATATGTACATAAGACAT**TAC**AAT**TAC**GATGCAAGGATAGACGAG	FOR
	CTCGTCTATCCTTGCATC**GTA**ATT**GTA**ATGTCTTATGTACATATTAGG	REV
CBM42A_S73Y	GGGTATTACTTAAGGCAT**TAC**AATTATGATTTAAGCCTG	FOR
	CAGGCTTAAATCATAATT**GTA**ATGCCTTAAGTAATACCC	REV

CBM42A_S91D	CTTCACTTTTTGCGGAA**GAC**GCAACATTTAAAATAG TACCC	FOR
	GGGTACTATTTTAAATGTTGC**GTC**TTCCGCAAAAAG TGAAG	REV
CBM42B	CTC**GCTAGC**ACGGGTGCCGATGGTGCTATA	FOR
	CAC**CTGGAG**ACTGTCAATCACTCTGAATGT	REV
CBM42B_D41A	GATAATGTTACACCGGAAACA**GCG**GCCCAATGGGT GCTTGTTCCC	FOR
	GGGAACAAGCACCCATTGGGC**CGC**TGTTTCCGGT GTAACATTATC	REV
CBM42B_D91A	CCAGAATTTTTGCGGAG**GCG**GCAACATTTAAAATG GTTCCG	FOR
	CGGAACCATTTTAAATGTTGC**CGC**CTCCGCAAAAA TTCTGG	REV
CBM42B_D138A	GCCCTTGACAGAGAG**GCG**GCCACATTCAGAGTG	FOR
	CACTCTGAATGTGGC**CGC**CTCTCTGTCAAGGGC	REV
CBM42C	CTC**GTCGAC**ACCCTTGACGGTGGTGTT	FOR
	CAC**GTCGAC**ATCGCTGATTATTAAAAAGG	REV
CBM42C_D41A	GAAAACGTAACTCCTCTGGAA**GCG**TCACAATGGAG GCTGGTTCCG	FOR
	CGGAACCAGCCTCCATTGTGA**CGC**TTCCAGAGGA GTTACGTTTTC	REV
CBM42C_D91A	CCACAATTTTTGCTGAG**GCG**GCAACCTTTAAACTG GTTCCG	FOR
	CGGAACCAGTTTAAAGGTTGC**CGC**CTCAGCAAAAA TTGTGG	REV
CBM42C_D138A	CCGATCTGGACAGGCAG**GCG**GCAACCTTTTTAATA ATCAGC	FOR
	GCTGATTATTAAAAAGGTTGC**CGC**CTGCCTGTCCA GATCGG	REV

3.1.2.2. Source of sugars used

All soluble polysaccharides were purchased from Megazyme International (Bray, County Wicklow, Ireland), except oat spelt xylan, laminarin and hydroxyethylcellulose (HEC), which were obtained from Sigma. Avicel (PH101) was obtained from Serva, while acid-swollen cellulose was prepared as described previously (Najmudin *et al.*, 2006).

3.1.2.3. Mutagenesis

Site-directed mutagenesis was carried out using the PCR-based NZYMutagenesis site-directed mutagenesis kit (NZYTech Ltd) according to the manufacturer's instructions, using DNA of plasmids pCBM42A, pCBM42B and pCBM42C as templates. The sequence of the primers used to generate these mutants is displayed in Table 3.1. The mutated DNA sequences were sequenced to ensure that only the appropriate mutations had been incorporated into the nucleic acids.

3.1.2.4. Affinity Gel Electrophoresis

The affinity of CBM42A, CBM42B and CBM42C and respective mutant derivatives for a range of soluble polysaccharides was determined by affinity gel electrophoresis (AGE). The method used was essentially that described by Tomme *et al.* (2000), using the polysaccharide ligands at a concentration of 0.1 % (w/v), unless stated otherwise. Electrophoresis was carried out for 4 h at room temperature in native 10% (w/v) polyacrylamide gels. The non-binding negative control reference protein was bovine serum albumin (BSA). Quantitative assessment of binding was carried out as described previously (Takeo, 2004), using polysaccharide concentrations ranging from 0.002 to 0.1% (w/v). Briefly, the migration distances of the CBMs and the reference protein were measured from the bottom of the protein bands evident on the gels and these data were used to determine the dissociation constants (K_D) from plots of $1/(R_0-r)$ versus $1/C$, according to the affinity equation shown in Equation 1:

$$1/(R_0-r)=1/(R_0-R_C)(1+K_D/C) \qquad \text{(Eq. 1)}$$

Where r is the relative migration distance of the CBM in the presence of ligand in the gel, R_0 is the relative migration distance of the free CBM in the absence of ligand, R_C is the relative migration distance of the complex at high excess of ligand where all CBM molecules are fully complexed, C is the concentration of the ligand in the gel and K_D is the dissociation constant of the CBM for the macromolecular ligand. K_D values were determined as the inverse of the absolute value of the intercept on the abscissa of data plotted according to the affinity equation.

3.1.2.5. Crystallization and Data Collection

Crystallization of CBM42A was performed using the hanging-drop, vapour-diffusion method. Initial crystallization conditions were determined using Crystal Screens I and II from Hampton Research, with drops of 1 µl of 40 mg/ml protein and 1 µl of precipitating agent. Small crystals grew in the presence of sodium acetate (pH 4.6) and sodium formate and further experiments were performed in order to improve crystal quality. The optimized crystallization conditions contained 0.1 M sodium acetate and 2.1 M sodium formate and crystals with 0.1 × 0.1 mm were obtained within two weeks. Crystals were cryo-cooled with 30% glycerol prior to data collection and diffracted beyond 1.7 Å resolution. A complete data set was collected at beamline ID29 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Data were integrated using MOSFLM (Leslie, 1992) in $P3_221$ space group, with cell constants $a = b = 106.37$ Å, $c = 237.56$ Å and scaled with SCALA (Kabsch, 1988) from the CCP4 site (COLLABORATIVE COMPUTATIONAL PROJECT NUMBER 4, 1994), to a maximum resolution of 1.8 Å with 99.9% completeness. Statistical data are summarized in Table 3.2. Matthews (Matthews, 1968) coefficient calculations suggested the presence of several molecules in the asymmetric unit, from six (3.55 $Å^3$/Da and 65% of solvent content) up to nine (2.36 $Å^3$/Da and 48% of solvent content).

Table 3.2|Data collection and structure statistics

Crystal		
Space Group		$P3_221$
Unit cell parameters (Å) a = b, c		106.370, 237.564
Mathews parameter ($Å^3$/Da)		2.93
Data collection statistics		
X-ray source		ESRF, ID29
Wavelength (Å)		0.976
No. of observed reflections		1414710
No. of unique reflections		144547 (20870)
Resolution limits (Å)		45.22 – 1.80 (1.90 – 1.8)
Completeness (%)		99.9 (99.8)
Redundancy		9.8 (9.2)
Multiplicity		9.8 (9.2)
Average I/σ(I)		19.8 (4.9)
Rsym (%)		0.089 (0.381)
Refinement statistics		
Resolution limits (Å)		45.22 - 1.80
R-factor (%) (No. of reflections)		0.169 (144547)
R-free (%) (No. of reflections)		0.197 (7239)
No. protein residues in the asymmetric unit		1103
No. water molecules in the asymmetric unit		864
No. atoms in the asymmetric unit		10307
rmsd bond length (Å)		0.013
rmsd bond angles (°)		1.416
Average temperature factor ($Å^2$)	protein main chain atoms	7.1
	protein side chain atoms	9.0
	water molecules	15.7
	Ca^{2+}	29.6
Ramachandran plot	Residues in most favoured regions (%)	87.6
	Residues in additionally allowed regions (%)	11.6
	Residues in generously allowed regions (%)	0.8
Overall G-factor		0.01

3.1.2.6. Structure determination and refinement

The number and position of the molecules in the asymmetric unit were progressively found by Program PHASER (McCoy *et al.*, 2007), using as a search model the CBM42 module of the α-L-arabinofuranosidase from *Aspergillus kawachii* (*Ak*CBM42, pdb accession code 1WD3). The model was first edited by CHAINSAW to remove non-conserved residues and side-chains and the high similarity between the two proteins (36% of sequence identity) allowed the program to find a total of eight molecules in the asymmetric unit. The *Ak*CBM42 model was first edited by CHAINSAW (Stein, 2008) to remove non-conserved residues and side-chains. The solution of each run was inputted in the next search and an increase in the rotation and translation z-score was observed in each step. With all the molecules in the asymmetric unit located, density modification, together with non-crystallographic symmetry averaging, was performed with NCSREF and the final electron density maps showed clear solvent boundaries and good quality electron density that allowed model building (figure of merit of 0.7). ARP/wARP (Perrakis *et al.*, 1999) was used to automatically build the protein model. Model completion, editing and initial validation were carried out in COOT (Emsley & Cowtan, 2004).

Restrained refinement of the molecular model was done using REFMAC 5.5 (Murshudov *et al.*, 1997) and water molecules were added using COOT. Apart from the first six to nine residues and the last five to six residues, excluding the six histidine tag, all atoms in the protein could be properly assigned and refined. The final model contains 1103 amino acid residues, belonging to eight polypeptide chains and 864 water molecules. In the last cycle of refinement, temperature factors were refined isotropically for protein atoms and anisotropically for the calcium atoms. R-work and R-free converged to 15.8 % and 19.2 %, respectively, and geometrical validation was carried out by PROCHECK (Laskowski *et al.*, 1993), STAN (Kleywegt & Jones, 1996; Nayal & Di Cera, 1996; Weiss & Hilgenfeld, 1999) and MOLPROBITY (Davis *et al.*, 2007). According to these programs, the final model contains 99.2% of the residues in mostly favored and allowed regions of the Ramachandran plot and only 0.8% of the residues in generously allowed regions of the plot. Refinement details are summarized in Table 3.2.

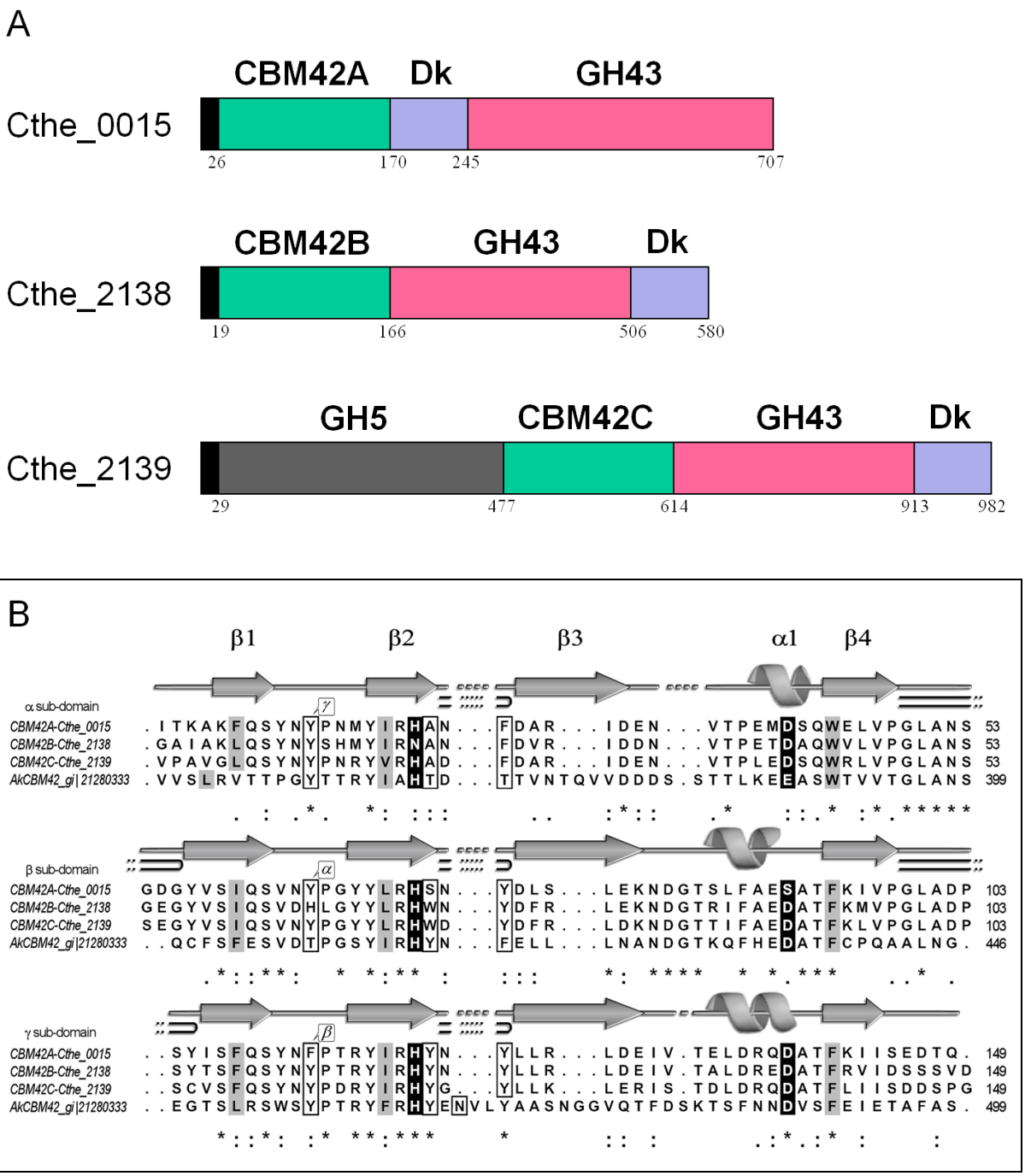
3.1.3. Results and Discussion

3.1.3.1. *C. thermocellum* family 42 cellulosomal CBMs bind to arabinoxylan

C. thermocellum proteome presents 72 polypeptides containing type I dockerins, which allowed assigning those proteins as cellulosomal components (Pinheiro *et al.*, 2009). Inspection of the primary sequence of those enzymes revealed that proteins with accession numbers Cthe_0015, Cthe_2138 and Cthe_2139 contain family 42 CBMs. The three

enzymes are putative GH43 arabinofuranosidases, although Cthe_2139 contains an additional GH5 catalyic module of unknown function (Figure 3.1, panel A).

Figure 3.1|Modular architecture of *C. thermocellum* enzymes containing CBM42 modules (panel A) and structural alignment of CBM42s (panel B).



The secondary structure displayed for each sub-domain is based on CBM42A structure. Three beta hairpins separate each β2 and β3 pair, with two additional ones between the α-β and β-γ sub-domains. The grey highlighted residues compose the hydrophobic nucleus. Crucial residues for ligand binding are highlighted with inverted colors and black boxes surround the residues providing an aromatic stacking environment. Residues in positions 18, 65 and 113, involved in aromatic stacking, display an extra α, β or γ symbol informing to which binding pocket they belong.

The primary sequences of the three cellulosomal CBM42s were aligned with the previously characterized family 42 CBM (*Ak*CBM42) of *A. kawachii* arabinofuranosidase GH54 (Miyanaga *et al.*, 2004; Miyanaga *et al.*, 2006), termed *Ak*Abf54 (Figure 3.1, panel B). *Ak*CBM42 was shown to bind the non-reducing end of the arabinose side-chains of arabinoxylan. Asp435 and Asp 488, located in *Ak*CBM42 binding pockets β and γ, respectively, form two pivotal hydrogen bonds with the O-2 and O-3 atoms of arabinose and thus play a key role in ligand recognition (Miyanaga *et al.*, 2006). Mutation of these two conserved residues of *Ak*CBM42 leads to a significant reduction in the arabinofuranosidase activity of the associated GH54 catalytic module against insoluble arabinoxylan (Miyanaga *et al.*, 2006). In addition, His416 (pocket β) and His463 (pocket γ) form an additional hydrogen bond with the O-5 atom of the arabinose moiety. The pocket of *Ak*CBM42 sub-domain α was, apparently, non-functional. Alignment of CBM42 domains, presented in Figure 3.1, revealed that all cellulosomal CBM42 display a strong conservation at the putative residues involved in ligand recognition. However, while CBM42B sub-domain α contains an asparagine instead of the conserved histidine, the β sub-domain of CBM42A lacks the conserved aspartate that is replaced by a serine. To investigate the biological function of the cellulosomal CBM42 modules, recombinant forms of the proteins were expressed in the soluble form in *E. coli* and purified to electrophoretic homogeneity. The capacity of CBM42A, CBM42B and CBM42C to bind a range of polysaccharides was assessed by affinity gel electrophoresis. The data (Table 3.3) show that all three CBM42 present similar ligand specificities and bind, preferentially, arabinoxylan and arabinan. The data reveal a lower affinity for oat spelt xylan, a less substituted carbohydrate. In contrast, the CBMs were unable to interact with insoluble forms of cellulose (data not shown). Thus, cellulosomal CBM42 domains comprise functional CBMs with a restricted specificity to arabinose containing polysaccharides. Homology shared by CBM42A, CBM42B and CBM42C with *Ak*CBM42 suggest that the identified CBMs will recognize the arabinose side chains of the complex hemicelluloses.

Table 3.3|Polysaccharide specificity of *C. thermocellum* CBM42A, CBM42B and CBM42C determined by affinity polyacrilamide gel electrophoresis

Ligand[a]	CBM42A[b]	CBM42B[b]	CBM42C[b]
Hydroxyethylcellulose (HEC)	-	-	-
Lichenan (Icelandic moss)	-	-	-
Laminarin	-	-	-
Crudlan	-	-	-
B-Glucan	-	-	-
Oat-spelt Xylan	+	+	+
Wheat arabinoxylan	+++	+++	++
Ferulic Xylan	++	++	++
Glucuronoxylan	-	-	-
Xyloglucan (Tamarind)	+	+	+
Mannan	-	-	-
Galactomannan	-	-	-
Galactomannan (Gal:Man, 21:79) (Carob)	-	-	-
Galactomannan (Gal:Man, 38:40) (Guar)	-	-	-
Galactan (Potato)	-	-	-
Arabinogalactan	+	+	+
Galactan (Lupin)	-	-	-
Arabinan	++	++	+++
Glucomannan	-	-	-
Rhamnogalacturonan (Soya bean pectic fibre)	-	-	-
Rhamnogalacturonan I	-	-	-
Pectic galactan (Potato)	-	-	-
Pustulan	-	-	-
Pullulan	-	-	-

[a] Ligands were screened at a concentration of 0.1 mg/ml.

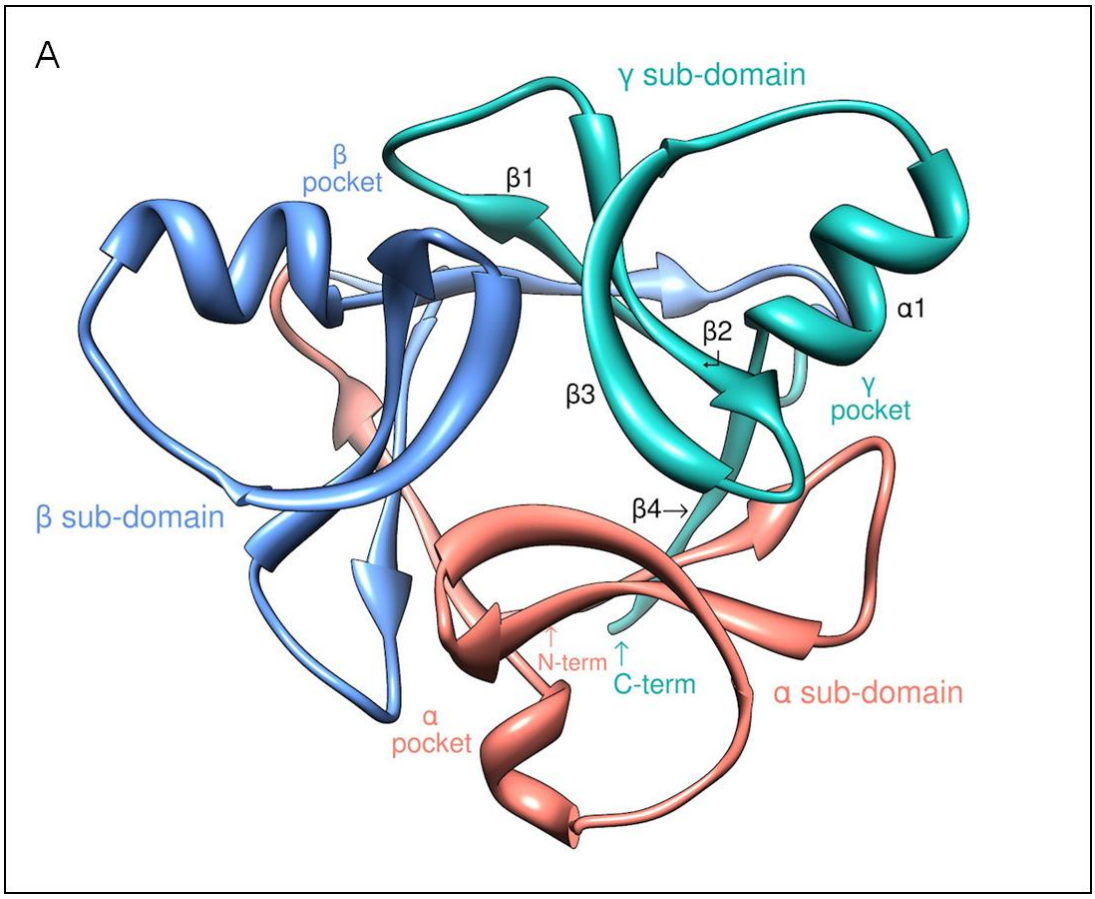
[b] No detectable binding, -; significant binding, ++; strong binding +++.

3.1.3.2. The crystal structure of CBM42A

To gain further insights into the molecular determinants of ligand specificity within CBM42 domains that recognize arabinoxylan, the crystal structure of CBM42A was determined. In common with members of CBM13 and CBM42 families, CBM42A structure adopts a β-trefoil architecture and can be divided into three discrete sub-domains (α, β and γ), each with approximately 45 residues (Figure 3.2, panel A). Each sub-domain is built up by four β-strands (β_1 to β_4) and one α-helix located between strands β_3 and β_4. The three sub-domains are involved in the formation of a central six-stranded antiparallel β-barrel capped by a triangular hairpin triplet. The structure has an internal three-fold axis and the three sub-

domains are very homologous, exhibiting *ca* 20% of sequence identity (Figure 3.1, panel B) and a rmsd of 0.76-1.22 Å upon superposition (Figure 3.2, panel B). Inspection of the primary sequence alignment and the structural overlay of the three pockets located in the CBM42A sub-domains (Figure 3.2, panel B) suggest that there are some important differences in the nature of key residues that decorate the putative ligand binding sites. Regarding the residues responsible for hydrogen bonding, CBM42A has three histidines distributed in the different pockets of the sub-domains.

Figure 3.2|Structure of *C. thermocellum* CBM42A.



In panel A, CBM42A β-trefoil architecture, seen from the triangular hairpin triplet face, is displayed revealing its internal three-fold pseudo-symmetry. The three trefoil sub-domains, each composed of four β strands (β1, β2, β3 and β4) and one α-helix (α1), are distinctively colored: α sub-domain (salmon), β sub-domain (blue) and γ sub-domain (green). β1 and β4 strands of each sub-domain compose the sides of a β-barrel structure. Each sub-domain contributes with a β-hairpin (β2-β3) to the triangular hairpin triplet structure, which is located at the bottom of the β-barrel.

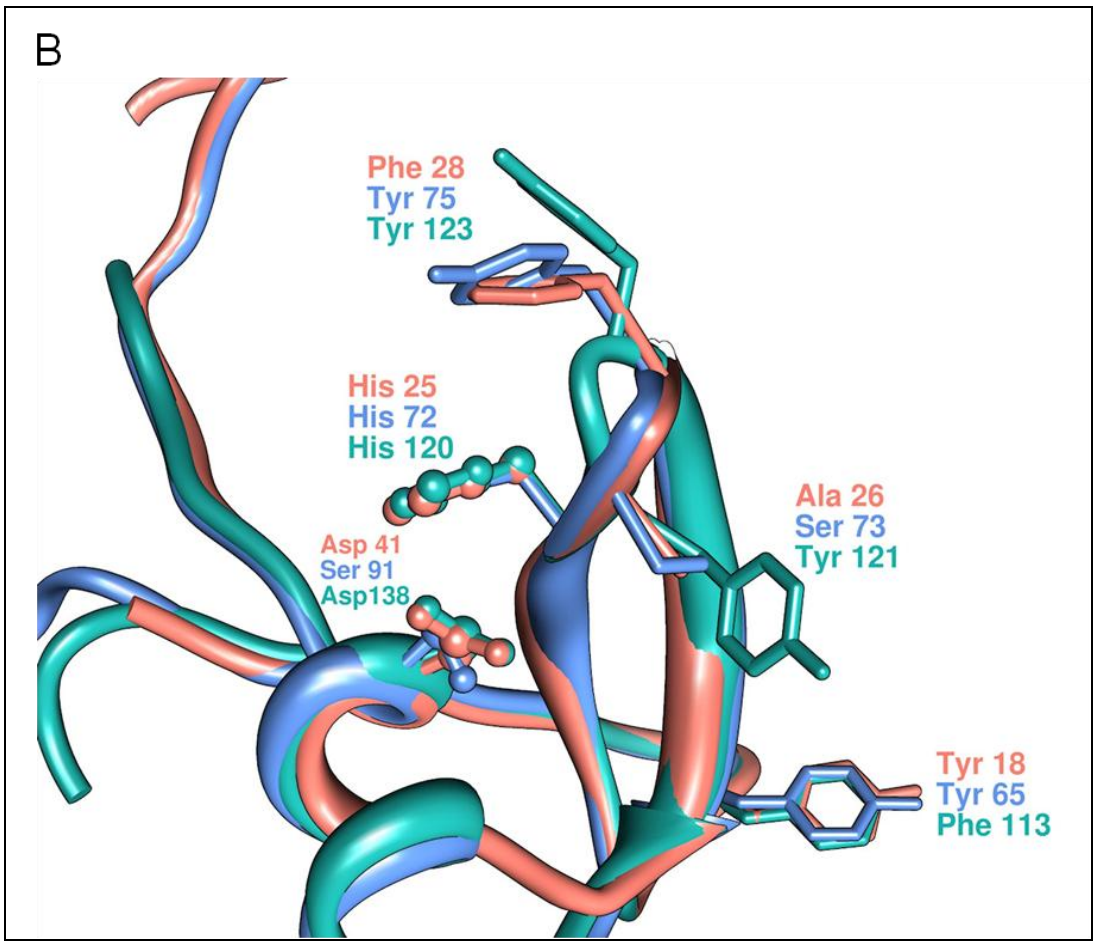
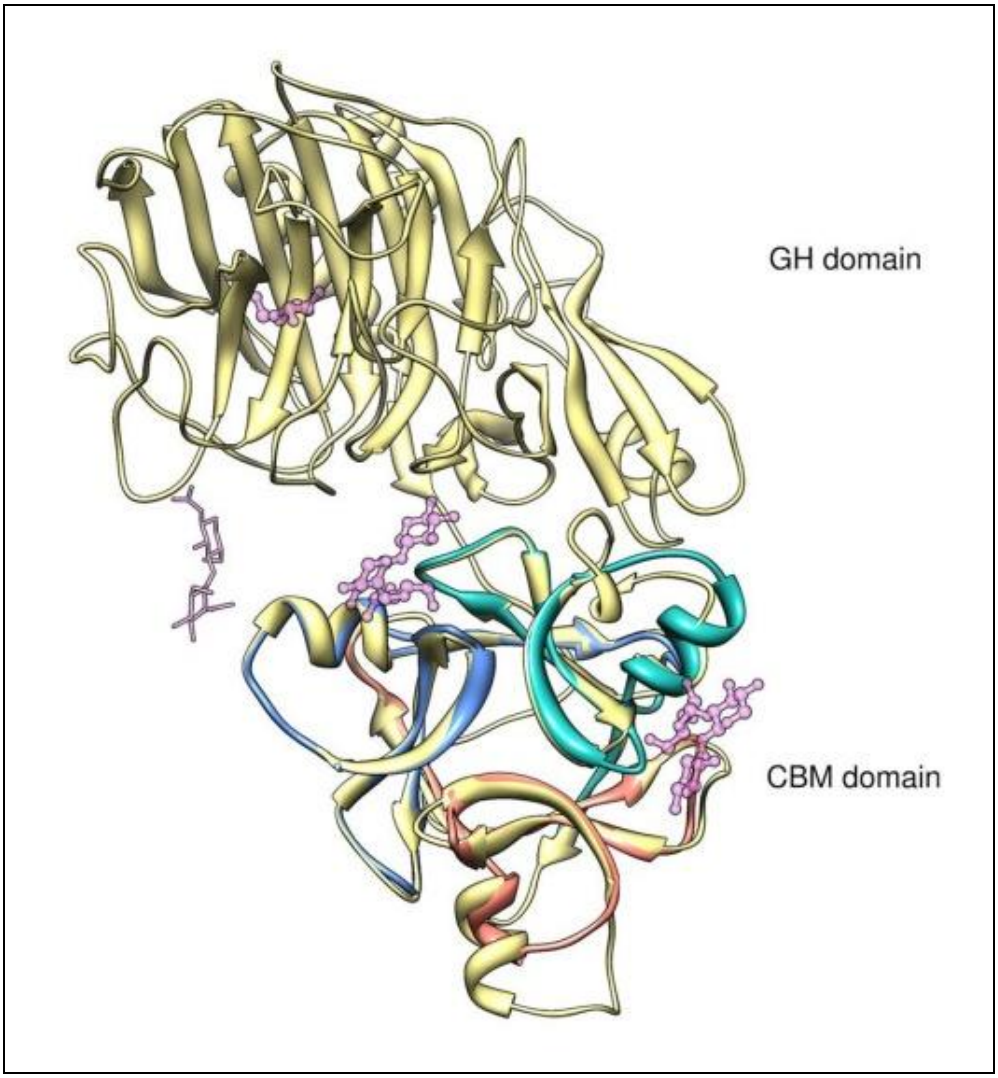


Figure 3.2 (continuation)| Structure of *C. thermocellum* CBM42A.

In panel B, superposition of CBM42A α (salmon), β (blue) and γ (green) sub-domains (rmsd Cα 0.75Å, 92%) showing the residues putatively involved in modulating stacking environment (stick representation) and ligand binding (ball & stick). The picture was prepared using Chimera (Pettersen *et al.*, 2004).

As mentioned previously, only two aspartates are present in the expected binding sites of the sub-domains since a serine is in position 91 of the β pocket. The aromatic stacking effect accomplished by an aromatic residue triad is complete in pocket γ but not in pockets α and β where a tyrosine is substituted by Ala26 and Ser73. The relevance of the referred residues for ligand recognition was analysed by comparing the architecture of the three pockets of cellulosomal CBM42A with those of *Ak*CBM42 (Miyanaga *et al.*, 2006).

Figure 3.3|Overlay of CBM42 modules.

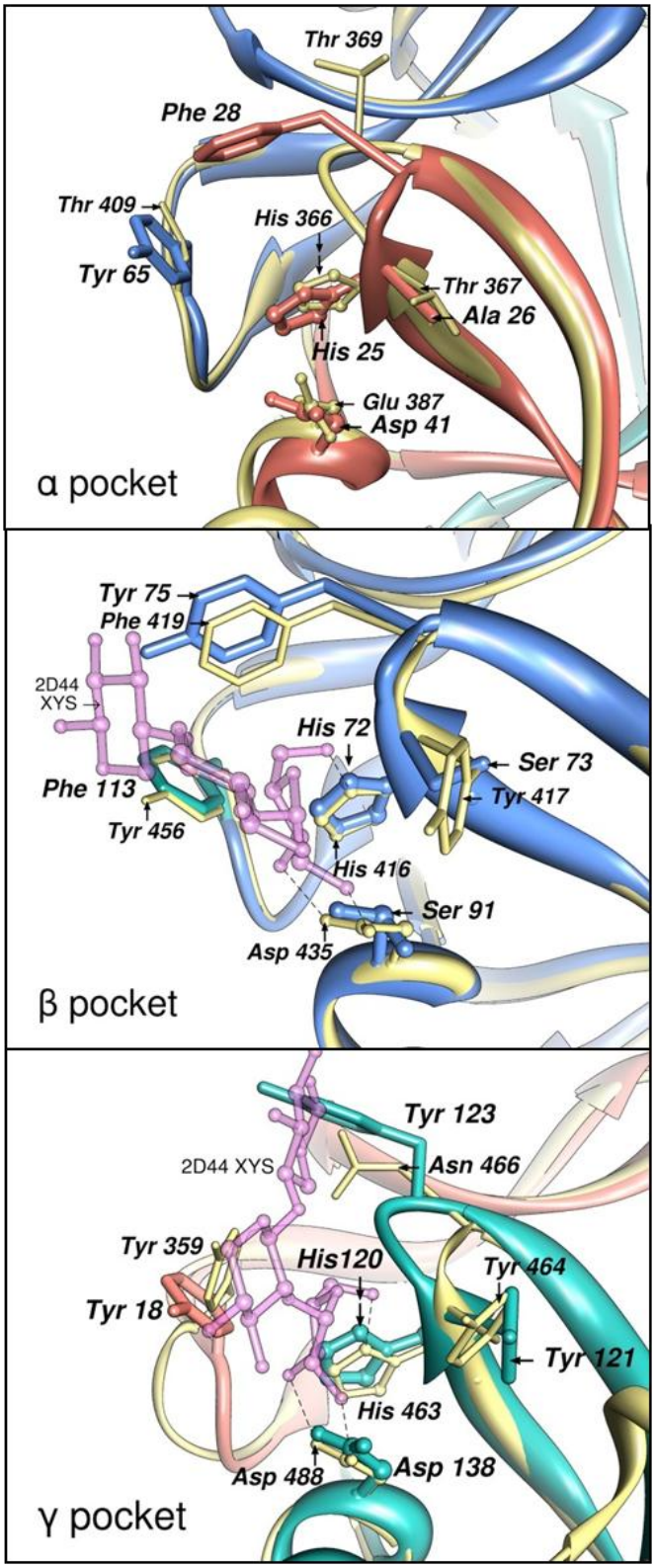


Superposition of *Ak*Abf54 (2D44) (yellow) with CBM42A (rmsd Cα 0.75Å, 92%). The later has the three trefoil sub-domains distinctively colored: α sub-domain (salmon), β sub-domain (blue) and γ sub-domain (green). The GH54 arabinofuranosidase module (*Ak*GH54) is displayed with an α-L-arabinofuranose sugar (purple, ball & stick) in the active site (position derived from 1WD4 model). Two arabinofuranosyl-α-1,2-xylobiose molecules (purple, ball & stick) are also observed attached to *Ak*CBM42 β and γ sub-domains. Furthermore, the glycosylation site of the GH54 domain (Asn202-Ser203-Thr204) is covalently bound to a N-acetylglucosamine disaccharide (purple, stick). The picture was prepared using Chimera (Pettersen *et al.*, 2004).

The structure of *Ak*CBM42 was solved at 1.75 Å resolution in conjunction with the appended GH54 arabinofuranosidase catalytic domain of *Ak*Abf54. Superposition of CBM42A and *Ak*CBM42 yields a low rmsd value of 0.75 Å for 92% of Cα atoms (125 residues out of 136 for molecule A) Figure 3.3)). The minor differences between the two structures involve the length of some of the loops connecting the β-strands. Also, *Ak*CBM42 contains two cysteine residues involved in a disulfide bond which are not present in CBM42A, although this difference has no consequence in the overall arrangement of the structures (Figure 3.3). The structural comparison between CBM42A and *Ak*CBM42 binding sites based on the overlay of the three CBM42 sub-domains, is illustrated in Figure 3.4. Even though the two proteins are very similar, details of the three sub-domains show important differences regarding the

constitution of the binding pockets. Considering the γ sub-domain, Asp138, His120, Tyr121 and Tyr18 are approximately in the same positions as the corresponding residues of *Ak*CBM42. The distance separating the phenolic oxygen atoms of the tyrosines is slightly larger in CBM42A, 12.5 Å, than in *Ak*CBM42, 10.2 Å. In addition, CBM42A has a third tyrosine residue, Tyr123, in the vicinity of the binding cleft. This residue, at 14.9 Å and 8.5 Å from the two other tyrosines, is confining the ligand binding arrangement and putatively influencing the site specificity. In the β sub-domain, the two CBM42 have a similar arrangement although Phe419 of *Ak*CBM42 is closer to the neighbouring tyrosines, decreasing the cavity volume. As mentioned previously, CBM42A β sub-domain lacks the crutial Asp91. In addition, the β sub-domain does not have the complete aromatic residue triad, since Tyr75 and Phe113 are not co-adjuvated by a third hydrophobic residue, replaced here by Ser73. In the α sub-domain, a reduction in the aromatic stacking effect is observed in both CBM42s since one of the tyrosine residues has been replaced by a threonine in *Ak*CBM42 and an alanine in CBM42A. The conserved aspartate residue necessary for ligand binding and present in CBM42A α sub-domain, is missing in *Ak*CBM42 α sub-domain. The importance of each of the described residue differences for ligand recognition by CBM42A will be further exploited below.

Figure 3.4|Overlay of the three pockets of CBM42 modules.

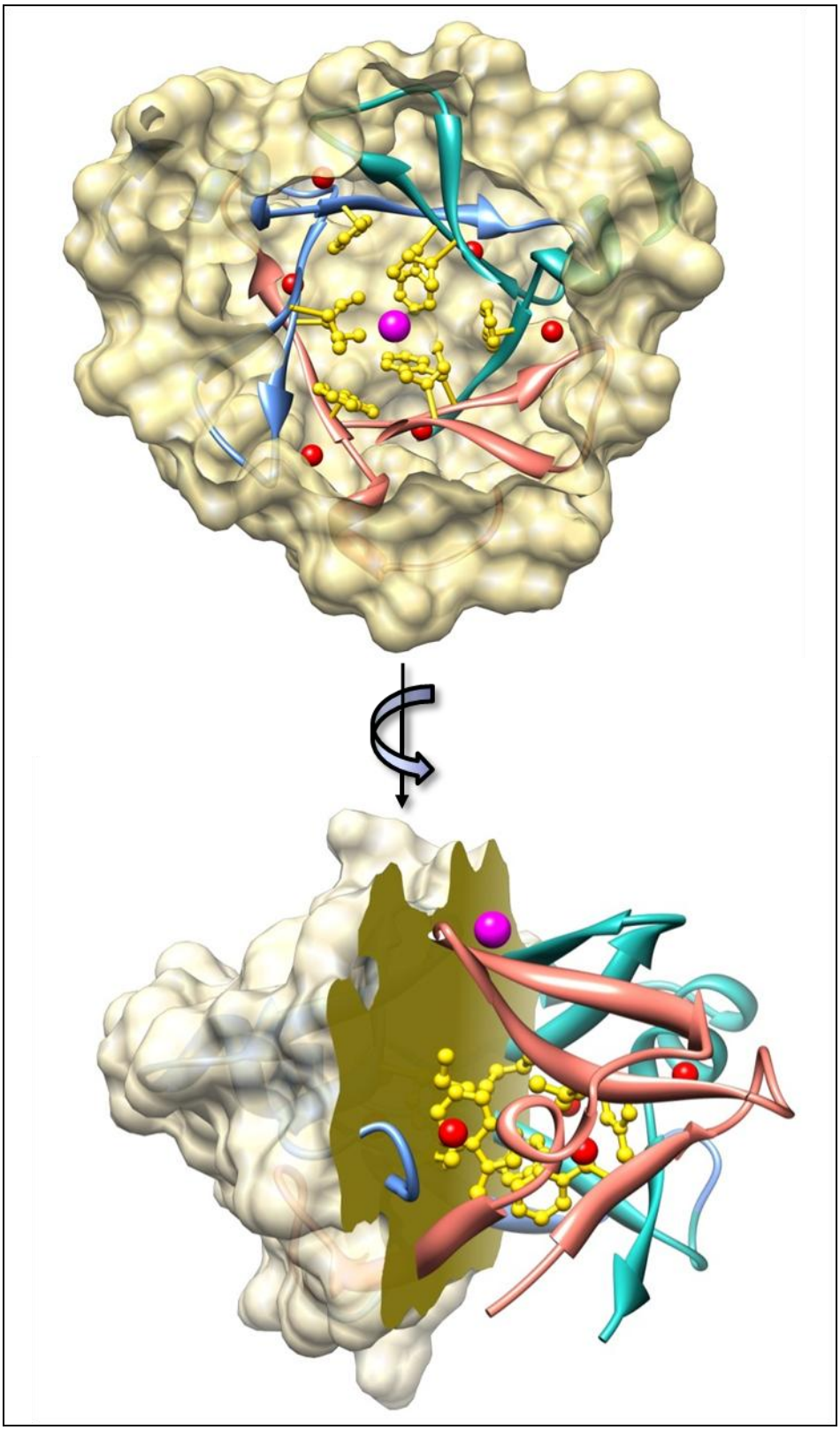


CBM42A (colored as in Figure 3.2) and *Ak*CBM42 (yellow) α, β and γ putative carbohydrate binding sites are overlaid. Residues putatively involved in modulating stacking environment are showed as “stick” and ligand binding residues as “ball & stick” representation. β and γ pockets display the 2D44 based arabinotriose ligands (AHR - alpha-L-arabinofuranose). The picture was prepared using Chimera (Pettersen *et al.*, 2004).

3.1.3.3. Identification of structural ions in CBM42A

*Ak*CBM42 displays a hydrophobic nucleus in the centre of the β-trefoil. This nucleus is composed of several leucine, isoleucine, phenylalanine residues and a tryptophan residue, belonging to the three sub-domains, with the side chains oriented to the core of the trefoil. CBM42A also displays a similar feature in its overall structure (Figure 3.5). However, at the bottom of the β-barrel and opposite to this hydrophobic core, a calcium ion with octahedral coordination has been identified in the eight molecules of the asymmetric unit of CBM42A crystal structure. The calcium ion is coordinated by Ala30, Leu77 and Leu125 main chain carbonyls and by Asp29 and Asp76 side chains and one water molecule. These residues belong to the triangular hairpin triplet cap and hold the calcium atom exactly in the centre of the cap at the protein surface. Although calcium is not present in the crystallization conditions, after the purification procedure the protein was maintained in a 2 mM $CaCl_2$ solution. Calcium ions are known to have a structural role in various CBM families and are usually not directly implicated in ligand recognition. However, functional calcium ions that mediate carbohydrate binding have been described in several CBMs with a β-sandwich fold. Several recent studies have shown that this metal ion does contribute to sugar binding in CBMs where the target carbohydrate(s) is accommodated within the loops connecting the β-sheets (Jamal *et al.*, 2004; Montanier *et al.*, 2009a). It is the first time a calcium ion is observed in a member of CBM42 where, due to its internal position within the protein, calcium is likely to fulfil a structural role. Another interesting feature in CBM42A is the presence of water molecules buried in the protein (Figure 3.5). In each sub-domain, two waters are found in these conditions, hydrogen bonded to main chain/side chain atoms, separated by one aromatic residue of the hydrophobic nucleus, and apart from each other by *ca* 7.6 Å. These waters, forty eight in total, are well conserved in the eight protein molecules of the asymmetric unit, with a very low B factor. These solvent molecules have no accessible surface area and might be important for CBM42 fold. Their proximity to the conserved histidine residue, which is involved in polysaccharide binding, suggests a putative contribution to constrain the binding pocket concavity.

Figure 3.5|Top and side view of CBM42A hydrophobic nucleus, calcium ion and structural waters.



The nine hydrophobic centre residues are depicted in yellow. The calcium ion (pink) is located at the center of the hairpin triplet cap, which was removed from the top view. Each sub-domain also features two structural water molecules (red). The picture was prepared using Chimera (Pettersen *et al.*, 2004).

3.1.3.4. Mapping CBM42 ligand binding sites by mutagenesis

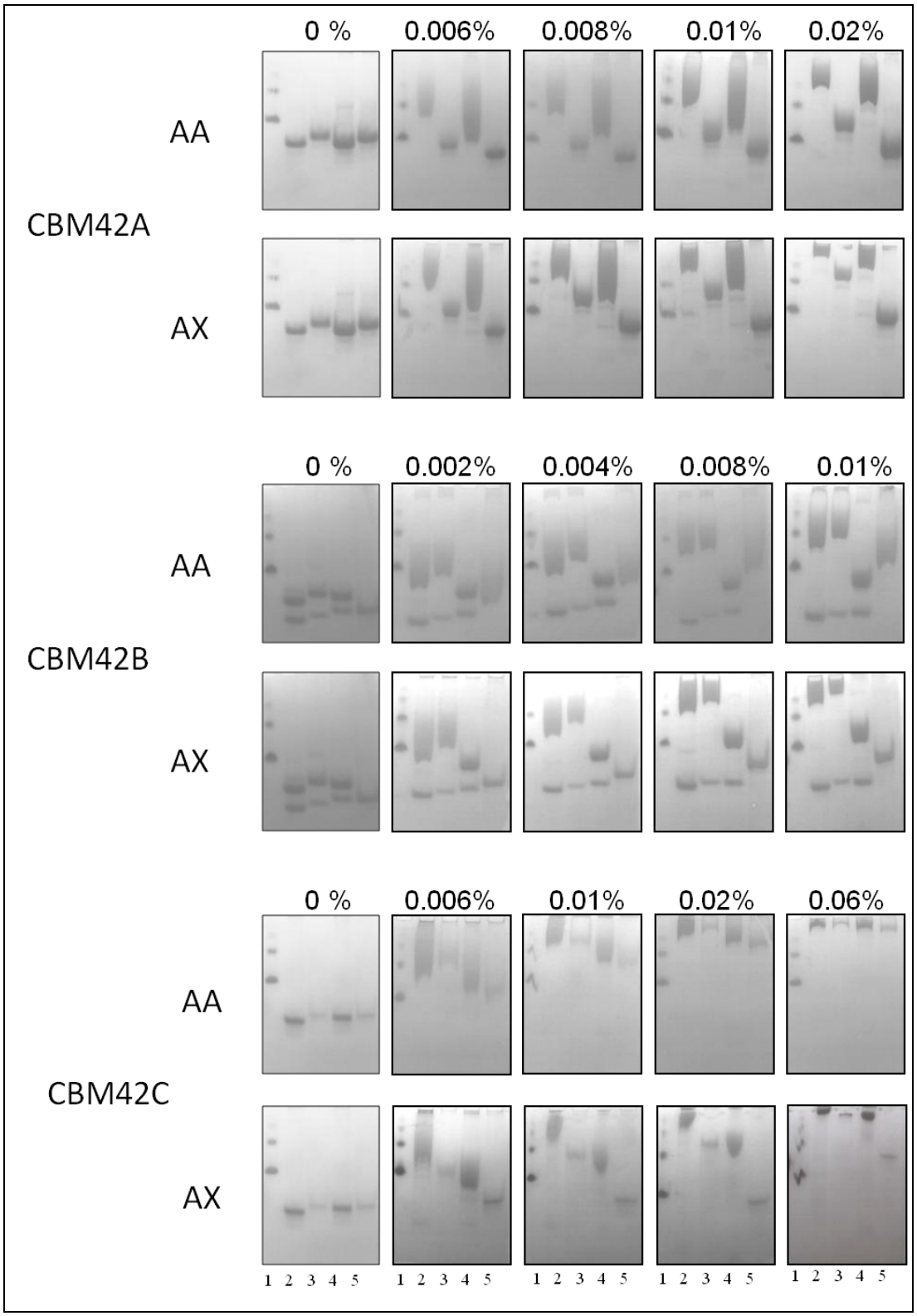
As described above, inspection of the three putative pockets of CBM42A, CBM42B and CBM42C reveals the presence in each of the three protein sub-domains of a highly conserved aspartate residue that was suggested to play a pivotal role in ligand recognition (Figure 3.1, panel B). To evaluate the importance of this residue for arabinan and arabinoxylan binding by the various CBM42s, mutant derivatives of CBM42A, CBM42B and CBM42C at each of the three sub-domains were produced and their biochemical properties compared to the wild type protein. The data for CBM42A, presented in Table 3.4 and Figure 3.6, revealed that mutation of Asp138 located in the γ pocket abolishes binding to both arabinoxylan and arabinan. Mutation of Ser91 (sub-domain β) resulted in a modest decrease on the affinity of CBM42A against both polysaccharides, while the change of acid residue at the α sub-domain leads to a considerable reduction in affinity. Thus, the data suggest that Asp138 dominates ligand recognition by sub-domain γ and CBM42A. Mutation of Tyr121, which potentially establishes a hydrophobic platform for stacking arabinose residues, had no effect in ligand affinity (Table 3.4). To identify the residue substitutions implicated in the reduced affinity expressed by CBM42A α and β sub-domains, Ala26 and Phe28 in sub-domain α and Ser73 and S91 in sub-domain β were replaced by the overlapping residues present in sub-domain γ (Figure 3.1, panel B), which are tyrosines except for Ser91 which is the conserved aspartate. These mutant derivatives were generated using CBM42A D138A mutant protein derivative, which has a non-functional γ sub-domain. Analysis of the affinities of the resulting mutant derivatives for both arabinan and arabinoxylan revealed that S91D mutation was able to partially restore the affinity of the D138A mutant thus suggesting that lack of an aspartate residue in the β pocket is responsible for its reduced affinity. Addition of a tyrosine in order to create an aromatic triad in this pocket was ineffective to improve ligand affinity. The same mutations at the α sub-domain have no effect in ligand recognition suggesting that potentially other unidentified topological differences are responsible for the impaired affinity revealed by the two sub-domains.

Table 3.4|Affinity of *C. thermocellum* cellulosomal CBM42 modules and their mutant derivatives for arabinoxylan and arabinan

Protein	Domain	K_a ($\%^{-1}$)	
		Arabinan	Arabinoxylan
CBM42A		114	135
CBM42A_D41A	Alpha	14	18
CBM42A_S91A	Beta	64	72
CBM42A_D138A	Gamma	NB	NB
CBM42A_Y121A	Gamma	128	155
CBM42A_D138A_A26Y	Alpha	NB	NB
CBM42A_D138A_F28Y	Alpha	NB	NB
CBM42A_D138A_A26YF28Y	Alpha	NB	NB
CBM42A_D138A_S73Y	Beta	NB	NB
CBM42A_D138A_S91D	Beta	81	109
CBM42A_D138A_S73YS91D	Beta	59	57
CBM42B		122	145
CBM42B_D41A	Alpha	112	162
CBM42B_D91A	Beta	21	29
CBM42B_D138A	Gamma	59	17
CBM42C		222	134
CBM42C_D41A	Alpha	224	78
CBM42C_D91A	Beta	102	26
CBM42C_D138A	Gamma	99	12

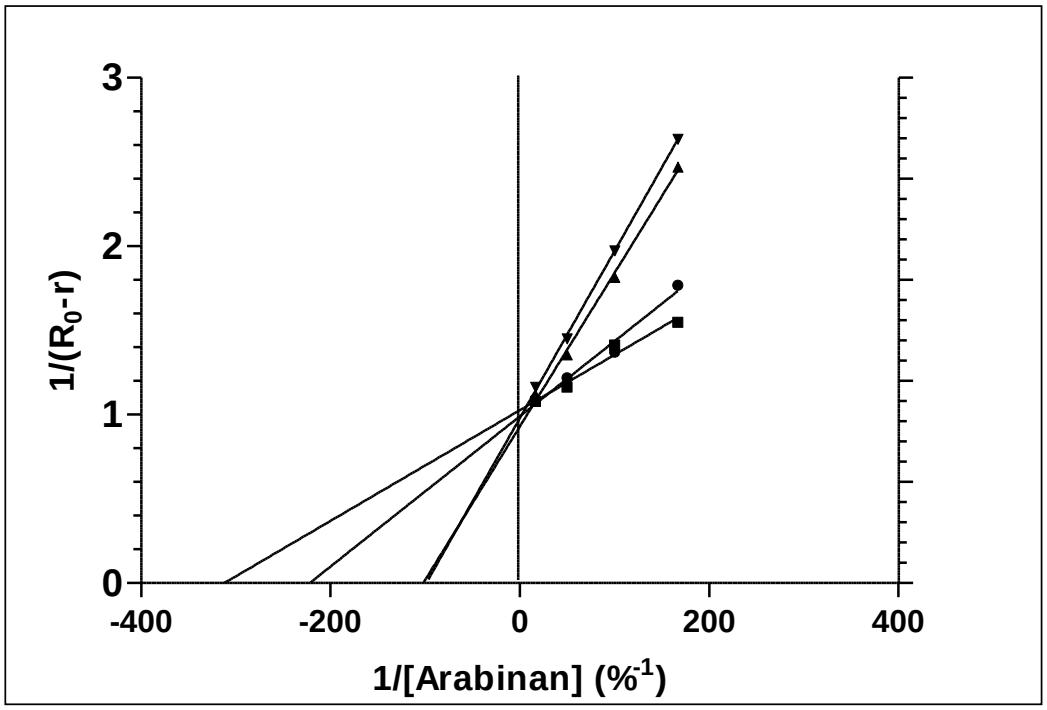
NB, affinity too low to allow calculation of K_a

Figure 3.6|Quantitative AGE analysis of the interaction of *C. thermocellum* CBM42A, CBM42B and CBM42C and its mutant derivatives with arabinan (AA) and arabinoxylan (AX).



Bovine serum albumin (lane 1), wild type CBM42s (lane 2), D41A (lane 3), D91A (lane 4) and D138A (lane 5) were subjected to AGE in gels containing a range of different concentrations of barley β-glucan (Panel A). The percentage concentrations of the soluble polysaccharides are displayed above the figures.

Figure 3.6 (Continuation)|Quantitative AGE analysis of the interaction of *C. thermocellum* CBM42A, CBM42B and CBM42C and its mutant derivatives with arabinan (AA) and arabinoxylan (AX).



The plot of the AGE data used to determine the affinity of CBM42C (•) and its mutant derivatives D41A (■), D91A (▲) and D138A (▼) for arabinan is displayed for exemplification.

*Ak*CBM42 has been crystallized in the presence of different oligosaccharides (Miyanaga *et al.*, 2004; Miyanaga *et al.*, 2006), such as α-L-arabinofuranose (PDB accession code 1WD4), arabinotriose (PDB accession code 2D43) and arabinofuranosyl-α-1,2-xylobiose (PDB accession code 2D44). Surprisingly, carbohydrates were only found bound to the β and γ sub-domains, hydrogen bonded to aspartate and histidine residues. Affinity gel electrophoresis studies using single, double and triple mutants revealed that mutation of Asp488 to an alanine in the γ sub-domain lead to an apparent lack of capacity to recognize arabinoxylan, similarly to what was described here for CBM42A. In addition, hydrophobic interactions between protein and ligands involving aromatic residues were also evident in the *Ak*CBM42 complexes. In the β and γ sub-domains, Tyr417-Tyr456 (the latest residue from the adjacent γ sub-domain) and Tyr464-Tyr359 (the latest residue from the adjacent α sub-domain) pair of residues, respectively, provide a perfect environment for stacking heterocyclic sugar rings, putatively modulating substrate specificity. However, *Ak*CBM42 α sub-domain and CBM42A α and β sub-domains do not present a clear hydrophobic stacking platform that could actively contribute to sequester the arabinose moiety at the binding site. Taken together, these data suggest that CBM42 present a different mechanism of binding when compared with the recently described examples of members of CBM13 that bind xylan. CBM13s from *Streptomyces lividans* and *S. olivaceoviridis* bind the xylan backbone internally and the three binding sites (α, β and γ), although expressing slightly different affinities for

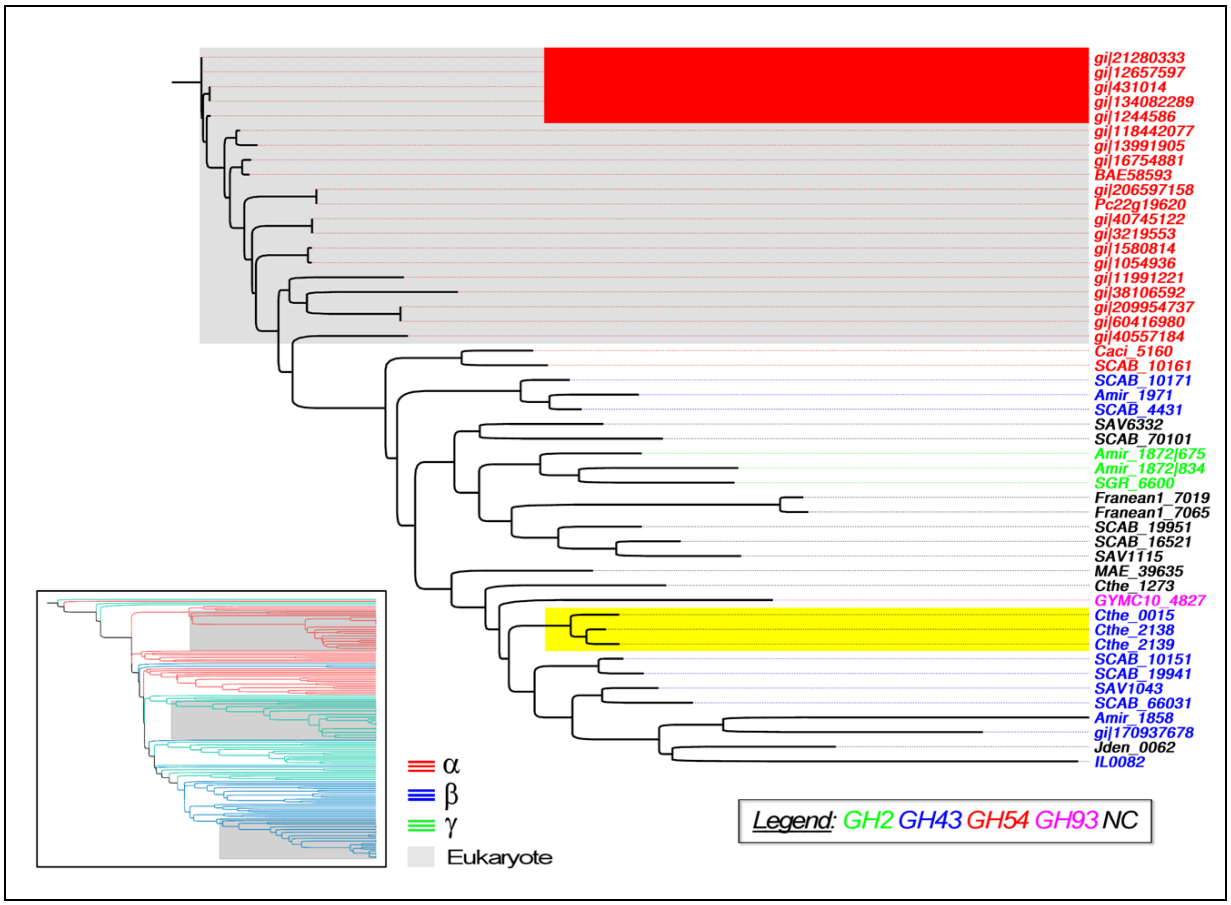
xylohexaose, cooperate during ligand recognition. Accommodation of xylooligosaccharides in a pocket-like binding site results from subtle changes in the amino acid residues that decorate the three binding interfaces. Data reported by Fujimoto *et al*. (2002), Notenboom *et al*. (2002) and Scharpf *et al*. (2002) suggest that the structure of a xylan molecule precludes the binding of a single carbohydrate chain by the three sub-domains at the same time, suggesting that the cooperative effects are mainly important for binding insoluble xylan clusters. It is thus intriguing why a molecular scaffold that originally displayed three functional binding sites, such as CBM42, has evolved a mechanism of binding that restricts ligand recognition primarily to a single binding pocket, such as it has been described here for CBM42A. Maybe ligand recognition through a single interface allows the correct positioning of the associated catalytic domain on the arabinoxylan molecule, thus efficiently directing the enzyme catalytic site into the substrate. In addition, it is possible that in CBM42A, the α and β sub-domains have an unknown ligand specificity, which could contribute to raise the ligand plasticity of CBM42A. However, the structural constrains identified in sub-domain β, particularly the lack of the conserved aspartate residue at the centre of the pocket, argue against this possibility.

To evaluate which of the CBM42B and CBM42C sub-domains interact with arabinoxylan and arabinan, the affinity of the wild type proteins and their various mutant derivatives for the two polysaccharides was quantified by AGE. The data, presented in Table 3.4 and Figure 3.6, revealed that CBM42B (and CBM42A) recognizes preferentially arabinoxylan, while CBM42C binds arabinan with a higher affinity. By analogy with CBM42A, Asp41, Asp91 and Asp138, in both CBM42B and CBM42C, are putatively the key residues involved in ligand recognition by pockets α, β and γ, respectively. Thus, the following CBM42B and CBM42C mutants were constructed: D41A (pocket α), D91A (pocket β), D138A (pocket γ). Similarly to what was described for CBM42A, Asp138 plays a key role in the recognition of arabinoxylan by both CBM42B and CBM42C. Intriguingly, Asp41 seems to play no direct role in the recognition of both polysaccharides by CBM42B. In contrast, this acid residue in CBM42C participates in ligand recognition since D41A of CBM42C displays a reduction in binding affinity. CBM42B and CBM42C D91A and D138A protein derivatives only displayed a partial reduction in the affinity for arabinan. Thus, the data suggest that both pockets β and γ contribute to the recognition of arabinan by CBM42B and CBM42C modules. In contrast, recognition of arabinoxylan by CBM42B and CBM42C seems to be dominated by the γ sub-domain.

3.1.3.5. Evolutionary relationships in CBM42 family

The evolutionary relationships among family 42 CBMs deposited within CAZy database are depicted in the phylogenetic tree presented in Figure 3.7.

Figure 3.7|CBM42 family phylogenetic tree.



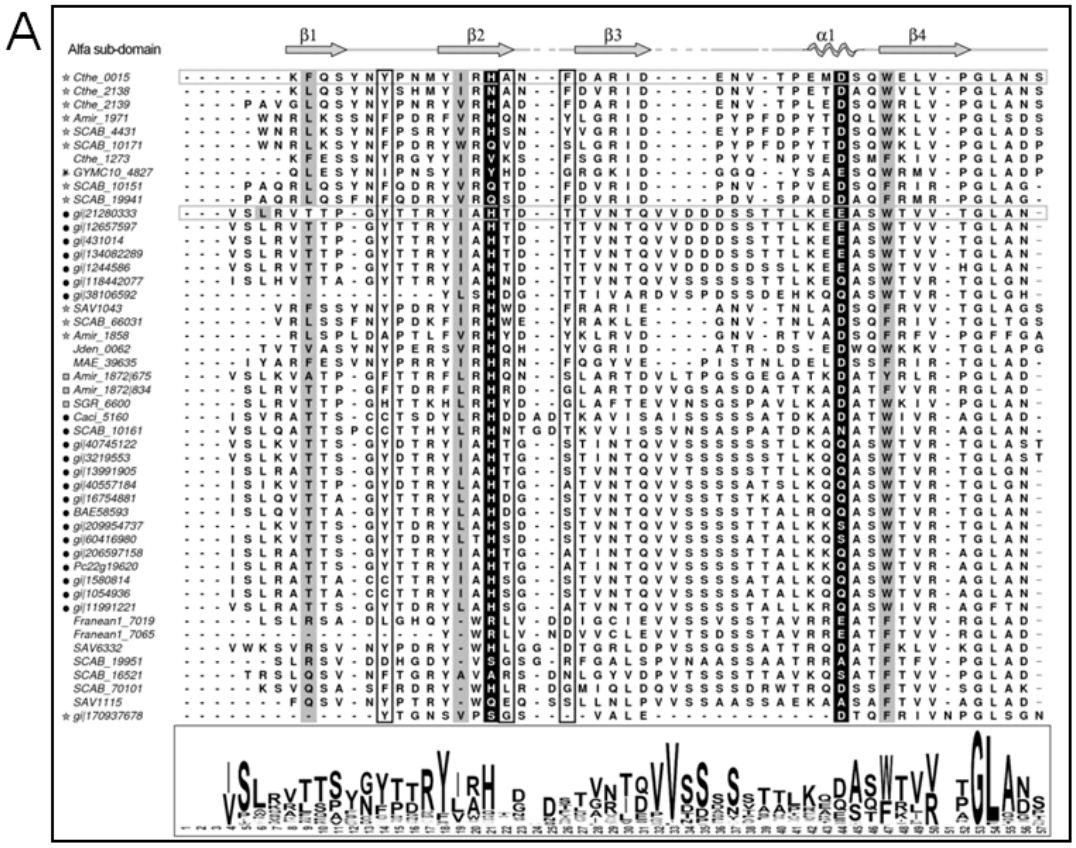
Phylogenetic tree based on a CBM42 family multiple sequence alignment. CBM42A, CBM42B and CBM42C are highlighted in yellow while AkCBM42 and four other related *Aspergillus* sequences are highlighted in red. The sequence name is color coded according to the catalytic Glycoside Hydrolase module family to which the CBM is attached: GH2 (green), GH43 (blue), GH54 (red), GH93 (magenta) and Not Classified (black). The small phylogram insert is based on an alignment considering α (red), β (blue) and γ (green) sub-domains as individual sequences. A grey box surrounds eukaryotic sequences (the figure was produced based on MAFFT/NJ-UPGMA (Katoh & Toh, 2008)).

Currently there are 50 available polypeptide sequences in CBM42, which include representatives from eubacteria and eukaryotes. Two members of CBM42 were excluded from the analysis (accession numbers ACR31368 and CAN91403) since their sequences diverged quite significantly from the family consensus. *C. thermocellum* CBM42A, CBM42B and CBM42C proteins, highlighted in yellow, are grouped together but are relatively distant from the eukaryotic sequences (grey box), where *Ak*CBM42 and four other related CBM42 *Aspergillus* sequences are included (red box). Overall, the analysis indicates that polypeptides originated from eubacteria or eukaryotes are grouped in different clusters. In addition, sequence clustering also parallels the nature of the catalytic domain to which the module is appended to. Thus, there are two clearly distinct groups of CBM42 modules: those

that are appended to GH43 bacterial enzymes and those that belong to GH54 eukaryotic enzymes. Although GH54 enzymes comprehend mainly arabinofuranosidases, GH43 enzymes that are bound to CBM42 modules remain to be characterized biochemically. In addition, two small clusters containing GH2 and unclassified catalytic domains were detected, while one of the enzymes is appended to a GH93 module.

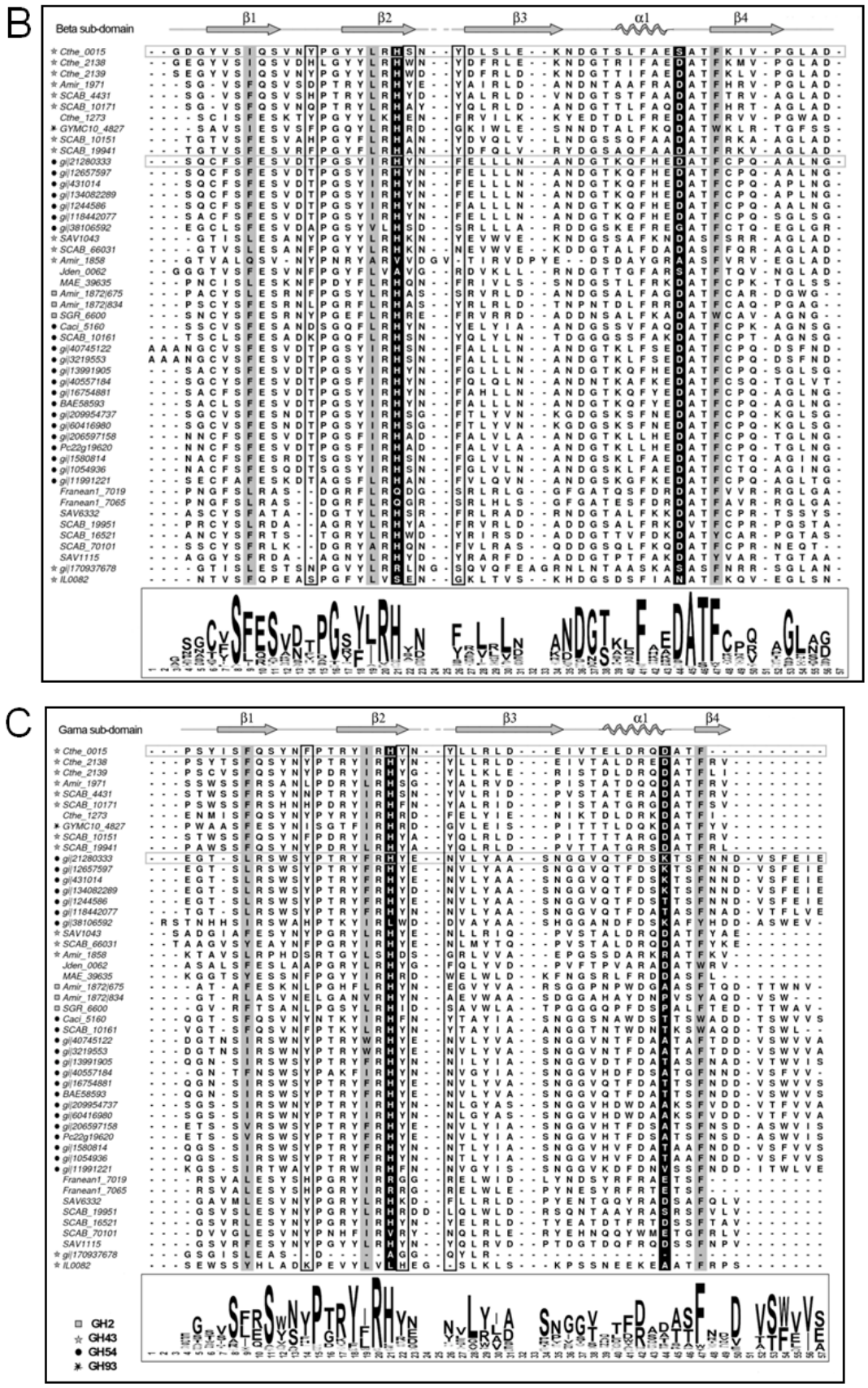
The small phylogram image inserted in Figure 3.7 depicts a genetic phylogeny based on an alignment that considers CBM42 α, β and γ sub-domains as individual sequences. Intriguingly, the figure suggests a marked clustering of each CBM42 sub-domain. Despite the relative evolutionary genetic distance between CBM42 family members, their multiple sequence alignment (Figure 3.8) reveals a high degree of sequence conservation, particularly at some of the key residues participating in ligand recognition. The alignment is separated among the three sub-domains, a grey horizontal rectangle highlights both CBM42A and *Ak*CBM42 and below each sub-domain, a sequence logo graphics represents the amino acid sequence conservation at that position.

Figure 3.8|Multiple sequence alignment of family CBM42. CBM42 family sequence alignment of α, β and γ sub-domains.



Panel A (alpha subdomain), panel B (beta subdomain) and panel C (gamma subdomain). The top secondary structure display is based on CBM42A (Cthe_0015). A grey horizontal rectangle highlights both CBM42A and AkCBM42 (gi|21280333). A Sequence Logo graphic, below each sub-domain, represents the amino acid sequence conservation at each position. On the left-hand side of each sequence database ID, a symbol represents the catalytic Glycoside Hydrolase module family to which the CBM is attached: GH2 (square), GH43 (star), GH54 (circle) and GH93 (ink blob) (the figure was produced based on ClustalW2 and WebLogo (Crooks *et al.*, 2004)).

Figure 3.8 (continuation)|Multiple sequence alignment of family CBM42. CBM42 family sequence alignment of α, β and γ sub-domains.



The importance of the histidine residue for ligand binding is patent by its high level of conservation in all sub-domains and across a wide range of family members. The same could be said about the acidic residue in alignment position 44, but here there is a noteworthy lesser conservation degree in α and γ sub-domains when compared with the β-sub-domain, with the notable exception of CBM42A, among very few others. Out of the already mentioned sequence features important in ligand binding, the three positions putatively participating in the ligand stacking effect present the least amount of sequence conservation. Taken together the data suggests that, in contrast with the observed variation in ligand specificity revealed by a variety of CBM families, CBM42 seems to present a much restricted capacity for recognizing plant cell wall polysaccharides.

3.1.4. Conclusions

Cellulosomes produced by anaerobic bacteria are highly efficient nanomachines that play a critical role in the biological deconstruction of plant cell walls. Cellulosomes are targeted to plant cell walls, which are remarkably heterogeneous macromolecule comprising a large variety of interacting polysaccharides, through a type A CBM3 located in the scaffoldin, which specifically bind crystalline cellulose. However, upon binding to crystalline cellulose, cellulosomes require additional supramolecular targeting to enable the catalytic components of the enzyme complex to be brought into proximity with their specific substrate. Fine tuning polysaccharide recognition is accomplished by a variety of type B and type C CBMs located in the cellulosomal enzymes. Here, the biochemical properties of the three family 42 CBMs of *C. thermocellum* cellulosome were characterized. Cellulosomal CBM42s are specifically located in GH43 enzymes, an enzyme family that usually display arabinofuranosidase activity, i.e. members of GH43 remove the arabinose side-chains of complex hemicelluloses. Thus, reflecting the putative binding specificity of the associated catalytic modules, cellulosomal CBM42s were shown to bind arabinose containing polysaccharides, particularly arabinoxylan and arabinan. The structure of CBM42A revealed a β-trefoil fold with the CBM containing three putative ligand interacting sub-domains with a pocket topology. However, mutagenesis experiments revealed that although CBM42A, CBM42B and CBM42C are built on a protein platform that could potentially accommodate three ligand–binding sites, binding of arabinoxylan is orchestrated primarily by the γ sub-domain. In contrast, CBM42B and CBM42C also bind arabinan and, in this particular situation, ligand recognition seems to depend on the cooperative interaction of both the β and γ pockets. Thus, this work confirms that CBM42 modules display a platform that could sustain three ligand binding sites although recognition of arabinose containing hemicelluloses is dominated by the γ sub-domain. This is in contrast with the mechanism of binding displayed by members of CBM13, which also display a β-trefoil fold, but where ligand recognition depends on the cooperative interaction of the three binding interfaces.

4. MANIPULATING BROILERS MEAT QUALITY

4.1. ENRICHMENT OF BROILERS MEAT WITH N-3 POLYUNSATURATED FATTY ACIDS BY DIETARY SUPPLEMENTATION WITH EXTRUDED LINSEED AND DHA GOLD™: EFFECTS ON PERFORMANCE AND MEAT QUALITY

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Abstract

Poultry meat is an important dietary source of beneficial n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) for humans. Levels of these molecules in poultry meat can be improved by increasing the incorporation of n-3 PUFA in poultry diets, usually through the supplementation with linseed oil, oily fish by-products or both. However, a decrease in flavor quality is usually associated with the dietary supplementation with n-3 PUFA, which is due to the intrinsic susceptibility of PUFA to oxidation. In this study the influence of two different n-3 fatty acid sources (extruded linseed and DHA gold™, a docosahexaenoic acid (DHA)-based marine microalgae product) on the productive performance and quality, oxidative stability and sensory traits of broiler meat was investigated. A total of 120 Ross 308 male broilers were fed 4 different diets (n=10 cages of 3 birds per treatment) between days 21 and 35 of age: a control group fed with a corn-based diet and 3 supplemented groups which diets contained 7.4% of DHA gold™ (rich in DHA), 15.4% of extruded linseed (LS) (rich in α-linolenic acid (LNA), the precursor of n-3 LC-PUFA) or 3.7% of DHA gold™ plus 7.7% of extruded linseed (DHALS). The four dietary treatments were formulated to contain 8% of fat with a total of 2% n-3 PUFA. DHA birds displayed a higher final body weight when compared with animals from other groups, particular animals fed on the conventional diet. In contrast, supplementation with extruded linseed increased the length and weight of the GI tract and resulted in a reduced feed intake and lower final body weights. The results suggest that meat from birds supplemented with 3.7% of DHA gold™ (DHALS), in contrast to the supplementation with 7.4% of the marine algae derivative, display a favorable overall appreciation of sensorial traits which is not significantly different from meat of non-supplemented animals

4.1.1. Introduction

Omega 3 (n-3) long chain-polyunsaturated fatty acids (LC-PUFA) are recognized as essential biomolecules for animal growth and development (Zhang *et al.*, 2010). Reports have consistently demonstrated that n-3 fatty acids, especially LC-PUFA, may delay tumor appearance and inhibit the rate of growth while eventually decreasing the size of tumors (Funahashi *et al.*, 2006). These important PUFA have also been shown to reduce the risk of several chronic disorders, including cardiovascular diseases (Lopez-Garcia *et al.*, 2004). The primary source of n-3 LC-PUFA is marine fish, seafood and some plants (Schmitz and Ecker, 2008), such as algae, that were shown to be the most prolific producers of eicopentaesanoic (EPA) and docosahexanoic (DHA) acids in the marine ecosystem (Arterburn *et al.*, 2006). However, studies developed by Alasalvar *et al*., (2002) revealed that n-3/n-6 ratios were higher in wild than in cultured sea bass. Thus, total lipid content, fatty acid proportions and trace mineral compositions of fish very much depend upon the diet consumed.

Western diets have become progressively poorer in fish and consequently in n-3 PUFA. This contrasts with an increase in n-6 PUFA intake that results from an improvement in vegetable oil consumption (Schmitz and Ecker, 2008). The essential α-linolenic (LNA) and linoleic (LA) acids are the fatty acid precursors of n-3 LC-PUFA and n-6 LC-PUFA, respectively. LA is converted to form arachidonic acid (AA, 20:4n-6) and then converted to docosapentaenoic acid (DPA, 22:5n-6) or eicosanoids (Schmitz and Ecker, 2008). LNA is converted to stearidonic acid and eicosatetraenoic acid (20:4n-3) to form EPA (20:5n-3). EPA is further metabolized to DHA (22:6n-3) or eicosanoids (Bezard *et al.*, 1994; Schmitz and Ecker, 2008). The conversion of these essential fatty acids, mainly LNA, is not an efficient process as a result of their poor dosage in diets (Lopez-Ferrer *et al.*, 2001a) and the competition for elongation by desaturase enzymes, which are the same for both the n-6 and n-3 pathways (Emken *et al.*, 1994). Thus, it is important to assure an adequate intake of LA/LNA ratio to obtain an efficient conversion of LNA into EPA and DHA.

Poultry meat annual *per capita* consumption in the United States, in 2006, was of 39 kg and represented 36.7% of the total meat and meat products intake (American Meat Institute, 2009). Thus, poultry meat has been considered one of the main potential sources of n-3 PUFA, including n-3 LC-PUFA, for humans (Givens and Gibbs, 2008; Rymer and Givens, 2005). Dietary supplementation with lipids and oils rich in n-3 PUFA can be an efficient method to increase the content of these fatty acids in animal muscle (Lopez-Ferrer *et al.*, 2001b) and, thus, to increase n-3 PUFA intake by humans. Despite the advantages of modifying the lipid profile of monogastric meats by modulating dietary fat, there are some disadvantages of using this route, which relate with meat oxidative stability. LNA, EPA and DHA are very susceptible to oxidation, producing off-flavors and odors in stored meat that are often associated with fish (Wood *et al.*, 2008). Thus, the use of fish oils at concentrations

greater than 1 to 2% in poultry diets entails several organoleptic problems in edible tissues and, consequently, contribute to decrease meat flavor quality (Bou *et al.*, 2001).

In a previous work, we showed that the effects of pasture intake (rich in LNA) on the fatty acid profile of broiler breast meat, while marginal, are statistically significant and result in an increased EPA content. In addition, low levels of the n-3 precursor LNA were observed in meat from these birds, suggesting a greater conversion of LNA into EPA in birds consuming fresh forages (Ponte *et al.*, 2008). Although it is well known that marine algae are excellent sources of n-3 LC-PUFA, the impact of novel algae-derived products, alone or in combination with other n-3 PUFA sources, on broiler performance and meat quality remains to be established. DHA gold™ is a product derived from *Schizochytrium* marine algae with the golden hue due to naturally occurring carotenoids, which may provide n-3 PUFA stabilization (Barclay *et al.*, 1994). Thus, a trial has been conducted in order to assess the impact of dietary supplementation with high levels of extruded linseed (rich in LNA, the precursor of n-3 LC-PUFA), DHA gold™ (rich in DHA) or a mixture of both n-3 PUFA sources (similar amounts of LNA and DHA) on bird performance, meat quality, oxidative stability, sensory traits, fatty acid composition and vitamin E homologues. Fatty acids and vitamin E contents in breast and thigh meats are presented in the companion paper. The aim of the study reported here was to investigate the impact of incorporating these two n-3 fatty acid sources in a corn-based diet, on performance, meat quality, meat oxidative stability and meat sensory traits in broilers.

4.1.2. Materials and Methods

4.1.2.1. Animals, Diets and Management

One hundred and twenty 1-day old male Ross 308 birds were housed in 40 battery brooders with 3 birds per cage, in a controlled environmental room under standard brooding practices. The animals were *ad libitum* fed with a corn-based diet during the first 21 days of the trial (starter diet described in Table 4.1). From days 21 to 35, animals were fed with four different treatments (finisher diet). All diets were formulated to achieve the National Research Council (1994) requirements. The four treatments consisted of a corn-based control diet (CN), and the CN diet supplemented with 15.4% of extruded linseed (LS; Reagro, Lisboa, Portugal), 7.4% of DHA gold™ (DHA; Novus, Brussels, Belgium) or 3.7% of DHA gold™ plus 7.7% of extruded linseed (DHALS) (Table 4.1). Feed was offered in the pelleted form. The four dietary treatments were formulated to obtain 8% of fat, from which 4% was from the different lipid supplementation sources with 2% of total n-3 PUFA in the three lipid supplemented diets. Extruded linseed (with levels of hydrocyanic acid (HCN) <10 mg/kg) and DHA gold™ determined content in crude protein, crude fiber, crude fat and crude energy were of 17%,

12%, 21% and 22 MJ/kg for linseed and 11%, 0.5%, 21% and 28 MJ/kg for DHA gold™, respectively. Body weight and feed consumption were recorded weekly for performance evaluation (body weight gain, feed conversion ratio and feed intake). Birds were slaughtered at day 35 in a commercial slaughterhouse after a fast of 24h. Gastro-intestinal (GI) contents were collected during evisceration and the size and weight of the GI tract was measured and weighted, respectively. Carcasses were weighted after 4 hour in the air-chilled circuit. Meat pH values and skin and meat color measurements were determined as described below. Carcasses were dissected after the air-chilled circuit and the *pectoralis major* muscle and whole thighs were obtained without skin. Thighs were deboned. The right breast and thigh of each animal were stored at -20ºC in a vacuum sealed bag until sensory analysis. The left breast and thigh were divided and frosted in a vacuum sealed bag at -20ºC, to determine meat shear force and oxidation susceptibility.

Table 4.1|Ingredient composition and calculated analysis of the experimental diets

Item	Starter/Grower (0 to 21 d)	Finisher (21 to 35 d)			
		CN	DHA	LS	DHALS
Ingredients (%)					
Corn	51.2	56.0	53.0	43.2	48.1
Soybean meal 47%	40.2	35.3	34.3	33.1	33.7
Soybean oil	4.98	5.65	2.34	5.26	3.80
NaCl	0.30	0.30	0.05	0.25	0.15
Calcium carbonate	0.76	0.91	0.65	0.57	0.61
Dicalcium phosphate 18%	2.19	1.59	1.94	1.94	1.94
DL-methionine	0.18	0.09	0.10	0.06	0.08
Mineral and vitamin premix[1]	0.20	0.20	0.20	0.20	0.20
DHA gold™	-	-	7.40	-	3.70
Extruded linseed	-	-	-	15.4	7.70
Calculated nutrient content					
ME (kcal/kg DM)	3100	3200	3200	3200	3200
CP (%)	23.0	21.0	21.0	21.0	21.0
Crude fat (%)	7.33	8.07	8.73	10.8	9.79
Crude fiber (%)	3.49	3.34	3.89	5.01	4.45
Ca (%)	1.00	0.90	0.90	0.90	0.90
P (%)	0.45	0.35	0.35	0.35	0.35
Met+Cys (%)	0.90	0.76	0.76	0.76	0.76
Met (%)	0.53	0.42	0.43	0.40	0.41
Lys (%)	1.28	1.15	1.11	1.14	1.13
Thr (%)	0.89	0.81	0.80	0.82	0.81
Try (%)	0.29	0.26	0.26	0.28	0.27

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS; [1]Mineral-vitamin premix provided the following per kilogram of feed: vitamin A, 9,000 IU; vitamin D3, 2,100 IU; α-tocopherol, 20 mg; nicotinic acid, 30 mg; vitamin B12, 0.12 mg; calcium pantothenate, 10 mg; vitamin K3, 2 mg; thiamin, 1 mg; riboflavin, 4.2 mg; vitamin B6, 1.7 mg; folic acid, 0.5 mg; biotin, 0.5 mg; Fe, 80 mg; Cu, 10 mg; Mn, 100 mg; Zn, 80 mg; Co, 0.2 mg; I, 1.0 mg; Se, 0.3 mg; monensin, 100 mg/kg.

4.1.2.2. Determination of Carcass Yield, pH and Color

Carcass yield was determined by the difference of live body weight and the carcass weight after removal of viscera, neck, head and feet (ready to cook carcass). The carcass was weighted after 4 hour in the air-chilled circuit. The pH was determined before the dissection of the whole carcass, in the right breast muscle (in triplicate), using a HI9025 potentiometer (Hanna instruments, Woonsocket, RI). Color measurements were determined with a Minolta™ Chroma Meter CR-300 Series (Osaka, Japan), with a data processor DP-301 attached, in homologue locations of the skin of all carcasses. After skin color measurements, the skin was removed and additional determinations were performed in homologue locations of the right breast muscle and thigh of all carcasses. All color measurements were replicated three times and the values were expressed in the International Commission on Illumination (CIELAB) system of lightness (L*), redness (a*) and yellowness (b*) (CIE (International Comission on Ilumination), 1978).

4.1.2.3. Determination of Meat Oxidation

Approximately 15 g of meat from the left breast and thigh of each animal were minced, divided in 4 portions and kept 0, 2, 4 or 6 days at 4ºC exposed to air in plastic bags. Meat oxidation was determined at day 0, 2, 4 and 6, by the technique of thiobarbituric acid-reactive substances (TBARS) based on Botsoglou *et al*. (1994) and Grau *et al*. (2000). Briefly, 1 ml of EDTA 0.3% (w/v), 5 ml of BHT 0.8% (w/v) in *n*-hexane and 8 ml of TCA 5% (w/v) were added to 2 g of fresh meat. The suspension was homogenized using a Ultra-Turrax T25 homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) for 30 seconds at 20000 rpm. The homogenate was centrifuged at 1900 rcf during 5 minutes. The *n*-hexane layer was removed and the remaining volume was filtered in Whatman™ nº1 paper. The filtered volume was adjusted to 10 ml with TCA 5% (w/v) solution. An aliquot of 2.5 ml was removed and 1.5 ml of TBA 0.8% (w/v) was added and was incubated during 30 minutes at 70ºC. After cooling the tubes in water, the absorbance was measured at 532 nm against a blank sample (2.5 ml TCA 5% + 1.5 ml BHT 0.8%). Duplicate measurements were obtained and the results were expressed as mg of malondialdehyde (MDA) per kg of meat.

4.1.2.4. Meat Sensory Analysis

Meat sensory analysis was performed only on breast muscle due to the difficulty to individualize muscles in thigh cuts. Right skinless breast muscles (only *pectoralis major*) were removed from whole carcass at slaughter day and were frosted in a vacuum sealed bag at -20ºC, as well as a portion of the left breast muscles until the sensory analysis day. For sensory analysis, left and right breast meat were defrosted at 4ºC during 36 hours and were grilled until an internal temperature of 80ºC was reached. The meat was turned the first time

when the internal temperature was 55ºC and from then on was turned frequently until the final temperature was reached. Before and after grilled, breast muscle was weighted to determine cooking loss. Left breast meat was cooled at room temperature during 2 hours after grilling to measure shear force. Shear force was determined as described by Ponte *et al*., (2008) in the maximum 2 cm wide-strips of the muscles that were subjected to a Warner-Bratzler blade, coupled to a texture analyzer TA.XT.plus® from Stable Microsystems™ (Surrey, UK). Measurements (cuts) were replicated extensively depending on the size of available meat sample (minimum of 5 replications).

For the sensory analysis, muscles were cut in cubes of 1 cm and added to a sensory box previously identified. The trained sensory panel (Faculty of Veterinary Medicine, Lisbon, Portugal), composed by 9 members, tasted the meat samples to investigate tenderness, juiciness, chicken flavor, presence of off-flavors and overall appreciation in 4 panel sessions, with 10 random samples per session including all the 4 animal treatments. The sensory tests were made by blind tasting. All the attributes were tested in a numeric scale from 1 to 8, where 1 is the low/negative score and 8 is the high/positive score. For off-flavor evaluation, the scale was from 0 (absence of off-flavor) to 8 (maximum of off-flavor).

4.1.2.5. Statistical Analysis

The experimental design was completely randomized and the cage with 3 birds was the experimental unit (4 treatments with 10 replicates of 3 birds each = 120 broilers). Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The results of the sensory panel were evaluated by the same procedure described above. The relationships between sensorial analysis and the most important fatty acid and some physical properties of breast meat were assessed by principal component analysis (PCA), using the statistica® program (Statsoft, Tulsa, Oklahoma, USA). The PCA was performed to reduce the dimensionality of the data set and to describe the variability of the data in two dimensions. It was used to examine the relationships between sensorial analysis and fatty acid composition and some physical variables considered, enabling not only a plot of the relationships between the variables but also attempting to explain those relationships. Differences were considered significant when $P<0.05$.

4.1.3. Results and Discussion

This study was conducted to determine the influence of a dietary supplementation with high levels of extruded linseed (rich in LNA, the precursor of n-3 LC-PUFA), DHA gold™ (rich in DHA, a product of the n-3 LC-PUFA pathway) or the mixture of both n-3 PUFA sources (similar amounts of LNA and DHA) on bird performance, meat quality, oxidative stability, sensory traits, fatty acid composition, cholesterol and vitamin E homologues. The content of total n-3 fatty acids was adjusted at 2% in the three lipid-supplemented diets. The different treatments used in this experiment did not affect the various serum biochemical indicators of animal health (data not shown). Data presented in the companion paper revealed a direct improvement in the fatty acid nutritional value of meat from birds supplemented with DHA gold™. In addition, the high levels of linseed supplementation resulted in a poor conversion of α-linolenic acid into LC-PUFA, suggesting that metabolization of LNA is not sufficiently extensive to improve meat quality (Ribeiro *et al*., submitted). In the current report broiler performance, meat quality, oxidative stability and sensory attributes were evaluated.

4.1.3.1. Productive Performance

Bird performance, expressed as body weight (BW), BW gain, feed intake and feed conversion ratio are summarized in Table 4.2. The dietary treatments started at day 21 and finished at day 35 of the trial. There were significant differences in BW and BW gain between treatments ($P<0.05$), namely in birds from the DHA and LS groups. Growth rates were significantly higher and lower for birds of the DHA and LS treatments, respectively, when compared with control animals. LS treatment was also different from the other treatments for feed intake and feed conversion ratio ($P<0.05$). Thus, birds subjected to the DHA treatment displayed the highest performance while incorporation of linseed oil severely affected growth and feed intake. The final body weight of birds from the DHA treatment was >10% greater than the control animals. Differences in body weight for birds of the LS treatment can, in part, be explained by a lower feed intake. Pellets from this treatment were less cohesive and very susceptible to disintegration into flour. This phenomenon was also observed by Haug *et al*. (2007) and was attributed to the melting point of linseed. Appearance of extruded linseed is significantly unctuous and viscous when compared with DHA gold™. Nevertheless, it is also possible that the presence of specific anti-nutritive factors in linseed, even at lower levels, might have affected feed intake and/or digestibility. Taken together these results suggest that while DHA gold™ improves body weight, incorporation of extruded linseed in diets decrease feed intake and consequently growth rates. Although there were no evidences in other studies that DHA improves growth in poultry, there is some controversial information about this effect on humans. Ryan *et al*., (1999) reported lower final body weights but only in male infants. Carlson *et al*. (1996) found a negative effect on growth when DHA was

supplemented *per se*. In contrast, other studies such as Foreman-van Drongelen *et al.,* (1996), shown that there were no negative effects when DHA is supplemented with arachidonic acid (AA). Nevertheless these discoveries were not corroborated in poultry studies.

Table 4.2|Performance of broilers *ad libitum* fed with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Body weight (g)						
21 d	962	970	971	931	15.4	0.241
28 d	1399^{b}	1494^{a}	1221^{c}	1377^{b}	19.9	<0.0001
35 d	1844^{b}	2048^{a}	1514^{c}	1840^{b}	45.1	<0.0001
Body weight gain (g)						
21-28 d	437^{b}	525^{a}	237^{c}	438^{b}	16.3	<0.0001
28-35 d	458^{b}	552^{a}	292^{c}	452^{b}	27.2	<0.0001
21-35 d	882^{b}	1077^{a}	537^{c}	906^{b}	40.7	<0.0001
Feed intake (g)						
21-28 d	875^{b}	927^{a}	805^{c}	828^{bc}	22.4	0.002
28-35 d	966^{b}	1041^{a}	849^{c}	944^{bc}	32.7	0.002
21-35 d	1841^{a}	1968^{a}	1654^{b}	1838^{a}	43.8	<0.0001
Feed conversion ratio						
21-28 d	2.01^{b}	1.77^{b}	3.53^{a}	1.92^{b}	0.128	<0.0001
28-35 d	2.13^{b}	1.93^{b}	3.00^{a}	2.14^{b}	0.106	<0.0001
21-35 d	2.11^{b}	1.84^{b}	3.16^{a}	2.10^{b}	0.117	<0.0001

Control diet, CN, and control diet supplemented with DHA gold^{TM}, DHA, extruded linseed, LS, or DHA gold^{TM} plus extruded linseed, DHALS

The relative weights of crop, gizzard and liver, as well as the relative lengths of duodenum, jejunum, ileum and caecum of broilers are presented on Table 4.3. The relative weights of gizzard and liver were affected (P<0.05) by lipid supplementation of diets, as well as the relative lengths of duodenum, jejunum, ileum and caecum. In addition, birds from the LS treatment when compared with those from the DHA gold^{TM} treatment have heavier compartments and longer GI tract. Barley, wheat or rye-based diets rich in soluble Non-Starch Polysaccharides (NSPs) also lead to an increase in the size and length of GI tract compartments (Choct, 1997) as a consequence of an increase in digesta viscosity. The effect of extruded linseed on intestinal length has not been reported previously. However, given the difficulty in pelletizing the extruded linseed diet, it is possible that the dietary incorporation LS contributes to change the physical/rheological properties of the digesta, decreasing feed passage rates and thus contributing to impose a pressure towards the increase in organ size.

Table 4.3|Relative weight (g/100 g body weight) and length (cm/100 g body weight) of gastrointestinal tract of broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Crop	0.307	0.342	0.348	0.276	0.026	0.210
Gizzard	1.02^{ab}	0.852^{b}	1.06^{a}	0.854^{b}	0.059	0.030
Liver	2.52^{a}	1.92^{b}	2.05^{ab}	1.65^{b}	0.184	0.017
Duodenum	1.43^{c}	1.39^{c}	1.89^{a}	1.65^{b}	0.074	<0.0001
Jejunum	3.71^{bc}	3.67^{c}	4.99^{a}	4.34^{b}	0.234	0.001
Ileum	3.98^{b}	4.10^{b}	5.48^{a}	4.53^{b}	0.249	0.001
Caecum	0.963^{b}	0.878^{b}	1.22^{a}	0.975^{b}	0.060	0.002

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS

4.1.3.2. Meat Quality Parameters

Carcass yield and breast meat pH, cooking loss and shear force is shown in Table 4.4. Lipid supplementation had no effect on breast meat pH, cooking loss and shear force value (P>0.05). In contrast, carcass yield was higher for birds of the DHA treatment when compared to the LS animals. This result is likely explained by the higher final body weight of birds from the DHA group and the larger size of the GI tract in LS birds. In addition, shear force was higher in breast meat of LS birds when compared with meat from DHA animals. Skin and meat color were only slightly affected by the dietary treatments (Table 4.5). Lightness (L*) of broiler skin tended to be negatively affected by DHA gold™ supplementation (P<0.1). In addition, thigh yellowness (b*) was higher in thighs of the DHA treatment, which may be explained by the β-carotenoids content of the DHA gold™ product.

Table 4.4|Carcass yield (%) and breast meat pH, cooking loss (%) and shear force (kg) of broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Carcass yield	70.7[a]	70.6[a]	66.5[b]	68.2[ab]	1.06	0.022
pH	6.01[ab]	5.94[ab]	5.94[a]	6.04[a]	0.04	0.142
Cooking loss	22.8	24.6	24.9	23.4	2.46	0.919
Shear force	29.1	25.4	32.8	29.5	2.26	0.150

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS)

Table 4.5|Skin, breast and thigh color of broilers fed *ad libitum* with the experimental diets

		Treatment				SEM	p(F)
		CN	DHA	LS	DHASL		
Skin	**L**	71.6	69.7	70.9	71.0	0.51	0.064
	a*	3.89	4.85	4.01	4.28	0.528	0.585
	b*	13.6	13.6	12.6	13.0	0.77	0.756
Breast	**L**	49.5	48.3	48.8	50.8	1.17	0.463
	a*	4.14	4.33	3.34	3.42	0.348	0.118
	b*	1.23	1.30	0.47	0.38	0.649	0.639
Thigh	**L**	50.3	49.8	50.4	49.0	0.75	0.561
	a*	4.55	5.16	4.94	4.68	0.373	0.662
	b*	1.60	2.52	0.59	1.20	0.494	0.061

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS

The oxidative stability of breast and thigh meats, after 2 or 4 days of storage at 4ºC, was affected by diets (P<0.1) (Table 4.6). Oxidative susceptibility of thigh meat of the DHA treatment immediately after slaughter was significantly different (P<0.05) from meat of the other treatments. At day 6, the oxidative stability of thigh meat from the supplemented groups was significantly higher when compared with meat from birds of the CN treatment. The reduced oxidative stability of meat from lipid supplemented birds was due to the enrichment of meat with LNA and n-3 LC-PUFA (see companion paper), which are very susceptible to oxidation. In addition, the higher susceptibility of thigh meat to oxidation, relative to breast meat, might result from the higher lipid content of this meat (Betti *et al.*, 2009; Leskanich and Noble, 1997). Oxidative stability of unsaturated fatty acids decreases as the degree of unsaturation increases. Thus, broiler meat with higher levels of long-chain-n-3 fatty acids is more susceptible to oxidation than meat with PUFAs with lower degrees of unsaturation, such as LA (Rymer and Givens, 2005). In spite the lower oxidative stability of DHA when compared with LNA, there were no differences in this parameter between meat enriched in LNA and that enriched in DHA, except a trend for thigh meat with 2 days of storage. This is likely explained by the compensation of the unsaturation degree of membrane phospholipids with other PUFA (Jerónimo *et al.*, 2009). Data presented in the companion paper also

showed that meat from DHA and LS treatments are less protected with natural antioxidants, especially α-tocopherol, when compared with the control group. Finally, it is unlikely that a major change in the oxidative stability of diets occurred upon storage. The diets were stored under vacuum at -20ºC while the source of PUFA, DHA Gold (contains high levels of carotenoids, 6.44 IU/100g) and extruded linseed (subjected to a treatment to reduce peroxide value to less than 10 meq O_2/kg), are believe to remain stable during storage as claimed by the manufacturers (shelf life of 2 years and 5 months, respectively). Taken together the results presented here suggest that meat from DHA or linseed-supplemented birds displayed comparable lipid stability parameters.

Table 4.6|Oxidative stability of breast and thigh meats, measured as TBA reactive substances (mg malondialdehyde/kg meat), of broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Breast						
0	0.03	0.05	0.01	0.01	0.022	0.690
2	0.04	0.21	0.09	0.15	0.051	0.098
4	0.09	0.49	0.20	0.17	0.125	0.134
6	0.09	0.97	0.42	0.21	0.280	0.071
Thigh						
0	0.07^{b}	0.34^{a}	0.12^{b}	0.09^{b}	0.067	0.026
2	0.09	0.32	0.13	0.33	0.075	0.063
4	0.13	0.74	0.36	0.34	0.170	0.095
6	0.19^{c}	1.28^{a}	0.66^{ab}	0.39^{b}	0.256	0.025

Control diet, CN, and control diet supplemented with DHA goldTM, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS)[1]Days of meat storage at 4ºC.

4.1.3.3. Meat Sensory Evaluation

Breast meat from lipid-supplemented birds had significant differences (P<0.05) in flavor, off-flavors and overall acceptability when compared to that from non-supplemented animals (Table 4.7). Meat tenderness and juiciness was not affected by the treatments. Nevertheless, meat from DHA animals is slightly tender than that from LS birds. The type of lipid supplementation strongly affected meat sensory traits. Thus, meat from DHA birds had lower flavor and overall acceptability (P<0.0001) scores, in addition to a high score for off-flavors, when compared with LS meat. The deterioration of flavor quality by dietary supplementation with fish oil and marine algae are well known and has been previously described (Mooney *et al.*, 1998). However, reduction in the flavor score is less marked with marine algae supplementation than fish oil probably because the oil droplets in algae are encapsulated within the algal cell, thus protecting them from oxidative deterioration (Mooney *et al.*, 1998). In a more recent study (Rymer *et al*., 2010) a higher content of aldehydes in meat supplemented with marine algae was described. Nevertheless, in this study there was no sensory evaluation of meat quality and the data was related to cooked meat. It is also

possible that algae contain more natural antioxidants, mainly carotenoids, than fish oil. Meat of the DHALS group had a score for off-flavor intermediate relative to the score for LS and DHA treatments, and similar flavor and overall acceptability scores than LS birds. These data suggest that meat from birds of the DHASL treatment, in contrast to the supplementation with DHA gold^{TM}, has a minimal compromise of flavor quality compared with the meat from non-supplemented animals. Overall appreciation shows that only meat from DHA treatment had negative appreciation (less than 4) and is different from all the other treatments. Although meat from non-supplemented birds display a higher overall appreciation (6 – "moderately positive") when compared with the supplemented groups, LS and DHALS meats have a favorable score of 5 ("slightly positive"). The data suggest that the presence of DHA gold™ is responsible for the decrease in appreciation. Rymer and Givens (2006) also showed that a supplementation with 4% of fish oil had no impact in meat sensory characteristics, which is a comparable supplementation rate (3.7% of DHA gold™) to the DHASL treatment. However, other studies revealed that an even lower inclusion rate of fish oil generates an unacceptable meat due to oxidation (Mirghelenj *et al.*, 2009).

Table 4.7|Sensory panel traits of breast meat of broilers fed ad libitum with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Tenderness	6.6^{ab}	6.8^{a}	6.1^{ab}	6.4^{b}	0.22	0.155
Juiciness	4.6	4.7	4.2	4.5	0.34	0.744
Flavor	4.8^{a}	3.6^{b}	4.4^{a}	4.7^{a}	0.20	0.001
Off-flavor	0.5^{b}	3.6^{a}	0.6^{b}	1.3^{b}	0.35	<0.0001
Overall	6.0^{a}	3.8^{b}	5.3^{a}	5.3^{a}	0.25	<0.0001

Control diet, CN, and control diet supplemented with DHA goldTM, DHA, extruded linseed, LS, or DHA goldTM plus extruded linseed, DHALS)[1]The different attributes were quantified on a rating scale from 1 to 8, with the exception of the flavor and off-flavor that were quantified from 0 to 8.

4.1.3.4. Principal Component Analysis

The results of the PCA of breast meat sensory traits and major fatty acids (see companion paper) are shown in Table 4.8. The five first PC explained 84.81% of the total variance. The first and second PCs were responsible for 55.23% of the total variance, respectively 33.95% (PC1) and 21.27% (PC2). PC3 explained 14.63%, PC4 9.53% and PC5 5.42% of the variability. As total variance explained by the first two PC is greater than 50%, the projection of sensory traits and major fatty acids in the plane defined by these PC is shown in Figure 4.1. The coefficients of the loadings (eigenvectors) for the first two principal components are presented in Table 4.9. Overall, PC1 was mainly characterized by 22:6n-3 (0.39), off-flavor (0.37) and 16:0 (0.24) variables on the right side and by 18:2n-6 (-0.38), overall (-0.33) and flavor (-0.30) variables on the left side. Therefore, PC1 revealed an inverse dependence between meat off-flavor and flavor, as well as a close relationship between meat flavor and

the overall appreciation. This suggest that when off-flavor is too strong (DHA treatment), chicken flavor cannot be detected and the meat overall appreciation decreases. In addition, meat off-flavor seems to be associated with the presence of 22:6n-3 (DHA). In contrast, LNA does not introduce detectable off-flavors in meat even at the highest levels of supplementation (LS treatment). As expected, tenderness is inversely proportional to shear force, which means that tender meat (higher scores in panel evaluation) requires less force to cut. Juiciness is also inversely proportional to water loss during grilling (cooking loss).

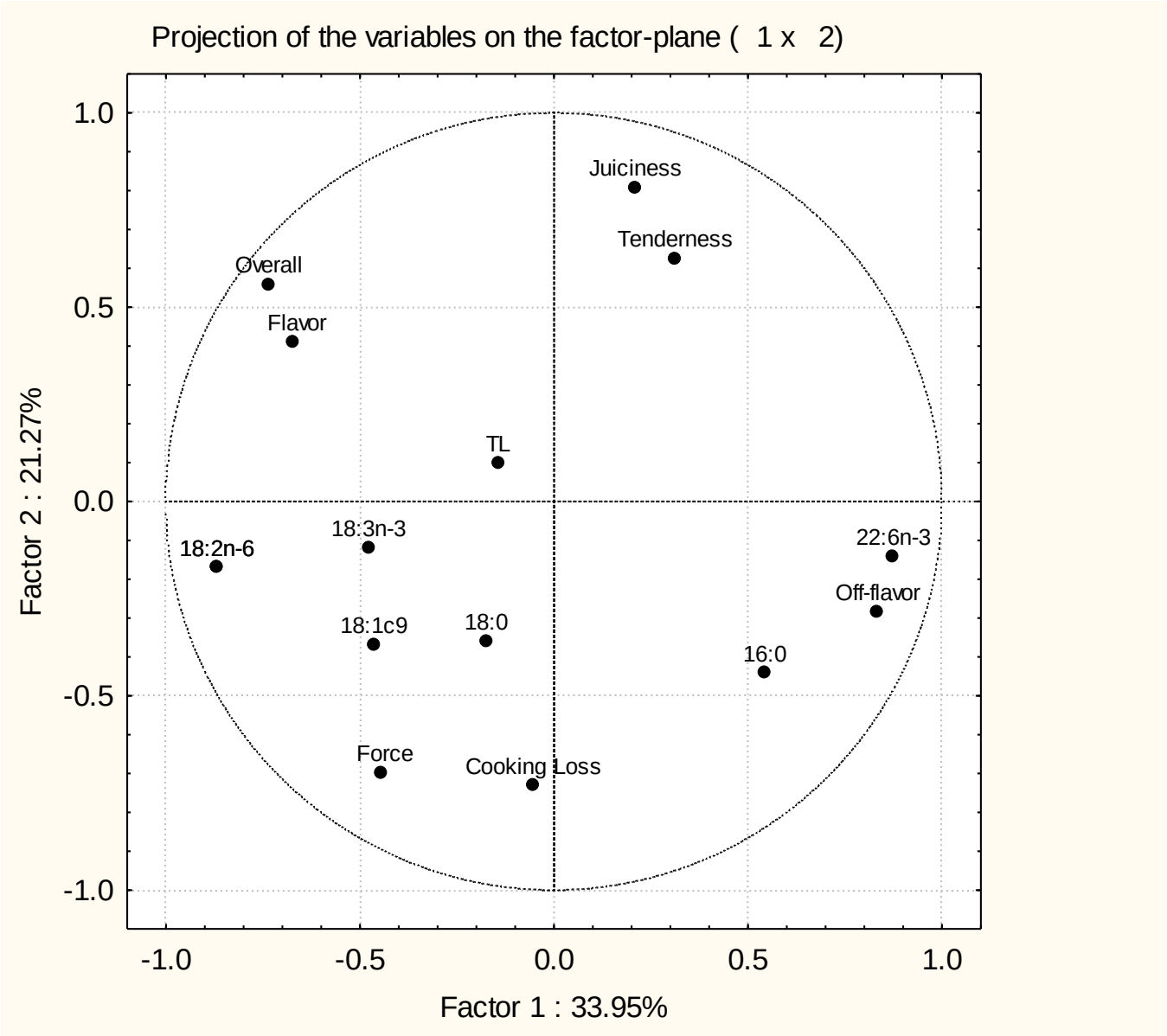
Table 4.8|Principal components (until 5% of the total variance)

	% Total variance	Cumulative %
1	33.95	33.95
2	21.27	55.23
3	14.63	69.86
4	9.53	79.39
5	5.42	84.81

Table 4.9|Coefficients of the loadings (eigenvectors) for the first two principal components

Component	Factor 1 (%)	Factor 2 (%)
Shear force	-0.20	-0.39
Cooking loss	-0.02	-0.41
Total lipids	-0.06	0.06
Tenderness	0.14	0.35
Juiciness	0.09	0.45
Flavor	-0.30	0.23
Off-flavor	0.37	-0.16
Overall	-0.33	0.31
16:0	0.24	-0.25
18:0	-0.08	-0.20
18:1c9	-0.21	-0.21
18:2n-6	-0.38	-0.10
18:3n-3	-0.21	-0.07
22:6n-3	0.39	-0.08

Figure 4.1|Projection of the variables in the plane defined by the two first principal components for breast meat of broilers fed *ad libitum* with the experimental diets



Treatments: control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS). Abbreviations: TL=Total lipids; Overall=overall appreciation of sensorial panel; Force=shear force.

4.1.4. Conclusion

Data presented here reveal that incorporation of DHA in broiler diets leads to an improvement in final BW and BW gain. In contrast, introduction of extruded linseed in diets reduced feed intake and negatively affected growth rates. Overall, the data reported here indicate that meat from birds supplemented with 3.7% of DHA gold™ (DHASL), in contrast to the supplementation with 7.4% of the same marine algae derivative, has a flavor quality that is comparable with the meat from non-supplemented animals. A dietary supplementation with DHASL (3.7% of dietary supplementation with the marine microalgae-derived product) may comprehend a better alternative for the enrichment of broiler meat with n-3 PUFA than other

traditional sources. The possibility of increasing the levels of DHA gold^{TM} (>3.7%) in broiler diets, when associated with an appropriate antioxidant compound, is presently under investigation.

4.2. ENRICHMENT OF BROILERS MEAT WITH N-3 POLYUNSATURATED FATTY ACIDS BY DIETARY SUPPLEMENTATION WITH EXTRUDED LINSEED AND DHA GOLD™: EFFECTS ON MEAT FATTY ACID PROFILE, CHOLESTEROL, TOCOPHEROLS AND TOCOTRIENOLS CONTENTS

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Abstract

This experiment was conducted to study the effect of introducing two different omega 3 (n-3) fatty acid sources (extruded linseed and DHA gold™, a docosahexaenoic acid (DHA)-based marine microalgae product) on the fatty acid profile and conversion efficiency of long-chain polyunsaturated fatty acids (LC-PUFA) in the breast and thigh meats of broilers. A total of 120 Ross 308 birds were fed four different diets (n=10 cages of 3 birds per treatment) between days 21 and 35 of age: a control group with a corn-based diet and 3 supplemented groups which diets contained 7.4% of DHA gold™ (rich in DHA), 15.4% of extruded linseed (rich in α-linolenic acid,LNA, the precursor of n-3 LC-PUFA) or 3.7% of DHA gold™ plus 7.7% of extruded linseed. The four dietary treatments were formulated to obtain 8% of fat with 2% of total n-3 PUFA. The data revealed a direct improvement in the fatty acid nutritional value of meat from birds supplemented with DHA gold™. Extruded linseed supplementation resulted in a poor conversion of α-linolenic acid into LC-PUFA, suggesting that metabolization of LNA *in vivo* is not sufficient to improve meat quality in n-3 LC-PUFA. In contrast, meat from birds submitted to the DHA gold™ treatment had the highest contents of DHA and total n-3 LC-PUFA, revealing that direct supplementation is the best alternative to modulate an improvement of broiler meat in beneficial fatty acids. Finally, the results indicate that the different dietary supplementation strategies have no impact on the levels of major antioxidants (vitamin E homologues).

4.2.1. Introduction

Western diets are rich in saturated fatty acids (SFA) and omega 6 (n-6) polyunsaturated fatty acids (PUFA), particularly linoleic acid (LA, 18:2n-6), and sub-optimal in n-3 PUFA, particularly in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Prates & Bessa, 2009). Thus, nutritional guidelines recommend a reduction in SFA (WHO, 2003), which are associated with an increased risk of hypercholesterolemia and thus cardiovascular diseases (CVD) (Wood *et al.*, 2008). Nutritionist advisers recommend a higher intake of PUFA, especially n-3 PUFA at the expense of n-6 fatty acids (British Department of Health, 1994). There is a growing evidence of the beneficial effects of n-3 PUFA, mainly the long-chain (LC-PUFA; >C18) EPA and DHA. Recent studies pointed out that the consumption of EPA and DHA may reduce the risk of CVD, some inflammatory and neurological diseases, breast cancer, obesity and type-2 diabetes (Prates & Bessa, 2009). More recently, the results of Griffin (2008) re-enforced the current recommendations for increasing α-linolenic acid (LNA, 18:3n-3) and decreasing LA intakes, in order to promote the endogenous synthesis of long chain n-3 fatty acids.

It is well known that low PUFA/SFA and high n-6/n-3 ratios of some meats contribute to the imbalance regarding the fatty acid intake of today's consumers (Wood *et al.*, 2008). Thus, some dietary strategies have been used to improve the nutritional and health value of the intramuscular fat of poultry. Major sources of n-3 PUFA sources include fish, seafood and some plant sources (Schmitz & Ecker, 2008), such as algae, that were shown to be the most prolific producers of EPA and DHA in the marine ecosystem (Arterburn *et al.*, 2006). Since worldwide consumers are increasing white meat intake, which displays a lower SFA content, an healthier unsaturated/saturated ratio (Brenna *et al.*, 2009) and a richer n-3 fatty acid profile, it is important to design novel strategies to improve the quality of poultry meat fatty acid profile. Modulation of lipid profile in poultry meat may be obtain through the dietary route (Poureslami *et al.*, 2010a) and it can promote health benefits beyond the improvement of meat nutritional value (Narciso-Gaytan *et al.*, 2011). Nevertheless, to ensure the efficacy of these strategies there is a need to understand the fundamental steps involved in lipid metabolism, especially in the biosynthesis of LC-PUFA, particularly n-3 LC-PUFA (Lopez-Garcia *et al.*, 2004).

LNA and LA are the essential fatty acids precursors of the n-3 LC-PUFA and n-6 LC-PUFA series, respectively, through a common biosynthetic pathway involving desaturases and elongase (Pereira *et al.*, 2003). Δ9, Δ6 and Δ5 desaturases catalyze, respectively, the first step in the PUFA biosynthetic pathway, the rate-limiting conversion of essential fatty acids to LC-PUFA, and the final step in the production of the eicosanoid precursors (arachidonic acid, AA, 20:4n-6, and EPA). Eicosanoids from n-6 pathway and AA are metabolically more potent in promoting inflammation, platelet aggregation and immune and vascular reactivity than

those derived from n-3 LC-PUFA and have been associated with CVD, some inflammatory disorders and some cancers (Emken *et al.*, 1994; Siddiqui *et al.*, 2005). In addition, the conversion of these essential fatty acids, mainly the LNA, is not highly efficient maybe due to their poor dosage in the diet (Lopez-Ferrer *et al.*, 2001a) and the competition for elongation and desaturase enzymes by LA and LNA, as these enzymes act in both the n-6 and n-3 pathways (Emken *et al.*, 1994).

Enriching meat in n-3 PUFA can improve the levels of oxidized fatty acids, especially PUFA that are prone to oxidative instability, thus decreasing meat quality. The main antioxidant in meat is alpha-tocopherol (McDonald *et al.*, 1995). Besides alpha-tocopherol, also beta-, gamma- and delta-tocopherols and the correspondent tocotrienols, all natural diterpenes with vitamin E activity, can be present in biological systems. However, the various vitamin E homologues are known to have different antioxidant potencies (Bourgeois, 1992), and tocotrienols are also known to help lower plasma cholesterol levels (Qureshi *et al.*, 1997). In addition, it is well established that some SFA (mainly palmitic acid) and PUFA increase and decrease the levels of cholesterol in plasma, respectively (Grundy, 1997; Katan *et al.*, 1995). Meat provides from one-third to one-half of the maximum recommended daily cholesterol intake (300 mg, World Health Organization), which seems to be directly associated with a greater risk of hypercholesterolemia (Chizzolini *et al.*, 1999). In spite of the high levels of incorporation of n-3 PUFA sources and the expected increase in meat oxidation, the quantitative antioxidant profile of diets supplemented with linseed and DHA gold^{TM} (a product derived from *Schizochytrium* marine algae) remains unknown.

The current trial has been implemented to assess the impact of the dietary supplementation with extruded linseed (rich in LNA, the precursor of n-3 LC-PUFA), DHA gold^{TM} (rich in DHA) or the mixture of both n-3 PUFA sources (similar amounts of LNA and DHA) on bird performance and meat quality, oxidative stability, sensory traits, fatty acid composition, cholesterol and vitamin E homologues. In a companion paper, we reported the effect of the dietary supplementation of broilers with these sources of n-3 fatty acids on bird performance and meat quality, oxidative stability and sensory traits (Ribeiro *et al*., submitted). The objective of the research reported here was to investigate the effect of supplementing bird diets with these two n-3 fatty acid sources, and its interaction (each treatment with 2% of total n-3 PUFA), on fatty acid composition, nutritional quality of fatty acids, cholesterol and vitamin E compounds of breast and thigh meats. Moreover, since the essential fatty acids can be desaturated and elongated to varying degrees depending on the animal species and tissue location (Pereira *et al.*, 2003), the rate of biosynthetic pathway of LC-PUFA was also assessed (for both n-3 and n-6 series), through the calculation of desaturation and elongation indices, for both breast and thigh meats.

4.2.2. Materials and Methods

4.2.2.1. Birds, Diets and Management

One hundred and twenty 1-day old male Ross 308 birds were housed in 40 battery brooders with 3 birds per cage, in a controlled environmental room under standard brooding practices, and were fed with a corn-based diet during the first 21 days of the trial (the starter diet is described in the companion paper). From day 21 to 35, animals were fed with four different treatments (finisher diet). All diets were formulated to achieve the National Research Council (1994) requirements. The four treatments consisted on a corn-based control diet (CN), and the CN diet supplemented with 7.4% of DHA gold™, Novus, Brussels, Belgium, from marine *Schizochytrium* algae (DHA), 15.4% of extruded linseed; Reagro, Lisboa, Portugal (LS) or with 3.7% of DHA gold™ plus 7.7% of extruded linseed (DHALS). Feed, in the pellet form, was offered *ad libitum*. The four dietary treatments were formulated to obtain 8% of fat, from which 4% was from the different lipid supplementation sources and with 2% of total n-3 PUFA. The fatty acid composition and vitamin E profile of the diets is given in Table 4.10. Birds were slaughtered at day 35 after a fast of 24 h, in a commercial slaughterhouse. Whole carcass was dissected after 4 h in the air-chilled circuit. Left breast and thigh of each animal were collected, minced, divided and frosted at -20ºC in a vacuum sealed bag to determine fatty acid composition and vitamin E homologues. Animal experiments were conducted in accordance with the principles and specific guidelines presented in European Union (1986), reviewed by the Ethics Committee of CIISA, Faculdade de Medicina Veterinária, and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Veterinária, Lisboa, Portugal).

Table 4.10|Total fatty acids (mg/g DM), fatty acid composition (% wt/wt), cholesterol (mg/g DM) and diterpenes (µg/g DM) of the experimental diets

Item	Starter/Grower	Finisher			
		CN	DHA	LS	DHALS
Total fatty acids	75.4	77.5	79.8	117.2	100.4
Fatty acids					
14:0	0.09	0.11	4.83	0.22	2.17
15:0	0.03	0.03	0.18	0.03	0.10
16:0	12.3	10.0	18.2	10.1	12.3
16:1c7	0.04	0.04	0.04	0.03	0.03
16:1c9	0.11	0.14	0.17	0.10	0.12
18:0	2.92	3.12	1.88	3.14	2.70
18:1c9	21.8	24.5	12.9	19.7	17.8
18:1c11	1.52	1.07	0.87	1.25	0.89
18:2n-6	54.8	58.1	32.3	40.3	38.3
18:3n-6	0.03	nd	0.09	0.09	0.05
18:3n-3	5.37	1.60	3.00	23.44	13.5
18:4n-3	nd	nd	0.11	nd	0.06
20:0	0.31	0.34	0.25	0.25	0.24
20:1c11	0.20	0.17	0.13	0.17	0.13
20:2n-6	0.04	0.03	nd	nd	0.03
20:3n-6	nd	nd	0.13	nd	0.07
20:3n-3	nd	nd	Nd	0.02	0.02
20:4n-7	nd	nd	0.61	nd	0.26
20:4n-6	nd	nd	0.17	nd	0.08
20:4n-3	nd	nd	0.33	nd	0.17
20:5n-3	0.16	0.11	0.58	0.08	0.3
22:0	0.34	0.53	0.22	0.30	0.34
22:5n-6	nd	0.04	5.81	0.18	2.58
22:5n-3	nd	nd	0.12	nd	0.06
22:6n-3	nd	0.1	17.0	0.51	7.58
Cholesterol	0.58	0.44	0.53	0.55	0.61
α-Tocopherol[1]	100.8	84.1	27.7	48.1	59.0
β-Tocopherol	1.22	1.51	0.75	1.26	1.35
γ-Tocopherol[2]	45.2	28.6	35.4	29.6	60.1
γ-Tocotrienol	5.89	6.51	6.01	6.6	5.04
δ-Tocotrienol	6.94	1.76	4.27	1.94	5.89

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS; nd = not detected; [1]Co-eluted with a small proportion of α-tocotrienol; [2]Co-eluted with a small proportion of β-tocotrienol.

4.2.2.2. Determination of Dry Matter and Total Lipids

Dry matter (DM) content of breast and thigh meats was determined, in duplicate, by microwave desiccation using a SMART System5 apparatus, model sp1141 (CEM Corporation™, Matthews, NC). Control analyses of DM were performed, in duplicate, by lyophilisation. The difference between the two methods for DM determination did not exceed 1%. Samples were lyophilized to constant weight using a lyophilisator Edwards Modulyo from Edwards High Vacuum International, Crawley, UK, at -60ºC and 2.0 hPa. After lyophilization samples were maintained in desiccators at room temperature and analyzed for fatty acids within 2 wk. For total lipid determination, intramuscular fat was extracted from feed and lyophilized breast and thigh muscles using the method of Folch *et al*., (1957) upon mincing

and homogenization. Total lipids were measured gravimetrically, in duplicate, by weighing the fatty residue obtained after solvent evaporation.

4.2.2.3. Determination of Fatty Acid Composition

Intramuscular fat of lyophilized meat (0.25 g) and feed (0.10 g of DM) samples were first dissolved in 1 mL of dry toluene. The neutral lipids (NL) and phospholipids (PL) fractions were eluted from the cartridges with dichloromethane and methanol, respectively, and transesterified by a basic/acid sequential reaction as described by Raes et al., (2001). In short, fatty acids were methylated at a temperature of 50°C with sodium methoxide in anhydrous methanol (0.5 mol/L) for 30 minutes followed by hydrogen chloride in methanol (1/1 vol/vol) for 10 minutes. Fatty acid methyl esters (FAMEs) were extracted twice with 3 mL of hexane, and pooled extracts were evaporated until 2 mL volume under a stream of nitrogen. The fatty acid composition was determined by gas chromatography of fatty acids methyl esters, performed by a chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame ionization detector and a CP-Sil 88 capillary column (100 m; 0.25 mm i.d.; 0.20 mm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA). The chromatographic conditions were as follows: injector temperature: 250ºC; detector temperature: 280 ºC; helium was used as carrier gas and the split ratio was 1:30. The gas chromatograph oven temperature was programmed to start at 50ºC (maintained for 4 minutes) followed by a 13ºC/min ramp to 175ºC (maintained for 20 minutes), followed by a 4ºC/min ramp to 275ºC (maintained for 40 minutes). Identification was accomplished by comparing the retention times of peaks from samples with those of FAME standard mixtures (Sigma® Aldrich Co, Buchs, Switzerland). Quantification of FAME was based on the internal standard technique, using nonadecanoic acid (19:0) as internal standard and on the conversion to relative peak areas to weight percentage. Fatty acids were expressed as gravimetric contents (mg/g of muscle) or as a percentage of the sum of identified fatty acids (% wt/wt).

4.2.2.4. Determination of Cholesterol, Tocopherols and Tocotrienols

The simultaneous quantification of total cholesterol, tocopherols, and tocotrienols in meat was performed as described by Prates *et al*. (2006). The method involved a direct saponification of the fresh meat (0.75 g) or feed (0.10 g of DM), a single *n*-hexane extraction, and analysis of the extracted compounds by normal-phase HPLC, using fluorescence (tocopherols and tocotrienols) and UV-visible photodiode array (cholesterol) detections in tandem. The contents of total cholesterol, tocopherols and tocotrienols were calculated in duplicate for each sample based on the external standard technique from a standard curve of peak area *vs.* compound concentration.

4.2.2.5. Statistical Analysis

The experimental design was completely randomized and the cage with 3 birds was the experimental unit (10 cages of 3 birds per treatment). Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Differences were considered significant when $P<0.05$.

4.2.3. Results and Discussion

This experiment aims to assess the influence of dietary supplementation with high levels of extruded linseed (rich in LNA, the precursor of n-3 LC-PUFA), DHA gold^{TM} (rich in DHA, a product of n-3 LC-PUFA pathway) or the mixture of both n-3 PUFA sources (similar amounts of LNA and DHA) on bird performance, meat quality, oxidative stability, sensory traits, fatty acid composition, cholesterol and vitamin E homologues. The content of total n-3 fatty acids was adjusted at 2% in the three supplemented diets. Data presented in the companion paper indicate that meat from birds supplemented with 3.7% of DHA gold^{TM}, in contrast to the supplementation with 7.4% of the same marine algae derivative, displays a flavor quality that is comparable with the meat from non-supplemented animals. Moreover, the meat from DHA birds (7.4% of dietary supplementation with DHA gold^{TM}) seems to have the same deterioration of lipid quality when compared with the meat from 7.7% of extruded linseed supplemented animals (DHALS) (Ribeiro *et al*., submitted). In this report, capacity of the two dietary supplements rich in n-3 PUFA to improve broiler the quality of meat fat and vitamin E composition was evaluated. The extension of conversion of the high levels of n-3 LC-PUFA precursor, LNA, into LC-PUFA was also estimated.

4.2.3.1. Fatty Acid Composition

The major fatty acid present in all dietary treatments was LA (32-58% of total fatty acids) (Table 4.10). Nevertheless, the percentage of this fatty acid decreased with the increase of the relative proportion of n-3 LC-PUFA in the diet (DHA treatment), which is in agreement with data reported by Komprda *et al.*, (2005). LA is the precursor of the n-6 pathway, while LNA is the precursor of the n-3 pathway. n-6 LA is converted to araquidonic acid (AA) and then converted to docosapentaenoic acid (DPA, 22:5n-6) or eicosanoids (Schmitz and Ecker, 2008). n-3 LNA is converted to stearidonic acid and eicosatetraenoic acid (20:4n-3) to form EPA. EPA is further metabolized to DHA or eicosanoids (Schmitz and Ecker, 2008). LNA competes very effectively with LA for common desaturases and elongase that convert C18 PUFA to their C20 and C22 derivatives (Emken *et al.*, 1994). For that reason, background diet influences the conversion of these fatty acids (Arterburn *et al.*, 2006) and there is a need to maintain an adequate ratio of LA/LNA to guarantee the LNA metabolization. Diet of the

DHA treatment had LC-n-3- PUFA levels similar to that described by Rymer and Givens (2010b) in a treatment supplemented with 8% of fish oil. Total n-3 fatty acids in the supplemented diets varied from 21.2 to 24.1% of total fatty acids (1.9-2.6% of feed DM), in contrast to the 1.8% in the non-supplemented diet (0.15% of feed). Compared with the control diet, the three supplemented diets had lower n-6/n-3 and LA/LNA ratios since supplemented groups had increased amounts of LNA and/or LC-PUFA. The LA/LNA ratio can affect the efficiency of metabolic pathways since there is competition between n-6 and n-3 desaturases involved in the conversion of LC-PUFA. The affinity of Δ6 desaturases is greater for LNA than for LA, although the typically greater concentrations of LA in diets and consequently in cells results in a greater conversion of n-6 LC-PUFA (Simopoulos, 2000). To achieve a better conversion of n-3 LC-PUFA, it is critical to increase LNA intake and to obtain a low LA/LNA ratio. Hence, diets containing linseed (DHALS and LS) displayed a lower LA/LNA ratio than the DHA diet, while this later diet had a lower LA/LNA ratio than the control group. Linseed supplementation increased LNA, especially in the LS treatment, where LNA content was 7.8-fold greater than the DHA treatment. Birds fed with DHA gold^{TM} had higher DHA contents in meat compared with the other diets. Levels of DHA were 33-fold higher in the DHA treatment than in meat from LS or control birds. Herber and van Elswyk (1996) revealed that it is possible to enrich eggs with DHA and EPA by supplementation with marine algae. Comparatively with the non-supplemented birds and animals supplemented with fish oil, eggs from the algae supplemented group had also the highest contents of total n-3 and significantly differences in PUFA. Moreover, Farrel (1998) concluded that consumption of enriched eggs produced by birds supplemented with fish oil contributes to the intake of almost the recommended daily dose of n-3 PUFAs.

Breast meat presented lower contents of total lipids (14.7-16.3 mg/g; Table 4.11) when compared with thigh meat (58.0-61.6 mg/g; Table 4.12). Dietary lipid supplementation did not affect ($P>0.05$) total lipid content in both meats. The content of total lipids in breast and thigh meats are similar to those detected by Rebole *et al.*, (2006) or Komprda *et al*., (2005) which suggest that lipid supplementation does not affect the total lipids of breast and thigh meat of broilers. Our values of total lipids are slightly higher when compared with those obtained by Ponte *et al*., (2008) using birds of a slow-growing genotype and with access to pasture. The fatty acid composition of breast and thigh meats is presented in Table 4.11 and Table 4.12, respectively. The effect of dietary lipid supplementation on meat FA composition was evident mostly for the individual FA, particularly on thigh meat, with the exception of some minor fatty acids (16:1c7, 18:1c11 and conjugated linoleic acids (CLA)). In breast meat, although an extensive modification on most FA was evident, some major fatty acids, like 18:0 and 18:1c9 were not affected ($P>0.05$) by dietary lipid supplementation. The general trend was to an increase in n-3 PUFA, reflecting the fatty acid composition of the diets. Nevertheless, differences between the types of dietary lipid supplementation were observed. As expected,

in both breast and thigh meats, extruded linseed supplementation resulted in a major increase in LNA (up to 10.4 % of total fatty acids), whereas DHA gold^{TM} supplementation resulted in a improved content in DHA (up to 5.6 % in thigh meat and 9.8% in breast meat). Sardi *et al*. (2006) evaluated the effects of dietary supplementation with a marine algae product containing high levels of DHA on heavy pig production performances. Pigs fed on marine algae diets showed a significant (P<0.01) improvement in DHA levels both in loin and in subcutaneous fat.

In DHA supplemented birds, there was a major depression of LA but not of AA in both meat types, whereas in linseed supplemented birds, the levels of LA remained elevated and close to the values observed in control. This is likely due to the compensation of the unsaturation degree of fatty acids in membrane phospholipids necessary to membrane fluidity and functionality (Jerónimo *et al.*, 2009). In both meat types, DHA gold^{TM} supplementation resulted in increased 14:0, 15:0, 16:0, 16:1c9, EPA and DHA compared with extruded linseed supplementation, reflecting the relative abundance of these FA in experimental diets as reported by Rymer and Givens (2010a). DHA content of DHA treatment is in accordance with the results of Mooney *et al*., (1998) and Rymer and Givens (2010a). The deposition of DHA and LNA, the major fatty acids in the lipid supplements tested, showed an additive response, presenting intermediate levels in DHALS when compared with LS and DHA animals. The 16:0, only in thigh meat, and LA, in both meats pieces, had higher proportions in DHALS (P<0.05) birds than the expected intermediate value obtained by additive interpolation.

The partial sums of fatty acids were calculated to detect any effect of diets on PUFA elongase and desaturase activities. Although the percentages of SFA, monounsaturated fatty acids (MUFA), PUFA, total n-3 and total n-6 seem similar in breast and thighs, some other fatty acids sums such as LC-PUFA, n-3 LC-PUFA and n-6 LC-PUFA were higher in breast meat as a consequence of the lower levels of intramuscular fat. Lower levels of total fat in meat decreases the triacylglycerol/phospholipid ratio since PUFA, mainly LC-PUFA, are mainly located in the phospholipid fraction (Rymer *et al.*, 2003). The partial sums of fatty acids reflect the pattern followed by the major individual constituents. Taken together, lipid supplements did not affect SFA, MUFA, PUFA, n-6 PUFA and n-6 LC-PUFA but increased n-3 PUFA in breast meat, whereas did not change SFA and n-6 LC-PUFA but affected all other partial sums in thigh meat. Differential effects between lipid supplements were observed for SFA, total PUFA, n-6 PUFA, and n-3 LC-PUFA in breast meat, and for all partial sums of fatty acids (excluding MUFA) for thighs. For both meat fractions, linseed supplementation resulted in lower (P<0.001) SFA and n-3 LC-PUFA, and higher (P<0.001) total PUFA and n-6 PUFA when compared with the DHA gold^{TM} supplementation. Birds fed with DHA accumulated more LC n-3 PUFA in breast and thigh meat when compared with the other

treatments as a result of the higher amount of DHA found in the diet (Rymer and Givens, 2010b; González-Esquerra and Leeson, 2000).

Meat from supplemented birds had similar PUFA/SFA ratios relative to the control group (1.4-2.4) but much lower n-6/n-3 ratios for breast (15 *vs.* 2-3) and thigh (14 *vs.* 3-4) meats. These differences reflect the pattern of the partial sums of fatty acids. For both meat fractions, PUFA/SFA ratio was affected by lipid supplementation and meat from LS animals had a higher value than DHA birds. The n-6/n-3 ratio was also affected by lipid supplementation in both muscles and higher in LS than in DHA breast meat. Current nutritional recommendations are that the PUFA/SFA ratio in human diets should be above 0.45 and, within the PUFA, the n-6/n-3 ratio should not exceed 4.0 (Burghardt *et al.*, 2010). In view of the above guidelines, n-6/n-3 ratios in the meat of breast and thighs of the supplemented birds, in contrast to that from control animals, are inside the recommended values for the human diet, which is favorable. However, the beneficial n-3 DHA is higher in breast (1.56 mg/g meat) and thigh (3.40 mg/g) meats of DHA gold^{TM}-treated animals, intermediate in meat of DHA gold^{TM} plus linseed-fed birds (0.83-1.63 mg/g) and lower in linseed-fed animals (0.14-0.30 mg/g). In addition, the percentage of the beneficial LC-PUFA EPA is only residual (<0.7%) in both meats analyzed.

Table 4.11|Total lipid content (mg/g meat), fatty acid composition and selected sums of fatty acids (% wt/wt) and nutritional ratios in breast meat of broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Total lipids	14.7	15.9	16.3	15.5	1.00	0.732
Fatty acids						
12:0	0.03^{c}	0.08^{a}	0.03^{c}	0.06^{b}	0.004	<0.0001
14:0	0.40^{c}	1.97^{a}	0.29^{c}	1.13^{b}	0.045	<0.0001
14:1c9	0.04^{c}	0.17^{a}	0.04^{c}	0.09^{b}	0.006	<0.0001
15:0	0.09^{c}	0.15^{a}	0.09^{c}	0.12^{b}	0.005	<0.0001
16:0	16.0^{bc}	20.0^{a}	13.9^{b}	16.4^{c}	0.58	<0.0001
16:1c7	0.32	0.32	0.28	0.29	0.016	0.437
16:1c9	1.27^{bc}	2.00^{a}	1.07^{b}	1.37^{c}	0.090	<0.0001
16:2	0.20^{bc}	0.15^{ab}	0.22^{c}	0.19^{c}	0.011	0.002
17:0	0.18	0.16	0.19	0.18	0.009	0.440
18:0	7.91	7.20	7.10	6.93	0.387	0.877
18:1c9	24.6	20.3	20.8	20.8	0.95	0.235
18:1c11	1.31^{b}	1.28^{b}	1.53^{a}	1.24^{b}	0.047	<0.0001
18:2n-6	34.7^{b}	23.3^{a}	32.8^{b}	29.3^{b}	1.29	<0.0001
18:3n-6	0.25^{a}	0.16^{b}	0.16^{b}	0.16^{b}	0.012	<0.0001
18:3n-3	1.75^{c}	1.99^{c}	10.4^{a}	6.50^{b}	0.41	<0.0001
18:4n-3	0.05^{a}	0.09^{b}	0.09^{b}	0.10^{b}	0.007	<0.0001
CLA[1]	0.03	0.05	0.03	0.06	0.015	0.347
20:0	0.12^{a}	0.11^{ab}	0.11^{b}	0.10^{b}	0.007	0.079
20:1c11	0.21^{a}	0.18^{ab}	0.17^{b}	0.21^{ab}	0.013	0.087
20:2n-6	0.41^{a}	0.31^{b}	0.31^{ab}	0.35^{b}	0.025	0.045
20:3n-6	0.46^{a}	0.34^{b}	0.38^{a}	0.35^{b}	0.022	0.006
20:3n-3	0.05^{a}	0.21^{b}	0.12^{c}	0.12^{c}	0.014	<0.0001
20:4n-6	3.33^{a}	2.17^{b}	3.10^{a}	2.38^{ab}	0.291	0.040
20:5n-3	0.11^{a}	0.66^{b}	0.34^{c}	0.48^{d}	0.019	<0.0001
22:4n-6	0.72^{a}	0.23^{b}	0.37^{c}	0.22^{b}	0.048	<0.0001
22:5n-6	0.31^{a}	1.95^{b}	0.14^{c}	0.98^{d}	0.036	<0.0001
22:5n-3	0.34^{b}	0.59^{a}	0.75^{c}	0.53^{a}	0.037	<0.0001
22:6n-3	0.76^{a}	9.82^{b}	0.83^{a}	5.37^{c}	0.249	<0.0001
Partial sums						
SFA	24.8^{b}	29.6^{a}	21.7^{bc}	24.9^{bd}	0.92	<0.0001
MUFA	27.7	24.2	23.9	24.0	1.06	0.539
PUFA	43.5^{a}	41.9^{a}	50.0^{b}	47.0^{b}	1.52	0.0002
n-3 PUFA	3.06^{b}	13.4^{a}	12.6^{a}	13.1^{a}	0.47	<0.0001
n-6 PUFA	40.2^{a}	28.4^{b}	37.2^{a}	33.7^{a}	1.37	<0.0001
LC-PUFA	6.19^{b}	16.3^{a}	6.35^{b}	10.8^{c}	0.58	<0.0001
n-3 LC-PUFA	1.18^{d}	11.3^{a}	2.04^{c}	6.50^{b}	0.28	<0.0001
n-6 LC-PUFA	5.01	4.99	4.30	4.27	0.38	0.341
Nutritional ratios						
PUFA/SFA	1.78^{b}	1.42^{c}	2.32^{a}	1.89^{b}	0.054	<0.0001
n-6/n-3	15.0^{a}	2.14^{b}	3.03^{b}	2.60^{b}	0.634	<0.0001

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS; [1]CLA, conjugated linoleic acid (18:2c9,t11 with vestigial amounts of other conjugated isomers

Table 4.12|Total lipid content (mg/g meat), fatty acid composition and selected sums of fatty acids (% wt/wt) and nutritional ratios in thigh meat of broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Total lipids	58.0	60.9	61.6	58.3	3.08	0.784
Fatty acids						
12:0	0.02^{a}	0.06^{ab}	0.02^{b}	0.04^{ab}	0.001	<0.0001
14:0	0.39^{a}	1.84^{ab}	0.36^{b}	1.06^{ab}	0.031	<0.0001
14:1c9	0.05^{a}	0.19^{ab}	0.05^{b}	0.10^{ab}	0.005	<0.0001
15:0	0.08^{a}	0.13^{ab}	0.07^{b}	0.10^{ab}	0.002	<0.0001
16:0	16.0^{a}	19.4^{b}	14.4^{a}	16.2^{a}	0.17	<0.0001
16:1c7	0.36^{a}	0.35^{ab}	0.33^{b}	0.34^{ab}	0.012	0.223
16:1c9	1.87^{bc}	2.70^{a}	1.78^{c}	2.08^{b}	0.091	<0.0001
16:2	0.26^{bc}	0.20^{a}	0.28^{c}	0.25^{b}	0.006	<0.0001
17:0	0.16^{c}	0.03^{a}	0.02^{b}	0.14^{a}	0.006	<0.0001
18:0	6.27^{a}	5.59^{b}	5.42^{b}	5.49^{b}	0.132	0.0002
18:1c9	27.5^{a}	24.4^{b}	24.7^{b}	24.6^{b}	0.29	<0.0001
18:1c11	1.37^{b}	1.35^{b}	1.57^{a}	1.33^{b}	0.020	<0.0001
18:2n-6	38.1^{a}	28.5^{b}	35.7^{c}	33.4^{d}	0.44	<0.0001
18:3n-6	0.29^{a}	0.20^{b}	0.19^{b}	0.19^{b}	0.011	<0.0001
18:3n-3	2.22^{a}	2.73^{a}	10.3^{b}	6.71^{c}	0.218	<0.0001
18:4n-3	0.05^{b}	0.09^{c}	0.10^{ac}	0.11^{a}	0.007	<0.0001
CLA[1]	0.02^{a}	0.02^{abc}	0.02^{b}	0.02^{c}	0.002	0.033
20:0	0.09	0.08	0.09	0.08	0.004	0.097
20:1c11	0.22^{a}	0.19^{b}	0.20^{b}	0.19^{b}	0.004	0.0002
20:2n-6	0.27^{b}	0.19^{a}	0.21^{a}	0.24^{b}	0.010	<0.0001
20:3n-6	0.30^{b}	0.24^{a}	0.20^{b}	0.23^{a}	0.008	<0.0001
20:3n-3	0.02^{a}	0.03^{a}	0.08^{b}	0.07^{b}	0.003	<0.0001
20:4n-6	1.40^{a}	1.13^{b}	1.05^{b}	1.09^{b}	0.056	0.0003
20:5n-3	0.07^{a}	0.60^{b}	0.17^{c}	0.41^{d}	0.017	<0.0001
22:4n-6	0.29^{a}	0.13^{b}	0.12^{b}	0.11^{b}	0.010	<0.0001
22:5n-6	0.17^{a}	1.43^{b}	0.09^{a}	0.65^{b}	0.028	<0.0001
22:5n-3	0.14^{c}	0.37^{b}	0.27^{a}	0.25^{a}	0.013	<0.0001
22:6n-3	0.39^{a}	5.58^{c}	0.49^{a}	2.79^{b}	0.128	<0.0001
Partial sums						
SFA	23.0^{a}	27.2^{c}	20.3^{b}	23.1^{a}	0.27	<0.0001
MUFA	31.4^{a}	29.2^{b}	28.6^{b}	28.6^{b}	0.36	0.539
PUFA	43.9^{a}	41.4^{b}	49.3^{c}	46.5^{d}	0.45	0.0002
n-3 PUFA	2.88^{a}	9.39^{b}	11.4^{c}	10.3^{d}	0.29	<0.0001
n-6 PUFA	40.8^{a}	31.8^{b}	37.6^{c}	35.9^{d}	0.47	<0.0001
LC-PUFA	3.03^{a}	9.70^{c}	2.68^{a}	5.83^{b}	0.205	<0.0001
n-3 LC-PUFA	0.61^{a}	6.57^{b}	1.01^{a}	3.51^{c}	0.150	<0.0001
n-6 LC-PUFA	2.42^{a}	3.13^{b}	1.67^{c}	2.32^{a}	0.080	0.341
Nutritional ratios						
PUFA/SFA	1.92^{a}	1.53^{b}	2.42^{c}	2.01^{a}	0.037	<0.0001
n-6/n-3	14.3^{a}	3.41^{b}	3.33^{b}	3.53^{b}	0.297	<0.0001

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS; [1]CLA, conjugated linoleic acid(18:2c9,t11 with vestigial amounts of other conjugated isomers).

4.2.3.2. Desaturase Activities

Estimation of Δ9 desaturase activities and of the two pathways of LC-PUFA conversion in breast and thigh meats were determinate by the ratio between the fatty acid product content for a given pathway over the sum of that product and its correspondent precursor (Table 4.13). Δ9 desaturase activity, the rate-limiting step of MUFA synthesis, in breast and thigh meats (Δ9 16:0 and Δ9 18:0) was not consistently affected in lipid supplemented broilers. Only Δ9 16:0 desaturase activity and not Δ9 18:0, was influenced by the dietary lipid supplementation in thigh meat and by the type of lipid supplementation in breast meat. Since Δ9 18:0 desaturase activities values were much higher and possibly more reliable than the Δ9 16:0 index, the dietary supplementation with n-3 PUFA sources does not seem to have an important inhibitory effect on the activity of the Δ9 desaturase enzyme.

The Δ6 desaturase activities of n-6 PUFA series were determined by the ratio between the amount of DPA and the sum of DPA and AA. This desaturase activity was higher for the DHA gold^{TM} treatment and lower for the linseed treatment, in both meats.. The presence of higher amounts of LNA in the LS treatment may have inhibited the n-6 pathway and the lower amounts of LNA in the DHA treatment may have promoted the preference for the n-6 series. Overall activity for the conversion of LNA to EPA (Δ6 and Δ5 desaturases) or DHA may be masked by the levels of EPA in this treatment that are higher than in other treatments and probably result from the retro-conversion of DHA into EPA and not from the conversion of LNA to EPA. Overall activity of desaturases involved in the conversion of LNA to DHA cannot be discussed for the treatments with DHA gold^{TM} due to high contents of DHA provided by the diet. Meat from the linseed treatment had lower desaturase activity in all n-3 series suggesting an inefficient and poor conversion of LNA into LC-PUFA. For comparison purposes, we calculated Δ5 (Dihomo-γ-linolenic acid,(DGLA)-AA) and Δ6 plus Δ5 (LA-AA) activities as described by Betti *et al*., (2009) (data not shown). Δ5 activity was found to be very similar to that described for 35 days of supplementation with 10% of flaxseed while Δ6+Δ5 activities were lower than previously reported. Betti *et al*., (2009) demonstrated that at day 35 the Δ5 activity was lower than the same activity measured in the first days of supplementation, indicating that LNA suppressed bioconversion of AA from LA.

Although the low levels of LC-PUFA in breast and thigh meats in birds where these molecules were not provided directly in the diet and, in some cases, the similar desaturase activities among treatments, data suggest some conversion of essential fatty acids. Comparison of levels of some LC-PUFA in meat and in diets suggests that some conversion of LA and LNA might have occurred. In the n-3 pathway, levels of EPA, DPA and DHA were in larger amounts in breast and thigh meats than in the diet, where in some cases these FA were undetectable, suggesting the conversion of LNA into n-3 LC-PUFA. In the n-6 pathway, there is also some evidence for the conversion of LA, especially to AA. Thus, levels of AA were approximately of 3.1% and 1% in breast and thigh meats, respectively, in absolute

contrast with its absence in diets. These conversions were larger in meat of the LS treatment but also occurred in the other groups. Some retro-conversion may have occurred in meat of the DHA treatment, from DHA to EPA, although at a lower degree, as described by Arterburn *et al*. (2006). Birds fed the LS diet displayed a 2-fold increase in levels of EPA and DPA in breast meat when compared with meat originated from birds not subjected to a supplementation, in spite the lower EPA content in the LS diet. However, the conversion from precursors of n-3 LC-PUFA was showed to be limited and inefficient such as it has been reported by other authors (Betti *et al.*, 2009; Lopez-Ferrer *et al.*, 2001; Rymer *et al.*, 2010a). These results agree with data reported in the literature suggesting that *in vivo* conversion of LNA into EPA and DHA is a relatively inefficient process. For instance, some studies in humans revealed that males convert LNA to EPA on a rate between 0.3 to 8% and LNA to DHA on a rate that is lower than 4%, while women convert up to 21% of LNA into EPA and up to 9% of LNA into DHA (Arterburn *et al.*, 2006).

Table 4.13|Estimation of desaturases and elongase activities in muscles of broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Breast meat						
Δ9 16:0	0.07^{b}	0.09^{a}	0.07^{b}	0.08^{b}	0.004	0.003
Δ9 18:0	0.76	0.74	0.75	0.75	0.010	0.597
Δ6+Δ5 LA-AA	0.09	0.09	0.09	0.07	0.009	0.502
Δ6 AA-DPA	0.09^{a}	0.48^{b}	0.04^{c}	0.30^{d}	0.011	<0.0001
Δ5 DGLA-AA	0.88	0.87	0.88	0.87	0.006	0.204
Δ5 GLA-AA	0.93	0.93	0.94	0.93	0.006	0.304
Δ6+Δ5 LNA-EPA	0.07^{a}	0.25^{b}	0.03^{c}	0.07^{a}	0.009	<0.0001
Δ6 EPA-DHA	0.85^{b}	0.94^{a}	0.69^{c}	0.92^{a}	0.016	<0.0001
Δ6+Δ5 LNA-DHA	0.28^{a}	0.83^{b}	0.08^{c}	0.45^{d}	0.020	<0.0001
Thigh meat						
Δ9 16:0	0.10^{a}	0.12^{b}	0.11^{ab}	0.11^{ab}	0.004	0.059
Δ9 18:0	0.81	0.81	0.82	0.82	0.004	0.673
Δ6+Δ5 LA-AA	0.04^{a}	0.04^{a}	0.03^{b}	0.03^{b}	0.001	0.0002
Δ6 AA-DPA	0.10^{a}	0.56^{b}	0.08^{a}	0.37^{c}	0.011	<0.0001
Δ5 DGLA-AA	0.82	0.82	0.83	0.82	0.006	0.397
Δ5 GLA-AA	0.83	0.85	0.84	0.85	0.010	0.328
Δ6+Δ5 LNA-EPA	0.03^{a}	0.18^{b}	0.02^{c}	0.06^{d}	0.004	<0.0001
Δ6 EPA-DHA	0.85^{a}	0.90^{b}	0.74^{c}	0.87^{a}	0.008	<0.0001
Δ6+Δ5 LNA-DHA	0.15^{a}	0.67^{b}	0.05^{c}	0.29^{a}	0.012	<0.0001

Control diet, CN, and control diet supplemented with DHA gold^{TM}, DHA, extruded linseed, LS, or DHA gold^{TM} plus extruded linseed, DHALS; Desaturases and elongase activities were measured as follows: Δ9 16:0= 16:1c9/(16:1c9+16:0); Δ9 18:0= 18:1c9/(18:1c9+18:0); Δ6+Δ5 LA-AA = AA/AA+LA ; Δ6 AA-DPA = DPA/DPA+AA; Δ5 DGLA-AA = AA/AA+DGLA; Δ5 GLA-AA = AA/AA+GLA; Δ6+Δ5 LNA-EPA = EPA/EPA+LNA; Δ6 EPA-DHA = DHA/DHA+EPA; Δ6+Δ5 LNA-DHA = DHA/DHA+LNA.

4.2.3.3. Cholesterol

Levels of cholesterol in diets and in breast (0.41-046 mg/g meat) and thigh (0.56-0.58 mg/g) meats are displayed in Table 4.14. Cholesterol contents found in diets and poultry meats are in accordance with other studies (Ayerza *et al.*, 2002; Ponte *et al.*, 2008; Ponte *et al.*, 2004b). Dietary lipid supplementation and composition did not affect ($P>0.05$) total cholesterol content for any type of meat analyzed. As expected, breast meat had lower cholesterol levels than thigh meat as thigh meat displayed higher amounts of fat due to a higher level of TAG. Overall the data suggest that the different supplementation strategies tested had no impact on meat cholesterol levels.

4.2.3.4. Tocopherols and Tocotrienols

Vitamin E profile of feedstuffs and breast and thigh meats are shown in Table 4.14. The quantification of diterpenes in diets and meats resulted in a complete profile of vitamin E compounds. In diets, the main diterpenes were α-tocopherol and γ-tocopherol, although with some differences among treatments. α-tocopherol was higher in the control group and lower in the DHA group, while γ-tocopherol was higher (approximately 2 fold than the other groups) in the linseed treatment and lower in the control diet. Beta-tocopherol and γ-tocotrienol displayed similar values in all treatments although lower in content. Levels of δ -tocotrienol were in general low but varied between treatments, revealing higher values in DHA and DHALS diets. Alpha-tocopherol co-eluted with small amounts of α-tocotrienol and γ-tocopherol co-eluted with small amounts of β-tocotrienol.

In general, meat diterpene profile was in agreement with diet profiles although the lower values incorporated in meat. In general, thighs had higher diterpene levels than breast meat, which is likely due to the higher fat content, although displaying a similar profile. α-tocopherol was the major diterpene, while the others vitamin E homologues were present at small levels. This meat profile is in agreement with that described by other authors (Prates *et al.*, 2006). Curiously, δ-tocotrienol was absent in breast meat unlike the thigh meat, where it was identified in very small amounts. Lipid-supplemented diets had lower contents of α-tocopherol in breast and thigh meats, as well as lower γ-tocopherol in thigh meat, relative to the control meat. However, the type of dietary lipid supplementation had no influence in any of the vitamin E homologues analyzed in the various meats. In fact, DHA gold^{TM} and linseed diets displayed lower contents of vitamin E homologues resulting in a decrease in meat α-tocopherol, the most important meat antioxidant compound. Barclay *et al*. (1994) indicated that microalgae DHA gold^{TM} is rich in other antioxidant compounds such as β-carotene and canthaxanthin responsible for the golden hue. Curiously β-carotene was not detected in this study.

Table 4.14|Cholesterol (mg/g meat) tocopherols and tocotrienols (µg/g meat) contents in meat from broilers fed *ad libitum* with the experimental diets

	Treatment				SEM	p(F)
	CN	DHA	LS	DHALS		
Breast meat						
Cholesterol	0.41	0.44	0.46	0.42	0.027	0.557
α-Tocopherol[2]	9.39[a]	5.93[b]	5.74[b]	8.86[ab]	1.134	0.053
β-Tocopherol	0.12	0.10	0.12	0.11	0.013	0.560
γ-Tocopherol[3]	0.91[a]	1.07[ac]	1.35[b]	0.84[ac]	0.091	0.002
γ-Tocotrienol	0.74	1.22	0.91	1.63	0.440	0.510
Thigh meat						
Cholesterol	0.58	0.56	0.58	0.58	0.014	0.466
α-Tocopherol[1]	18.4	11.4	11.0	12.8	1.48	0.004
β-Tocopherol	0.17	0.14	0.12	0.16	0.016	0.232
γ-Tocopherol[2]	1.14[a]	1.54[bc]	1.72[b]	1.32[c]	0.130	0.018
γ-Tocotrienol	1.64	1.95	1.41	1.82	0.497	0.881

Control diet, CN, and control diet supplemented with DHA gold™, DHA, extruded linseed, LS, or DHA gold™ plus extruded linseed, DHALS; [1]Co-eluted with a small proportion of α-tocotrienol; [2]Co-eluted with a small proportion of β-tocotrienol.

4.2.4. Conclusion

This study compared different supplementation approaches in order to improve the levels of broiler meat in n-3 fatty acids, especially in n-3 LC-PUFA. Taken together, the data presented here suggest that direct supplementation may be a more appropriate strategy when compared with the indirect supplementation with a n-3 LC-PUFA precursor, such as linseed. Thus, data presented here suggest that the conversion of precursor n-3 LC-PUFA into n-3 LC-PUFA seems to be very limited and inefficient. Although the n-6/n-3 ratio was similar for the meat of all supplemented broilers, n-3 LC-PUFA (mainly DHA) were much higher in DHA-treated birds, followed by the DHALS-fed animals. Moreover, the different supplementation strategies have no impact on the major lipid-soluble antioxidant vitamins. Taken together the data suggest that supplementation with the marine algae sub-product (DHA gold™) is highly efficient to improve broiler meat quality. Finally, the results reported here together with those described in the companion paper indicate that dietary supplementation with 3.7% of DHA gold™ (DHA) may be a better alternative to improve broiler meat with n-3 PUFA than the traditional supplementation with fish products that dramatically affect poultry meat flavor quality.

5. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Diets for monogastric animals are based on cereals, mainly maize, and in some cases wheat or barley, depending primarily on the price of raw-materials. Wheat and barley are rich in soluble non-starch polysaccharides, which affect nutrient digestibility and intake. Since simple stomach animals are not able to degrade plant cell wall polysaccharides, there is a need to incorporate these biocatalytic activities directly in the diets through the provision of exogenous enzymes. Degradation of the anti-nutritive soluble carbohydrates improve feed intake and makes the digestive process more efficient increasing nutrient availability. Degradation of structural polysaccharides is accomplished by CAZYmes, particularly glycoside hydrolases, carbohydrate esterases and pectate lyases which activity can be optimized through the action of appended CBMs. Complete degradation of plant cell wall carbohydrates requires the concerted action of a large range of enzymes displaying diverse substrate specificities. In addition, it is also well established that different organism inhabiting different ecological niches have developed different strategies to organize CAZYmes during plant cell wall hydrolysis. Thus, aerobic microorganisms usually secrete large quantities of individual cellulases and hemicellulases to the extracellular space and the enzymes act individually during polysaccharides degradation. In contrast, anaerobic microorganisms secrete organized multi-enzymatic complexes containing different cellulases and hemicellulases that are usually produced at considerably lower levels. These complexes are known as cellulosomes, due to their high specific activity against cellulose, and the most well characterized bacterial cellulosome is the one produced by *Clostridium thermocellum*. Cellulosomes are targeted to plant cell walls by family 3 (CBM3), which bind to crystalline cellulose, but other CBMs are present within the complex to fine-tune polysaccharide recognition.

In chapter 2, a CBM11 from *C. thermocellum*, was used to evaluate the capacity of CBMs to improve the efficacy of exogenous feed β-glucanases. Each animal trial was conducted with different catalytic modules attached to CBM11: a GH26 coupled with a GH5 from *C. thermocellum*, a GH16 from *C. thermocellum* or a GH5 from *C. mixtus*. The trial with CBM11 in association with Lic26A-Cel5E revealed that birds fed on Lic26A-Cel5E-CBM11 diets present a performance (body weight and weight gain) similar to or better (feed conversion ratio) than the positive control group. It was concluded that the accessory enzymes included in the commercial mixture (enzymes that do not cleave β-1,3-1,4-glucans) were obsolete and that CBM11 potentiates catalytic activity by targeting the appended catalytic domains to β-glucans. Comparing the enzymatic dose used in this experiment with the one used in the experiment performed by Guerreiro *et al*. (2008), it can be concluded that CBMs are particularly effective when feed enzymes are used at lower dosages. Thus, by contributing to reduce the levels of feed supplementation with exogenous CAZYmes, CBMs may lead to a

significant increase in the economic parameters of poultry production. In general, feed enzymes contribute to decrease the weight and size of the GI tract of broilers by decreasing digesta viscosity of cereal-based diets. In contrast, results from chapter 2 revealed that enzymatic supplementation had no effect on the weight and length of poultry GI compartments. However, viscosity data demonstrated a similar decrease in the duodenum, jejunum and ileum viscosities of the treatments supplemented with enzymes when compared with the negative control group. The low viscosity of barley lots used in these experiments may explain the lower efficacy of the feed enzymes in reducing the size of the GI tract of broiler chicken. Recombinant enzymes could be detected in most GI compartments and seemed to be more prevalent than the commercial enzyme mixtures. However, molecular integrity of the recombinant enzymes was shown to be affected by proteolysis, mainly at gizzard level. Thus, linker regions that separate the different enzyme modules were shown to be prone to proteolysis, resulting in the release of individual modules that can still, however, maintain catalytic activity. This observation suggest that the effect of CBMs in the function of exogenous enzymes is particular important in the upper part of the GI tract. The only GH16 enzyme of *C. thermocellum* cellulosome was characterized in this work. GH16 has a high specific activity for barley β-glucans with an optimal temperature of 70 ºC and a broad optimum pH range with maximal activity at pH 7. The capacity of the GH16 enzyme to improve the nutritive value of a high viscosity barley-based diet displaying low endogenous β-glucanase activity was evaluated. There were no differences between the supplemented treatments and recombinant enzymes, suggesting that a single enzyme is effective to remove the anti-nutritive β-glucans in barley-based diet. Birds of the negative control group revealed very poor performances, as a result of the highly viscous barley. At the dosage rate used in this experiment, which is relatively high, the CBM11 had no impact in broiler performance. In contrast with what was described above, the three exogenous enzymes used in this experiment significantly reduced the weight and length of duodenum, jejunum, ileum and caecum, when compared with the negative control group. This may result from a dramatic decrease in digesta viscosity as a consequence of enzyme supplementation. Recombinant proteins used in this experiment were also susceptible to proteolysis in the linker regions, resulting in two treatments with enzymes with similar molecular architectures. The last article of chapter 3 explores the use of cellulase GH5 from *C. mixtus*, coupled or not with a family 11 CBM. Previously, *Cm*Cel5A (Fontes *et al.*, 1997) was shown to have endo-β-1,4-glucanase activity and to be resistant to proteolysis and, despite its mesophilic origin, to be slightly thermostable. These findings suggested that this catalytic module could be of interest to improve the nutritive value of barley-based diets for broilers, particularly because its optimum temperature seems to be more appropriate to function at the conditions of the GI tract. Two bird trials were conducted to test the efficacy of this cellulase *in vivo*. In the first experiment, addition of the exogenous enzymes had no effect on broiler performance. A

second trial was performed with a different barley lot and results revealed that only the commercial mixture had the capacity to improve the nutritive value of the barley-based diet. *Cm*Cel5A was shown to be ineffective in reducing the anti-nutritive effects of barley, particularly because it demonstrated limitations while acting at lower pH. Since the two different barley lots displayed different responses to enzyme supplementation, it was suggested that there are intrinsic properties in this cereal that may differ between lots and affect its nutritive value. Analysis of β-glucan content, cereal viscosity and endogenous β-glucanase activity was performed in the two barleys and it was apparent that endogenous enzymatic activity could potentially explain the differential response to enzyme supplementation. Similar analyses were performed in a large range of barley lots revealing significant variations in the three parameters, although the differences were wider for the endogenous enzymatic activity. Thus, a third animal trial was performed to test the response to enzyme supplementation of barleys with high or low endogenous β-glucanase activities. The data revealed that supplementation is not effective when endogenous enzymatic activity of the barley lot is high. Under this condition, the endogenous activity is sufficient to improve broiler performance, reduce GI size and digesta viscosity. Enzymatic supplementation had a positive effect in barley nutritive value exclusively with the barley lot expressing low endogenous β-glucanase activity.

Taken together, the experiments described in chapter 2 have many implications to poultry nutrition. In the trials where enzyme supplementation had a positive impact in poultry performance, it was clear that the first 2 weeks of supplementation were the ones where the differences in the grow rate were more dramatic. This has been described by other authors using barley-based diets (Brenes *et al.*, 1993; Franceschi *et al.*, 1995; Hesselman & Aman, 1986) and future work should evaluate if restricting β-glucanase supplementation to the first two weeks of growth is sufficient to improve barley nutritive value. In addition, the data revealed that the impact of CBM11 should be more apparent at lower dosage rates as sugested by Guerreiro *et al* 2008. CBM11 may function by targeting the appended catalytic domains to their target substrates, the soluble β-glucans. Thus, at lower enzyme dosages the CBM will effectively direct the enzyme to the soluble glucans improving the rate of catalysis; this effect is not apparent when enzymes are used at high rates when the anti-nutritive glucans may be saturated with the enzyme. However, enzyme dosage level should also be dependent on β-glucan content and endogenous β-glucanase activity, which has been showed to be the most important parameter affecting enzymatic supplementation. Therefore, in the near future the best way to rationalize enzyme incorporation in poultry diets may be to analyze barley before incorporation in diets and to adequate the enzyme cocktail and dosage levels to the presence of the anti-nutritive carbohydrates. In addition, studies with the genes encoding endogenous β-glucanases and cellulases in different varieties and lots of barley grains could reveal which genes are more expressed in barley grains with a higher β-

glucanase endogenous activity. The experiments described here also confirm that linker sequences in recombinant enzymes are prone to proteolysis after crop passage, which suggest that CBM function may primarily be restricted to the crop. Due to the proved capacity of CBMs to improve the function of the associated catalytic domains, it would be interesting to develop novel ways to protect linker regions in recombinant enzymes from proteolysis to guarantee a better integrity of the enzymes. This could be accomplished either by reducing linker size or by producing the recombinant enzymes in eukaryotic hosts that could proper glycosylate linker regions, thus conferring protection to proteolysis.

In chapter 3, the biochemical properties of the three cellulosomal family 42 CBMs (A, B and C) were evaluated aiming to characterize their role in carbohydrate recognition by the multi-enzyme complex. The structure of CBM42A revealed that these modules are typical type C CBMs, with affinity for the arabinose side chains of hemicelluloses. In general, type C CBMs bind small sugar chains (mono- or di-saccharides) present in complex carbohydrates. All cellulosomal CBM42s revealed affinity exclusively for arabinose side chains and were unable to recognize the xylan backbone *per se*, a function that is common for type B CBMs. The three CBM42s are attached to family 43 GHs, that usually display arabinofuranosidase activity (remove the arabinose moieties from complex carbohydrates). Thus, the ligand specificities of the cellulosomal CBM42 may reflect the substrate specificities of the appended GH43 modules and the CBMs may function by directing the catalytic domains to their substrates. Ichinose and colleagues (2008) also studied an arabinofuranosidase from *Streptomyces avermitilis* with a modular structure consisting of a N-terminal GH43 and a C-terminal CBM42. These authors showed that the rate of hydrolysis of arabinan was improved by the presence of CBM42. However, CBM42 domains were initially found associated to a GH54 α-L-arabinofuranosidase from *Trichoderma reesei* as a xylan-binding domain (Nogawa *et al.*, 1999). In 2004 (Miyanaga *et al.*), the crystal structure of *Aspergillus kawachii* GH54 α-L-arabiofuranosidase was solved in the presence of the associated C-terminal CBM42 that was found to bind arabinose. Among the *C. thermocellum* CBM42s described in the present work, CBM42A and CBM42B were shown to display a higher affinity for arabinoxylan, while CBM42C revealed more affinity for arabinan. CBM42's display a β-trefoil fold, presenting three subdomains α, β and γ. Studies in other CBMs presenting the β-trefoil fold, such as CBM13, revealed that interaction of the three subdomains is required for ligand recognition. In contrast, mutagenesis studies presented here revealed that the γ subdomain dominates arabinoxylan recognition in CBM42A, CBM42B and CBM42C, while the β and γ subdomains are involved in the binding to arabinan by CBM42B and CBM42C. For the first time, data presented here revealed the importance of calcium ions and water molecules in maintaining the structure and the binding pocket concavity, respectively, of CBM42 members. Taken together, these data clarified the importance of *Ct*CBM42 in cellulosome function. It is anticipated that this knowledge may be used to improve the efficacy of arabinoxylan

degradation in wheat and rye based diets for broilers. Fontes *et al.* (2004) demonstrated that a xylan-binding CBM6 from *C. thermocellum* potentiates the action of a modular xylanase used to supplement wheat-based diets for broilers. CBM6 have an exclusive affinity for the xylan backbone. Since arabinoxylan and not undecorated xylans are the anti-nutritive components of cereal-based diets, it will be interesting to test the efficacy of *Ct*CBM42 in comparison with the CBM6 in promoting the action of exogenous xylanases used to supplement wheat-based diets for broilers.

In chapter 4, our focus was on improving the levels of benefic fatty acids in breast and thigh broiler meat through a variety of dietary supplementation strategies. Today, consumers are more aware to the impact of diets in health. There is evidence that long-chain n-3 PUFA have positive effects on human health by decreasing the cardiovascular risk factors, metabolic syndrome and neurological disorders. EPA and DHA long-chain n-3 PUFA are present in marine-derived foods and LNA, the precursor of LC-n-3 PUFA, is found in plant oils, being linseed one of the richest sources known. However, current LC-n-3 PUFA intake is lower than the recommended for humans because levels of meat intake are higher than fish or other marine foods. Poultry meat has been described as a potential good candidate to accommodate PUFA on their lipid fraction. Following these ideas, we have tested different dietary sources of LNA and a DHA on broiler meat quality and their involvement in modifying the fatty acid profile of meat. Extruded linseed was shown to increase substantially LNA content of breast and thigh meats, decreasing the LA:LNA ratio and leading to healthier nutritional ratios for human consuming. However, the precursor of LC-n-3 PUFA was not effectively converted to their long chain homologues. In contrast, direct supplementation with DHA was shown to be an efficient strategy to increase LC-n-3 PUFA. Nevertheless, due to PUFA oxidative instability, the DHA treatment showed the highest oxidation levels on breast and mainly in thigh meat. Sensorial panel evaluation revealed that all supplemented groups lead to off-flavor presence in breast meat, mainly the DHA treatment. However, DHALS and LS treatments did not express differences between each other. Lipid supplementation did not affect levels of cholesterol and diterpens of breast and thigh meats. Taken together, the data suggest that future work is required to study DHA supplementation with antioxidants to guarantee the consumers acceptance and meat shelf life. Regarding PUFA metabolism, it would be interesting to determine the elongation and desaturation activities of the n-3 and n-6 pathways in breast and thigh tissues. Experimental design modifications could be made in order to gather more data on PUFA metabolism quantification to clarify some unresolved questions concerning the conversion of LNA or LA to their LC-PUFA homologues on broilers. In particular, determination of digestibility and intake of each fatty acid *in vivo*, as suggested by Turchini and Francis (2007), would allow a more real insight on the lipid metabolism and LC-PUFA accumulation.

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